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Effect of Excess Application of Nitrogenous Fertilizer on Postharvest Quality of Spinach during Storage

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ABSTRAK (ENGLISH)

The quality of vegetables depends upon the preharvest and postharvest practices. The preharvest practices include type, amount, form of fertilizers, harvesting period, growing season, and conditions that influence the yield of the crop and affect its chemical, biochemical, and mechanical properties. Moreover, postharvest practices such as storage conditions and processing methods also affect the quality of vegetables. Therefore, the present study was conducted to study the effect of different doses of nitrogen fertilizer (0–400 kg·N/ha) and storage conditions on the quality attributes of spinach leaves such as respiration rate, physiological loss in weight (PLW), chromatic properties, chlorophyll, puncture strength, TSS, pH, and nitrate during the storage studies. The respiration rate of leaves decreased with an increase in fertilizer dose. The a⁎and ΔE values of spinach leaves significantly ($p<0.01$) increased with an increase in storage temperature (T) and storage time (t) and decreased significantly ($p<0.01$) with an increase in fertilizer dosage (F). The puncture strength (PS) of leaves significantly ($p<0.01$) decreased with an increase in F, T, and t. The T, t, and their interaction had a significant ($p<0.05$) negative effect on the chlorophyll content of spinach leaves for all treatments. However, the T, t, and their interaction had a significant ($p<0.01$) positive effect on pH and PLW and a negative effect on the nitrate content. The colour change, nitrate, and chlorophyll degradation were higher with storage time for leaves which have a higher amount of nitrate content initially. The TSS, pH, PLW, and mechanical properties degradation were higher with storage time for leaves that initially had a lower amount of nitrate content. Therefore, application of the recommended dosage (as per the soil nutrient status) of nitrogen fertilizer can produce leaves with minimum nitrate content, and storage of leaves under refrigerated conditions can enhance the shelf life.

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1. Introduction

Vegetables play an important role in meeting the needs of human beings for vitamins and minerals. Spinach (*Beta vulgaris* L.) is an important leafy vegetable commonly grown in India. It is a rich source of vitamins, minerals, lipids, antioxidants, and phytochemicals. On account of its nutritional value and properties such as antimicrobial, anticarcinogenic, and antioxidant activity [1], spinach is often utilized as raw (salads and smoothies), cooked (soups, curries, casseroles, and steamed), boiled, frozen, canned, pureed form, or dehydrated and used as a food fortifying agent or food colouring agent [2]. Spinach also forms an ingredient in a wide variety of foods such as bread, noodles, tofu, cheese, deep-fat fried products, chapati, and biscuits [3, 4].

For better yields and brighter colour of leafy vegetables, the farming community tends to overuse nitrogenous fertilizer. The addition of nitrogen to the soil, more than the recommended dose, generally results in an increase in grain protein till an upper threshold is reached [5]. The concentration of nitrogen varies from plant to plant and it is present in the form of nitrate, which is the most stable oxidation state [6].

The overall characteristics and attributes of vegetables are predominantly determined by the preharvest and postharvest practices that are implemented. These practices encompass a multitude of factors, including but not

limited to the type, amount, and form of fertilizers utilized, the specific period in which the harvest is conducted, and the environmental conditions in which the crop is grown. It is essential to recognize that these preharvest practices hold a significant influence over the overall yield of the crop, as well as exert a profound impact on the chemical, biochemical, and mechanical properties of the vegetables. Some previous studies reported the effect of different forms of nitrogenous fertilizers and their dose on the quality of different fruits and vegetables such as purslane [7], lettuce [8], ginger [9], and cauliflower [10]. It has been reported that an excess (more than the recommended dosage) of nitrogenous fertilizer application results in undesirable changes, such as an increase in nitrate content, acid-to-sugar ratio, titratable acidity, carotenes, and vitamin-B1, on the other hand, a decrease in vitamin-C concentration, soluble solids, total antioxidant activity, soluble sugar, polyphenols, Ca, and Mg content in many vegetables (Liu et al. [8]; Singh et al. [9]). Besides the fertilizer dosage, the quality of vegetables is also affected by the postharvest practices, such as the storage conditions. The rate of conversion of nitrate into nitrite increases with an increase in storage temperature [11]. The effect of different storage conditions such as storage temperature, storage period, packaging material, and gas composition within the packages on the quality and shelf life of different vegetables has been reported in different studies [12–14]. However, there were no previous studies on the effect of excess nitrogen fertilizer dose on the shelf life and quality attributes of leafy vegetables during storage. It is essential to study the changes in respiration rate, chromatic properties, mechanical properties, physiological loss in weight (PLW), TSS (total soluble solids), total chlorophyll, pH, and nitrate content of leafy vegetables during storage. These parameters collectively help in assessing the quality, shelf life, and nutritional value of the crops during storage. The respiratory rate affects metabolic activity, which in turn impacts shelf life and freshness. Chromatic properties enhance consumer acceptance by reflecting visual quality. Mechanical properties, which measure texture, influence palatability. PLW affects marketable yield by measuring weight loss. TSS influences taste and energy values by indicating the sugar content. The total chlorophyll level links photosynthetic activity, nutritional quality, and green pigment levels. pH changes influence taste and safety by affecting microbial growth levels. Monitoring nitrate content is crucial as high levels can pose health risks. These parameters also help in optimizing storage conditions, ensuring safety, and prolonging shelf life while maintaining nutritional and sensory qualities.

Therefore, the present study focused on the effect of storage temperature, storage period, and nitrate content of leaves (due to varied fertilizer application rates) on the respiration rate, chromatic properties, physical parameters, and mechanical and chemical parameters of spinach leaves. The relationship between storage conditions and quality attributes of spinach has been expressed by using mathematical models.

2. Materials and Methods

2.1. Experimental

The spinach (*var. All Green*) plants were cultivated in plastic containers of size 1 × 1 × 0.3 m in the open environment during the winter season (December-February). These containers were filled up with soil up to a depth of 0.2 m. This soil was a top 10 cm layer of soil sample that was collected from the same field. The seeds were sown in all trays with an application rate of 40 kg/ha. The plants were administered with different levels of nitrogen (0, 50, 100, 150, 200, 250, 300, 350, and 400 kg·N/ha), in three stages, 50% at the time of showing, 25% after 15 days, and the remaining 25% after 25 days [1]. The application rates of P (60 kg/ha) and K (40 kg/ha) were kept constant for all the trays. The plants were watered thrice a week with a watering can. Leaves were collected early in the morning, every day from each treatment after 35 days of sowing.

The polypropylene (PP) material of uniform thickness (30 microns) was purchased from a local manufacturer, M/s Supreme Plastic Industries, Govindpura, Bhopal, India. Spinach leaves were weighed and packed in packs of 50 g each in 25 × 20 cm^2 PP packaging material. Five packets of each of the 9 treatments were stored at each storage temperature. One packet from each storage temperature was collected after 3, 5, 7, and 10 days of storage and used for quality analysis. The storage studies were conducted in triplicate at room temperature (22 ± 2°C), refrigerated temperature (4°C), and at 12 ± 2°C (Figure 1).

[figure(s) omitted; refer to PDF]

2.2. Postharvest Quality Parameters

The quality parameters such as respiration rate, chromatic properties (a* and ΔE), physical parameters (PS and PLW) and chemical parameters (TSS, total chlorophyll, pH, and nitrate) were studied after 3, 5, 7, and 10 days of storage.

2.2.1. Respiration Rate

The respiration rate of spinach leaves were measured at different storage temperatures selected from the previous studies such as room temperature (22 ± 2°C) and refrigerated temperature (4°C and 12°C). 100 g of spinach leaves was placed inside the glass jar of 2900 ml volume and sealed with a polyethylene-lined lid. A self-sealing septum was provided at the top of the lid to facilitate measurement of the concentration of gas at different intervals of time by means of an injecting needle using a headspace analyzer (Systech-GS3/P). All the samples were placed in respective jars and placed at a particular temperature. The instrument was calibrated before starting the gas analysis for each sample. The experimental respiration rate in terms of O_2 consumption and CO_2 emission at a constant temperature is calculated by using the following equations [15]:(1)RO2=YO2t-YO2t+1ΔtVfW,(2)RCO2=Z CO2t−ZCO2t+1ΔtVfW,where RO2 and RCO2 are the respiration rates (ml O_2 or CO_2/kg h); YO2 and ZCO2 are the gas concentrations for O_2 and CO_2, respectively, t is the storage time in h, Δt is the time difference between two gas measurements, Vf is the free volume of the respiration chamber in ml, and W is the weight of the leaves in kg.

2.2.2. Chromatic Properties

The colour values of spinach leaves were measured using the colorimeter (Hunter Lab, LabScan XE, Hunter Associates Laboratory Inc., Reston). The equipment was calibrated before conducting the experiment using the black-and-white reference. The leaves were placed on the port (30 mm) and covered with a black opaque cover. The leaves were scanned three times at the same position and three positions within the leaf and averaged to one value. For each treatment, nine leaves were taken, and the values were averaged. The readings were obtained in terms of lightness (L*), greenness (a*), and redness (b*). The colour change (ΔE) was calculated by using the following expression:(3)ΔE=L*−L*+a*−a*+b*−b*.

2.2.3. Mechanical Properties

The puncture strength of the spinach leaves was assessed using a texture analyzer (TAXT.T2-Stable Microsystems, Godalming, UK). This test was conducted with a 2 mm cylindrical probe with a pretest speed of 2 mm/s, a test speed of 1 mm/s, and a posttest speed of 10 mm/s. The spinach leaves were placed between two clamped HDP/FSR plates (Figure 2) with coinciding holes (area of 19.63 mm^2). The clearance between the probe and the holes of the plates was 1.5 mm. As a result of this test, the force-displacement graph for spinach leaves was generated for each treatment. The puncture strength (PS) was measured from the graph. The leaves with identical weights were used for testing, and a total of 15 leaves per treatment were used for testing. Measurement was made on the left/right side centre portion of the leaf blade relative to the midrib and a test was conducted on the adaxial side. The test was conducted on blade tissue without striking the major veins.

[figure(s) omitted; refer to PDF]

2.2.4. Physiological Loss in Weight (PLW)

The PLW of packed spinach during storage at different storage temperatures was measured using the electronic weighing balance (Precisa 310M, Precisa Gravimetrics AG, Switzerland) with a least count of 0.0001 g.

2.2.5. pH and Total Soluble Solids (TSS)

The pH of the spinach leaves was measured by preparing a semisolid blend of the leaf by mixing 10 g of a leaf with 100 ml of deionized water and blended using a domestic mixture [16]. The pH of this blend was measured using a pH meter (AE MAX-ME-73, Automatic Electronics Limited, Mumbai, India) by dipping the pH electrode inside the blend. The electrode was cleaned thoroughly with distilled water and wiped with the tissue paper every time before changing the sample. The TSS of spinach leaves was measured by using a digital handheld pocket refractometer (ATAGO INDIA Instruments Pvt. Ltd.).

2.2.6. Total Chlorophyll

The total chlorophyll of spinach leaves was measured destructively by using a UV spectrophotometer (Shimadzu Corp, UV-1800, Shimadzu Schweiz GmbH Analytical and Measuring Instruments, Switzerland). The weight of the

leaves was measured using the weighing balance before the extraction process. Methanol of 99.9% purity was used as a solvent to extract the chlorophyll from the leaves. After that, samples were placed at 4°C in a dark room overnight [17]. After immediate extraction, the absorbance of samples was measured at 652.4 and 665.2 470 nm using a UV spectrophotometer. The total chlorophyll was estimated by using the following equation:(4)total chlorophyll=1.44A665.2−24.93A652.4.

2.2.7. Nitrate

The spinach leaves were weighed using a digital weighing balance and transferred into test tubes containing 2 ml of distilled water. The test tubes were closed with three layers of aluminium foil to avoid the evaporation of water and then boiled in a water bath for 20 min. These test tubes were cooled to room temperature, and the nitrate content was measured using a nitrate meter (HORIBA Scientific LAQUATwin Nitrate Meter, Spectrum Technologies, Inc., Aurora).

The nitrate meter works on the principle of the ion-selective conductive method with a measurement range of 16–4000 ppm (mg of NO_3^-/L), with an accuracy of ±10%. Calibration of the nitrate meter was carried out by using 150 ppm and 2000 ppm standard solutions. The accuracy of the nitrate meter was checked by preparing different concentrations of nitrate solution using potassium nitrate. The following equation was used to calculate the nitrate content of the sample [6]:(5)NCS=NClWs×Vl,where NCS is the nitrate content of the sample in mg/kg; NCl is the nitrate concentration of the extract in mg/mL; Ws is the weight of the leaf in kg; and Vl is the volume of the extraction liquid in mL.

2.3. Statistical Tools

The relationship between independent variables (storage temperature (*T)*, storage period (*t)*, and fertilizer dose (*F)*) and dependent variables (a⋆, ΔE, PS, TSS, pH, total chlorophyll, nitrate, and PLW) was developed using the quadratic model. The experiments were replicated thrice, which is necessary to estimate the variability of measurements. The following equation was adopted to predict the response variables:(6)Y=β0+∑i=0kβiXi+∑i=0kβjXi 2+∑i=1k−1∑j=i+1kβijXiXj,where *Y* is the predicted responses; β0 is the constant; βi is the linear coefficient; βj is the squared coefficient; βij is the cross product coefficient; and *k* is the number of factors.

The coefficient of estimates and statistical parameters for each and every parameter were calculated from the regression analysis. The performance evaluation of the developed models was evaluated based on the statistical parameters such as RMSED (root mean squared error of deviation), SED (standard error of deviation), and R2 (coefficient of determination). The regression analysis was conducted using Design-Expert v12 software. The contour plots were developed to depict the individual and interaction effect of each independent variable on the dependent variables of spinach leaves.(7)R2=∑YP−YP¯YO−YO¯2∑YP−YP¯2∑YO−YO¯2,(8)RMSED=1n∑i=1nyi−xi 2,(9)SED=1n−1∑y−x−bias2,(10)bias=1n∑y−x.

3. Results and Discussion

3.1. Respiration Rate

The optimum gas composition is necessary to enhance the shelf life of the product by lowering the metabolic activity without losing any quality attributes (Wills et al. [18]). Therefore, the optimum O_2 concentration should be available to maintain aerobic respiration, and the CO_2 level should be below the threshold limit. High CO_2 (>20%) concentration in the package leads to anaerobic respiration, and it is not beneficial [18]. The influence of CO_2 on respiration rate is not yet evident because it depends on the type and maturity stage of the commodity, the concentration of CO_2, and exposure time [19]. Some studies stated that CO_2 lowers the intercellular pH which influences enzymatic activity. Other studies explained that CO_2 inhibits the production of ethylene which in turn lowers the respiration rate; thus, CO_2 does not have a direct effect on the respiration rate [19].

The oxygen concentration of the harvested spinach grown with different doses of nitrogenous fertilizer and contained in the packets decreased with an increase in storage time from an initial value of 20.9 to 14.2–18.4%, and CO_2 increased from 0.3 to 3.8–7.9% after storage of spinach leaves for 90 h at 4°C (Figure 3(a)). Similarly, at 12°C storage temperature for 70 h, the O_2 and CO_2 concentrations in the packets varied in the range of 13-13.8% and 7.6–8.8%, respectively (Figure 3(b)). On the other hand, for a temperature-time combination of 22 ± 2°C and 50 h, the

O_2 and CO_2 concentration ranges were 6.6–10.7 and 11.0–15.1%, respectively (Figure 3(c)). Generally, for every 10°C rise in temperature, the biological reactions increased by two to three times, and this led to a decrease in O_2 along with a subsequent increase in the concentration of CO_2 [20]. Similar results are reported for avocado [19], baby spinach [21], and diced red onions [22]. The management of temperature during storage therefore assumes utmost importance to delay the deterioration by lowering the respiration rate and metabolic activity [22].

[figure(s) omitted; refer to PDF]

It has already been mentioned that the rate of O_2 consumption and CO_2 generation was high at higher storage temperatures across all the fertilizer treatments. The respiration rate of spinach leaves at any instance of time can be gauged by the slope of the tangent at that point for the plotted graphs (Figure 3). It is a common observation that the respiration rate of spinach leaves increased with an increase in storage temperature (Figures 3(a)–3(c)) and decreased with storage time for all treatments. The respiration rate of spinach at 12°C was around two times the respiration rate of spinach stored at 4°C, while it was around 3.25 times at 22°C as compared with that at 4°C. The respiration rate of spinach leaves decreased with an increase in fertilizer dose. This can be attributed to the decrease in TSS of leaves with an increase in fertilizer dose. This is specifically because of the decrease in carbohydrates, proteins, and fats due to their participation in the respiratory pathway [23]. Similar findings were reported for tomato [24]. It was observed in the present study that the respiration rate of spinach at 4°C and 12°C storage was high initially and gradually became constant when the O_2 concentration decreased to 16 and 14%, respectively, after about 50 h of storage. Similarly, for 22°C storage temperature, the respiration rate remained almost constant till 55 h of storage.

3.2. Chromatic Properties

The chromatic properties of spinach leaves grown under different fertilizer doses (F, kg N/ha) were evaluated during storage at different storage temperatures (*T*, °C) for a varying period of time (*t*, days) in terms of greenness (a*) and change of colour (ΔE). It was observed that a second-order polynomial model could express the relation between the variables significantly ($p<0.01$). The coefficient of estimates and statistical parameters of developed models for spinach leaves are tabulated in Table 1. The a* and ΔE values of spinach leaves significantly ($p<0.01$) increased with an increase in *T* and *t* and decreased significantly ($p<0.01$) with an increase in *F*. Chlorophyll is the colour pigment present in the leaves which is responsible for the green colour of the leaves, and during storage, the decrease in greenness (a*) of spinach leaves is due to the degradation of chlorophyll due to the enzyme chlorophyllase [25]. This enzyme helps in the conversion of chlorophyll to pheophytin-a and pheophytin-b, respectively [26]. Mattos et al. [27] stated that the chlorophyll degradation and subsequent yellowing of leaves might be associated with a change in pH.

Table 1

Coefficient of estimates and statistical results of the significant ($p<0.01$) quadratic model developed with nitrogen fertilizer dose, storage temperature, and storage time for different quality attributes of spinach leaves.

Predictors	Coefficients							
a*	ΔE	PS	PLW	TSS	Chlorophyll	pH	Nitrate	Intercept
−10.939	5.260	0.116	2.663	5.144	79.891	7.001	1828.81	*T*
0.247***	1.756***	−0.006***	3.390***	−0.194***	−12.900***	0.151***	−254.71***	*t*
0.785***	3.598***	−0.011***	3.613***	−0.523***	−14.234***	0.223***	−293.57***	*F*

−0.051	−1.655***	−0.017***	−0.523***	−1.417***	47.914***	0.217***	1295.70***	*T*× *t*
0.554***	1.337***	−0.011***	3.061***	−0.129***	−12.536***	0.062***	−199.60***	*T*× *F*
−0.707***	−1.883***	−0.004**	−0.678***	0.092***	−6.386***	0.027**	−185.75***	*t*× *F*
−0.765***	−1.478***	0.009***	−0.385**	0.146***	−7.421***	−0.025*	−211.97***	T2
0.471***	1.040***	−0.005**	2.293***	0.069*	−3.471**	0.039***	−118.09**	t2
0.326***	−1.796***	−0.002	−1.156***	0.090**	2.109	−0.081***	85.61**	F2
0.810***	−0.298	−0.011***	1.191***	0.558***	2.149	0.017***	−106.98**	
	ANOVA							
–								
R2	0.598	0.552	0.508	0.899	0.847	0.807	0.870	0.901
RMSED	1.116	3.107	0.020	0.343	14.971	0.120	351.88	1.431
SED	1.117	3.111	0.020	0.343	14.990	0.120	352.35	1.429

T, temperature; *t*, time; F, fertilizer dosage; R2, coefficient of determination; RMSED, root mean squared error of deviation; SED, standard error of deviation. ***p<0.01; **p<0.05; *p<0.1.

All three interactions were found to have significant (p<0.01) effect on a* and ΔE values (Figures 4(a)–4(f)). This demonstrates that within the experimental range, for the same fertilizer dose, spinach stored at a higher *T* loses colour more rapidly than the one stored for a longer duration of time (*t*) (Figures 4(a) and 4(d)). The rate of chlorophyll degradation was higher at higher storage temperatures; therefore enhanced ΔE in leaves was observed at higher *T*. The effect of *T* and *t* on the colour change of spinach was similar to the previous studies reported for baby spinach [21] and fresh-cut romaine lettuce [28]. In the present study, chlorophyll degradation was higher during the initial stage due to the high concentration of $O_{2,}$ and the degradation rate decreased with time because of the increase in CO_2 content in the headspace of the package.

[figure(s) omitted; refer to PDF]

3.3. Puncture Strength (PS)

The epidermal thickness is the major parameter that affects the PS of a leaf, however, the presence of veins on the leaf lamina creates a major problem in illustrating the PS of the leaves. Apart from the epidermal thickness, the leaf anatomy in terms of the midrib also affects the PS [29]. This problem was avoided in the present study by excluding the midrib of the leaf during the evaluation of the PS.

The individual effect of *F*, *T*, and *t* on PS of spinach leaves reflected a significant (p<0.01) negative effect on PS. The results agree with the results reported for lettuce [30]. This negative effect of *F* on PS is due to the changes in leaf anatomy in terms of alteration in cell size and shape, and the development of more extensive intercellular air

spaces between veins is presumably due to excessive growth or expansion under high nitrogen fertilizer dose. While under low *F*, the isodiametric cells of spinach leaves are spaced closely which results in an elevated PS [29].
The interactive effect ($p<0.01$) of *T* was negative with *t* and *F*, while *t* had a positive ($p<0.01$) interaction with *F*. In all interactions, the behaviour of PS was in line with the individual effects of the variables (Figures 4(g)–4(i)). PS decreased as *T* and *t* increased linearly (Figure 4(g)), and the increase was more pronounced with *T* than with *t*. The PS of leaves mainly depends on the moisture content, the subtle skew (Figure 4(h)) demonstrated by the PS of the leaf can be attributed to the loss in moisture and increase in the lignifications [31] due to the increase in the temperature. As *F* and *T* increased, the PS decreased (Figure 4(i)). An elongated storage period might have led to the leakage of electrolytes due to the rupture of the cell membrane caused by the increase in CO_2 concentration, pH, and ammonia [32]. Again, the increase in ammonia, due to the increase in fertilizer, also caused an excess rise in CO_2 resulting in an anaerobic metabolism causing a loss of tissue integrity [33].

3.4. Physiological Loss in Weight (PLW)

The PLW increased significantly ($p<0.01$) with an increase in *T* and *t*, while it decreased ($p<0.01$) with an increase in *F*. The maximum PLW was observed when the leaves were stored at higher *T* for a longer *t* (Figure 4(j)). The weight loss in the packets is due to the respiration-induced consumption of carbohydrates present in the leaves. The rapid increase in PLW at high temperatures was due to the increase in respiration rate and loss of moisture to the surrounding environment through the packaging film. A similar trend in PLW with respect to temperature and storage period was reported for iceberg lettuce [12], lettuce [13], and purple cabbage [14]. The negative effect of *F* on PLW may be due to the lower respiration rate of spinach leaves grown under a high fertilizer dose due to its lower TSS content as compared to leaves grown under a lower fertilizer dose. The interaction effect ($p<0.01$) of *F* with *T* (Figure 4(k)) and *t* (Figure 4(l)) had a significant negative effect on PLW. The contour plot indicates that within the experimental range, for the same *F*, the PLW was higher for spinach leaves stored at a higher *T* than the one stored for a longer duration of time. This may be due to the high respiration rate of leaves grown under lower N fertilizer dose because of higher TSS content, which results in higher PLW.

3.5. Chemical Parameters

3.5.1. Total Soluble Solids (TSS)

The TSS content of spinach leaves significantly ($p<0.01$) decreased with an increase in *T*, *t* and *F*. There is a gradual decrease in TSS content during storage due to the consumption of sugar through the respiration process [34]. The increase in temperature leads to an increase in respiration rate; this entailed a higher loss in TSS at higher storage temperatures. The results obtained in the present study are in agreement with the results reported for *Sesamum indicum* L [35]. The interaction of *T* with *t* had a significant ($p<0.01$) negative effect, but *F* with *T* and *t* had a significant ($p<0.01$) positive effect on TSS. The negative individual effect of the variables on TSS was reflected in the interactions as well. The value of coefficients of linear terms had a bearing on the behaviour of TSS vis-à-vis interaction of *T* (0.194) and *t* (0.523). TSS decreased sharply with respect to an increase in *t* as compared to *T* (Figure 5(a)). It was observed that the effect of *F* with respect to *T* (Figure 5(b)) and *t* (Figure 5(c)) was overwhelming. TSS increased sharply for every unit fall in *F* as compared with *T* and *t*, and similar results are reported for cabbage [36] and purple cabbage [14].
[figure(s) omitted; refer to PDF]

3.5.2. Chlorophyll

The *T* and *t* had a negative ($p<0.01$) effect on the total chlorophyll content of spinach leaves, on the other hand, *F* had a positive ($p<0.01$) effect. There was a sharp decrease in total chlorophyll as the *T* and *t* moved towards their respective maximum values. The interaction effect of *T* with *t* had a significant ($p<0.01$) negative effect on total chlorophyll content (Figure 5(d)). A similar effect of temperature, storage time, and interaction effect was reported for Chinese cabbage [37]. The degradation of chlorophyll during storage was due to the chlorophyllase enzyme. This enzyme helps in the conversion of chlorophyll to pheophytin-a and pheophytin-b, respectively [26]. On the other hand, some researchers attributed this chlorophyll degradation to the senescence and peroxidation of the cell membranes because of the direct effect of oxygen on the enzymatic degradation activity on chlorophyll pigments

[37]. The effect of *T* and *t* on the chlorophyll content of spinach leaves obtained in the present study is in further agreement with the fenugreek [38], lettuce [27] and iceberg lettuce [12]. The interaction of *F* with *T* (Figure 5(e)) and *t* (Figure 5(f)) had a significant ($p<0.01$) negative effect on the total chlorophyll content. The effect of *F* on *t* was by far more defined than it was with *T*. This depicts that within the experimental range, for the same *F*, the rate of total chlorophyll degradation was higher for spinach leaves stored for a longer duration (*t*) than the one stored at higher temperature (*T*).

3.5.3. pH

The pH of spinach leaves significantly ($p<0.01$) increased with an increase in *T*, *t,* and *F*. The positive effect of F on the pH of the leaves was due to the decrease in ascorbic acid (AA) content with an increase in N fertilizer dose. The increase in pH was due to a decrease in AA and an increase in carotenoids or flavonoids' content with an increase in N fertilizer dose [39]. The positive effect of *T* and *t* on pH was due to the decrease in AA content of the leaves during storage. The decrease in AA during the storage was due to the reduction in the antioxidant potential of fruits and vegetables [40]. The increase in pH of spinach with an increase in *T* and *t* in the present study agreed with previous studies reported for baby spinach [32]. The interaction effect of *T* with *t* had a significant ($p<0.01$) positive effect on the pH (Figure 5(g)). The interaction effect of *F* with *T* ($p<0.05$) and *t* ($p<0.1$) was also found to be significant. The contour lines in the *F vs T* interaction graph (Figure 5(h)) were more curved than the *F* vs *t* interaction graph (Figure 5(i)) of spinach. This depicts that within the experimental range, for the same *F*, the rate of pH change was higher for spinach stored at a higher *T* than the one stored for a longer duration. According to Tudela et al. [32], the increase in pH of spinach leaves during storage was because of ammonia generation due to injury of leaves by CO_2.

3.5.4. Nitrate

The nitrate content of spinach leaves significantly ($p<0.01$) decreased with an increase in *T* and *t*. However, *F* had a significant ($p<0.01$) positive effect on the nitrate content of spinach leaves. The interaction effect of *t* with *T* (Figure 5(j)) had a significant negative effect on the nitrate content. The nitrate content of spinach leaves reached a minimum value when the leaves were stored at a higher temperature (*T*) for a longer duration of time (*t*). The nitrate content in the leaves after immediate harvesting was very high as compared to nitrite, and with time, the nitrate converts into nitrite due to enzymatic/microbial activity. The results obtained in the present study agree with the previous results reported by Wu et al. [41]. At lower temperatures, the nitrate reductase was inactivated as well as bacterial activity was prevented. When the vegetables were stored under refrigeration conditions (5°C), the nitrate content was almost unaffected for 7 days [42]. However, the nitrate gets converted into nitrite after a prolonged storage of more than 12 h, and this may be due to the release of endogenous nitrate reductase that leads to the formation of nitrite, especially in vegetables containing a large amount of nitrate [42]. The interaction effect of *F* with *T* and *t* had a significant ($p<0.01$) negative effect on the nitrate content of leaves. An increase in *F* led to a massive increase in the nitrate content in relation to *T* (Figure 5(k)) and *t* (Figure 5(l)).

4. Conclusions

The present study investigated the effect of excess application of nitrogen fertilizer dose on the quality attributes of spinach leaves during storage. The results revealed that the fertilizer dose had a negative effect and storage temperature had a positive effect on the respiration rate. The greenness of leaves increased with an increase in *F* and decreased with an increase in *T* and *t*. Similarly, the puncture strength deceased with an increase in *F*, *T*, and *t*. The chlorophyll content increased with an increase in *F* and started degrading during the storage. The pH, PLW, and nitrate decreased with an increase in *F* and increased with an increase in *T* and *t*. From the results, it can be stated that the leaves grown under high fertilizer doses result in high amounts of nitrates and lower mechanical strength, hence, the application of the recommended dosage of nitrogen fertilizer can meet the food safety concern and yields better quality products with optimal mechanical strength. Storage of leaves under refrigerated conditions can minimize nitrate to nitrite conversion and enhance the shelf life with minimal quality loss.

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Comparative Analysis of Antioxidant Potency and Phenolic Compounds in Fruit Peel of Opuntia

robusta , Opuntia dillenii , and Opuntia ficus-indica Using HPLC-DAD Profiling

Marhri, Ahmed; Youssef Rbah; Allay, Aymane; Boumediene, Mehdi; Aziz Tikent; dkk.

ABSTRAK (ENGLISH)

The edible part of the prickly pear fruit is most commonly consumed, while the peel is often discarded, leading to the loss of valuable bioactive components, such as phenolic compounds. This research aims to assess the antioxidant activity, total phenolic content, and quantities of phenolic and flavonoid contents of fruit peel of *Opuntia ficus-indica*, *Opuntia robusta*, and *Opuntia dillenii*. Ten phenolic compounds were identified, with isorhamnetin being the most abundant flavonoid measuring 7184.09 μg/g, while sinapic acid was identified as the major phenolic acid measuring 1806.36 μg/g. *Opuntia robusta* exhibited the highest total phenolic content, total flavonoids, and total phenolic acids measuring 5583.19 mg GAE 100 g^{-1}, 21041.03 μg/g, and 3265.17 μg/g, respectively. *Opuntia robusta* and *Opuntia dillenii* demonstrated the higher antioxidant capacity in scavenging 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) compared to *Opuntia ficus-indica*, whereas *Opuntia dillenii* and *Opuntia ficus-indica* exhibited the highest concentration of antioxidants capable of reducing the Fe^{3+} complex of ferric ions $(TPTZ)^{3+}$ to the ferrous complex Fe $(TPTZ)^{2+}$. In contrast, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC) assays showed no significant difference between the examined species. Compared to the local species *Opuntia ficus-indica*, the two newly introduced *Dactylopius opuntiae*-resistant species are richer in phenolic compounds and exhibit greater antioxidant activity.

TEKS LENGKAP

1. Introduction

Nopal cactus, commonly referred to as prickly pear, belongs to the cactaceae family, comprising around 2000 distinct species [1]. According to [2], Mexico, Italy, and South Africa are the largest producers and consumers of prickly pear, accounting for 45%, 12.2%, and 3.7% of the world's production, respectively. This versatile plant serves various purposes, such as fodder for livestock, pharmaceutical, and also, its fruits and young cladodes are highly suitable for human consumption [3]. Furthermore, the cactus has also applications in the industry and medicine. This drought-resistant perennial plant provides economic benefits especially to rural communities. The fruits and young cladodes can be sold in local markets, providing an additional source of income for farmers. Consequently, prickly pear emerges as an excellent choice for preserving livelihoods, alleviating poverty, and generating employment opportunities due to its properties [4]. According to a report by the Food and Agriculture Organization (FAO) and the International Center for Agricultural Research in Arid Zones (ICARDA), cultivating prickly pear cactus can enhance resilience to drought and soil impoverishment, addressing challenges faced by rural communities [5]. In Mexico, the pear sector presents significant employment potential, particularly in arid regions, with approximately 20000 families relying on this agriculture [2]. Cultivating cactus pears proves to be an effective strategy for combating climate change and promoting rural development, especially in arid and semiarid areas [2].

The fruit is the most consumed and appreciated component in the prickly pear plant, earning widespread recognition across the globe. In some regions, particularly in North America, young cladodes claim place in consumption, in which the young cladodes are frequently prepared by grilling or broiling [6]. The low fiber content in prickly pear cladodes makes them easily decomposable, contributing to their reputation as substantial methane producers [7]. The edible portion of the fruit is the most used part of the plant, whereas the fruit peel is rarely used except as

livestock feed (Potgieter and Mulaudzi). Either the consumption or industrial processing of the fruit generated a large amount of solid waste, which is mainly fruit peel. Currently, the issue of food waste has escalated to a significant global concern, emerging as a recent focal point within academic discussions [8]. The predominant disposal method involves burying food waste in landfills, resulting in the loss of its energy content. In fact, waste landfilling continues to be viewed as a viable waste management solution in numerous countries worldwide. Unfortunately, this inadequate management of food waste has profound and detrimental effects on the environment, particularly contributing to climate change [9]. Apart from environmental pollution, the disposal of waste is progressively becoming more costly.

Many studies primarily focus on the characteristics of the fruit's edible portion, often overlooking by-products, such as peels, which constitute 44.51% of the fruit [10]. However, there should be a growing interest in harnessing by-products generated from industrial processing or human consumption [11]. In effect, the disposal of fruit peel could lead to loss of valuable components like phenolic compounds, which possess a wide range of biological activities, such as anticancer, cardioprotective, anti-inflammatory, antimicrobial, and antioxidative [12]. In the last three decades, the determination of biological activity, especially antioxidant activity, has gained attention of researches [13]. Additionally, an oxidative process can generate free radicals, which has a harmful effect on health, such as hypertension, cancer, atherosclerosis, cardiovascular diseases, nitrosative stress, diabetes mellitus, and neurological disorders [14]. Consequently, antioxidants and especially natural substances exhibiting antioxidant properties have attracted the attention of scientific community [15].

Polyphenols, categorized as secondary metabolites predominantly present in plant tissues, are synthesized through shikimic acid and phenylpropanoid pathways [16]. These polyhydroxy phytochemicals, characterized by aromatic compounds containing at least one phenol group in their structure, represent the most prevalent secondary metabolites in plants, boasting over 8000 identified structures [17]. Based on their chemical structures, phenolic compounds are divided into various subclasses like flavonoids, phenolic acids, coumarins, curcuminoids, quinones, tannins, lignans, and stilbenes [16]. For the past few years, polyphenolics become a subject of interest in a scientific fraternity and many investigations were elaborated on these compounds. Epidemiological studies have demonstrated that the consumption of beverages and foods rich in phenolic content can reduce the risk of heart disease by acting as antioxidants against low-density lipoprotein [18]. Numerous investigations within the scientific community have focused on the bioactivities of polyphenolics, revealing their diverse properties, including anti-inflammatory, antimicrobial, antioxidant, and antiproliferative activities [19]. Presently, there is heightened interest among researchers and food manufacturers in the antioxidant properties of phenolics and their potential preventive role in various oxidative stress-related conditions [20]. The shift toward exploring natural sources of antioxidants has gained momentum, particularly with the elimination of synthetic antioxidants in food applications. Nutritionists have recently directed their attention to the nutritional and health benefits of polyphenols, recognizing their capacity to influence oxidative stability, color, bitterness, odor, astringency, and flavor [16, 21]. Various conventional and nonconventional methods are employed to extract phenolic compounds. The most commonly used traditional extraction techniques include liquid-liquid extraction, solid-liquid extraction (Soxhlet extraction), and maceration, whereas the alternative techniques encompass multiple methods, such as supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, and countercurrent chromatography.

The spectrophotometric assessment of antioxidant efficacy is carried out by measuring the ability of biological sample to scavenge the synthetic-colored radical. Two kinds of assays can be performed, the first based on hydrogen atom transfer, while the second, of particular interest to us, encompasses electron transfer, including DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), and FRAP (ferric reducing antioxidant power) assay. These chemical assays are commonly used due to their simplicity, cost-effectiveness, easy accessibility, and independence from advanced laboratory equipment [22].

To the best of our knowledge, rare studies have investigated the phenolic compounds and antioxidant activity of fruit peel, particularly for the species *O. dillenii* and *O. robusta*. Most research on fruit peel has focused only on *O. ficus-*

indica, which creates a research gap. The objective of the present contribution is firstly to highlight the importance of valorization peel fruit by studying its components, and this involves examining total phenolic content, phenolic compounds, and conducting antioxidant tests (DPPH, FRAP, TAC, and ABTS) as potential reservoirs of natural antioxidants. The statistical exploration of the correlation between phenolic content and antioxidant activity was also undertaken. Secondly, it is to compare the local species (*O. ficus-indica*) with the two recently introduced resistant species to the *D. opuntiae* (*O. robusta* and *O. dillenii*).

2. Materiel and Methods

2.1. Plant Material

The research study concentrated on three distinct groups of populations collected from the Oujda region in the northeastern part of Morocco (Spring 2023), located at coordinates 34°41′ 12.001″ N and 1° 54′ 41″ W. This geographic area is known for its Mediterranean climate, characterized by cool winters, hot summers, and irregularly distributed precipitation [23]. Healthy fruits used in the current study (intact of infection and infestation) were selected and carefully cut at their bases from the chosen plant.

2.2. Sample Preparation

The fruits were subjected to a completely cleansing with tap water, and the peels were separated manually from the rest of the fruit. Subsequently, the peels were cleaned and shade-dried at room temperature and finely ground to powder suitable for extraction. Then, oven drying occurred in an oven drier maintained at a consistent temperature of 30°C for a duration of 48 hours. The resulting dried samples were then processed in an electric grinder (Type ME 2B, 800 W; INTERMITTENT 15 s/1 min; manufactured in France). Finally, the resulting powder was preserved in an air-tight bottle.

2.3. Extraction and Determination of Total Phenolic Content

As per reference [24], phenolic compounds were extracted by combining 0.1 g of powder with 5 mL of 50% acetone (v/v). Subsequently, the acquired mixture underwent vortexing for a period of 10 minutes and was then subjected to centrifugation at 5000 rpm for 15 minutes. This extraction procedure was iterated three times to guarantee the complete extraction of phenolic compounds. Then, a filtration using a 45 μm microfilter was carried out. For the assessment of total polyphenol content, 50 μL of the obtained supernatant was used. Absorbance measurements were conducted with a RAYLEIGH UV1800 Ultraviolet-Visible spectrophotometer (Beijing Rayleigh Analytical Instrument Corporation, China) at a wavelength of 760 nm. Utilizing gallic acid as a reference standard, a calibration curve was established, and the outcomes were presented as milligrams of gallic acid equivalent per 100 grams of dry matter (mg GAE100 g^{-1}·DM).

2.4. Antioxidant Activity Determination

The evaluation of antioxidant capacity across diverse species under study involved employing various methodologies focused on neutralizing free radicals. These methods included determining total antioxidant capacity (TAC), the [2.2-di(4-tert-octylphenyl)-1-picrylhydrazyl] (DPPH) assay, an evaluating ferric reducing antioxidant power (FRAP) and the 2.20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test. The outcomes were measured in milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM). Optical density measurements were conducted using a UV-Vis spectrophotometer, specifically the RAYLEIGH UV1800 model.

2.4.1. Total Antioxidant Capacity Assay

The determination of total antioxidant capacity (TAC) followed the procedure outlined in [25]. The phosphomolybdenum reagent was created by mixing equal amounts of 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid. The experimental process involved mixing 1.5 mL of this reagent with 50 μL of the extract, followed by thorough vortexing and an incubation period in a water bath at 95°C for 90 minutes. Subsequently, absorbance was promptly recorded at 695 nm, with a blank used as a reference for baseline correction. The results were expressed in milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM).

2.4.2. Radical Scavenging Activity of 1,1-Diphenyl Picrylhydrazyl—DPPH Assay

The assessment of DPPH free radical scavenging activity in the samples was conducted using spectrophotometry, following the outlined procedure in reference [26]. This assay relies on the antioxidant's electron transfer ability to

neutralize the DPPH radical, causing a measurable color change, which is quantified at 517 nm. There is a reciprocal relationship between the absorbance of the reaction mixture and the extent of DPPH free radical scavenging activity. In this study, 50 μL of each sample was introduced into 2 mL of a 0.13 mM methanolic solution of DPPH. The resulting mixture was vigorously vortexed and then incubated in a dark environment at room temperature for 30 minutes. The outcomes were presented in milligrams of Trolox equivalent per Gram of dry matter.

2.4.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric-reducing antioxidant power (FRAP) assay is based on the measurement of an antioxidant's ability to reduce ferric ions (Fe^{3+}) and form an intensely blue ferrous complex (Fe^{2+}), as described in reference [25]. In this procedure, each sample, comprising 50 μL, was mixed with 0.65 mL of 0.2 M phosphate buffer (pH 6.6) and 0.65 mL of a 1% ferricyanide solution. After 20 minutes of incubation at 50°C in a water bath, the reaction was stopped by adding 0.65 mL of 10% trichloroacetic acid. Following this, 0.65 mL of the extract was combined with 0.65 mL of water and 0.25 mL of a 0.1% solution of $FeCl^{3+}$. The reducing power of the tested antioxidants can be assessed spectrophotometrically, where increased absorption at 700 nm indicates strong antioxidant activity. The results are expressed in milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM).

2.4.4. ABTS [2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)] Free Radical Scavenging Activity Assay

The evaluation aimed to assess the ability of antioxidants to counteract the stable radical cation, specifically 2,2-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS•+). This radical cation presents as a blue-green chromophore with its highest absorption peak at 734 nm, and the presence of antioxidants leads to a reduction in the intensity of this chromophore.

To generate the ABTS•+ radical, 2.45 mM potassium persulfate was mixed with 7 mM of ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)], and the mixture was allowed to incubate at room temperature in the absence of light for 16 hours. Following this, the working solution, composed of 50 μL of the extract mixed with 2 mL of the ABTS•+ solution, underwent a 30-minute incubation at room temperature in a light-shielded environment. Finally, absorbance was measured at 734 nm using a spectrophotometer, with a blank used as a reference [25]. The results are stated in milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM).

2.5. HPLC-DAD of Phenolic Compounds

Chromatographic analyses were performed using an Agilent 1260 Infinity II high-performance liquid chromatography system equipped with a diode array detector. The chromatographic separation utilized an Eclipse XDB-C18 column (3.5 μm particle size, 150 × 4.6 mm internal diameter, USA). The elution solvent consisted of a mixture of water (A) and acetonitrile (B), with the addition of formic acid (1%, v/v) in water. The chromatographic separation employed a gradient elution process, starting at 2% B and held constant from 0 to 6 minutes. Subsequently, it increased to 12.5% B between 23 and 33 minutes, further rising to 30% B from 33 to 38 minutes, then to 45% B from 38 to 42 minutes. This was followed by a stepwise increase to 75% B from 42 to 47 minutes and maintaining 100% B from 47 to 49 minutes. The solvent composition reverted to 2% B from 49 to 50 minutes and remained constant at 2% B from 50 to 51 minutes. The flow rate was set at 0.6 mL/min, with a 10 μL injection volume. Extract separation was monitored at multiple wavelengths (254, 280, 300, and 340 nm), and UV/visible spectra for each compound were recorded over a range from 190 to 800 nm. To identify the compound within the sample, we examined the retention time of the detected peaks and the UV spectra, comparing them with the standards from Sigma-Aldrich in Saint-Louis, United States. Quantification was conducted using an external standard curve of isorhamnetin (20–200 μg/mL), chlorogenic acid (20–200 μg/mL), sinapic acid (20–200 μg/mL), gallic acid (6.25–100 μg/mL), kaempferol (6.25–100 μg/mL), ferulic acid (8–60 μg/mL), and hydroxycinnamic acid (8–60 μg/mL) based on their respective commercial standards. The HPLC profiles were visualized and analyzed using the Agilent OpenLab CDS software. Results are expressed as milligrams of standards per 100 grams of dry matter [27].

2.6. Statistical Analysis

In this study, all analyses of the prickly pear cladode were conducted in triplicate to ensure the robustness and reliability of the findings. A one-way analysis of variance (ANOVA) was performed to identify variations among the means of different samples. Statistical significance was considered established when the p value was below 0.05,

indicating a statistically significant difference. Following this, the Tukey post hoc test was employed to conduct multiple comparisons of means, allowing for a more detailed assessment of differences among groups. The results are presented as the mean ± standard deviation, and the statistical analysis was carried out using IBM SPSS Statistics 25 software. Additionally, correlations among the obtained data were calculated using Pearson's correlation coefficient (*r*), providing insights into the relationships between the variables under consideration.

3. Results

3.1. Total Phenolic Content of Fruit Peel

The assessment of total phenolic content (TPC) was conducted for each of *O. robusta*, *O. dillenii*, and *Opuntia ficus-indica* (Figure 1). The results revealed significant variations ($p<0.02$) among the species. *Opuntia robusta* exhibited the highest TPC, measuring 5583.19 milligrams of gallic acid equivalent per 100 grams of dry matter (mg GAE100 g^{-1} DM). While *O. dillenii* displayed moderate TPC results at 5513.55 mg GAE100 g^{-1} DM, *O. ficus-indica* showed the lower results, measuring 5464.97 mg GAE 100 g^{-1} DM (Supplement Table 1). This indicates noteworthy differences in the phenolic content among the three *Opuntia* species.

[figure(s) omitted; refer to PDF]

3.2. Assessment of Antioxidant Activity of *Opuntia robusta*, *Opuntia dillenii,* and *Opuntia ficus-indica* Fruit Peel

The use of four distinct techniques aims to comprehensively evaluate the antioxidant activity and measure the antioxidant capacity of fruit peels of the various species studied. This multifaceted approach allows for a comprehensive understanding of how antioxidant properties vary among different plant species.

3.2.1. DPPH Radical Scavenging Activity

The DPPH radical scavenging activities of the species under investigation ranged from 1.19 to 1.2 milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM) (Supplemental Table 2). Notably, no significant difference was observed between the studied species (Figure 2). In this specific antioxidant test, *O. robusta* and *Opuntia dillenii* exhibited a superior antioxidant capacity but without any significant difference with *Opuntia ficus-indica* with exhibited 1.19 mg TE g^{-1} DM.

[figure(s) omitted; refer to PDF]

3.2.2. ABTS Free Radical Scavenging Activity Assay

The ABTS values varied from 0.3 to 0.38 mg Trolox equivalent per Gram of the sample, as detailed in Supplemental Table 2. Importantly, significant variations in antioxidant activity were observed among the examined species (Figure 2). *O. robusta* and *O. dillenii* demonstrated the greatest antioxidant activity, with 0.37 and 0.38 milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM), respectively. Notably, there was no significant difference between the antioxidant activities of *O. robusta* and *O. dillenii*. Conversely, *O. ficus-indica* displayed the lowest antioxidant activity, registering 0.3 mg TE g^{-1} DM.

3.2.3. Ferric-Reducing Antioxidant Power (FRAP) Assay

The ferric-reducing capacities of the species under investigation ranged from 0.49 to 0.83 milligrams of Trolox equivalent per Gram of dry matter (mg TE g^{-1} DM) (Supplemental Table 2). Notably, significant differences were observed between the studied species ($p<0.05$). Without any significant difference, *O. dillenii and O. ficus-indica* exhibited the highest antioxidant capacity registering 0.83 and 0.76 (mg TE g^{-1} DM), respectively, whereas *O. robusta* exhibited the lowest ferric reducing capacities, registering 0.49 (mg TEg^{-1} DM) (Figure 2).

3.2.4. Total Antioxidant Capacity (TAC) Assay

The obtained results of TAC assay varied between 18.9 and 19.16 milligrams of Trolox equivalent per Gram of dry matter (mg TEg-1 DM) (Supplemental Table 2). *O. dillenii* demonstrated the highest value at 19.16 mg TE g^{-1} DM, followed by *O. ficus-indica* with a recorded value of 18.98 TE g^{-1} DM. Conversely, *O. robusta* showed the lowest value with 18.9 mg TE g^{-1} DM. However, no significant differences were observed between the species under examination (Figure 2).

3.3. Quantification of Phenolic Compounds by HPLC-DAD

In the examination of phenolic compounds, ten distinct compounds were identified, encompassing seven flavonoids and three phenolic acids (Table 1). HPLC analysis allows the identification of isorhamnetin as the most abundant

flavonoid, with levels ranging from 2429.737 to 7184.09 μg/g. Following closely were quercetin and rutin. Naringenin represented the least abundant flavonoid, measuring from 95.40 to 242.83 μg/g. *O. robusta* displayed the highest concentration of four flavonoids, including isorhamnetin, epicatechin, quercetin, and rutin, while *O. dillenii* showed the highest concentration of two flavonoids: kaempferol and naringenin. On the other hand, *O. ficus-indica* demonstrated the highest concentration of only gallocatechin.

Table 1

Identification and quantification of phenolic compounds of *O. robusta*, *O. dillenii,* and *O. ficus-indica*.

N	RT (min)	Phenolic compounds	Structural formula	Fruit peel (μg/g)		
O. ficus-indica (μg/g)	*O. robusta* (μg/g)	*O. dillenii* (μg/g)			*Flavonoids*	
1	11.032	Isorhamnetin		2429.73	7184.09	6571.72
–						
2	13.60	Kaempferol		181.43	236.72	303.84
–						
4	18.87	Gallocatechin		3194.26	1420.25	2404.88
–						
5	18.97	Epicatechin		583.37	669.81	448.38
–						
6	20.21	Rutin		1350.78	5440.53	2131.25
–						
9	24.06	Quercetin		1915.04	5994.19	1580.83
–						
10	25,20	Naringenin		230.43	95.40	242.83
–						
		Total flavonoids		**9885.06**	**21041.03**	**13683.77**
–						

		Phenolic acids				
3	16.55	Hydroxybenzoic acid		761.65	1235.94	1563.49
–						
7	22.21	Ferulic acid		98.07	222.86	80.14
–						
8	22.49	Sinapic acid		592.17	1806.36	1410.92
–						
		Total phenolic acids		**1451.89**	**3265.17**	**3054.56**
–						
		Total phenolic compounds		**11336.96**	**24306.2**	**16738.33**

Values in bold represent the total flavonoids, total phenolic acids, and total phenolic compounds.

In contrast to flavonoids, only three phenolic acids were identified, namely, sinapic acid, ferulic acid, and hydroxybenzoic acid. Sinapic acid represents the most abundant phenolic acid, with levels ranging from 592.17 to 1806.36 μg/g, followed by hydroxybenzoic acid, which ranged from 761.65 to 1563.49 μg/g. Ferulic acid, in contrast, constituted the lowest concentration ranging from 80.14 to 222.86 μg/g. Regarding the total phenolic acids, *O. robusta* displayed the highest results measuring 3265.17 μg/g, followed by *O. dillenii* with 3054.56 μg/g, while *O. ficus-indica* exhibited the lowest concentration of total phenolic acids with 1451.89 μg/g.

Concerning the total phenolic compounds, *O. robusta* demonstrated the highest concentration measuring 24306.2 μg/g, followed by *O. dillenii*, which exhibited moderate results with 16738.33 μg/g, while *O. ficus-indica* showcased the lowest content of total phenolic compounds measuring 11336.96 μg/g.

4. Discussion

Due to the limited research conducted on fruit peel of *Opuntia* species, we have focused primarily on comparing the results among the different species studied. The acquired results reveal significant differences among the studied species ($p<0.024$). *O. robusta* exhibited the highest total phenolic content, measuring 5583.19 mg GAE 100 g^{-1} DM. The substantial increase in polyphenolic components in *O. robusta* emphasizes the nutritional importance linked to this particular species. Considering that the examined species were collected from the same location, indicating identical environmental conditions, it confirms that the noted distinctions among these species mainly arise from their inherent biochemical characteristics.

Our results show that the total phenolic content (TPC) of the studied species is notably higher than the values reported in previous studies [28] which documented levels ranging between 785 and 1759 mg GAE 100 g^{-1} DM in *O. ficus-indica*. Also, our findings are so higher than those reported by [29, 30] where the total phenolic content of *O. ficus-indica* peel was reported as 1078 and 1439 mg GAE 100 g^{-1} DM, respectively. Compared to [31] who used cyclohexanone for the extraction, declared a TPC of 5111 mg GAE 100 g^{-1} DM for the fruit peel of *O. ficus-indica*, our results are slightly higher. This slight difference could be attributed to the environmental conditions or the method of extraction, in which they used cyclohexanone while we used acetone for the extraction. According to [29], the

extraction efficiency of polyphenols from plant tissue depends on the solubility of the phenolic compounds in the chosen solvent, and higher amounts of phenol compounds are extracted with more polar solvents. It is worth noting that the TPC content of fresh peel of *Opuntia megacantha* was reported to be much lower, with 243.79 and 226.2 mg GAE $100\,g^{-1}$ DM, respectively [32, 33]. The observed lower TPC content could be related to the studied species, the extraction method, and also the nature of the tissue. We assume that more polyphenols could be extracted from dried tissue compared to fresh tissue.

The oxidation process can occur through various pathways, such as thermal oxidation, autoxidation, enzymatic oxidation, and photooxidation, with many of these involving free radicals. Among the numerous approaches to control oxidation, the use of antioxidants proves to be the most effective, convenient, and economical method [34]. Antioxidants are substances that, when present in very low concentrations, can delay, control, or completely prevent oxidative processes. They encompass free radical scavengers, metal ion chelators, peroxide inactivators, inhibitors of pro-oxidative enzymes, and singlet oxygen quenchers [34]. Consequently, the inhibitory effect against oxidation processes manifests through various mechanisms. For that, to assess antioxidant activity accurately, a range of assays employing different mechanisms should be employed, including single electron transfer (ET), hydrogen atom transfer (HAT), metal chelation, and reducing power. As emphasized by [35], evaluating antioxidant ability necessitates the use of multiple in vitro antioxidant methods, relying solely on one method does not provide an accurate reflection of antioxidant activity. At least three different methods should be conducted simultaneously to determine antioxidant activity comprehensively.

To assess the antioxidant activity of the fruit peel of the studied species, four bioanalytical assays were employed, namely, DPPH, TAC, ABTS, and FRAP. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) removal assay, which assesses antioxidant capacity, stands out as one of the most commonly employed techniques. It is characterized by its speed, simplicity, and cost-effectiveness when compared to alternative testing approaches. The DPPH test measures the transfer of hydrogen atoms from antioxidants to the free radical DPPH, resulting in the radical reduction and the formation of hydrazine (DPPH-H). This reaction is accompanied by a color change from violet to yellow that consequently can be monitored by spectrophotometry, which correlates with the antioxidant capacity. Conversely to the reported results [36], which indicated that the highest antioxidant activity was measured by the ABTS assay, followed by DPPH and FRAP test, the current study demonstrates that the DPPH assay exhibits the highest antioxidant capacity, followed by FRAP and ABTS assay.

Regarding the antioxidants capable of proficient in quenching the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, no significant differences were recorded between the studied species. All the examined species measured almost 1.2 mg TE g^{-1} DM of DPPH. The DPPH assay necessitates the dissolution of the radical in an organic solvent. Consequently, this method finds its primary applicability in the evaluation of hydrophilic antioxidants [37]. This method is not restricted to a specific antioxidant; rather, it extends to assessing the overall antioxidant capacity of the sample. As indicated by the obtained results, no significant variations were noted. Therefore, within the group of investigated species, the studied species demonstrates a consistent concentration of hydrophilic antioxidants.

Polyphenols are recognized as important natural reducing agents due to their redox properties (donation of a hydrogen atom and/or an electron to free radicals, singlet oxygen quenchers, and metal-chelating potential), leading to break the chain reaction of oxidation. Subsequently, a correlation analysis was conducted between the DPPH test and the total phenolic content (Supplemental Table 3). In the case of *O. dillenii* and *O. robusta*, moderate positive correlations were recorded, with values of 0.56 and 0.40, respectively, while, for *O. ficus-indica*, fort correlation was registered between polyphenols and antioxidant capacities measured by DPPH measuring 0.99. This strong correlation underscores a direct and pronounced connection between phenols and their capacity to scavenge the DPPH free radical. This finding aligns with the conclusions drawn by a prior study conducted by [38], which also highlighted the substantial scavenging effects of phenolic compounds against the DPPH free radical.

The FRAP assay, rapidly performed, inexpensive, and robust, does not require specialized equipment. In addition, it demonstrates high reproducibility and highest correlations with total phenolics, which make it an appropriate assay for determining antioxidant activity [39]. The FRAP assay distinguishes itself from other methods as it does not

involve free radicals; instead, it focuses on monitoring the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) [32]. Moreover, to ensure iron solubility and promote electron transfer, the FRAP assay is conducted under acidic pH conditions (pH 3.6). This adjustment enhances the redox potential, leading to a shift in the dominant reaction mechanism [40].

In contrast to the DPPH test, the FRAP assay enables differentiation among the studied species. *O. dillenii* exhibited the highest concentration of antioxidants capable of effectively reducing the Fe^{3+} complex of ferric ions $(TPTZ)^{3+}$ to the ferrous complex Fe $(TPTZ)^{2+}$. Following closely, *O. ficus-indica* also demonstrated substantial reducing power. Conversely, *O. robusta* displayed the lowest antioxidant activity among the studied species. The antioxidant properties are mainly attributed to the presence of phenolic compounds, indicating that the effectiveness of a fraction is proportional to its phenolic concentrations [41, 42]. A strong correlation of 0.89 was registered between polyphenols and antioxidant capacities measured by FRAP assay in *O. robusta*. Meanwhile, moderate and lower positive correlation was recorded for *O. dillenii* and *O. ficus-indica*, respectively (Supplemental Table 3). The FRAP assay showed a lower correlation between phenolic content and the antioxidant capacity compared to DPPH and ABTS. Our results contrast with those reported by [39], who asserted that the correlation between total phenolic contents and antioxidant capacity is stronger when employing the FRAP assay compared to the DPPH or ABTS assay. Although the FRAP assay is effective and reproducible, the results can vary based on the analysis time for the reaction between antioxidants and Fe^{3+}, which can range from a few minutes to several hours. Consequently, a single-point absorption endpoint may not completely capture the reaction, as different antioxidants may necessitate different reaction times for detection [43, 44]. Therefore, it is recommended to complement the FRAP assay with other methods to identify dominant mechanisms for different antioxidants [45].

The ABTS test stands out as a crucial method for assessing antioxidants in samples. In contrast to the other assays, the ABTS test is characterized by its applicability, which differs from the two previously discussed tests: DPPH and FRAP. In effect, the ABTS assay enables the assessment of both the hydrophilic and hydrophobic antioxidant capacity [46, 47]. In addition, the ABTS antioxidant assay is applicable across a broad pH range. The obtained finding from the ABTS test differed from the second assays previously discussed assays (DPPH and FRAP). *O. robusta* and *O. dillenii* demonstrated a higher antioxidant capacity in scavenging 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) than *O. ficus-indica*, which displayed the lowest antioxidant capacity. This observed difference could be attributed to the characteristic of ABTS assay, which allows to gauge both hydrophilic and hydrophobic antioxidants. Our results are lower than those documented by [48], who stated that the ABTS values range from 1.6 to 9.26 mg TE g^{-1} DM of Spanish *O. ficus-indica* cultivars. As our study comports *O. ficus-indica*, the observed difference may be attributed to the variations in the solvents employed and also to the edaphoclimatic conditions. Notably, a strong positive correlation was observed between the total phenolic content and the ABTS assay of *O. dillenii*. Additionally, a moderate to strong positive correlation was found between the total phenolic content and the ABTS test of *O. robusta*. In contrast, low correlation was observed between the total phenolic content and the ABTS of *O. ficus-indica*.

Concerning the phosphomolybdenum assay (TAC), no significant difference was observed between the examined species. The TAC assay assesses the antioxidant capacity in both fat-soluble and water-soluble antioxidants, highlighting the distinctions between TAC and other examined antioxidant assays. Regarding the correlation between the studied species and total phenolic content, a strong positive correlation was evident for *O. ficus-indica* (Supplemental Table 3), while *O. robusta* and *O. dillenii* displayed a moderate positive correlation.

According to [49, 50], the antioxidant activity is influenced by the solvent utilized, agricultural practices, and geographic location. However, in our investigation, the samples were collected from identical locations, and the same assays and a uniform experimental protocol were applied to all the species under scrutiny. Therefore, the observed variations among the examined species can be predominantly attributed to genetic factors.

Phenolic compounds, the most extensively distributed secondary metabolites, originate biogenetically from either the shikimate/phenylpropanoid or the “polyketide” acetate/malonate pathway [51]. Phenolic compounds, boasting more than 8000 identified structures, stand out as the most prevalent secondary plant metabolites. Recently, researchers

have shown heightened interest in these compounds due to their intriguing bioactivities, including anti-inflammatory, antioxidant, antiproliferative, and antimicrobial properties [14, 17].

According to the findings, *O. robusta* stands out as the species with the higher concentration of phenolic compounds, closely followed by *O. dillenii*. In contrast, *O. ficus-indica* exhibits the lowest level of these compounds. It is crucial to emphasize that all the examined species were collected from the same geographic region and underwent a consistent extract preparation procedure, underscoring consistent edaphoclimatic conditions. Therefore, the observed variations in secondary metabolites cannot be attributed to external factors like abiotic or biotic influences but are mainly linked to the inherent biochemical characteristics of the species under investigation. In investigating phenolic compounds, a direct comparison with other species has been conducted, mainly due to the limited number of studies conducted on the peel of prickly pear species, particularly *O. dillenii* and *O. robusta*. Concerning the phenolic acids, the HPLC analyses allow the identification of ferulic acid, sinapic acid, and hydroxybenzoic acid. Our finding differs from those obtained by [31], who conducted a study on *O. ficus-indica* and reported that the main constituent is catechin followed by gallic acid, caffeic acid, chlorogenic acid, and ellagic acid. Regarding flavonoids, seven compounds were identified, including isorhamnetin, kaempferol, epicatechin, gallocatechin, naringenin, quercetin, and rutin. According to the same author previously mentioned, only three flavonoids were identified, including quercetin, kaempferol, and rutin. The observed differences could be attributed to the method of extraction and also to the edaphoclimatic conditions.

Flavonoids are an important class of natural products that are broadly distributed throughout the plant kingdom, with approximately 4000 flavonoids identified in the plants [52], and are classified as low-molecular-weight secondary metabolites with a polyphenolic structure. Now, flavonoids are recognized as an essential component in various applications, including pharmaceutical, cosmetic, nutraceutical, and medicinal [53, 54].

Isorhamnetin, having a polyphenolic structure and specifically classified as a flavonol, is a direct metabolite of quercetin and exhibits a broad range of pharmacological activities [55]. Isorhamnetin is an immediate metabolite of quercetin, with a polyphenolic structure and is specifically categorized as a flavonol, possesses extensive pharmacological activities. Isorhamnetin possesses a wide range of pharmacological effects on neurodegenerative, tumors, cardiovascular diseases, and pharmacodynamics against pulmonary fibrosis and hyperuricemia [56–58]. In the present study, isorhamnetin emerges as the predominant flavonoid, ranging from 2429.73 to 7184.09 μg/g. The substantial quantity of isorhamnetin identified in the fruit peel adds significant value to the utilization of this by-product.

Quercetin is one of the abundant flavonoids found with levels ranging from 1580.83 to 5994.19 μg/g. Quercetin is a common flavonol found mostly as glycosides, with a sugar group linked at the 3-position by a glycosidic bond [59]. Quercetin is recognized for its diverse biological effects, which encompass antitumor and anti-inflammatory properties in malignant cancer cells. Recent research indicates that quercetin has the potential to modify the morphology and trigger apoptosis in gastric cancer cells [60].

Rutin was also found in higher amount ranging from 1350.78 to 5440.53 μg/g. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, depicted in Figure 1) is a crucial nutritional component, also referred to as rutoside, quercetin-3-rutinoside, and sophorin. The “rutin” name is derived from the plant Ruta graveolens. Rutin has exhibited various pharmacological activities, including vasoprotective, antioxidant, neuroprotective, cytoprotective, cardioprotective, and anticarcinogenic effects [61].

The term “phenolic acids” typically refers to phenolic compounds with a single carboxylic acid group [62]. They are categorized into two subgroups: hydroxybenzoic and hydroxycinnamic acid [63]. Hydroxycinnamic acid compounds are frequently formed as simple esters with glucose or hydroxycarboxylic acids [64]. Phenolic acids are synthesized through the phenylpropanoid pathway from shikimic acid, as by-products during the monolignol pathway, and some are produced by microbes and through the breakdown of cell wall polymers like lignin [65]. Dietary phenolic acids, present widely in plants, play a crucial protective role under oxidative stress conditions [66]. The content of phenolic acids is influenced by the extraction method, which, in turn, is regulated by factors, such as the sample-solvent volume ratio, pH, temperature, and the number and duration of each extraction step [67, 68]. Conversely, the

observed variations among the studied species are primarily attributed to genetic factors.

5. Conclusion

The current study provided information about antioxidant properties and bioactive compounds of *O. robusta*, *O. dillenii,* and *O. ficus-indica* fruits peel. *O. robusta* registered the highest total phenolic compounds, which plays an important role against oxidation, as well as the highest antioxidant activity by ABTS method. Among the four tests of antioxidant activity, ABTS and FRAP assays demonstrate that they are better estimates of the antioxidant capacity. In contrast, no significant difference was recorded between the studied species using DPPH assay and TAC assay. Consequently, the applicability of the antioxidant test to both hydrophilic and lipophilic antioxidants is found an important factor. Consequently, various assays should be applied for testing the antioxidant properties. The noted distinctions among these species mainly arise from their inherent biochemical characteristics. Concerning the newly introduced species, *O. robusta* and *O. dillenii* were demonstrated that they have the more richness of phenolic compounds compared to the local *O. ficus-indica*. In addition, it showed important antioxidant capacity in all performed assays. The peel of the studied species shows that it contains important amounts of phenolic compounds, which can be used in various fields, such as pharmaceutical and cosmetic and food industries. However, additional research is necessary to investigate other vital components within these species, such as fatty acids and minerals. This exploration would contribute to a more comprehensive understanding of their nutritional and medicinal capabilities [44, 45, 69].

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DETAIL

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Suitability of Almond Bagasse Powder as a Wheat Flour Substitute in Biscuit Formulation

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ABSTRAK (ENGLISH)

Almond bagasse, a by-product derived from the production of almond vegetable drink, contains antioxidants, fibre, protein, and a high-fat content, presenting itself as a potential functional ingredient for the food industry. This study aimed to assess the powder derived from almond bagasse as a suitable alternative in the formulation of bakery goods. Various formulations substituting wheat flour with almond bagasse powder, obtained by air drying or freeze-drying at 10%, 15%, and 25%, were analysed in terms of technological and rheological properties. Furthermore, the physical and antioxidant attributes of biscuits with superior nutritional and functional values produced using these blends were examined. The results revealed significant changes in oil retention capacity, stability, and emulsifying activity, influenced by both the level of wheat flour replacement and the drying method used to obtain the almond bagasse powder. The most significant changes were observed in the emulsifying activity, which was zero in the wheat flour and showed values of 20% in the hot air-dried almond bagasse powder and 59% in that obtained by freeze-drying. In the blends, the values of this variable ranged from 1.8% to 7.1%. The highest value was obtained with a 25% replacement of wheat flour by freeze-dried almond powder. On the other hand, the lack of starch and the high concentration of fat (around 25%) and insoluble fibre (higher than 20%) in the almond bagasse powder determined the viscoelastic behaviour of the hydrated blends. As the percentage of substitution with the almond bagasse powder increased, the final viscosity decreased, being reduced from 2302 MPa·s in the wheat flour to 873 MPa·s in the blend containing 25% hot air-dried almond powder. It is worth noting that, the use of these blends for biscuit preparation resulted in a final product with a higher content of antioxidant components. The highest increase in antiradical capacity was 33% and was observed in the biscuits obtained with the mixture containing 25% hot air-dried almond powder.

TEKS LENGKAP

1. Introduction

Bakery products, such as bread [1], biscuits [2], cakes [3], and breadsticks [4], among others, widely consumed in the human diet and in Western countries, are usually made mainly from wheat flour [12]. Unfortunately, for some people who are intolerant to gluten, the wheat proteins can cause digestive or allergic problems [5]. Moreover, when refined, wheat flour loses crucial nutrients during processing, resulting in products with lower contents of minerals, vitamins, and fibre, higher caloric value, and an increased glycaemic index [6]. In addition, the overproduction of wheat-based products has led to a significant expansion of wheat cultivation for flour, which may have a negative impact on the environment [7].

On the other hand, there is currently a growing interest in valorising and reintegrating waste from the food industry into the food chain. Significant amounts of waste and by-products are typically generated during the processing of plant-based foods, leading to economic and environmental issues due to the high volumes and associated costs of disposal [8]. These waste materials may contain high levels of proteins, dietary fibre, essential fatty acids, antioxidant compounds, vitamins, and minerals and can be easily stabilised by transformation into powdered products [9]. Therefore, the incorporation of powdered by-products as ingredients with high nutritional and/or functional values in the development of new products could lead to healthier processed products. Functional powders made from vegetable, legume, or nut waste could provide a nutritional supplement to other types of flours with lower nutritional content. In addition, they could contribute to reduce intolerance or allergy problems and to lower the glycaemic index, thus supporting the prevention of diseases such as celiac disease, type 2 diabetes, coronary heart diseases, or certain cancers [9, 10].

Many researchers have conducted studies employing revalorized by-products from fruits, vegetables, cereals, legumes, or nuts as ingredients in the production of bakery products. Those from fruits, vegetables, and cereals are added to increase the fibre content or certain bioactive components (such as polyphenols or carotenoids) in the final

product [11]. In addition, those from legumes or nuts can also be used to improve the protein content or the polyunsaturated fatty acid profile. In most cases, revalorized by-products include pods, bran, skins, hulls, and shells. Thus, Aguilar et al. [12] used powdered tiger nut beverage by-product to make gluten-free bread. Gaglio et al. [13] incorporated almond skin powder in traditional semolina sourdough bread production. Ojeda et al. [14] used flours from legume pods to improve wheat bread. Christ-Ribeiro et al. [15] used fermented rice bran for the formulation of gluten-free biscuits. Villasante et al. [16] evaluated wheat bread and tortilla with pecan nut shell powder. Bento et al. [17] developed instant noodles and baked snacks without gluten from black bean and carioca bean by-product flours. Owiredu et al. [18] used cashew nut flour to make more nutritious biscuits. However, there are other by-products that are of great interest and have not yet been researched in extent. These include press cakes from oil extraction processes or from the production of vegetable drinks [19]. The almond press cake by-product obtained from almond vegetable drink production could be a suitable raw material to replace part of the wheat flour in the production of bakery products. It contains monounsaturated fatty acids and essential amino acids, and when powdered, it has good water and oil interaction properties [20]. Although the fatty acid and essential amino acid profile of almond bagasse has not been determined, Duarte et al. [20] estimated the total fat and protein content of almond bagasse powder to be around 25% and 16%, respectively. In addition, Duarte et al. [21] studied the effect of drying and *in vitro* gastrointestinal digestion on the polyphenolic profile of almond bagasse and found that polyphenols such as apigenin-7-glucoside were retained at 83.3% after hot air drying at 70°C. Despite this, the scientific literature about composition and uses of press cake from almond vegetable drink is scarce.

The incorporation of alternative ingredients to wheat in the baking process involves technological challenges to achieve acceptable quality products. Generally, the substitution of bread-making flours with ingredients derived from by-product valorisation varies between 2% and 20%, with 10% being the most accepted level by consumers due to their organoleptic characteristics [22–24]. It should be noted that the inclusion of gluten-free or starch-free ingredients can influence the elasticity and gas retention capacity in the dough, which would weaken its structure affecting the quality of the final product [25]. On the other hand, the substitution percentage of by-products in blends significantly impacts the nutritional and functional properties of bakery products, such as protein, fat content, dietary fibre, vitamins, minerals, or antioxidant compounds. Furthermore, it also influences technological and functional properties, including water-holding capacity, oil absorption capacity, swelling, gelling, and thickening [8]. Technological and functional properties play a key role in determining the suitability of a blend in the production of bakery products, exerting a significant impact on the texture, structure, and overall quality of such products. The flour's ability to interact with water is essential in forming an appropriate dough, as it should be able to absorb and retain water to achieve an elastic and malleable dough [26]. In addition, the ability to interact with oil is also important, as it is essential for incorporating fat into the dough, which could affect the softness, moisture, and sponginess juiciness of the final product. This interaction can also influence the texture and shelf life of the product [27–29].

The evaluation of the suitability of almond bagasse powder as a partial replacement of wheat flour in the production of bakery products is an innovation that, to the authors' knowledge, has not been published by any other author. Therefore, the aim of this work is to determine the technological properties of blends of wheat flour combined with almond bagasse powder, either hot air dehydrated or freeze-dried, in percentages of 5, 10, and 15%. Furthermore, the pasting and textural properties of the resulting doughs and the effect on the main physical properties and on the antiradical capacity of the biscuits made with the blends have been also evaluated.

2. Materials and Methods

2.1. Process of Obtaining Almond Bagasse and Almond Bagasse Powder

Local supermarket-bought almonds (*Prunus dulcis* var. *dulcis*) were hydrated with tap water at 1 : 2 (w:w) ratio for approximately 12 h. The weight gain was approximately 40%. The rehydrated almonds were then mixed with water at a ratio of 1 : 9 (w : w) and ground into a fine mixture using a household food processor (Thermomix®, Vorwerk, Spain) running at 10,000 rpm for 20 seconds. The resulting grind was later sifted at atmospheric pressure through a stainless-steel sieve with a mesh size of 500 μm. The almond bagasse, left behind on the sieve, was collected for

further analysis and processing. It was found that the weight of the collected almond bagasse was approximately 82% of hydrated almonds' weight.

To obtain the dehydrated almond bagasse, the almond bagasse was first evenly distributed forming a thin bed of 5–7 mm thickness, on plastic grids with a nominal opening of 2 mm. It was then subjected to hot air drying in a convective dryer (Pol-Eko Aparatura, Katowice, Poland) with a cross-flow of air at a speed of 10 m/s and a temperature of 60°C for 10 hours until it reached a water activity (aw) below 0.3. This process resulted in the air-dried almond bagasse (HAD). In addition, a freeze dryer (Telstar, Lioalta-g) was used to obtain the lyophilized (LYO) product from the almond bagasse previously frozen at −40°C for 24 hours and followed by sublimation of water at −45°C (condenser temperature) and 0.1 mbar for 48 h. Afterwards, both dehydrated almond bagasse were ground using a food processor (Thermomix®, Vorwerk, Spain) at 4,000 rpm for 20 seconds with 5-second intervals, and then at 10,000 rpm for 20 seconds with 5-second intervals, resulting in the almond bagasse powders. Lastly, the powder was stored at 20°C in opaque glass jars to prevent any deterioration and oxidation reactions. The almond bagasse powders (HAD and LYO) were mixed in proportions of 10, 15, and 25% with refined wheat flour to obtain blends, which were characterized.

2.2. Physicochemical Analysis of Wheat Flour and Almond Bagasse Powder Blends

The official procedure from AOAC (1996) was employed to determine moisture content [30] and fat content [31] in the blends. For fat content determination, a Soxhlet extraction with petroleum ether using a 5 g sample to 90 mL solvent ratio at 290°C was applied. The ash content was determined by subjecting the material to incineration in a muffle furnace at 550°C, following the [32] protocol. For bulk density, the method proposed by Amandikwa et al. [33] was followed with some modifications. A known sample weight was added to a graduated cylinder, tapped gently to compact the sample, and the occupied volume was registered. Analyses were performed in triplicate, and the results were expressed in g/mL.

2.3. Water Interaction and Emulsifying Properties

Water-holding capacity (WHC) is the quantity of water that the sample can retain without the application of external force. To determine this, 0.2 g of the sample was mixed with 10 mL of distilled water; the mixture was allowed to sit at 25°C for 18 hours [34]; the excess water was removed; and the water content of the resulting solid was determined. Water absorption capacity (WAC) refers to the ability of a sample to absorb and hold water even under external forces, such as centrifugation [34]. To determine WAC, 1 g of the sample was weighed into a graduated conical tube, and 10 mL of distilled water was added, allowing it to stand at 25°C for 18 hours. Subsequently, the mixture was subjected to centrifugation at 2,000 rpm for 30 minutes. The liquid portion was separated, and the weight of the sedimented residue was measured. The method suggested by Garau et al. [35] was employed to assess the oil absorption capacity. A combination of 0.2 g of the sample and 1.5 g of sunflower oil was prepared and allowed to stand at 20°C for an entire night. Following this, the mixture underwent centrifugation at 3,416 g for 5 min, during which the supernatant was extracted using a Pasteur pipette, and the weight of the residue was measured. The evaluation of oil absorption capacity was based on the rise in the sample weight, and the outcomes were presented in terms of g of absorbed oil per g of the initial sample (g_o/g_s). The assessment of emulsifying activity was carried out following the approach outlined by Yasumatsu et al. [36]. To perform the procedure, a solution consisting of 2% (w/v) sample and water was prepared. Subsequently, 7 mL of this solution was mixed with 7 mL of sunflower oil, and the mixture was homogenized for 5 minutes using a vortex at a speed of 2,400 rpm. Finally, the mixture was subjected to centrifugation at 10,000 rpm for 5 minutes, and the volume of the resulting emulsion was determined by calculating the ratio between the emulsion volume and the total fluid volume. The assessment of emulsifying stability was conducted following the procedure proposed by Yasumatsu et al. [36]. To accomplish this, a solution containing 2% (w/v) sample and water was prepared. Subsequently, 7 mL of this solution was combined with 7 mL of sunflower oil and homogenized for 5 minutes using a vortex at a speed of 2,400 rpm. Following that, it was subjected to heating at 80°C for 30 minutes, allowed to cool, and then centrifuged at 2,000 g for 5 minutes. Emulsifying stability was determined by calculating the ratio between the emulsion volume and the total fluid volume. Analyses were performed in triplicate.

2.4. Pasting Properties

The viscometric profile of the blends was obtained following the procedure described by Harasym et al. [37] in accordance with the ICC Standard method 162 and using a rapid visco analyser (RVA-4500, Perkin Elmer, USA). A quantity of 3.5 g of the sample was transferred to an RVA container, and distilled water (as the solvent) was added to achieve a total weight of 28.5 g. Each hydrated blend was kept at 50°C for 1 minute to reach equilibrium. Subsequently, the temperature was gradually increased to 95°C at a rate of 5°C per minute, held at 95°C for 5 minutes, then cooled to 50°C at a rate of 5°C per minute, and finally maintained at 50°C for the last 4 minutes. The stirring was initiated at 960 rpm for the first 10 seconds and then maintained at a constant 160 rpm for the rest of the analysis. Each sample underwent a duplicate analysis. The TCW3 software (Perkin Elmer, United Kingdom) was used to calculate the parameters of peak viscosity (PV), trough viscosity (TV), final viscosity (FV), setback (ST = FV − TV), gelatinization temperature, and time to peak viscosity. Analyses were performed in triplicate.

2.5. Rheological Measurements

For rheological determinations, dynamic oscillatory tests were performed on hydrated blends (3.5 g blend/25 g water). An Anton Paar MC102 rheometer (Anton Paar, Stuttgart, Germany) was used, employing serrated parallel plates (40 mm in diameter) made of steel, with a 1 mm gap, at a controlled temperature of 25°C using a KNX2002 thermal controller. The samples were placed onto the plate, trimmed, and allowed to equilibrate for 5 minutes before each test. Viscoelastic behaviour was assessed through the storage modulus (G') and loss modulus (G'') with a frequency sweep ranging from 10 to 1 Hz, within the linear viscoelastic region and under a constant stress of 1 Pa. All rheological tests were conducted in triplicate.

2.6. Texture Analysis

The hydrated blend was deposited into cylinders with a diameter of 2 mm and refrigerated at 4°C for 12 hours until a compact gel formed. The texture of the gels was determined in triplicate using an AXIS texture analyser (Axis, Gdansk, Poland) equipped with FM AXIS software. A double compression test of the texture profile analysis (TPA) was conducted. The gels underwent a 50% deformation test at a speed of 1 mm/s with 30-second intervals between the first and second compression. Analyses were performed in triplicate, and the results were expressed as maximum force (N) for both compressions.

2.7. Preparation of Dough and Biscuits

For the dough preparation used in biscuit production, wheat flour and almond bagasse powder were combined in varying proportions. In addition, other ingredients such as salt, sugar, water, and fat were incorporated according to the specifications outlined in Table 1.

Table 1

Composition of doughs for biscuit production.

Sample	Wheat flour (g)	Almond bagasse (g)	Salt (g)	Sugar (g)	Water (g)	Fat (g)
Control	100	—	1.0	20	25.7	30
HAD-10%	95	10	1.0	20	25.7	28.7
HAD-15%	90	15	1.0	20	25.7	27.5
HAD-25%	85	25	1.0	20	25.7	26.2
LYO-10%	95	10	1.0	20	25.7	28.7
LYO-15%	90	15	1.0	20	25.7	27.5

LYO-25%	85	25	1.0	20	25.7	26.2

The mixtures were kneaded manually on a kneading board and subsequently rolled out to a uniform thickness of 5 mm using a household rolling pin. The dough was then shaped using a 45 mm diameter round cutter mold. Finally, the shaped biscuits were baked (Rational, SCC 62, Germany) on greased trays at 180°C for 15 min. After baking, the biscuits were allowed to cool for 20 min before being stored in an airtight container for further analysis.

2.8. Determination of Physical Properties in Biscuits

The thickness (T) and diameter (D) of batches containing 10 biscuits each were measured using a vernier caliper. To calculate the relative weight (RW), relative diameter (RD), relative thickness (RT), specific volume (VE), and relative specific volume (REV), equations (1)–(5) were used. The dispersion ratio was calculated as D/T. The colour meter (Minolta, CM-3600D, Japan) was used to determine the CIE⁎L⁎a⁎b⁎ coordinates, considering the standard light source D65, the 10° standard observer, and the surface reflectance spectra ranging from 400 to 700 nm. The chroma (Cab) and colour differences (ΔE) between the wheat flour biscuits and those containing almond bagasse powder were calculated using (6) and (7), respectively. Analyses were performed in triplicate.(1)RW=weightcontrol weight,(2)RD=diametercontrol diameter,(3)RT=thicknesscontrol thickness,(4)VE=π·diameter2·thickness4·weight,(5) REV=VEcontrol VE,(6)Cab=a2+b2,(7)ΔE=ΔL⁎2+Δa⁎2+Δb⁎2,where control is referred to the wheat flour.

2.9. Antiradical Capacity and Total Phenol Content in Biscuits

To extract phenols and other constituents with antiradical capacity, an 80 : 20 (v/v) mixture of methanol and water was prepared and used as a solvent in a ratio of 1 g of the sample per 100 mL solvent. After 1 hour of magnetic stirring, the mixture was centrifuged (Selecta, “Medrifriger BL-S) at 10,000 rpm for 5 minutes at 20°C. The analyses were carried out on the resulting supernatant, referred to as the extract.

2.9.1. Antiradical Capacity by DPPH and ABTS Methods

The determination of antiradical capacity was conducted according to the DPPH method outlined by Stratil et al. [38] with certain modifications. A mixture of 0.1 mL of the extract and 2.9 mL of methanol-DPPH solution (0.394 of DPPH reagent/mL methanol) was prepared, and the absorbance was measured at 517 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The outcomes were presented as milligrams of Trolox equivalent per gram of dry matter (mg TE/g dm), utilizing the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥7%, Sigma-Aldrich) as the reference standard antioxidant, across a concentration range from 0 to 500 mg/L.

The evaluation of antioxidant activity was also carried out by the ABTS radical method (2,20-azobis-3-ethyl-benzothiazoline-6-sulfonic acid) [39]. A solution containing the ABTS radical (7 mM) and potassium persulfate (2.45 mM) in distilled water was prepared and allowed to incubate in the dark at room temperature overnight. Following this incubation period, a dilution with methanol was performed to achieve an absorbance of 0.7 ± 0.02 at 734 nm. Subsequently, the reaction was initiated in a spectrophotometer cuvette by combining 0.1 mL of the extract with 2.9 mL of the ABTS solution. For comparison, a blank sample was prepared by replacing the extract with distilled water. Absorbance was measured after 0, 3, and 7 minutes of reaction at a wavelength of 734 nm using a spectrophotometer (Thermo Scientific, Helios Zeta UV/Vis). The results were expressed as mg of Trolox equivalent/g of dry matter (mg TE/g dm), utilizing the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥7%, Sigma-Aldrich) as the reference standard antioxidant, across a concentration range from 0 to 500 mg/L.

2.9.2. Total Phenol Content

The quantification of total phenols was carried out using the Folin–Ciocalteu colorimetric method [40]. In a spectrophotometer cuvette, 0.125 mL of the extract, 0.125 mL of the Folin–Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany), and 0.5 mL of distilled water were sequentially added and allowed to react for 6 minutes. After that, 1.25 mL of a 7% (w/v) sodium carbonate solution and 1 mL of distilled water were added. A comparative reference was prepared replacing the sample by distilled water and left to react for 90 minutes. Ultimately, the absorbance was measured at 765 nm using a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results were correlated with a standard gallic acid curve (purity ≥98%, Sigma-Aldrich) and expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g dm).

2.10. Statistical Analysis

The results were statistically analysed using Statgraphics Centurion XVI.I software (Statpoint Technologies, Inc., Warrenton, VA, USA) at a 95% confidence level (p value <0.05). Normality of the data was assessed using the Shapiro–Wilk test ($p>0.05$). Analysis of variance (ANOVA) was then performed. Fisher's LSD test was used to identify significant differences between groups (p value <0.05).

3. Results and Discussion

3.1. Proximal Composition and Technological Properties of Wheat Flour and Almond Bagasse Powder

Table 2 shows proximal composition and water and oil interaction properties of wheat flour and almond bagasse powders dehydrated by hot air at 60°C and lyophilized. Regarding moisture, the dehydration process has significantly reduced the available water content in almond bagasse dried using either hot air or freeze-drying methods. Unlike these samples, wheat flour maintains a higher moisture level, as it was commercial flour with any additional treatment. Nevertheless, the moisture level remains below critical levels, which is advantageous for its preservation. Considering the fat and protein content, the almond bagasse powders exhibit a significant fat content (25%). Almond fat composition consists of mono- and polyunsaturated fatty acids, such as oleic, linoleic, palmitic, stearic, and palmitoleic acids [41]. Adequate consumption of these fatty acids offers several health benefits, including cholesterol reduction and a lowered risk of cardiovascular disease [42]. Spiller et al. [43] reported that consumption of 100 g of almonds daily for 4 weeks in a randomized parallel design study decreased total cholesterol (9%) and LDL cholesterol (12%) in 26 free-living hypercholesterolemic patients. The benefits were attributed to the fatty acid profile. Lipids represent a minor fraction in wheat flour compared to its other primary nutritional components. Thus, Prabhasankar and Haridas Rao [44] reported the free lipid content varied from 0.1 to 1.9% (dry basis) and bound lipid content from 0.2 to 2.1% (dry basis) in different wheat flour streams. The amount of protein found in both almond bagasse powder and wheat flour falls within a similar range, approximately between 0.16 and 0.12 g of protein/g, respectively. Similar findings have been observed in other products, such as oat bran (0.17 g protein per gram) [45] or soybean residue (0.15 g protein per gram) [10]. Despite this commonality in protein content, almond bagasse powders and wheat flour differ significantly in fibre and fat content which has an impact in technological properties as demonstrated below. The absence of starch in almond bagasse powders is worth noting, which undoubtedly determines the technological properties of the mixtures and the most recommended applications for them. Starch is fundamental for the structure and texture of bakery products. The absence of starch, when replacing part of the wheat flour with almond bagasse powder, can impact dough elasticity and gas retention, and result in a denser texture. Moreover, almond bagasse powder, with higher fat content, may affect the consistency and structure of the final product, leading to notable alterations in the quality of the baked final product [46].

Table 2

Proximal composition and technological properties of wheat flour and almond bagasse powders dehydrated by hot air at 60°C (HAD60) and lyophilized (LYO).

	Wheat flour	HAD60[1]	LYO[1]
Xw (g/g_{dm})	0.108 ± 0.003	0.014 ± 0.002	0.02 ± 0.08
Fat (g/g_{dm})	0.01 ± 0.02	0.252 ± 0.002	0.250 ± 0.006
Ashes (g/g_{dm})	0.0203 ± 0.0002	0.031 ± 0.007	0.030 ± 0.012
Protein (g/g_{dm})	0.12 ± 1.07[2]	0.16 ± 0.04	0.165 ± 0.008
Fibre Van Soest (g/g_{dm})	0.034[2]	0.45 ± 0.02	0.50 ± 0.03

Cellulose and lignine (g/g_{dm})	0.012[2]	0.20±0.05	0.21±0.02
Hemicellulose (g/g_{dm})	0.021[2]	0.260±0.014	0.295±0.002
Total starch (g/100g)	68.06[2]	—	—
Water-holding capacity (g_w/g_{dm})	0.56±0.04	2.9±0.5	8.4±1.8
Water absorption capacity (g_w/g_{dm})	0.71±0.01	4.5±0.2	5.91±0.08
Oil absorption ability (g_o/g_s)	0.144±0.004	2.3±0.5	4.2±006
Emulsifying stability (%)	—	19±2	34±2
Emulsifying activity (%)	—	20±2	59±2

[1]Duarte et al. [20]; [2]Hager et al. [46]; dm, dry matter; Xw, water content.

3.2. Functional Properties of the Powder Blends Prepared by Replacing Wheat Flour with Almond Bagasse Powder

As it has been shown by other authors [47], technological properties, such as water-holding capacity (WHC) and water absorption capacity (WAC), are primarily influenced by particle size, starch and fibre content, the amount of fat, and the type of proteins. Table 1 shows higher values for almond bagasse powders when compared to wheat flour. This could be attributed to the more soluble fibre (some of hemicellulose in this case), which demonstrates a high capacity to retain water and expand. Conversely, insoluble fibre also possesses the ability to retain and absorb water within its fibrous matrix, albeit to a lesser degree [48]. However, the freeze-dried powder demonstrates higher values than the hot air-dried powder, attributable to the particle size distribution [20]. According to Bai et al. [49], as particle size increases, the ability to absorb and retain water also increases due to the possibility of water molecules to traverse the larger gaps between particles. To date, there remains a dearth of substantial studies on the interaction properties with oil. Nonetheless, according to Devnani et al. [50], almond protein isolate demonstrates foaming and emulsifying properties that could be akin to those found in soy protein isolate [51]. Meanwhile, wheat flour plays a vital role in the creation of bakery items owing to its gluten content. This constituent provides elasticity and structure to the dough, enabling optimal retention of the gas generated by the yeast and resulting in fluffier texture [52].

Table 3 includes the results for water, fat, and ashes' content; bulk density; and water and oil interaction properties of the blends prepared replacing wheat flour by hot air-dried or lyophilized almond bagasse in the range from 10 to 25%. Regarding moisture, it was significantly affected ($p \leq 0.05$) by the percentage of substitution. As the percentage of substitution increases, the moisture content decreases. This is due to the inherent low moisture content of almond bagasse powder. A similar trend was observed in biscuits formulated with different substitution levels using Moringa leaf powder [53]. On the other hand, the same trend was observed in relation to the final fat content. Since almond bagasse contains around 25% fat, this means that the higher the percentage of substitution, the higher the amount of fat in the mixture. Regarding ash content, no significant differences were observed. However, concerning bulk density, notable differences ($p \leq 0.05$) were detected in the substitution percentage. Higher values were recorded in HAD60-10% and LYO-10%. It could be attributed to the substantial amount of fat in almond bagasse. Similar results were documented in wheat flour blends with yam powder for biscuit manufacturing by Amandikwa et al. [33].

Table 3

Water, fat and ashes' content, bulk density, and water and oil interaction properties of the blends prepared replacing wheat flour by hot air-dried almond bagasse in 10% (HAD60-10%), 15% (HAD60-15%), or 25% (HAD60-25%) and replacing wheat flour by lyophilized almond bagasse in 10% (LYO-10%), 15% (LYO-15%), or 25% (LYO-25%).

	HAD60-10%	HAD60-15%	HAD60-25%	LYO-10%	LYO-15%	LYO-25%	p value		
A	B	A-B	Xw (g/g_{dm})	0.103 ± 0.004^{c}	0.097 ± 0.002^{b}	0.087 ± 0.004^{a}	0.104 ± 0.002^{c}	0.101 ± 0.002^{bc}	0.089 ± 0.002^{a}
0.09	0.00	0.42	Fat (g/g_{dm})	0.091 ± 0.006^{a}	0.159 ± 0.009^{c}	0.19 ± 0.085^{d}	0.094 ± 0.009^{b}	0.161 ± 0.004^{c}	0.189 ± 0.005^{d}
0.00	0.00	0.82	Ashes (g/g_{dm})	0.0206 ± 0.0004^{a}	0.0205 ± 0.0002^{a}	0.0202 ± 0.0002^{a}	0.0203 ± 0.0001^{a}	0.0204 ± 0.002^{a}	0.0202 ± 0.003^{a}
0.45	0.20	0.98	Bulk density (g_{dm}/mL)	0.787 ± 0.009^{d}	0.784 ± 0.002^{cd}	0.771 ± 0.006^{ab}	0.774 ± 0.005^{bc}	0.770 ± 0.005^{ab}	0.762 ± 0.007^{a}
0.03	0.10	0.57	WHC (g_w/g_{dm})	0.62 ± 0.07^{a}	0.61 ± 0.02^{a}	0.58 ± 0.05^{a}	0.86 ± 0.02^{b}	0.91 ± 0.01^{b}	0.85 ± 0.04^{b}
0.00	0.44	0.30	WAC (g_w/g_{dm})	0.708 ± 0.015^{a}	0.71 ± 0.02^{a}	0.72 ± 0.02^{b}	0.72 ± 0.05^{b}	0.73 ± 0.02^{c}	0.74 ± 0.009^{d}
0.15	0.02	0.28	OAC (g_o/g_s)	0.20 ± 0.02^{ab}	0.18 ± 0.02^{a}	0.182 ± 0.007^{a}	0.22 ± 0.03^{b}	0.182 ± 0.005^{a}	0.193 ± 0.006^{ab}
0.00	0.00	0.00	ES (%)	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	1.8 ± 0.1^{c}	1.1 ± 0.1^{b}	1.8 ± 0.1^{c}	3.6 ± 0.1^{d}

0.00	0.00	0.00	EA (%)	1.8 ± 0.1[a]	1.8 ± 0.1[a]	3.6 ± 0.1[b]	3.6 ± 0.1[b]	3.6 ± 0.1[b]	7.1 ± 0.1[c]

dm, dry matter; Xw, water content; WHC, water-holding capacity; WAC, water absorption capacity; OAC, oil absorption capacity; ES, emulsifying stability; EA, emulsifying activity. A, treatment; B, replacement percentage. Mean ± standard deviation of three repetitions. Different superscript letters in the same line indicate statistically significant differences with a confidence level of 95%.

Regarding water-holding capacity (WHC) and water absorption capacity (WAC), significant differences were observed between drying methods, with higher values in mixtures containing freeze-dried powder. As previously mentioned, WHC and WAC are influenced by particle size, suggesting that mixtures containing freeze-dried almond powders will exhibit increased interaction with water (WHC and WAC), as water molecules can penetrate to the larger spaces [49]. However, it is important to note that, despite the percentage of substitution with almond bagasse powder, the water interaction properties did not exhibit significant improvements. This could be attributed to the fact that the substitution percentages with almond powders are not sufficiently high. Furthermore, the high-fat content present in almonds could impede the interaction with water molecules [12].

Significant differences ($p \leq 0.05$) were evident in oil interaction properties, including oil absorption capacity (OAC), emulsifying activity (EA), and stability (ES), concerning both the percentage of substitution and the drying method. These properties tended to improve as the substitution percentage increased. It could be attributed to the high protein content present in almond bagasse powder, likely accounting for its remarkable characteristics in oil interaction. Comparable outcomes were noted with dehydrated almond bagasse powder [20]. Regarding the dehydration method, blends containing freeze-dried powder displayed higher values compared to those dried using hot air. This effect is attributable to the freeze-drying process, which intensifies structural damage. Consequently, it could lead to the fracture of complex molecules, increasing the availability of hydrophilic and hydrophobic groups for enhanced interaction, thereby improving the oil interaction properties [54]. The use of blends as opposed to wheat flour in the industry could offer several advantages. Firstly, these blends will enhance the nutritional profile by incorporating polyunsaturated fatty acids, vitamins, and minerals [55, 56]. Moreover, they will provide an opportunity to enhance dough functionality, thereby improving factors such as water and oil interaction properties [37, 57]. In addition, their utilization could potentially decrease production costs, contingent upon the specific product used [12]. Lastly, they will enable the creation of a broader range of products with distinct properties, thereby augmenting versatility in the production line [12]. However, the utilization of these blends will also come with certain drawbacks. For instance, the complexity involved in formulating, issues related to labelling and consumer perception, or the potential for these mixtures to generate undesirable odours and flavours in the final product, which are a crucial consideration.

3.3. Pasting and Rheological Properties of the Hydrated Blends

Figure 1 shows pasting temperature, pasting curves, and the pasting of the blends prepared replacing wheat flour by hot air-dried or lyophilized almond bagasse. The measured parameters included peak viscosity (PV), trough viscosity (TV), breakdown viscosity (BV), final viscosity (FV), setback viscosity (SV), peak time, and pasting temperature. It can be observed that replacing wheat flour by almond bagasse powder had, in all cases, a noteworthy effect on viscosities measured along the pasting process. Although reducing the viscosity may improve the ease of handling in the manufacturing process, it will alter the textural and sensory properties of the final product.

[figure(s) omitted; refer to PDF]

The most significant differences are a decrease in BV and pasting temperature. Also, a slight increase in the time at which maximum PV is observed. The PV is an indication of the thickening power of the sample, the higher the peak viscosity, the higher the thickening power [58]. The low peak viscosity values of the blends may be suitable for products requiring low gel strength and elasticity. The differences can be attributed to the reduction in starch content and the increase in fibre and fat content as the replacing percentage of wheat flour by almond bagasse powder

increases. Starch granules together with gluten proteins are the main components of wheat flour with the ability to retain water and form a firm structure with the ability to swell, resulting in high dough viscosity. Replacing part of the wheat flour with almond powder, which is rich in insoluble fibre and fat, considerably weakens the gel structure and at the same time hinders the ability to interact with fat. Njapndounke et al. [59] also reported a reduction in PV values with increasing cowpea flour substitution when using it to make a gluten-free biscuit based on cowpea and banana cochon flour. They stated that this was due to the lower starch and higher protein content of cowpea flour in the composite flour, which resulted in a low gelatinization and swelling index.

Although pasting temperature has been reported to be related to water binding capacity, it does not seem to be related to these blends, as WHC is higher in blends with lyophilized almond bagasse than in wheat flour. It may be that the high-fat content determines the relationship between temperature and WHC. However, there was no significant change in the structural characteristics of the gel formed by mixing the wheat flour with the almond bagasse powder as, in all cases, the shape of the curve is maintained. It is the structure from the starch molecules and proteins in the wheat flour that continues to determine the behaviour during pasting, although this results in a softer dough as the percentage of wheat flour replaced by almond bagasse powder increases.

In relation to the effect of the dehydration treatment applied to the almond bagasse, it can be considered negligible, since the corresponding curves appear practically superimposed, regardless of the percentage of substitution applied. These results are consistent with the results obtained for the water interaction properties of the blends, which show only slightly different values compared to those of wheat flour.

The viscoelastic properties of the different blends are depicted in Figure 2. An oscillatory dynamic test was applied to analyse this behaviour. Both the elastic modulus (G′) and the viscous modulus (G″) exhibited a slight increase as the frequency increased, indicating a dependency on this variable. Across all the tested blends, G′ was consistently higher than G″ (G′ > G″), suggesting a prevalence of the elastic component over the viscous one. The control (Figures 2(a) and 2(b)) exhibited the highest values of the elastic modulus G′, while the lowest values were observed in the HAD60-25% and LYO-25% mixtures. Similarly, the trend seen in the elastic modulus was mirrored in the viscous modulus G″, with lower values also found in the HAD60-25% and LYO-25% blends. There is a clear impact of substitution evident in both moduli, as the substitution percentage increases, both G′ and G″ tend to decrease. This decline can be attributed to the interaction between water and the proteins present in wheat flour (gluten). Gluten plays a crucial role in imparting elasticity and viscosity to the dough [29]. Therefore, if the blends contain a reduced amount of wheat flour, their elasticity (G′) and viscosity (G″) will be lower.

[figure(s) omitted; refer to PDF]

The textural properties of the hydrated blends with varying percentages of almond bagasse powder substitution are presented in Figure 3. According to the texture measurements, it was observed that all the gels resisted the double compression and could restore their original height and shape. However, the results indicated that the force needed to deform the gels containing almond bagasse powder blends (HAD60 and LYO) was lower than that of the control (wheat flour gel). An increase in the substitution percentage correlated with a decrease in gel hardness. This decrease might be linked to the retrogradation of starch, enabling the formation of a more gelatinous and soluble structure when mixed with water [60]. Furthermore, this reduction in hardness could stem from the reorganization of amylose present in wheat flour, as it plays a crucial role in gel structure formation. This, coupled with the fact that stiffness correlates with amylose content, could elucidate the observed variation [61]. The decline in stiffness might be due to the reduced quantity of wheat flour owing to the substitution of almond bagasse powder. Previous studies have reported analogous outcomes when substituting apricot kernels for wheat flour, demonstrating a decrease in hardness and the energy required to deform the gel [60].

[figure(s) omitted; refer to PDF]

3.4. Physical Properties and Antiradical Capacity of Biscuits Prepared with Wheat Flour and with the Different Blends of Wheat Flour and Almond Bagasse Powder

Table 4 shows the absolute and relative weights, dimensions, and specific volumes of the biscuits prepared with wheat flour and with the different blends of wheat flour and almond bagasse powder. Both the treatment applied,

and the percentage of substitution had a significant effect on the weight and dimensions of the biscuits and, consequently, on the specific volume. However, the differences were not very large and although in all cases they implied a decrease in weight and volume (both thickness and diameter decrease), in some cases the specific volume increased; the biscuits obtained with the blend containing freeze-dried almond powder or hot air-dried almond powder at 25% had a higher specific volume and were therefore less compact. Although pasting and rheological analysis showed a lower viscosity and hardness for the hydrated mixtures containing almond bagasse powder, this did not have a major impact on their suitability for biscuit preparation. The incorporation of almond bagasse powder significantly weakened the structure of the gel formed, but its firmness was sufficient to provide a structured biscuit with a similar specific volume to that obtained with wheat flour. These results were different from those reported by Olaimat et al. [62], who replaced part of the corn flour with walnut or peanut flour in percentages ranging from 5 to 20% in the biscuit formulation. They observed that in all cases, the weight of biscuits increased compared to those formulated with corn flour and attributed this change to the increase in protein and fibre content.

Table 4

Absolute and relative weights, dimensions, specific volumes, and CIE-L*a*b* coordinates of the biscuits prepared with wheat flour and with the blends prepared by replacing wheat flour by hot air-dried almond bagasse in 10% (HAD60-10%), 15% (HAD60-15%), or 25% (HAD60-25%) and replacing wheat flour by lyophilized almond bagasse in 10% (LYO-10%), 15% (LYO-15%), or 25% (LYO-25%), and colour differences (Δ*E*) compared to the wheat flour biscuits.

	Wheat flour	HAD60-10%	HAD60-15%	HAD60-25%	LYO-10%	LYO-15%	LYO-25%	p value		
A	B	A-B	Weight (g)	8.5 ± 0.4^{d}	8.01 ± 0.14^{cd}	7.4 ± 0.7^{bc}	7.43 ± 0.15^{bc}	6.7 ± 0.2^{ab}	6.3 ± 0.5^{a}	7.2 ± 1.4^{b}
0.00	0.08	0.07	Diameter (mm)	52 ± 2^{d}	48 ± 3^{abc}	49.2 ± 0.7^{bcd}	51 ± 3^{cd}	46 ± 2^{ab}	46 ± 2^{a}	49.7 ± 0.9^{bcd}
0.06	0.04	0.50	Thickness (mm)	5.43 ± 0.13^{b}	5.5 ± 0.5^{b}	4.81 ± 0.11^{ab}	4.9 ± 0.2^{ab}	4.68 ± 0.08^{a}	4.9 ± 0.5^{ab}	5.1 ± 0.7^{ab}
0.47	0.69	0.11	VE (cm^3/g)	1.36 ± 0.37^{d}	1.24 ± 0.25^{cd}	1.24 ± 0.35^{abc}	1.36 ± 0.12^{bc}	1.16 ± 0.25^{a}	1.29 ± 0.06^{ab}	1.37 ± 0.43^{bcd}
0.09	0.22	0.16	RW	1.00 ± 0.01^{d}	0.94 ± 0.06^{cd}	0.86 ± 0.04^{bc}	0.87 ± 0.04^{bc}	0.79 ± 0.06^{ab}	0.73 ± 0.06^{a}	0.88 ± 0.12^{bc}

0.01	0.16	0.14	RD	1.00 ± 0.01^{d}	0.92 ± 0.05^{abc}	0.95 ± 0.05^{bcd}	0.98 ± 0.06^{cd}	0.89 ± 0.01^{ab}	0.88 ± 0.03^{a}	0.96 ± 0.04^{bcd}
0.08	0.04	0.51	RT	1.00 ± 0.01^{bc}	1.0 ± 0.2^{c}	0.89 ± 0.04^{ab}	0.91 ± 0.02^{abc}	0.86 ± 0.02^{a}	0.91 ± 0.08^{abc}	0.95 ± 0.11^{abc}
0.43	0.66	0.09	REV	1 ± 0.01	0.91 ± 0.11^{a}	0.91 ± 0.05^{a}	1.02 ± 0.08^{a}	0.85 ± 0.09^{a}	0.95 ± 0.09^{a}	1.007 ± 0.011^{a}
0.26	0.52	0.38	-							
Colour										
–										
L	62.4 ± 0.4^{e}	58.9 ± 0.2^{bc}	58.4 ± 0.2^{ab}	57.8 ± 0.3^{a}	59.8 ± 0.6^{d}	59.2 ± 0.7^{cd}	58.0 ± 0.3^{a}	0.01	0.00	0.30
a*	12.5 ± 0.2^{a}	13.6 ± 0.4^{b}	13.90 ± 0.08^{bc}	15.0 ± 0.4^{d}	13.4 ± 1.0^{b}	13.65 ± 0.09^{b}	14.6 ± 0.2^{cd}	0.29	0.00	0.90
b*	33.5 ± 0.3^{a}	33.7 ± 0.3^{a}	33.82 ± 0.11^{a}	34 ± 2^{a}	34.1 ± 0.7^{a}	33.1 ± 0.5^{a}	34.4 ± 0.3^{a}	0.23	0.11	0.89
C	35.8 ± 0.4^{a}	35.9 ± 0.2^{ab}	35.85 ± 0.09^{ab}	36 ± 1^{ab}	36.7 ± 1.0^{bc}	35.8 ± 0.4^{ab}	37.4 ± 0.2^{c}	0.01	0.01	0.30
Δ*E*	—	3.7 ± 0.3^{ab}	4.3 ± 0.2^{bc}	5.5 ± 0.3^{d}	2.9 ± 0.9^{a}	3.5 ± 0.7^{ab}	5.0 ± 0.3^{cd}	0.01	0.00	0.79

VE, specific volume; RW, relative weight; RD, relative diameter; RT, relative thickness; REV, relative specific volume. A, treatment; B, replacement percentage. Mean ± standard deviation of three repetitions. Different superscript letters in the same line indicate statistically significant differences with a confidence level of 95%.

The colour evaluation of the different formulated biscuits was also carried out, and the CIE-L*a*b* values together with the colour differences (Δ*E*) compared to the control biscuits are presented in Table 3. A significant decrease in the L* parameter can be observed in all biscuit formulations that have a percentage of almond bagasse powder substitution, resulting in slightly darker biscuits compared to the control. In addition, a significant increase was observed in the a* coordinate, which was slightly higher as the percentage of substitution with almond bagasse powder increased, while no significant differences were observed in the b* coordinate. Consequently, these changes were reflected in colour differences (Δ*E*) following the same increasing trend as the percentage of substitution with almond bagasse powder increased, with values between 3 and 5 that could be perceptible to the human eye [63,

64]. Finally, considering the different drying methods, no significant differences were observed between hot air drying at 60°C and lyophilization for each percentage of flour substitution.

Antioxidant activity assessments using the DPPH and ABTS methods of biscuits are shown in Figure 4. Significant differences were noted in both the drying methods (hot air at 60°C and freeze-drying) and the percentage of almond bagasse powder substitution in the biscuit production. Moreover, a discernible trend was observed. As the percentage of almond bagasse powder substitution increased (10, 15, and 25%), the DPPH antiradical activity rose within the range of 1.01 to 1.12 mg Trolox/g dry sample in the samples dried using hot air at 60°C. Slightly higher values were reported by [65] on linseed seeds (1.35 mg TE/g) or on sunflower seeds (2.28 mg TE/g). Conversely, in the freeze-dried samples, an increase ranging from 0.87 to 0.97 mg Trolox/g dry sample was recorded, with higher antiradical DPPH activity in the samples dried using hot air. This discrepancy might be attributed to reactions due to the drying temperature. High temperatures could potentially induce the generation of Maillard's reaction by-products, known for their remarkable antioxidant capacity [66]. Conversely, while assessing antiradical activity using the ABTS method, no significant differences were noted concerning the drying method or the percentage of almond bagasse powder substitution. Similar results were reported by Kamiloglu et al. [67] for almond kernels (0.91 mg TE/g) or figs (1.63 mg TE/g) and higher values for hazelnut (5.61 mg TE/g). Nevertheless, it is noteworthy that, despite the absence of significant differences, an upward trend was observed as the percentage of almond bagasse powder substitution increased across various formulations.

[figure(s) omitted; refer to PDF]

In addition, the total phenolic content in different biscuit formulations was evaluated. As depicted in Figure 4, coinciding with the ABTS antiradical activity determination, an increase in total phenolic content was evident with an increased percentage of almond bagasse powder in the biscuit formulation. No significant differences were observed between the drying methods. Importantly, in both the assessment of antioxidant activity and total phenolic content, control samples made solely with 100% wheat flour exhibited the lowest values compared to samples containing almond bagasse powder substitution. This difference is attributed to the likelihood that wheat flour may possess a less diverse profile of antioxidant compounds [68].

4. Conclusions

The bagasse resulting from the production of the vegetable almond drink, when properly processed, has good technological and functional properties to partially replace wheat flour in the production of bakery products such as biscuits. However, the absence of starch and the high-fat content conditioned their suitability during kneading. The incorporation of almond bagasse powder significantly weakened the structure of the gel formed, although its hardness was sufficient to provide a structured biscuit with a specific volume like that obtained with wheat flour and with a higher antiradical capacity.

Processing almond bagasse in the form of freeze-dried powder resulted in better technological properties, such as emulsifying activity and stability. It would be relevant to the industrial applications of these blends. For example, the use of other, possibly less healthy fats could be reduced. However, the hot air-dried powder at 60°C gave the biscuits a higher antiradical capacity.

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Nutritional Potential of Ziziphus lotus (L.) Lam. Almonds as Compared to Some Oilseeds from Morocco: Evidence from Proximate Composition, Mineral Profiling, and Oil Physicochemical Traits

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ABSTRAK (ENGLISH)

Nowadays, there is a great interest in the search for new sources of vegetable oils, natural bioactive compounds, and essential nutrients for human health. Oleaginous fruits and seeds are a good source of nutrients such as oil, carbohydrates, fatty acids, sterols, tocopherols, fibers, vitamins, minerals, and proteins for the human diet. The main objective of this study was to compare the nutritional composition of *Ziziphus lotus* almonds (ZLA) with some well-known seeds (argan kernel, nigella seed, cactus seed, and sesame seed) and to classify them by the mean of principle component analysis (PCA). The samples’ proximate composition, mineral profiling, and some physicochemical parameters of extracted oil were evaluated. Our results revealed that ZLA composition was as follows: oil yield (26.99 ± 1.52 g/100 g), proteins (30.79 ± 0.07 g/100 g), ash (6.5 ± 0.06 g/100 g), and moisture (3.62 ± 0.10 g/100 g). The mineral profile of ZLA consists mainly of P, K, Mg, and Ca. The main fatty acids in ZLA oil were oleic acid (60.73 ± 0.10 g/100 g), linoleic acid (18.75 ± 0.10 g/100 g), and palmitic acid (9.86 ± 0.10 g/100 g). *β*-Sitosterol (67.92 ± 0.46 g/100 g) was the major sterol in ZLA, followed by stigmasterol (15.34 ± 0.35%) and campesterol (8.75 ± 0.12%). Total tocopherols were present at 523 ± 9.23 mg/kg in the oil. PCA analysis demonstrated that the ZLA

composition was found to be close to that of sesame seeds. In conclusion, ZLA could be considered an alternative source of vegetable oils and protein as well as other various applications.

TEKS LENGKAP

1. Introduction

In addition to water and oxygen, humans need an energy source, which can be provided by a combination of carbohydrates, lipids, proteins (amino acids), minerals, and vitamins [1]. Some nutrients, such as linoleic and linolenic acids, cannot be produced by humans and must be obtained through diet from plants [2]. Plant seeds are considered a good source of oil, carbohydrates, minerals, and vitamins, depending on the type of vegetable plant [3]. They also contain phytochemicals that may have antioxidant, antibacterial, antifungal, antiviral, and anticarcinogenic properties [3]. Oilseeds are an agricultural product worldwide with great interest because of their high oil yield and their multipurpose use in several sectors [4]. Among these oilseeds, nigella, sesame, argan, and cactus are sources of virgin oils.

Nigella (*Nigella sativa* L.) is from the Ranunculaceae family and is also known as black cumin or black seeds. It is renowned for its culinary uses and is popular in traditional medicine [5]. Its nutritional value can be linked to the presence of a considerable amount of vegetable protein, fiber, minerals, and vitamins [6]. Sesame (*Sesamum indicum* L.) is a plant of the Pedaliaceae family. It is one of the most ancient and famous traditional oil crops, appreciated for its high-quality oil seeds [7]. Argan (*Argania spinosa* (L.) Skeels) is a species of the *Sapotaceae* family, and argan oil is an important food prepared from roasted argan kernels [8]. Cactus (*Opuntia ficus indica* L.) belongs to the family of Cactaceae; its fruits are consumed freshly and have several uses (syrups, alcoholic drinks, juices, and so on) [9]. The fruit seeds constitute about 10 to 15% of the edible pulp; they are considered as waste left after fruit consumption [10].

Ziziphus lotus (L.) Lam. (ZL), a plant belonging to the Rhamnaceae family, is used in several fields such as nutrition, health, and cosmetics. It is utilized in various forms, including honey, tea, jam, juice, oil, loaf, and cake [11]. ZL is an important source of fresh, inexpensive, and nutritious edible fruits [11] and contains various natural product classes with biological activities such as antifungal and antioxidant properties [11–13]. Different parts of ZL fruit contain important essential nutrients and phytochemical compounds [14] and this diverse composition gives it a high nutritional value. For instance, the fruit pulp is rich in sugars, fibers, vitamin A, vitamin C, fatty acids, mineral matter, tannins, and antioxidant compounds [15], while almonds are rich in oil and proteins [15, 16]. ZL almond (ZLA) oil predominantly comprises oleic acid (60.00 ± 0.10 to 69.99 ± 0.10%), linoleic acid (10.66 ± 0.10 to 20.10 ± 0.10%), and palmitic acid (9.00 ± 0.10–10.22 ± 0.10%) as the major fatty acids. The primary sterols present are *β*-sitosterol (65.94 ± 0.28 to 71.99 ± 0.82%), stigmasterol (10.96 ± 0.30 to 16.20 ± 0.26%), and campesterol (8.36 ± 0.10 to 9.52 ± 0.20%) [16]. A large portion of oils and fats, for human consumption or industrial purposes, are now derived from vegetable sources. Seed oils are functionally important constituents of foods. They not only contribute to flavor, odor, color, and texture but also confer a feeling of satiety to foods [17].

This study aims to assess whether ZLA may be used as an alternative source of vegetable oil for nutritional or industrial reasons. To achieve this, the nutritional composition of ZLA, including proximate composition, mineral profiling, physicochemical characteristics, fatty acid content, tocopherols, phytosterols, and oxidative stability, was determined and compared to that of other seed oils commonly used in Morocco.

2. Materials and Methods

2.1. Plant Material and Chemical Reagents

The samples of ZL fruits were collected from the Taroudant region (Morocco) in October 2020. Nigella seeds and argan fruits were harvested in the region of Beni Mellal (32°20′14″ North, 6°20′59″ West) and in Taroudant (30°28′13″ North, 8°52′37″ West) in August 2020, respectively. Sesame and cactus seeds were purchased from the Taroudant Market and the cooperative (Taroudant), respectively. The samples were then dried in the ambient air.

ZLA and argan kernels were manually isolated from fruits, and all samples were grounded until a homogeneous powder was obtained. Chemical agents and solvents were of analytical grade.

2.2. Proximate Composition and Energy Value

2.2.1. Oil Yield

A Soxhlet system was used to extract the oils from each seed powder. For oil extraction from the biomass, n-hexane was used as a solvent with a solid-solvent ratio of 1 : 10. The extraction was carried out for 8 h. The solvent was evaporated under reduced pressure in a rotary evaporator. The oil was purged with nitrogen and stored at 4°C until further analysis, and the oil content (OC) was calculated following the formula and expressed as a percentage of extracted oil related to the initially used powder (g/100 g) [18]:(1)Oil content g 100 g = mass of extracted oil g mass of powder g × 100.

2.2.2. Proximate Composition

Moisture content (MC) was determined using an oven Memmert model (Schwabach, Germany). 5 g of the sample powder was dried at 103°C until reaching a constant weight. MC was determined using the following formula:(2)MC % = P2 − P3 P2 − P1 × 100 where P1 is the weight of the crucible, P2 is the weight of the crucible and sample powder before drying, and P3 corresponds to the weight of the crucible with the sample after drying. The weight difference was used to determine the water content.

Ash content (AC) was determined using a muffle furnace (Nabertherm GmbH, Germany). In brief, 5 g of dried powder was incinerated for 4 h at 525°C, and the obtained ash was weighed.

The crude protein content (PC) was estimated based on the nitrogen content (N). N content was determined using an elementary analyzer model LECO (LECO FP628, USA). The measured nitrogen was then converted to PC expressed in percent using a factor of 6.25 [19].

2.2.3. Minerals' Determination

The mineral elements have been determined using an inductively coupled plasma optical emission spectrometer (ICP-OES) Perkin Elmer Model Optima 8000 DV. In brief, 1 g of the powder was treated in a muffle furnace at 500°C for 2 h, then 4 mL of 65% NHO_3 and 10 mL of HCl were added to the obtained ash and then injected into the device [20]. Ten minerals (K, Ca, P, Mg, Na, Fe, Mn, Cu, Zn, and B) were analyzed according to the parameters and emission lines shown in Table 1. The wavelengths used during the analysis were as follows: K 766.490 nm, Mg 285.213 nm, Na 589.592 nm, Ca 317.933 nm, P 213.617 nm, Zn 213.857 nm, Cu 324.752 nm, Fe 239.562 nm, B 249.772, and Mn 257.610 nm.

Table 1

Operating conditions of ICP-OES during mineral determination.

Condition	Description/value
Plasma gas flow rate	14 $L.min^{-1}$
Auxiliary gas flow rate	0.2 $L.min^{-1}$
Nebulizer gas flow rate	0.8 $L.min^{-1}$
Plasma gas	Argon
Sample flow rate	1.3 $ml.min^{-1}$
Time flush	7 s

Analysis mode	Axial
RF power	1300 W

2.2.4. Carbohydrates Content (CC)

Carbohydrates content (CC) was calculated by subtracting the sum of oil OC, AC, MC, and PC from 100 as [12] follows:(3)CCg100g=100−%MC+%PC+%AC+%OC.

2.2.5. Energy Value (EV)

The energy value (EV) is a parameter of the nutritional value of a food or product. It is calculated from the values of PC, OC, and CC and expressed as kcal/100 g of the dry powder using the following equation [19]:(4)EVKcal100g= 2.62×PC+8.37×OC+4.2×CC.

2.3. Physicochemical Properties of Oils

Physicochemical quality parameters of oils were determined according to the official analytical methods.

Iodine value (IV) is defined as the total unsaturation of an oil; it was determined based on the relative percentage of fatty acids using the following equation and expressed as g (I2)/100 g of oil:(5)IVgI2100g=0.998×C16:1+0.899×C18: 1+1.811×C18:2+2.736×C18:3+0.818×C20:1.

The saponification value is expressed as the content of ester linkages; it was determined according to the standard method ISO 3657: 2020 [21]. 2 g of oil and 25 mL of ethanolic potassium hydroxide solution (0.5 M) were placed in a 250 mL round-bottomed flask and allowed to react under reflux for 1 h. The mixture was then cooled to room temperature, and the excess potassium hydroxide was titrated with hydrochloric acid (0.5 M) using phenolphthalein. The endpoint was marked by the discoloration of the solution. A blank test was carried out under the same conditions. It is expressed as mg KOH/g of oil.

The density and the refractive index were measured at 20 ± 0.2°C according to the AOCS Cc 7–25 [22].

2.4. Fatty Acid Determination

Fatty acids' (FAs) composition was determined according to the standard analytical method ISO 12966-2: 2017 [23]. FAs were transformed into fatty acid methyl esters by reacting 60 mg of oil with 0.3 mL of a 2 M methanolic potassium hydroxide solution under reflux for 10 minutes. After cooling at room temperature, 2 mL of hexane was added to the mixture and washed with distilled water. The hexane layer containing fatty acid methyl esters (FAMEs) was collected and analyzed using an Agilent 6890 GC-system gas chromatography (USA, Santa Clara) coupled to a flame ionization detector (GC-FID) on a CPWax 52CB column (60 m × 0.25 mm i.d., 0.25 μm film thickness). Helium (flowing at a rate of 1 mL/min) was employed as the carrier gas. Temperatures for the oven, injector, and detector were 185, 200, and 230°C, respectively. The samples were injected in split mode with a 1 μL injection volume (split ratio). The results were expressed as a relative percentage of the area of each fatty acid methyl ester.

2.5. Oxidizability Value COX

To investigate the impact of fatty acid composition on the oxidative stability of the oils, the oxidizability value (COX) was calculated. It is based on the percentage of the unsaturated fatty acids according to the following formula [24]: (6)COX=1×C16:1+C17:1+C18:1+C20:1+10.3×C18:2+21.6×C18:3+C20:3100.

2.6. Sterol Determination

The composition of sterols was assessed using the recognized analytical procedure ISO 12228-1, 2014 [25]. Trimethylsilylated sterols were analyzed in an Agilent 6890 GC-System (USA, Santa Clara) coupled to a flame ionization detector (GC-FID), with a VF-1 column (30 m × 320 mm i.d., 0.25 μm film thickness) and helium (flow rate 1.6 mL/min) as the carrier gas. The temperature program for the column was set to range from 200 to 280°C (5°C/min). The temperature of the injector and detector was 300°C. The injection was made in a split mode with a volume 1 μL (split ratio 1 : 50). The results were expressed as the relative percentage of area of each individual sterol peak, and the total sterol was expressed in mg/100 g.

2.7. Tocopherols Determination

Tocopherol content was determined by HPLC following the official analytical method (ISO 9936: 2016) [26] using

Shimadzu instruments equipped with a C18-Varian column (25 cm × 4 mm; Varian Inc., Middelburg, Netherlands). A fluorescence detector (excitation wavelength 290 nm and detection wavelength 330 nm) was used; the eluent was a 99 : 1 isooctane/isopropanol (v/v) mixture and the flow rate was 1.2 mL/min. Identification was based on retention time, and quantification of tocopherols was carried out using α, γ, and δ tocopherols (Sigma-Aldrich, St-Louis, USA) as external standards. Results are expressed as mg of tocopherols per 1 kg of oil.

2.8. Statistical Analysis

All determinations and measurements were performed in triplicate. Values were represented as means ± SD (standard deviations). Principle component analysis (PCA) was carried out on the mean values of parameters of the studied plant seeds (ZLA, argan, cactus, nigella, and sesame). All statistical analyses were done using R software version 4.2.2 and Origin software 2018.

3. Results and Discussion

3.1. Proximate Composition and Energy Value

The oil yield is of great importance for the economic value of oilseeds [22]. The oil yield of ZLA and other studied seeds is presented in Table 2. ZLA oil yield was 28.98 ± 1.49%. This percentage shows that ZLA is rich in oil. Similar results were reported in the literature (29.25 ± 0.67 and 32.92 ± 0.293%) [27, 28]. In comparison with other oils, ZLA oil content is lower than that found in argan oil (57.12 ± 0.10 g/100 g), nigella oil (37%), and sesame oil (56.56 ± 0.62%), but it is higher than that of cactus seed oil (5.4–9.9%). Similar values for oils were reported by other authors [29–31]. The fact that ZLA contain a significant amount of fat is of great economic interest and could allow their use in several new applications. ZLA can be considered as an oleaginous seed (seed containing an important content of oil) such as nigella and sesame seeds.

Table 2

Mean values of oil content (OC), protein content (PC), ash content (AC), moisture content (MC), carbohydrate content (CC), and energy value (EV) of ZLA as compared to the studied seeds.

Proximate composition (g/100 g)/vegetable seed	ZLA	Argan kernel	Cactus seed	Nigella seed	Sesame seed
OC	26.99 ± 1.52^{b}	52.00 ± 1.50s^{d}	5.00 ± 0.40^{a}	37.83 ± 0.69^{c}	57.62 ± 0.01^{d}
PC	30.79 ± 0.07^{d}	10.81 ± 0.84^{b}	8.24 ± 0.29^{a}	21.66 ± 0.08^{c}	22.01 ± 0.01^{c}
AC	6.5 ± 0.06^{c}	5.41 ± 0.41^{b}	1.63 ± 0.00^{a}	4.86 ± 0.10^{b}	5.23 ± 0.01^{b}
MC	3.62 ± 0.10^{c}	0.45 ± 0.01^{a}	8.97 ± 0.30^{d}	3.10 ± 0.10^{c}	1.75 ± 0.01^{d}
CC	38.59 ± 1.45^{c}	31.33 ± 0.69^{b}	55.47 ± 0.44^{d}	32.55 ± 0.25^{b}	13.39 ± 0.01^{a}
EV (kcal/100 g)	468.69 ± 6.84^{b}	595.14 ± 15.72^{c}	296.41 ± 5.10^{a}	510.09 ± 6.38^{b}	596.18 ± 10.01^{c}

Same letters within the same line indicate no significant differences resulted from ANOVA ($P<0.05$).

In addition to serving as a source of energy, protein also aids in the activity of enzymes and the transport of biochemicals across cellular membranes [32]. Concerning PC, ZLA were rich in proteins compared to other seeds; ZLA protein content was 29.62 ± 1.52 g/100 g, followed by sesame seeds (22.01 ± 0.01%), nigella seeds (21.66 ± 0.08%), then argan kernels (10.81 ± 0.84) and cactus seeds (10.00 ± 0.17%). These results are different from values found in the literature [33–35]. A study on the isolated protein of ZLA demonstrates that they contain 91.30 ± 1.41% of proteins and have most of the essential amino acids. This isolated protein might find primary potential in protein

fortification for a variety of food products [36].

The MC is one of the main constituents of foods, and it has a significant influence on food preservation. As can be seen in Table 2, nigella seed, cactus seed, and ZLA enclose, respectively, are 3.10 ± 0.10%, 4.17 ± 0.00%, and 5.13 ± 0.08% of water, while small contents were found in sesame seed and argan kernel (1.75 ± 0.01 and 0.45 ± 0.01%). Food products' total AC can be used to determine their nutritional value [37]. AC in the analyzed samples was found to be higher in ZLA, argan kernel, sesame seed, and nigella seed with values of 6.5 ± 0.06%, 5.4 ± 0.41%, 5.23 ± 0.01%, and 4.86 ± 0.1% (4.4 ± 0.3%), respectively. Cactus seed contains less ash with a value of 1.63 ± 00%, and it is in line with previously published literature [31].

Carbohydrates are an important part of the diet of people around the world. Fruits, vegetables, cereals, grains, and dairy products provide carbohydrates [38]. Consecutively, in an increasing way, the carbohydrate contents in the studied seeds are as follows: sesame seed (13.39 ± 0.01%), ZLA (19.74 ± 0.60%), argan kernel (31.33 ± 0.69%), nigella seed (32.55 ± 0.25%), and cactus seed (55.47 ± 0.44%).

The energy value associated with sesame seeds was found to be the highest of the studied seeds, accounting for about 596.18 ± 10.01 kcal/100 g, followed by argan kernel with 595.14 ± 15.72 kcal/100 g, nigella seeds with 510.09 ± 6.38 kcal/100 g, then ZLA and cactus seeds with 468.69 ± 6.84 kcal/100 g and 296.41 ± 5.10 kcal/100 g, respectively.

3.2. Mineral Composition

Elemental analysis of food provides more accurate nutritional information. Many different metabolic and physiologic processes in the human body involve minerals [20]. Macronutrients such as K, Ca, Na, Mg, and P are present in large amounts in living organisms [39]. Table 3 shows the mineral profile of the studied seeds. A wide variety of important mineral elements were analyzed, including five macroelements (K, Ca, Na, Mg, and P) and five microelements (Zn, Fe, B, Mn, and Cu). P was found to be the major macroelement in ZLA (6528.88 ± 33.45 mg/kg), followed by K (6118.09 ± 8.32 mg/kg), Mg (2994.73 ± 15.01 mg/kg), and Ca (816.96 ± 8.57 mg/kg). For microelements, ZLA were rich in Zn (93.50 ± 0.35 mg/kg), then Fe (71.50 ± 0.23 mg/kg), Mn (59.26 ± 0.01 mg/kg), B (23.19 ± 0.11 mg/kg), and Cu (9.13 ± 0.03 mg/kg). These results are different from those reported in many previous papers [27, 40]. Argan and cactus were dominated by K, followed by P, Ca, and Mg. High amounts of K, P, Mg, Na, and Fe were found in argan kernel and small amounts were found in cactus seeds except for Na which had a small amount in ZLA. ZLA were found to be less rich in Ca, P, and Mg compared to argan kernels, nigella, and sesame seeds but contain higher amounts than cactus seeds (Table 3). Zn (93.50 ± 0.35 mg/kg) was the most abundant microelement in ZLA, as compared to other studied samples. Zn deficiency is generally indicated by blood plasma/serum zinc concentration, dietary intake, and the prevalence of stunting [41]. As a source of Zn, ZLA could be used in the diet to address this deficiency.

Table 3

Mean values of minerals of ZLA and other seeds.

Minerals (mg/kg)/vegetable seed	ZLA	Argan kernel	Cactus seed	Nigella seed	Sesame seed
Ca	816.96 ± 8.57[a]	5186.88 ± 120.13[c]	1559.84 ± 0.05[b]	5230.06 ± 35.50[c]	6563.55 ± 16.79[d]
K	6118.09 ± 8.32[b]	11130.37 ± 601.55[d]	3056.06 ± 6.82[c]	8496.37 ± 12.03[a]	5442 .69 ± 38.94[b]
P	6528.88 ± 33.45[c]	6992.04 ± 336.35[c]	1964.48 ± 8.77[a]	4823.58 ± 10.24[b]	5248.52 ± 10.19[b]

Mg	2994.73 ± 15.01[c]	3798.25 ± 107.32[e]	1442.87 ± 7.82[b]	2589.04 ± 9.85[d]	3370.79 ± 8.29[a]
Na	17.26 ± 2.01[a]	697.16 ± 65.36[b]	20.31 ± 0.17[a]	110.42 ± 5.12[a]	70.91 ± 1.29[a]
Zn	93.50 ± 0.35[c]	68.36 ± 3.53[b]	17.37 ± 0.01[a]	60.26 ± 4.45[b]	53.93 ± 0.05[b]
Fe	71.50 ± 0.23[b]	100.44 ± 6.13[c]	22.95 ± 0.04[a]	93.06 ± 5.36[c]	71.94 ± 1.13[b]
B	23.19 ± 0.11[b]	28.11 ± 2.63[b]	9.52 ± 0.01[a]	29.13 ± 3.32[b]	12.32 ± 0.19[a]
Mn	59.26 ± 0.01[d]	nd	42.1849 ± 0.12[a]	nd	10.86 ± 0.01[b]
Cu	9.13 ± 0.03[b]	12.11 ± 0.96[bc]	4.24 ± 0.01[a]	13.35 ± 1.10[c]	20.71 ± 0.08[d]

Same letters within the same line indicate no significant differences resulted from ANOVA ($P<0.05$). nd: not determined.

3.3. Physicochemical Properties of Oils

Vegetable oil quality is commonly assessed using various physical parameters (moisture content, refractive index, viscosity, specific gravity, color, and so on) and chemical parameters (saponification value, acid value, iodine value, ash content, and peroxide value). These parameters are influenced by the oil's source, processing, and storage conditions [42]. Table 4 summarizes key parameters (refractive index, density, saponification, and iodine values).

Table 4

Mean values of physical and chemical parameters of ZLAO and other vegetable oils.

Parameter	ZLAO			Argan oil	Cactus oil	Nigella oil	Sesame oil
Our study	[27]	[43]	Density at 20°C	0.880 ± 0.020[a]	0.900 ± 0.000	nd	0.906–0.919[a]
0.906 ± 0.001[a]	0.83 ± 0.01[a]	0.91 ± 0.01[a]	Refraction index at 20°C	1.471 ± 0.010[a]	1.470 ± 0.010	1.46	1.470 ± 0.010[a]
1.461 ± 0.001[a]	1.473 ± 0.001[a]	1.472 ± 0.001[a]	Saponification value (mg KOH/g)	225.45 ± 1.57[d]	184.67 ± 2.08	122	189.00–199.10[b]
186.63 ± 0.50[b]	74.61 ± 0.20[a]	191 ± 0.36[c]	Iodine value g (I2)/100 g)	90.27[a]	nd	86	100.98 ± 1.35[ab]

Same letters within the same line indicate no significant differences resulted from ANOVA ($P<0.05$). nd: not determined. Results are presented as means ± SD.

The density and the refraction index depend on the temperature and the fatty acid composition of the oil [42]. As the oil's unsaturation level increases, the refractive index also rises [44]. The values for density and refractive index of ZLA oil (ZLAO) were found to be comparable to those of other vegetable oils (argan, cactus, nigella, and sesame oil, Table 4).

The saponification value serves as an indicator of the average molecular weight of the fatty acids present. A higher saponification value indicates shorter fatty acids on the glycerol backbone [44]. For ZLAO, this value was measured at approximately 225.45 ± 1.57 mg KOH/g of oil. This value is higher than those reported in other studies [27, 43]. Smaller saponification values were observed in other oils compared to ZLAO (74.61, 186.63 ± 0.5, 189.0–199.1, and 19 mg KOH/g oil, respectively, for nigella, cactus, argan, and sesame oils). ZLAO's high saponification value suggests its suitability for soap making as well as for use in oil-based ice cream and shampoos [45].

The iodine value quantifies the amount of double bonds present in fats and oils, reflecting the oil's sensitivity to oxidation [45]. ZLAO showed a low iodine value (90.27 g (I2)/100 g), which is lower than that of argan oil (100.98 ± 1.35 g I2/100 g), sesame oil (110.741 ± 0.1 g I2/100 g), nigella oil (126 ± 4 g (I2)/100 g), and cactus oil (130.5 ± 0.3 g I2 /100 g).

3.4. Fatty Acids

The extracted oil of the samples was analyzed to determine its profile of fatty acids, the main abundant components present in vegetable oils [46]. The characteristics, stability, and nutritive value of a given vegetable oil depend strongly upon the fatty acid composition [47]. ZLAO contains 83.26 g/100 g of unsaturated fatty acids and 16.53 g/100 g of saturated fatty acids.

Unsaturated fatty acids account 60.73 ± 0.1 g/100 g of oleic acid, the major component of the lipophilic fraction of ZLAO, 18.75 ± 0.1 g/100 g of linoleic acid, and 3.59 ± 0.1 g/100 g of eicosenoic acid. Oleic acid is known to decrease blood pressure, oncogenes, autoimmune diseases, inflammation, and the apoptosis of carcinogenic cells [48]. The content of this acid is higher than that of argan oil (48.31 ± 0.05 g/100 g), sesame oil (43.4 ± 0.1 g/100 g), nigella oil (23.14 ± 0.09 g/100 g), and cactus oil (13.5 ± 0.03 g/100 g). This oil can be classified as high oleic. Among the polyunsaturated acids, linoleic acid, the second major acid percentage, is inferior to other oils. It was abundant in cactus oil (63.8 ± 3.3 g/100 g) followed by nigella oil (58.06 ± 0.08 g/100 g), sesame oil (39.3 ± 0.1 g/100 g), and argan oil (31.45 ± 0.09 g/100 g). ZLAO was characterized by the presence of eicosenoic acid (3.59 ± 0.1 g/100 g); this compound, also called paullinic acid, is very rare in the plant kingdom. It was discovered in higher concentrations (44 g/100 g) in the seed lipid fractions of *Paullinia elegans* from which its name is obtained as paullinic acid [49].

Saturated fatty acids of ZLAO were presented especially by palmitic acid (9.86 ± 0.1 g/100 g), stearic acid (4.99 ± 0.1 g/100 g), and arachidic acid (1.41 ± 0.1 g/100 g). The obtained results are close to those found by Chouaibi et al. (2012) in Tunisian ZL seeds [27]. The content of palmitic acid was not very different from other oils, while the stearic acid value (4.99 ± 0.1 g/100 g) was higher than that of cactus oil (3.1 ± 0.01 g/100 g) and nigella oil (3.12 ± 0.01 g/100 g), but smaller than the values found in argan and sesame oils (5.82 ± 0.01 g/100 g and 5.9 ± 0.1 g/100 g).

3.5. COX Value

A higher COX value indicates the lower oxidative stability of oils [50]. It is generally taken to evaluate the oil's tendency to undergo autoxidation [24]. The COX values of the oils are as follows: ZLA (2.51 ± 0.17), argan oil (3.74 ± 0.23), sesame oil (4.52 ± 0.36), nigella oil (6.26 ± 0.45), and cactus oil (6.75 ± 0.66). Among the studied oils, cactus oil was the most sensitive to oxidation, where COX was the highest and ZLA was most likely to overcome oxidative reactions. The COX values are in agreement with the results of oxidative stability determined by the Rancimat test at 120°C, which revealed the induction times of argan (31 ± 2 h) [51], sesame (27.82 h) [47], nigella (9 h) [22], and cactus (7 ± 1 h) [51]. The study of oxidative stability based on fatty acids composition demonstrates that ZLAO presents good stability expressed by a low value of COX. This order of oxidative stability was also confirmed by iodine values (Table 4).

3.6. Phytosterols

Phytosterols are organic substances that are present in all diets derived from plants [52]. They were used as pharmacological agents, and the most important benefit is their blood cholesterol-lowering effect via partial inhibition of intestinal cholesterol absorption [53]. In addition, phytosterols are usually used to prove authenticity since they can be considered a fingerprint of vegetable oils such as olive and argan oils [8].

In ZLAO, the content of phytosterols was 332.06 ± 2.01 mg/100 g. It is close to the value published in the literature by Chouaibi et al. [27]. In comparison with the studied oils, it is higher than that of argan and nigella oils and smaller than that of sesame and cactus oils (Table 5).

Table 5

Mean values of fatty acids, sterols, COX value, and total tocopherols of ZLAO and other vegetable oils.

	ZLAO			Argan oil	Cactus oil	Nigella oil	Sesame oil
Fatty acid (g/100 g)	Our study	[16]	[33]				
–							
C14: 0	0.10 ± 0.01^{a}	0.06 ± 0.00	0.08	0.14 ± 0.01^{a}	nd	0.14 ± 0.01^{a}	0.10 ± 0.01^{a}
C16: 0	9.86 ± 0.10^{a}	9.14 ± 0.43	10.27	13.29 ± 0.08^{d}	11.40 ± 0.04^{b}	12.05 ± 0.09^{c}	10.05 ± 0.10^{a}
C16: 1	0.18 ± 0.10^{a}	0.13 ± 0.00	0.12	0.10 ± 0.01^{a}	0.50 ± 0.01^{b}	0.21 ± 0.01^{ab}	nd
C18: 0	4.99 ± 0.10^{b}	4.84 ± 0.36	6.48	5.82 ± 0.01^{c}	3.10 ± 0.01^{a}	3.12 ± 0.01^{a}	5.90 ± 0.10^{c}
C18: 1	60.73 ± 0.10^{e}	61.93 ± 0.95	62.49	48.31 ± 0.05^{d}	13.50 ± 0.03^{a}	23.14 ± 0.09^{b}	43.10 ± 0.10^{c}
C18: 2	18.75 ± 0.10^{a}	18.31 ± 0.31	16.00	31.45 ± 0.09^{b}	63.80 ± 3.3^{c}	58.06 ± 0.08^{c}	39.30 ± 0.10^{b}
C18: 3	0.30 ± 0.10^{a}	1.35 ± 0.06	0.26	0.07 ± 0.02^{a}	0.20 ± 0.01^{a}	0.22 ± 0.01^{a}	0.20 ± 0.01^{a}
C20: 0	1.41 ± 0.10^{a}	0.17 ± 0.00	1.25	0.31 ± 0.09^{a}	0.40 ± 0.02^{a}	0.14 ± 0.01^{a}	0.90 ± 0.01^{a}
C20: 1	3.59 ± 0.10^{b}	3.20 ± 0.01	2.90	0.38 ± 0.01^{a}	0.20 ± 0.06^{a}	0.32 ± 0.01^{a}	nd
SFA	16.35 ± 0.10^{a}	14.95 ± 0.01	15.94	19.56 ± 0.19^{b}	15.20 ± 0.40^{a}	15.45 ± 0.12^{a}	16.30 ± 0.20^{a}
MUFA	64.51 ± 0.10^{d}	65.29 ± 0.01	65.57	48.79 ± 0.07^{c}	18.80 ± 2.10^{a}	23.67 ± 0.11^{a}	41.90 ± 0.10^{b}
PUFA	18.75 ± 0.10^{a}	19.66 ± 0.01	16.26	31.52 ± 0.11^{b}	64.00 ± 1.10^{e}	58.28 ± 0.90^{d}	42.30 ± 0.10^{c}

COX value	2.51±0.17[a]			3.74±0.23[a]	6.75±0.66[b]	6.26±0.45[b]	4.52±0.36[ab]
–							
Sterols (g/100g)	[16, 31]						
–							
Cholesterol	0.18±0.01[a]	0.12		nd	0.90±0.10[a]	0.80±0.40[a]	0.20±0.01[a]
Campesterol	8.75±0.12[b]	8.48		0.15±0.05[a]	13.95±0.40[d]	11.61±0.03[c]	17.80±0.10[e]
Stigmasterol	15.34±0.35[c]	11.32		nd	2.24±0.10[a]	nd	6.40±0.10[b]
β-Sitosterol	67.92±0.46[c]	71.7		nd	71.14±0.80[d]	49.24±0.05[a]	59.90±0.10[b]
Δ-5-Avenasterol	4.05±0.03[a]	4.24		nd	3.84±0.10[a]	11.10±0.04[c]	7.50±0.10[b]
Δ-7-Stigmasterol	0.22±0.01[a]	0.3		nd	3.93±0.12[c]	0.78±0.08[b]	0.30±0.02[a]
Δ-7-Avenasterol	0.25±0.01[a]	0.34		0.24±0.09[a]	2.55±0.10[b]	2.47±0.07[b]	0.10±0.01[a]
Stigmasta-8.22-dien-3b-ol	nd	nd		5.00±0.06	nd	nd	nd
Schotenol	nd	nd		46.22±0.07	nd	nd	nd
Spinasterol	nd	nd		38.39±0.09	nd	nd	nd
Total sterols mg/100 g	332.06±2.01[b]	285.03		119.45±0.60[a]	1085.90±30.6[d]	156.9±0.50[a]	540±6.20[c]
Total tocopherols (mg/kg)	523±9.23[b]			747.20±7.99[d]	679.70±4.00[c]	453.70±9.12[a]	446±7.14[a]

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, and nd: not determined. Same letters within the same row indicate no significant differences resulted from ANOVA ($P<0.05$). Results are presented as means±SD.

β-sitosterol, stigmasterol, campesterol, and Δ-5-avenasterol were the major sterols in ZLAO, respectively. The amount of *β*-sitosterol (67.92±0.46g/100g) was close to that of cactus (71.14±0.80g/100g). This sterol was also the major component of nigella and sesame oils but with a lower percentage than ZLA. Numerous in vitro and in vivo studies have demonstrated that *β*-sitosterol has a variety of pharmacological effects, including sedative and anxiolytic effects, analgesic, immunomodulatory, antibacterial, anticancer, anti-inflammatory, lipid-lowering impact,

and hepatoprotective benefits [54]. This phytosterol has the potential to be used as a supplement in the fight against life-threatening diseases [54], highlighting the significance of the oil's richness in this sterol. Stigmasterol, the second principle sterol in ZLAO (15.34 ± 0.35 g/100 g), was detected in large quantities compared to sesame and cactus oils and not detected in argan and nigella oils. In the third rank, campesterol was about 8.75 ± 0.12 g/100 g. It is found in high amounts in sesame, cactus, and nigella oils and is absent in argan oil. Finally, Δ-5-avenasterol (4.05 ± 0.03 g/100 g) is close to the value found in cactus oil but smaller than the percentages in sesame and nigella oils, while it is not detected in argan oil. Other sterols are found in amounts less than 1 g/100 g.

The composition of ZLAO sterols is similar to that found by other authors [27, 43] and presents some similarities with cactus, nigella, and sesame oils, while it is entirely different from argan oil (Table 5).

3.7. Tocopherols

Tocopherols are lipophilic antioxidant chemicals of the vitamin E group, only produced by plants [55]. They are a key factor in determining the quality of edible oils as plant endogenous antioxidants, with a greater effect on the stability of oil constituents [56]. In the human body, they maintain normal human muscle metabolism and the integrity of the central nervous and vascular systems [56]. The tocopherol content in ZLAO is about 523 mg/kg, which is more important than the content in sesame and nigella oils. A high content of tocopherols was found in argan and cactus oils (747.20 ± 7.99 and 679.70 ± 4.00 mg/kg) (Table 5). On one hand, the content of tocopherols in oil is important to protect the oil from oxidation, but on the other hand, the nature of fatty acids is particularly important in relation to oxidative stability. For example, linolenic acid is oxidized the fastest, followed by linoleic and oleic acids [57]. This can explain the high oxidative stability of ZLAO compared to argan and cactus oils.

3.8. Principle Component Analysis and Hierarchical Clustering

Principle component analysis was carried out to visualize similarities among various plant seeds' composition (ZLA, argan kernel, cactus seed, nigella seed, and sesame seed). The PCA analysis is shown in Figure 1. The points plotted on the surface were delimited by the first two components, which accounted for more than 70% of the total variability. As can be seen in Figure 1, argan kernel interacted with high values of scho, spin, stig-8,22, SFA, C16 : 0, SFA, TT, Na, K, and Ca. ZLA were associated with best scores of C20:0, C20:1, stigmasterol, and PC. Sesame seeds were correlated with medium values of C20:0 and stigmasterol, while cactus seeds were linked to the highest content of cholesterol, Δ-7- stigmasterol, Δ-7-avenasterol, campesterol, total sterols, PUFA, C16:1, C18:2, and CC. PCA and cluster analyses are allowed to classify ZLA among four commonly used seeds for vegetable oils in Morocco. ZLA composition was found to be near to that of sesame and nigella seeds.

[figure(s) omitted; refer to PDF]

Cluster analysis (CA) examined the relationship between different seeds based on the Euclidean distance. As can be seen in Figure 2, the seeds were separated into 4 clusters. The first cluster comprised cactus seed, the second contains argan kernel, the third cluster contains ZLA, and the last one combined nigella and sesame seeds. PCA and CA were used as multivariate techniques to explore data variation in foods [58, 59].

[figure(s) omitted; refer to PDF]

4. Conclusions

ZLA, argan kernels, sesame, nigella, and cactus seeds are of great interest, as evidenced by their nutritional composition and oil profiling. The present study investigated and compared the compositional attributes of ZLA with other seeds. As a result, ZLA could be a source of fat, protein, and minerals. Analysis of the oil composition reveals a notable abundance of oleic acid and *β*-sitosterol in ZLAO. Considering its classification among the studied seeds, some similarities in nutritional benefits were found, and PCA analysis classified ZLA's composition as close to that of sesame seeds. Overall, ZLA are a nutritious food source, on the same level as other widely used seeds and fruits, and could be a valuable by-product that could be incorporated into a healthy diet.

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DETAIL

Subjek:	Physicochemical properties; Phytochemicals; Vegetable oils; Carbohydrates; Fatty acids; Antioxidants; Potassium; Gas flow; Vitamins; Oils &fats; Dietary minerals; Nitrogen; Vegetables; Bioactive compounds; Solvents; Seeds; Principal components analysis; Oilseeds; Fruits; Potash; Fibers; Essential nutrients; Physical properties; Nutritional status
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Leverage of Matricaria chamomilla L. Oil Supplementation over Ochratoxin A in Growing Quails

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ABSTRAK (ENGLISH)

Ochratoxin A (OTA) is one of the mycotoxins in the agriculture and livestock sectors. The poultry sector suffered from significant economic losses due to the adverse impacts of OTA on the growth rate, feed conversion ratio, and livability. Thus, the present investigation aimed to determine the impact of chamomile essential oil supplementation against OTA toxicity in growing quails. 360 one-week-old growing quails were distributed into six groups ($n=60$) with four replicates of 15 birds. The groups were G1 (control negative), G2 (OTA 1 mg/kg diet, control positive), G3 (chamomile oil 0.5 g/kg diet), G4 (chamomile oil 1 g/kg diet), G5 (OTA 1 mg/kg diet + chamomile oil 0.5 g/kg diet), and G6 (OTA 1 mg/kg diet + chamomile oil 1 g/kg diet). Adding OTA significantly ($P<0.05$) reduced live body weight and weight gain at 5 weeks. Feed intake at 5 weeks was nonsignificantly reduced in G3 and G4 compared to G1. G4 showed a significant ($P<0.05$) increase in weight gain and the lowest feed conversion ratio. The G2 showed the lowest superoxide dismutase (SOD), total antioxidant capacity (TAC), glutathione transferase (GST) activity, and the highest levels of malondialdehyde (MDA). Moreover, they showed a significant improvement in liver enzymes and kidney function tests and a significant ($P<0.05$) reduction in the levels of total cholesterol and triglycerides. Chamomile supplementation alone or with OTA significantly ($P<0.05$) increased immunoglobulin M, G, A, and complement 3 than OTA alone. Chamomile oil with an OTA diet or alone reduced the negative effects of OTA and improved the performance, antioxidant status, lipid profile, and immunological state of growing Japanese quails.

TEKS LENGKAP

1. Introduction

The prevalence of mycotoxins is a global problem that has negative impacts on humans, animals, and poultry [1–3]. Several researchers have established that ochratoxin A (OTA) is one of the mycotoxins in the agriculture and livestock sectors [4–8]. Certain types of fungi, such as *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus niger*, and *Penicillium verrucosum*, are responsible for the production of OTA as a result of improper storage of products under deviated temperature and relative humidity [9, 10].

Ochratoxins have been detected in poultry feed and feed additives worldwide [11], and their immunotoxic, liver-toxic, neurotoxic, teratogenic, and mutagenic effects have been extensively documented in several poultry and mammal species [12–14]. When broilers were fed feed contaminated with OTA, the weight of immune organs was reduced [15], and the responses of humoral and cellular immune systems were suppressed [16]. It was also observed that chicks administered OTA at dosages of 2 and 4 mg/kg feed for 15 days exhibited a decrease in the number of immunoglobulin-bearing cells in both their immune organs and serum [17, 18]. Tinelli et al. [19] reported further detrimental consequences of OTA, which included elevated lipid peroxidation, impairment of mitochondrial function, and reduced synthesis of macromolecules.

Scientific investigations have demonstrated the potential efficacy of various naturally occurring feed additives, including phytochemicals, prebiotics, and probiotics, in ameliorating the severity of ochratoxicosis-related symptoms [20–22]. Broiler chicks supplemented with OTA exhibited a decrease in body weight gain (BWG), suboptimal feed efficiency, atypical relative organ weights, and abnormal serum biochemistry [23]. Due to antibiotics' detrimental effects on human and avian health, medicinal plants have been used as a potential substitute for antibiotics [24–26]. Most active components in medicinal plants are rapidly absorbed, and their half-life is shorter than antibiotics [27]. Consequently, they are completely absent from the animal tissue [28]. For thousands of years, chamomile was used for medicinal treatments in Greece, Rome, and Ancient Egypt [29]. According to several studies, chamomile is effective as an anti-inflammatory, antioxidant, sedative, wound-healing, antibacterial, and antifungal agent [29–31]. Reda et al. [32] suggested that the antimycotic, antibacterial, and anti-inflammatory activities and the antioxidant properties of chamomile could enhance body weight and improve feed conversion. Göger et al. [33] found that chamomile contains sesquiterpenoid compounds with antibiotic-like properties. Chamomile has been observed to

donate protons and exhibit antioxidant properties through scavenging or inhibiting the activity of free radicals [34]. To the best of our knowledge, there are some studies on the role of herbal plants as natural additives in reducing the detrimental effects of mycotoxins, but no studies on the role of chamomile oils in growing quail diets. Therefore, we aimed to investigate the augmenting influences of chamomile in reducing the negative impact of OTA and improving performance, antioxidants, liver and renal functions, immunity, and serum biochemical markers of Japanese quails.

2. Materials and Methods

2.1. Preparation of OTA

The strain *Aspergillus ochraceus* (CGMCC 3.4412) was employed to produce OTA. This strain was sourced from the Central Laboratory of Residues of Agricultural Products, located at the Agriculture Pesticides Residues Centre in Dokki, Egypt. To synthesize OTA, the fungal organism was cultured for 8 days in a liquid medium containing 2% yeast extract and 20% sugar. The percentage of OTA in the media was determined according to the Association of Official Agricultural Chemists guidelines [35].

2.2. Tested Oil

Chamomile oil ChO was obtained from Harraz Co., a local supplier based in Egypt. A gas chromatography-mass spectrometry (GC-MS) investigation was used to ascertain the active ingredients in the ChO used in the current investigation [36]. GC-MS analysis detected several active ingredients in the ChO with different peak area % and retention times. Retention times and peak areas (%) of the natural constituent in ChO analyzed by GC-MS were 14.27 mn and 0.47% E-2-undecen-1-ol, 15.63 mn and 0.98% Valeric acid, 15.63 mn and 0.98% -Octanol, 2,7-dimethyl-, 17.45 mn and 0.68% cis-á-Farnesene, 22.23 mn and 0.23% acetic acid, 24.21 mn and 3.83% 2H-Pyran-3-ol, 27.14 mn, and 1.48%1,6-Dioxaspiro[4.4]non-3-ene, 29.18 mn and 14.98% Hexadecanoic acid (CAS), and 32.92 mn and 62.64% 6-Octadecenoic acid, respectively.

2.3. Housing and Animals

The trial was conducted in the poultry farm at the Faculty of Agriculture, Poultry Department, Zagazig University, Zagazig, Egypt. Quails were one week old and had similar average body weights (30.82 g) across all groups. The experiment complies with the Zagazig University Ethics Committee's regulations for using experimental animals (Approval No. ZU-IACUC/2/F/313/2023). The recommendations of the ARRIVE guidelines in animal research were also consulted and considered [37]. Quails were housed in traditional cages with drinking water and mash feed provided ad libitum. The trial conditions were a 23 h light–1 h dark cycle in an open-door building with 24–26°C daily temperature and 60–70% humidity. The medical program was conducted according to the different age stages under veterinarian supervision.

2.4. Experimental Design, Diets, and Treatments

The experimental method used in this research was a randomized complete block design. It consisted of six treatments (4 replicates of 15 birds), resulting in 360 growing quails. The study was designed to last for five weeks. The experimental groups were: G1 (negative control) supplemented with basal diet (BD), G2 supplemented with BD with OTA (1 mg/kg of diet), G3 supplemented with BD with chamomile (0.5 g/kg of diet), G4 supplemented with BD with chamomile (1 g/kg of diet), G5 supplemented with BD with OTA (1 mg/kg of diet) and chamomile (0.5 g/kg of diet), and G6 supplemented with BD with OTA (1 mg/kg of diet) and chamomile (1 g/kg of diet). All bird specimens were raised in identical management and hygienic environments and provided nutritionally balanced diets to meet their dietary needs, as outlined in the National Research Council guidelines (Table 1).

Table 1

Ingredients and nutrient contents of the basal diet of growing Japanese quail.

Item	(%)
Ingredient (%)	

Maize 8.5%	51.80
Soybean meal 44%	36.70
Maize gluten meal 62%	5.21
Soybean oil	2.90
Limestone	0.70
Di-calcium phosphate	1.65
Salt	0.30
Premix[1]	0.30
L-Lysine	0.13
Dl-Methionine	0.11
Choline chloride (50%)	0.20
Total	100
–	
Calculated composition[2] (%)	
ME (Kcal/Kg)	2995
Crude protein	24.00
Calcium	0.80
Nonphytate P	0.45
Lysine	1.30
TSAA	0.92

[1]Provides per kg of diet: Vitamin A, 12,000 I.U; Vitamin D3, 5000 I.U; Vitamin E, 130.0 mg; Vitamin K3, 3.605 mg; Vitamin B1 (thiamin), 3.0 mg; Vitamin B2 (riboflavin), 8.0 mg; Vitamin B6, 4.950 mg; Vitamin B12, 17.0 mg; Niacin, 60.0 mg; D-Biotin, 200.0 mg; Calcium D-pantothenate, 18.333 mg; Folic acid, 2.083 mg; manganese, 100.0 mg; iron, 80.0 mg; zinc, 80.0 mg; copper, 8.0 mg; iodine, 2.0 mg; cobalt, 500.0 mg; and selenium, 150.0 mg. [2]Calculated according to NRC (1994).

2.5. Growth Performance

Body weight (BW) measurements were taken for all birds at 1, 3, and 5 weeks of age using a Digital Micro Scale (model MAB250, IndiaMART C ompany, India). Additionally, BWG was measured using mathematical calculations. The feed intake was consistently measured throughout the trial periods in a duplicated manner to estimate the feed conversion ratio.

2.6. Carcass Traits

At the end of the experiment, quails ($n=30$; 5 birds randomly distributed per group) were weighed and then were anesthetized by using intramuscular injection with 1 ml/kg of ketamine xylazine mixture (2 : 1), slaughtered by sharp knife to determine carcass traits, and their blood was collected in sterile tubes for blood analysis. The carcass weight, and giblets (gizzard, heart, and liver) were quantified and expressed as a percentage of the total weight at slaughter.

2.7. Blood Parameters

Blood samples were obtained from five quails that had been sacrificed for carcass traits for each group. These samples were collected in sterilized tubes. Subsequently, the samples were allowed to undergo coagulation and centrifuged at 4000 rpm for 10 min. The collected serums were kept until they were ready for analysis. The spectrophotometric analysis of various factors was conducted using kits supplied by the Biodiagnostic Company (Giza, Egypt). These factors included total protein (TP), albumin (ALB), aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol levels and very low-density lipoprotein (VLDL) cholesterol levels. Determining serum globulin (GLOB) levels involves subtracting albumin concentration from the total serum protein concentration. The albumin-to-globulin ratio (A/G) was calculated. The levels of immunoglobulins G (IgG), M (IgM), A (IgA), and complement 3 (C3) were determined using kits manufactured by Spectrum Company in Cairo, Egypt. The concentrations of superoxide dismutase (SOD), malondialdehyde (MDA), total antioxidant capacity (TAC), and glutathione transferase (GST) were measured in plasma samples using commercially available kits and read spectrophotometrically (UV-2600i/2700i, Shimadzu, Japan).

2.8. Microbiological Analysis

Five quails per treatment were randomly selected and slaughtered postexperiment (5 weeks) to estimate the total microbial population in the caecum content. Caecal digesta samples (1 g/quail) were rapidly prepared, placed in bottles, exposed to a stream of CO_2, and transported to the lab for microbiological analysis. According to Xia et al. [38], microbial counts (total bacterial count, lactobacilli count, *salmonella spp.*, *E. coli*, and *Coliform*) were performed.

2.9. Statistical Analysis

Data were analyzed using GraphPad Prism8 software (GraphPad Software, Inc., La Jolla, CA, USA) and were first checked for normality using the D'Agostino-Pearson normality test. Differences in performance, carcasses, serum components, and oxidative stress were analyzed by one-way ANOVA (with the diet as the fixed factor) followed by the Newman–Keuls multiple comparison test. $P<0.05$ was considered significant.

3. Results and Discussion

The findings demonstrated a significant ($P<0.001$) difference in LBW across groups at 5 weeks. However, LBW was considerably impacted by the various dietary supplements, and G2 had the lowest BW. On the other hand, chamomile-supplemented groups showed the highest BW levels. Moreover, G5 and G6 improved BW more than the OTA-supplemented group. BWG revealed that OTA significantly ($P<0.001$) reduced between 1 and 5 weeks compared to the control and other groups. G3 and G4 obtained a better improvement. Dietary supplements of chamomile and OTA-chamomile mix enhanced the BWG more than OTA. Moreover, during the 3–5 weeks, there were significant ($P<0.05$) differences in BWG between the various experimental groups. Data in Table 2 revealed a nonsignificant decrease in feed intake (FI) in G3 and G4 over the experimentation period (1–5 weeks).

Table 2

Effects of treatments on growth performance of growing quails.

Items	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	Body weight (g)		
1 wk	29.08	29.39	29.37	29.35	29.1	29.01	0.399	0.9732
3 wk	104.34^{b}	89.95^{d}	109.66^{a}	113.57^{a}	97.00^{c}	100.67^{bc}	1.685	<0.0001
5 wk	206.10^{b}	185.11^{d}	215.12^{a}	220.65^{a}	193.70^{c}	199.80^{bc}	2.332	<0.0001
–								
Body weight gain (g/day)								
1–3 wk	5.38^{bc}	4.33^{e}	5.74^{ab}	6.02^{a}	4.85^{d}	5.12^{cd}	0.117	<0.0001
3–5 wk	7.27^{abc}	6.80^{c}	7.53^{ab}	7.65^{a}	6.91^{c}	7.08^{bc}	0.121	0.0129
1–5 wk	6.32^{b}	5.56^{d}	6.63^{a}	6.83^{a}	5.88^{c}	6.10^{bc}	0.082	<0.0001
–								
Feed intake (g/day)								
1–3 wk	15.19	15.99	15.12	14.83	14.44	14.65	0.345	0.1308
3–5 wk	21.43	21.96	21.12	21.23	22.56	21.95	0.655	0.7295
1–5 wk	18.31	18.97	18.12	18.03	18.5	18.3	0.501	0.8388
–								
Feed conversion ratio (g feed/g gain)								
1–3 wk	2.83^{b}	3.70^{a}	2.65^{bc}	2.47^{c}	2.98^{b}	2.86^{b}	0.089	<0.0001
3–5 wk	2.95	3.25	2.80	2.79	3.27	3.10	0.135	0.1420
1–5 wk	2.90^{bc}	3.41^{a}	2.73^{c}	2.64^{c}	3.15^{ab}	3.00^{bc}	0.103	0.0034

Means in the same column within each classification bearing different letters are significantly different ($P<0.05$ or $P<0.01$). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet.

FI in the G5 and G6 was similar to the control. While, G2 revealed the highest level of feed intake. Likewise, treatments had a significant (P<0.01) increase in FCR in G2 (1–3 and 1–5 weeks) compared to all other groups, while G4 had the best FCR.

According to the results, there are no significant differences in carcass percentage, and G4 had the lowest carcass percentage when in comparison to the other treatments, followed by G2 (Table 3).

Table 3

Effects of treatments on carcass traits of growing quails.

Items	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	Percentage of slaughter weight (%)		
Carcass	80.00	79.62	79.99	79.09	81.65	81.32	1.033	0.5975
Liver	2.41^{b}	2.95^{a}	2.25^{b}	2.25^{b}	2.68^{ab}	2.54^{ab}	0.127	0.0203
Gizzard	2.03	1.83	1.95	2.06	2.01	1.84	0.105	0.7529
Heart	0.95	0.81	0.80	0.93	0.81	0.92	0.049	0.1547
Giblets	5.39	5.59	5.00	5.24	5.51	5.30	0.167	0.3663

Means in the same column within each classification bearing different letters are significantly different (P<0.05 or P< 0.01). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet.

Dietary supplementation revealed a significant (P<0.05) impact on liver weight percentage, and G2 showed the highest levels in comparison to the other groups. Moreover, dietary supplementation with OTA or chamomile to quail diet revealed a nonsignificant impact on gizzard, heart, and giblet percentage. Furthermore, G5 and G6 increased the carcass percentage to be comparable to G1 but insignificant.

The findings revealed that G3 and G4 had a significant (P<0.05) elevation in total protein, albumin, and globulin in comparison to control and other supplements, while G2 showed the lowest levels of total protein, albumin, and globulin. The A/G levels were significantly (P<0.05) increased in the G2 and G4. Furthermore, G2 presented a significant (P<0.05) elevation in levels of ALT and AST, while G4 revealed the lowest ALT and AST levels. In Table 4, G2 represented a significant (P<0.01) increase in LDH level while revealed the lowest level.

Table 4

Effects of treatments on lipid profile of growing quails.

Items[2]	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	TC (mg/dL)	158.95^{c}	220.40^{a}
155.97^{c}	148.49^{c}	196.65^{ab}	175.80^{bc}	7.827	0.0006	TG (mg/dL)	161.60^{d}	344.05^{a}

158.35[d]	144.55[d]	288.85[b]	215.25[c]	9.502	<0.0001	HDL (mg/dL)	51.63[b]	28.76[d]
53.42[b]	62.51[a]	34.66[cd]	39.60[c]	2.702	<0.0001	LDL (mg/dL)	75.01[c]	122.83[a]
70.89[c]	57.07[d]	104.23[b]	93.16[b]	4.184	<0.0001	VLDL (mg/dL)	32.32[d]	68.81[a]

Means in the same column within each classification bearing different letters are significantly different (P<0.05 or P< 0.01). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet. [2]TC: total cholesterol; TG: triglycerides; HDL: high-density lipoprotein; LDL: low density lipoprotein; VLDL: very low-density lipoprotein. Creatinine, urea, and uric acid levels were significantly (P<0.05) impacted in quail when OTA and chamomile were added to their diets. In comparison to the other groups, G4 (1 g/kg) showed the lowest plasma creatinine, urea, and uric acid level, while G2 revealed the highest plasma creatinine, urea, and uric acid. Table 5 shows that the addition of chamomile at a level (1 g chamomile/kg diet) to the diet decreased plasma total cholesterol levels (P<0.001) and raised plasma HDL levels (P<0.001).

Table 5

Effects of treatments on liver and kidney functions of growing quails.

Items[2]	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	TP (g/dL)	3.20[ab]	2.14[c]
3.68[a]	3.52[a]	2.71[b]	2.93[b]	0.153	0.0006	ALB (g/dL)	1.35[cd]	1.20[d]
1.69[ab]	1.86[a]	1.53[bc]	1.39[cd]	0.069	0.0011	GLOB (g/dL)	1.86[a]	0.94[c]
1.99[a]	1.67[ab]	1.18[bc]	1.54[ab]	0.142	0.0030	A/G ratio	0.73[b]	1.31[a]
0.86[b]	1.13[ab]	1.29[a]	0.96[ab]	0.097	0.0305	ALT (IU/L)	10.21[c]	15.05[a]
9.55[c]	7.24[d]	12.73[b]	11.02[bc]	0.663	<0.0001	AST (IU/L)	139.45[c]	210.40[a]
145.77[c]	103.90[d]	173.90[b]	159.80[bc]	7.629	<0.0001	LDH (IU/L)	222.65[b]	372.38[a]
236.90[b]	199.25[b]	335.10[a]	287.45[ab]	25.747	0.0040	Creat (mg/dL)	0.61[b]	0.82[a]
0.36[c]	0.35[c]	0.52[b]	0.42[c]	0.031	<0.0001	Urea (mg/dL)	0.96[c]	3.91[a]
1.04[c]	0.94[c]	2.60[b]	2.10[b]	0.238	<0.0001	Uric acid A	9.32[b]	12.21[a]

Means in the same column within each classification bearing different letters are significantly different (P<0.05 or P< 0.01). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet. [2]TP: total protein; ALB: albumin; GLOB: globulin; A/G ratio: albumin: globulin ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase, LDH: lactate dehydrogenase, Creat: creatinine.

Triglyceride, LDL, and VLDL readings in the G4 were significantly less (P<0.05) than in the G1 group. The G5 revealed the highest levels of total cholesterol, triglyceride, LDL, and VLDL and the lowest levels of plasma HDL.

The results of the plasma's antioxidant status are shown in Table 6. There were no statistically significant differences observed between treatments in terms of the activity of SOD in the plasma. The G4 showed the highest level of SOD, while G2 revealed the lowest levels. Compared to the G1 group, the levels of MDA were significantly (P<0.001) reduced in G4 and increased in G2. TAC was higher (P<0.001) in G4 than in G1, while G2 revealed the lowest levels. GSH levels were higher in G4 than in the G1.

Table 6

Effects of treatments on immunity and antioxidants of growing quails.

Items[2]	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	Antioxidants		
SOD (U/mL)	0.17	0.16	0.20	0.19	0.17	0.16	0.029	0.9458
MDA (nmol/mL)	0.52^{c}	1.28^{a}	0.40^{cd}	0.30^{d}	1.05^{b}	0.90^{b}	0.050	<0.0001
TAC (ng/ml)	0.56^{b}	0.24^{c}	1.17^{a}	1.19^{a}	0.54^{b}	0.75^{b}	0.063	<0.0001
GST (ng/ml)	0.17	0.15	0.20	0.21	0.16	0.14	0.016	0.0538
–								
Immunity								
IgM (mg/dl)	0.46^{c}	0.23^{d}	0.79^{a}	0.64^{b}	0.38^{c}	0.43^{c}	0.023	<0.0001
IgG (mg/dl)	0.83^{b}	0.47^{c}	1.15^{a}	1.06^{a}	0.76^{b}	0.70^{b}	0.059	<0.0001
IgA (mg/dl)	0.65^{b}	0.28^{c}	1.14^{a}	1.31^{a}	0.59^{b}	0.63^{b}	0.055	<0.0001
Complement 3 (mg/dl)	131.00^{bc}	105.50^{d}	140.00^{ab}	150.50^{a}	127.00^{c}	122.50^{c}	3.801	<0.0001

Means in the same column within each classification bearing different letters are significantly different (P<0.05, or P< 0.01). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet. [2]SOD: superoxide dismutase; MDA: malondialdehyde; TAC: total antioxidant capacity; GST: glutathione transferase; IgG: immunoglobulin G: IgM: immunoglobulin M; IgA: immunoglobulin A.

As shown in Table 6, there were statistically significant (P<0.001) variations in plasma concentrations of IgM, IgG, IgA, and complement 3 in each group, and G3 revealed a significant (P<0.01) elevation in the plasma

concentrations of IgM and IgG, while G4 revealed a significant (P<0.01) elevation in the plasma concentrations of IgA and complement 3 in comparison to the other groups.

The G2 revealed a significant (P<0.001) decrease in IgM, IgG, IgA, and complement 3 plasma concentrations.

Data in Table 7 demonstrates the impact of OTA and chamomile on growing quail's gut microbial composition.

Table 7

Effects of treatments on the microbial count of growing quails.

Items	Treatments[1]						SEM	P value
G1	G2	G3	G4	G5	G6	Microbiological count (log CFU/g)		
Total bacterial count	6.85^{ab}	6.71^{bc}	6.88^{a}	6.92^{a}	6.48^{d}	6.65^{c}	0.047	0.0002
Total yeasts and molds count	4.37	4.35	4.21	4.18	4.25	4.26	0.074	0.4809
E. coli	5.67^{a}	5.75^{a}	5.42^{bc}	5.38^{c}	5.53^{b}	5.45^{bc}	0.033	<0.0001
Coliform	6.73^{a}	6.75^{a}	6.58^{b}	6.53^{b}	6.60^{b}	6.65^{ab}	0.036	0.0078
Salmonella spp.	2.09^{a}	2.19^{a}	1.64^{c}	1.41^{d}	1.83^{b}	2.11^{a}	0.043	<0.0001
Lactic acid bacteria	6.47^{b}	5.20^{e}	6.77^{a}	6.85^{a}	5.93^{c}	5.41^{d}	0.052	<0.0001
Enterococcus spp.	5.54^{c}	5.90^{a}	5.38^{d}	5.18^{e}	5.69^{b}	5.63^{bc}	0.043	<0.0001

Means in the same column within each classification bearing different letters are significantly different (P<0.05, or P< 0.01). [1]Treatments: G1 = control, G2 = OTA 1 mg/kg diet, G3 = chamomile 0.5 g/kg diet, G4 = chamomile 1 g/kg diet, G5 = OTA 1 mg/kg diet + chamomile 0.5 g/kg diet, G6 = OTA 1 mg/kg diet + chamomile 1 g/kg diet.

G4 showed a nonsignificant reduction in total yeast and mold count levels and a significant (P<0.05) reduction in *E. coli,* coliforms, *Salmonella*, and *Enterococcus* count compared to the other groups. Moreover, the same group revealed a significant (P<0.001) elevation in levels of total bacterial count and lactic acid bacteria. In addition, G2 showed a significant (P<0.05) elevation in *E. coli*, coliforms, *Salmonella*, and *Enterococcus* count compared to G1.

OTAs are the most well-known food and feedstuff pollutants due to their potential to induce adverse health effects and economic losses [39, 40]. The dose rate and the length of exposure both play a role in determining the seriousness of symptoms and the clinical signs associated with OTA toxicity. The most prominent symptoms that birds display include weakness, a reduced FCR, impaired growth, compromised egg and feather quality, increased mortality rates, and elevated weight of internal organs such as the liver, spleen, pancreas, proventriculus, gizzard, and testes in male birds [41].

Studies displayed that OTA hurts laying hens' ADG, ADFI, and productivity [42]. In the current investigation, quail given a diet that contaminated OTA showed a statistically significant reduction in their LBW and BWG. The reduction in quail body weight caused by ochratoxicosis was consistent with the findings of several earlier studies in broilers that used dietary OTA supplementation at 567 ppb [43], 0.5 to 2 parts/10^6 [44–46]. OTA negatively impacts the digestive tract, causing a decrease in feed absorption and body weight and weight gain [45]. In contrast, Prior et al. [44] revealed that the decrease in body weight observed during ochratoxicosis was not primarily caused by the direct impact of OTA. However, it was attributed to the reduced consumption of feed, which subsequently resulted in a decline in total serum proteins or hypoproteinaemia. Elaroussi et al. [13] reported that broilers' decreased feed

consumption, FCR, and body weight may have resulted from their elevated serum T3 and decreased T4. The findings of our study showed that the inclusion of chamomile as a dietary supplement for growing quails resulted in a significant rise in live body weight at 5 weeks of age compared to the control group.

Furthermore, quails in G3 and G4 exhibited increased BWG and reduced feed conversion ratio. Our findings contrast the findings of Dada et al. [47], who noted no positive effects in growing quail at chamomile concentrations of 0.002 and 0.004% in feed and 0.0018 and 0.0036% in water. This lack of favorable effects could be attributed to the low dosages in their study. Moreover, including chamomile at different concentrations (0.25, 0.50, 0.75, and 1%) showed a significant reduction in broiler chickens' final body weight and weight gain [48]. According to McCrea et al. [49], the active constituents in chamomile flowers can potentially prevent the proliferation of unfavorable intestinal microorganisms. These compounds have antimicrobial, antifungal, and anti-inflammatory effects, similar to what probiotics do in the gut, which may improve nutrient absorption, preserve the normal microbiota, and inhibit the excessive spread of disease-causing bacteria in the gut [50]. According to Abaza et al. [51], chamomile's antimicrobial, antifungal, and anti-inflammatory properties can potentially enhance productive efficiency. Moreover, chamomile enhances the activity of thyroxin hormones, which accelerates food metabolism and raises body weight [52]. However, Tenório et al. [53] observed that the performance of the birds was not significantly affected by the chamomile extract.

The current findings indicate that OTA has detrimental effects on carcass traits, as evidenced by a decrease in carcass and giblet percentages and a decrease in the relative weight of some organs, such as the gizzard and heart. The observed reduction in growth may be due to several factors, including a decline in food intake, the redirection of nutrients, and a decrease in the synthesis of proteins necessary for the regeneration of organs affected by OTA poisoning. According to our results, we only observed statistically significant differences ($P<0.05$) in liver weights among carcass features, and the G2 revealed a significant increase in liver weights compared to the other groups. Our results agree with Stoev [54], who reported many pathological changes were observed in the liver of chicks treated with OTA, including granular deterioration, swelling, and infrequently fatty modifications of the liver cells.

The current study demonstrates that exposure to OTA in a quail diet significantly reduced total protein, albumin, and globulin levels compared to control and other supplements. Moreover, OTA caused a significantly high level of liver enzyme activity (AST and ALT) and LDH, creatinine, uric acid, and urea levels. The increased levels of liver enzymes can be attributed to tissue damage and the subsequent release of enzymes into the bloodstream [55]. Our findings were consistent with Sakhare et al. [56], who documented that OTA elevated uric acid levels and creatinine in broiler chicks. Moreover, exposure to OTA resulted in renal damage and increased uric acid and creatinine levels [45]. Incorporating chamomile as a dietary supplement for growing quails in the current study led to a significant increase in total protein, albumin, and globulin levels compared to control and other supplements.

Furthermore, chamomile supplementation for growing quails resulted in a significant decrease in the levels of liver enzymes (AST and ALT), LDH, creatinine, urea, and uric acid compared to the other groups. Our result disagrees with Akbari et al. [57], who observed that broilers fed with *Matricaria chamomilla* 0.6% and 1.2% exhibited significantly higher uric acid levels than control. Furthermore, laying Japanese quail that received chamomile supplementation significantly increased total protein and albumin serum levels and reduced glucose levels [58]. There were no variations in the total bilirubin, direct bilirubin, creatinine, and ALT levels compared to the control group. The current findings match El-Galil et al. [24], which examined the impact of including chamomile powder into the diet on serum biochemical markers in laying Japanese quail. Additionally, adding chamomile to the diet of Japanese quails at a concentration of 0.3% led to a significant rise in total protein and globulin levels and a decrease in cholesterol [59].

The current study revealed that when quail were fed a diet containing OTA, total cholesterol, triglyceride, LDL, and VLDL were significantly elevated. Our findings were in contrast with those reported by Abo El-Fetouh et al. [60], which indicated that OTA caused a significant reduction in the levels of serum total fat, cholesterol, and triglycerides in ducks and with the finding reported by Elaroussi et al. [13], who observed that OTA induces a decrease in total lipid, triglyceride, and cholesterol levels in chickens. The trial conducted by Schaeffer et al. [61] showed a decrease

in blood total lipid, cholesterol, and triglyceride levels in broiler chickens exposed to OTA. In contrast, chamomile supplementation to the quails' diet revealed a significant decrease in total cholesterol, triglyceride, LDL, and VLDL levels. Our findings demonstrated that OTA exposure in growing quail led to a notable reduction in SOD, TAC, and glutathione transferase (GST) while concurrently causing a significant elevation in the levels of MDA. However, the administration of the chamomile resulted in an increase in SOD activity and TAC, along with a significant decrease in MDA levels, as also observed by other authors [62, 63]. The results of our findings also agree with Sinha [64], who indicated that OTA induced reductions in SOD and CAT activity and an increase in the level of MDA in rats [64], and Sohail et al. [65]; who found a modification in the antioxidant enzyme levels following the supplementation of anti-OTA.

Our findings demonstrated that the administration of OTA revealed a significant reduction in IgM, IgG, IgA, and complement 3 levels. According to Ruan et al. [66], OTA has been found to lower the synthesis of the anti-inflammatory cytokine IL-10 and reduce the IgA concentration in poultry jejunum. Likewise, Tong et al. [67] found that the mRNA expression of IL-1β and tumor necrosis factor α was elevated, and the phosphorylation of nf-kB was induced in one-day-old broiler hens fed 50 μg of OTA/kg body weight. In addition, the administration of OTA to broilers at a concentration of 4 parts/10^6 reduced humoral immunity. Moreover, when OTA was combined with aflatoxin at a concentration of 2 parts/10^6, both humoral and cellular immunity were shown to be lowered [16]. The immunological response of chickens following immunization against the B1 strain of Newcastle disease virus [54, 68] or the Lasota strain [69] is reduced by OTA, leading to immunity suppression at both the humoral and cellular levels. This suppression also creates an opportunity for subsequent bacterial infections [12, 46]. The present investigation showed that either 1 or 0.5 g of chamomile in the OTA diet significantly reduced or prevented certain adverse effects caused by OTA and increased levels of IgM, IgG, and IgA. The immunomodulatory effects observed in chamomile may be due to the activation of immunostimulatory abilities of macrocytes, the stimulation of immunoregulatory cells in peripheral circulation, and the enhanced susceptibility of effector cells to support signals [70].

The present study indicates that the administration of OTA revealed a significant increase in quail gut total yeast and mold count, *E. coli*, coliforms, *Salmonella*, and *Enterococcus* compared to control and other animals. According to Yang et al. [71], introducing OTA in the diet at a concentration of 50 μg/kg BW for 21 days in White Feather Broilers decreased the diversity and abundance of caecal microbiota. Several recent research studies have documented the antibacterial properties of chamomile, showing that the lactobacilli levels in the intestinal digesta were significantly increased in broilers that were administered *Matricaria chamomilla* at concentrations of 0.6 and 0.9% in their diets, in comparison to the control diet [72]. The present investigation demonstrated that including chamomile at doses of 0.5 or 1 g/kg in a growing quail diet containing OTA significantly reduced or prevented certain adverse effects of OTA on the gut microorganisms.

4. Conclusions

The administration of chamomile oil combined with OTA revealed a reduction in the negative impacts of OTA, improving the growth performance, antioxidant capacity, liver and kidney function, immune response, and gut microbiota of growing Japanese quails. Using chamomile oil up to 1 g/kg diet could also be useful in solving the problem of OTA in poultry farms.

Disclosure

No persons or third-party services were involved in the research and manuscript preparation. Moreover, no AI softwares have been used to prepare the manuscript.

Authors' Contributions

R.S.M., M.M.A., and M.A. conceptualized the study. A.I.A., M.M.E.-M., and A.S.S. proposed the methodology. A.D.C. and M.N. performed formal analysis. F.S.A.I. and M.M.A. investigated the study. A.D.C. and M.A. provided resources. M.N., A.I.A., R.S.M., and M.M.A. contributed to data curation. A.D.C., M.N., M.A., and R.S.M. wrote the original draft. A.D.C., R.S.M., and M.A. reviewed and edited the article. A.D.C. and M.A. supervised the study. A.D.C., M.A., and R.S.M. performed project administration. All authors agree to be accountable for the content and conclusions of the article.

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DETAIL

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Effect of Blending Ratio and Extrusion Operating Conditions on Nutritional Quality and Functional and Sensory Acceptability of Teff-Bulla-Based Complementary Food: A Response Surface Methodology Approach

Chewicha, Getu Weya; Muhammed Adem Abdullahi; Wudineh, Tadele Andargie; Tatek Tamiru Geletu; Satheesh, Neela.

ABSTRAK (ENGLISH)

The purpose of this study was to identify possible locally accessible cereals, tubers, and legumes for use in the extrusion of teff, bulla, and haricot beans to prepare inexpensive supplemental foods. This study used central composite rotatable design (CCRD) to assess the effects of specific processing parameters on response variables such as proximate composition and physical, functional, and sensory qualities of produced complementary food. The processing parameters included haricot bean blending ratios (BR) (10–30%), feed moisture content (FMC) (15–21%), and barrel temperature (BT) (120–160°C). The proximate composition of the complementary food ranged from 9.25 to 12.36% moisture content, 7.82 to 14.04% protein, 2.1 to 3.28% ash, 1.13 to 3.93% fiber, 1.17 to 3.12% fat, 65.40 to 78.25% carbohydrate, and 338.42 to 354.86 kcal/100 g energy. The predominant minerals and antinutritional factors ranged from 2.40 to 10.37 mg/100 g, 58.70 to 146.26 mg/100 g, 0.8 to 4.74 mg/100 g, 113.21 to 325.00 mg/100 g, and 16.54 to 44.62 mg/100 g for Fe, Ca, Zn, phytate, and tannin, respectively. The blending ratio and extrusion parameters demonstrated significant ($p<0.05$) linear, quadratic, and interaction impacts on the physical and functional responses, according to analysis of variance (ANOVA). Mothers and caregivers largely approved all the trial porridges. The best blending and extrusion parameters, according to numerical optimization, were a BR of 28.2837%, FMC of 15.0007%, and BT of 120.001°C. The predicted responses for optimization were water solubility index (WSI) (32.4462 g/g), water absorption index (WAI) (3.69849%), viscosity (2016.47 cP), protein (14.046%), and energy (348.034 kcal/100 g) with a desirability value of 0.856. This research leads us to believe that producing complementary meals with excellent nutritional value for developing nations may be achieved by evidence-based selection of locally accessible plant-based components.

TEKS LENGKAP

1. Introduction

Protein-energy malnutrition (PEM) and micronutrient deficiencies are prevalent among infants and children residing in impoverished communities in the developing world, with a particular emphasis on Sub-Saharan African nations [1]. In Ethiopia, the persistent high rates of child malnutrition pose significant public health challenges [2]. According to the 2019 Ethiopian Mini Demographic and Health Survey (EMDHS) conducted by the Ethiopian Public Health Institute (EPHI) and ICF, 37% of children under the age of 5 exhibit stunted growth, with 12% classified as severely stunted. In addition, 7% of children are wasted, including 1% classified as severely wasted. Moreover, 21% of all children in Ethiopia are deemed underweight, with an additional 6% classified as severely underweight (Ethiopian

Public Health Institute (EPHI) and ICF, 2019). The challenge of malnutrition is particularly pronounced during the complementary feeding period (6–24 months), marking this phase as a “critical window” for fostering optimal infant growth [3]. Urgent measures are warranted to introduce external sources of “complementary foods” into the child’s diet during this crucial developmental stage. These complementary foods play a vital role in supporting children’s growth and development by addressing nutritional and developmental needs beyond what breast milk alone can provide [4].

Numerous studies indicate that a significant proportion of complementary foods consumed by infants across various regions lack essential macronutrients and micronutrients. This nutritional deficiency is a significant contributor to malnutrition, a severe public health concern prevalent in developing nations such as Ethiopia [3, 5, 6]. PEM and micronutrient malnutrition can be reduced by improving the nutrient status of staple foods. In order to address these nutritional concerns, it is preferable to identify locally available, sustainable, and relevant food sources. As a result, teff, bulla, and haricot beans have been chosen to prepare complementary foods for infants to meet their recommended nutrient intake, particularly for protein, iron, calcium, and zinc.

Teff, an ancient and nutritionally rich grain, serves as a primary cereal in Ethiopia, contributing to 19 percent of the total cereal production. In Ethiopian cuisine, teff flour is a fundamental ingredient used to create staple foods such as injera (flatbread) and porridge and incorporated into homemade alcoholic beverages [7]. Despite being underutilized historically, teff has garnered international attention in recent years owing to its promising nutritional characteristics [8, 9]. Teff is rich in nutrients, and it has elevated levels of minerals such as calcium, magnesium, iron, and folate, along with essential fatty acids, fiber, and beneficial phytochemicals such as polyphenols and phytates [7, 10]. Beyond its traditional use in injera, teff can be diversified into various forms including instant porridge to elevate its nutritional value. This versatility positions teff to potentially contribute significantly to both Ethiopian food security and agriculture.

Ensete ventricosum, commonly known as enset, is a native Ethiopian plant often referred to as the “fake banana” due to its striking resemblance to the banana plant [11]. Bulla, a versatile ingredient in traditional Ethiopian cuisine, is derived from the enset plant by extracting pulp from the leaf sheath, peduncle, and grated corm. The process involves squeezing liquid, rich in starch, from the pulp, allowing the starch to concentrate into a white powder, which is later rehydrated with water [12]. Given enset’s abundance in calcium, zinc, and iron, it serves as a staple food for a significant portion of Ethiopia’s population, playing a crucial role in the country’s food security [13]. While enset is a valuable source of starch, it is relatively low in protein content, necessitating dietary protein supplementation.

Legume flour stands out not only as a valuable protein source but also holds promise for creating novel complementary foods enriched with essential vitamins and minerals. Haricot beans (*Phaseolus vulgaris* L.) play a significant role in the nutrition and economic landscapes of both rural and urban areas in Ethiopia [14, 15]. The proximate composition of haricot bean protein, crude fat, crude fiber, ash, carbohydrates, and moisture content falls within the ranges of 17.96–22.07 g/100 g, 1.27–3.02 g/100 g, 4.66–5.95 g/100 g, 2.86–4.26 g/100 g, 56.53–61.56 g/100 g, and 9.08–11.00 g/100 g, respectively [16]. Haricot beans exhibit several advantageous qualities, including high nutritional value, extended storage capability, and relatively low cost compared to animal products [17]. However, antinutritional factors and inadequate community awareness of the nutritional benefits contribute to the low consumption of haricot beans for food purposes. Therefore, the best way to address this problem is by germination, which unlocks important opportunities for using flour in different food systems by providing a unique combination of high nutrition and gluten-free [18]. As a result, the inclusion of haricot beans in complementary foods is strongly recommended whenever possible [17].

As outlined by Klang et al. [19], the combination of cereals, legumes, and tubers results in superior nutrient content compared to these products that are consumed separately. Low-income households encounter a challenge where high-nutrition food supplements provided by the food industry are deemed luxuries beyond their reach. Consequently, identifying affordable and locally accessible sources of high protein and micronutrient materials becomes a crucial undertaking in this context.

Extrusion plays a crucial role in the preparation of complementary foods vital for the nutrition and development of

infants and young children. In recent times, extrusion processing has garnered considerable attention as a manufacturing technique for crafting complementary foods, owing to its capacity to enhance nutrient bioavailability, improve functional properties, and achieve favorable sensory attributes [3]. The operational parameters of extrusion, including temperature, moisture content, screw speed, and die design wield significant influence over the physicochemical and nutritional traits of extruded products [20]. The systematic exploration of these conditions using response surface methodology (RSM) enables a comprehensive understanding of variable interactions, facilitating the development of products characterized by superior nutritional quality and sensory acceptability.

Comprehending the impact of BR and extrusion operating conditions on the nutritional quality, functional properties, and sensory acceptability of complementary foods is pivotal to crafting nutritious and appealing products. Utilizing RSM, researchers can systematically assess and fine-tune these factors, thus facilitating the development of complementary foods tailored to meet the precise dietary requirements of infants and young children.

The aim of this paper was to conduct a thorough assessment of the impacts of BR and extrusion processing conditions on the proximate composition, physical attributes, functional properties, and sensory acceptability of extruded complementary food created from locally sourced materials (bulla, teff, and haricot bean).

2. Materials and Methods

2.1. Raw Materials Procurement and Preparation

The haricot bean (Nasir variety released in 2003) was acquired from the Melkasa Agricultural Research Center (MARC), while the teff (Eragrostis teff (Zucc.) Trotter) “Felagot” variety was sourced from the Debre Zeit Agricultural Research Center (DZARC). Bulla powder was obtained from the open market in the Worka woreda of the West Arsi zone. The teff underwent manual cleaning to eliminate damaged grains, stones, dust, light materials, glumes, stalks, undersized and immature grains, and other impurities. The grains were subsequently milled into fine flour using a small-scale commercial mill, following the procedure outlined in [1]. Postgrinding, the flour underwent sifting through a 710 μm test sieve [21]. The sifted flour was then sealed in polyethylene plastic bags and stored at room temperature (27 ± 2°C) for subsequent laboratory analysis until the extrusion cooking experiment was conducted. Haricot beans underwent a cleaning process and were germinated for 48 hours, followed by drying at 60°C for 8 hours, as detailed in the method outlined by Wodajo and Emire, [18]. Germination was employed to enhance mineral content while reducing phytate, trypsin, and condensed tannin levels [22, 23]. In addition, this process led to improvements in crude protein, carbohydrate, and energy values [18, 23, 24]. Subsequently, the germinated beans were dehulled, milled using a mechanical local miller, and the resulting flour was sifted through a 710 μm sieve. The sifted flour was then packed into plastic bags and stored at ambient temperature in the food process laboratory. The bulla was solar-dried until completely dehydrated, then milled using a mechanical local miller, sifted through a 710 μm mesh sieve, and similarly sealed in polyethylene plastic bags for storage at ambient temperature in the food process laboratory.

2.2. Composite Flour Formulation

Following preliminary trials, teff flour and bulla powder were mixed in a fixed ratio of 75 : 25% (Tolesa, 2014) to establish a consistent base for subsequent formulation development. Meanwhile, the haricot bean blending compositions varied at 10%, 20%, and 30% (Table 1). The three distinct flour mixtures underwent a thorough mixing in a ribbon-type blender (Model AB, Alvan blanch Type ribbon blender, England, 2007) for a duration of 20 minutes. The resulting blends were then packed into polyethylene bags and stored in a refrigerator at 4°C until they were ready for the extrusion process.

Table 1

Independent variables and their levels used in the present study.

Variables	Variables coded levels

$-\alpha$	−1	0	1	$+\alpha$	Blending ratio (%) A
3.15	10	20	30	36	Feed moisture (%) B
12.95	15	18	21	23.05	Barrel temperature (°C) C

2.3. Design of Experiments

The investigation employed response surface methodology with a central composite experimental design to assess the impact of the BR of haricot bean (A) and extrusion-cooking parameters, including moisture content (B) and barrel temperature (C). Each independent variable was determined through preliminary trials and existing literature. The central composite rotatable design (CCRD) comprised 20 experimental runs, incorporating 6 central points and 6 axial points (α=±1.682) for a 2^3 full factorial design. CCRD was chosen due to its versatile approach for understanding the overall influence of multiple factors. The actual factor levels were varied across an equidistant range defined by $-\alpha$ and $+\alpha$.

The independent variables encompassed the percentage of teff: bulla and haricot bean blend ratio (10–30 g/100 g blend), BT (120–160°C), and FMC (15–21%). The upper and lower levels of these variables were established based on insights from various cereal-legume composite extrusion studies and preliminary observations. The data obtained from the study were fitted to a second-order polynomial regression model [25] as follows:(1)Y=b0+b1A+b2B+b3C+b11A2+b22B2+b33C2+b12AB+b13AC+b23BC,where *A*, *B*, and *C* represent the BR feed composition (haricot bean flour), feed moisture, and barrel temperature, respectively. The variables b0, b1, b2, and b3 denote the regression constant and linear regression terms. The terms b11, b22, and b33 correspond to quadratic regression terms, while b12, b13, and b23 signify the cross-product regression terms.

2.3.1. Extrusion Cooking and Adjustment of Process Parameters

The extrusion process was executed by configuring the process parameters according to the experimental design, utilizing a twin-screw extruder (model Clextral, BC-21 No 124, Firminy, France). The prepared samples underwent extrusion at all combinations of the specified operating conditions, including blend ratios, barrel temperature, and feed moisture.

Before commencing the extrusion process, essential calibration and adjustments were made to the flour feed rate and water flow rate. Extrusion was then carried out at a constant flour feed rate of 60 g/min (3.6 kg/h) and a screw speed of 200 revolutions per minute (rpm), which remained consistent throughout the experiment [26].

The BT of the three zones in the extruder barrel was varied between 120, 135, and 150°C. These temperature ranges were chosen based on the outcomes observed in preliminary extrusion trials. Temperature measurements were conducted using a thermocouple positioned deep into the barrel walls for each zone. The signals from these measurements were sent to controllers that regulated the corresponding heaters [27, 28].

The moisture content of the dough within the barrel underwent adjustment by manipulating the water injection rate of the pump. Water was introduced into the extruder near the material feed port. The feed moisture was modulated by injecting water at levels of 14%, 16%, and 18% (chosen based on findings from preliminary extrusion trials) into the mixes, while maintaining a consistent material feed rate of 60 g/min, following hydration (2) and [25].(2)Wa=Swm−m0100−m,where Wa is the weight of water added (g); Sw is the sample flour weight (g); m0 is the original flour moisture content (% weight base); and *m* is the required dough moisture level (% weight base).

Samples from the extrusion process were gathered once the process parameters stabilized to a steady state.

Steady state was confirmed when there was no observable fluctuation in torque and die pressure. Subsequently, the extruded products were labeled, allowed to cool for 30 min at room temperature (27 ± 2°C), sealed in plastic bags, and stored at room temperature (27 ± 2°C). These samples were reserved for analysis of physical, functional, and nutritional properties, as well as subsequent sensory evaluation. Additional samples were processed for the preparation of complementary food following a designated procedure.

2.4. Complementary Food Preparation

The extruded samples underwent oven drying at 105°C for 15 min, followed by milling for use in the preparation of gruels, as outlined by Mezgebo et al. [29]. Complementary porridges were prepared in a cooking vessel, combining 50 g of flour with 150 mL of water (Figure 1). The mixture was cooked at 96°C for a duration of 10 min.

[figure(s) omitted; refer to PDF]

2.5. Quality Evaluation of Complementary Food

2.5.1. Proximate Composition, Mineral, and Antinutritional Factor Analysis Proximate Composition

The moisture content of the sample was assessed using the hot air oven method (method no. 925.10), while the protein content was determined by applying a conversion factor of 6.25 through the Kjeldahl crude protein analysis method (method no. 979.09). The determination of crude fat employed the Soxhlet extraction method (method no. 2003.06). Crude fiber was determined using the nonenzymatic gravimetric method (method no. 920.168), and ash content was determined by the gravimetric method (method no. 923.03), following the AOAC official methods [20]. The total percentage of carbohydrate content was determined by the difference method, as outlined by Adem et al. [25]. Gross energy was calculated according to the method developed by Osborne and Voogt [30].

2.5.2. Mineral Content Determination (Fe and Zn)

Selected mineral quantities of the prepared extrudates were determined by ICP-MS (inductively coupled plasma mass spectrometer) by using AOAC [20], a standard method. The methods were succinctly outlined as follows: crucibles and glassware underwent washing with 6N HCl and 10% nitric acid. Afterward, crucibles were dried for 30 min at 100°C, cooled in desiccators, and meticulously weighed. Approximately 2.5 g of the sample was measured in the predried, ignited crucibles and ashed at 550°C until complete ashing. The resultant ash was moistened with deionized water, evaporated on a hot plate, and subjected to an additional 30 min of ashing at 550°C. Deionized water and concentrated HNO_3 were introduced, and the sample underwent another 30 min of ashing at 550°C. The cooled ash was weighed, treated with 6N HCl, and evaporated on a hot plate. Subsequently, the ash was treated with 3N HCl, brought to a boil, cooled, and filtered into a graduated flask. The crucible was rinsed with 3N HCl, and the rinsings were filtered into the flask. Finally, the solution underwent ICP-MS analysis for iron and zinc at wavelengths of 259.93 nm and 213.85 nm, respectively.

The calcium content was determined using the official method outlined in AOAC [20] for flame atomic absorption spectrophotometry. In this process, a 1.0 g sample was treated with 10 mL of concentrated HNO_3 and 4 mL of 70% $HClO_4$. The resulting solution was evaporated to 7 mL and then transferred to a 50 mL volumetric flask. To this solution, 1 mL of $SrCl_2.6H_2O$ was added, and the flask was filled to volume with distilled water. Subsequently, the solution was sprayed into an atomic absorption spectrophotometer at 422.7 nm to determine the calcium concentration. For calibration purposes, calcium standards ranging from 0 ppm to 30 ppm were utilized.

2.5.3. Antinutritional Factors' Analysis

The phytate content was assessed by utilizing the modified method described by Latta and Eskin [31]. Fresh samples underwent extraction with HCl, and the supernatant was collected postcentrifugation. The Wade reagent facilitated a colorimetric reaction with phytate, and the UV-VIS spectrophotometer measured the absorbance of the sample solution at 500 nm. A standard curve, derived from standard solutions of phytic acid, provided the basis for calculating the phytate content of the sample, utilizing the slope and intercept.

For the determination of tannin content, the modified method detailed by Burns [32] was employed. Approximately 2 g of the sample was extracted with 1% HCl in methanol for 24 hours at room temperature (27 ± 2C) with mechanical shaking. Following centrifugation, the supernatant was mixed with vanillin-HCl reagent. D-catechin served as the standard for condensed tannin determination. A calibration curve was established using standard solutions of D-

catechin, and the slope and intercept were utilized to compute the tannin content of the sample.

2.5.4. Physical and Functional Properties Analysis

Bulk density (BD) was determined by a method illustrated by Kavitha and Parimalavalli [33] by using the following equation:(3)bulk density BD=weight of the sample volume occupied.

The viscosity of the prepared complementary porridge was determined by combining 15 g of flour with 100 mL of water in a glass beaker. This mixture was then cooked at 96°C for 10 min. The resulting gruel was transferred to a water bath maintained at 40°C (heating temperature), and its viscosity was measured at the same temperature. A Brookfield Viscometer (model: DV-II Rheometer V2.0 RV; Middleboro, Massachusetts, USA) was employed for viscosity measurement. In the viscometer beaker, the cooked gruel was poured and cooled to 40°C, and viscosity (measured in centipoises, cP) was determined using spindle number 5 at a shear rate of 200 revolutions per minute (rpm). The average reading of maximum viscosity and minimum viscosity was recorded within 2 min, following the procedure outlined by Tizazu and Emire [34].

The water absorption index (WAI) was assessed following the procedure outlined by Adem et al. [25]. Approximately 2.5 g of the finely ground sample was suspended in 30 mL of distilled water within a tarred 50 mL centrifuge tube and shaken for 30 min. Afterward, the sample underwent centrifugation for 10 min at 3000 rpm. The transparent supernatant from the centrifugation was transferred into a predried and weighed glass beaker for the determination of the water solubility index (WSI). The gel residue remaining in the centrifuge tube was weighed, and the WAI was subsequently calculated.(4)WAI=WgWds,where WAI is the water absorption index, Wg is the weight of the gel, and Wds is the weight of the dry sample.

The supernatant preserved from the WAI measurement was evaporated at 96°C overnight. The WSI is calculated as (5)WSI=WrWs∗100,where WSI is the water solubility index (%), Wr is the weight of the residual supernatant after evaporation (g), and Ws is the weight of the sample (g).

The oil absorption capacity was calculated using the method in [35] with minor modifications. The OAC is calculated as(6)OAC=V1−∗ρoilWs∗100,where OAC is the oil absorption capacity (%), V1 is the initial volume of oil added (mL), V2 is the volume of supernatant after centrifugation (mL), ρoil is the density of sunflower oil, which was taken as 0.895 g/ml, and Ws is the weight of the sample (g)

2.5.5. Sensory Evaluation

The techniques outlined by Forsido et al. [3] were employed to evaluate the sensory acceptability of porridge samples. A group of fifteen untrained panelists, consisting of community-level mothers with their children, was selected from the residents of Bahir Dar City in accordance with the sensory assessment protocol by Adegbanke et al. [4]. Just before the taste session, the panelists underwent an orientation on the sensory assessment protocol. The panelists were then instructed to convey their perceptions of the products by assigning scores to the sensory attributes, including aroma, taste, color, flavor, texture, and overall acceptability. This was performed by using a nine-point hedonic scale, where 9 represented the highest score (i.e., like extremely) and 1 indicated the lowest score (i.e., dislike extremely).

2.6. Statistical Data Analysis

The statistical analysis was conducted based on the set of 20 treatments, focusing on the extrusion independent variables such as teff-bulla:haricot bean (T-B:H) blend ratio, feed moisture content, and barrel temperature. Design-Expert v11 (Stat-Ease Inc., Minneapolis, MN, USA) was utilized for the design of the experiment, data analysis, and the generation of second-degree polynomial models. Analysis of variance (ANOVA) was employed to pinpoint significant terms within the models, following the approach outlined by Myers et al. [36]. The adequacy of the model was assessed using the coefficient of determination (R2), adjusted *R*-squared (Adj R2), predicted *R*-squared (Pred R2), and the p value of the *F*-statistic for the lack-of-fit test. For each response variable, a response surface plot was created from the regression equations, holding one variable at its middle value while changing the other two variables.

2.7. Optimization of Processing Parameters

Numerical optimization and interactive graphs were employed to optimize multiple input variables and responses in

the production of teff-bulla: haricot bean complementary food. The optimal processing conditions were ascertained through numerical multiresponse optimization, allowing for a comprehensive evaluation of the extrudate's viscosity, WAI, WSI, protein content, and energy.

3. Results and Discussion

3.1. Proximate Composition of Raw and Composite Flour

The proximate composition of bulla flour, teff flour, haricot bean flour, and blend flour is shown in Table 2.

Table 2

Proximate composition, minerals content, phytate, and tannin of raw materials and composite flours.

Code	Proximate composition (%)							Minerals content and antinutrients				
Ash (%)	Crude fat (%)	Crude fiber (%)	Moisture (%)	Crude protein (%)	CHO (%)	Gross energy (kcal/100 g)	Iron mg/100 g	Calcium mg/100 g	Zinc mg/100 g	Phytate mg/100 g	Tannin mg/100 g	UGHB
4.74 ± 0.14	5.88 ± 0.16	3.79 ± 0.14	8.20 ± 0.01	14.19 ± 0.27	63.20 ± 0.14	362.47 ± 0.16	2.4 ± 0.14	110 ± 0.16	4.4 ± 0.14	165.7 ± 0.57	44.62 ± 0.69	GHB
4.27 ± 0.14	5.85 ± 0.14	2.44 ± 0.15	8.31 ± 0.01	13.45 ± 0.29	65.68 ± 0.14	369.14 ± 0.15	3.1 ± 0.14	120 ± 0.71	3.2 ± 0.14	156.92 ± 0.81	27.19 ± 0.42	Bulla
5.20 ± 0.17	0.40 ± 0.14	1.12 ± 0.01	7.80 ± 0.16	0.90 ± 0.28	84.58 ± 0.15	345.53 ± 0.28	10.37 ± 1.70	146.26 ± 0.33	4.74 ± 0.17	325 ± 0.33	24.77 ± 0.74	Teff
6.33 ± 0.14	3.07 ± 0.13	6.90 ± 0.14	12.21 ± 0.04	6.68 ± 0.25	64.81 ± 0.14	313.59 ± 0.13	4.99 ± 0.16	58.7 ± 0.28	0.8 ± 0.14	197.06 ± 0.11	16.54 ± 0.44	BR1
5.12 ± 0.15	3.29 ± 0.08	3.49 ± 0.05	9.16 ± 0.12	11.40 ± 0.07	67.54 ± 0.03	345.37 ± 0.20	7.62 ± 0.12	77.35 ± 0.04	3.6 ± 0.14	127.12 ± 20	30.22 ± 1.8	BR2
4.84 ± 0.16	3.85 ± 0.09	3.27 ± 0.12	9.47 ± 0.05	12.76 ± 0.04	65.81 ± 0.14	348.93 ± 0.11	7.35 ± 0.01	77.16 ± 0.14	3.86 ± 0.09	122.34 ± 1.5	30.10 ± 1.40	BR3
4.89 ± 0.15	4.21 ± 0.04	3.19 ± 0.12	9.53 ± 0.11	13.11 ± 0.01	65.07 ± 0.02	350.61 ± 0.09	6.11 ± 0.12	78.23 ± 0.09	3.99 ± 0.14	113.21 ± 0.99	22.7 ± 3.20	Control (0 : 75 : 25)

UGHB, ungerminated haricot bean; GHB, germinated haricot bean; T, teff; B, bulla; BR1, 10% of haricot bean; BR2, 20% of haricot bean; BR3, 30% of haricot bean; control, 75% of bulla and 25% of teff flour.

3.1.1. Proximate Composition of Bulla Flour

The results of the proximate composition of bulla flour used for complementary food were 5.20 ± 0.170 ash, 84.58 ± 0.15 carbohydrates, 0.40 ± 0.141 crude fat, 1.12 ± 0.014 crude fiber, 7.80 ± 0.156 moisture, 0.90 ± 0.283 crude protein, and 345.52 ± 0.284 kcal/100 g gross energy. The results of the present study are in agreement with Shufa and Taye [37], who reported 2.85%, 5.56%, 0.16%, 3.26%, 1.14%, 92.59%, and 376.36 (kcal/100 g) moisture, crude fiber, crude fat, ash, crude protein, carbohydrates, and energy, respectively. The results of this study suggest that roots and tubers are not abundant sources of essential macronutrients, and the food sources from root crops should be cosupplemented with legumes in complementary food formulations.

3.1.2. Proximate Composition of Germinated Haricot Bean Flour

The proximate composition of germinated haricot bean flour was determined to be 4.27% ash, 64.31% carbohydrates, 5.84% crude fat, 2.44% crude fiber, 8.31% moisture, 13.44% crude protein, and 364.85 kcal/100 g gross energy. These findings are corroborated by the work of [18].

In this study, the composite flour with 30% germinated haricot bean (BR3) had the highest protein content (13.11%). The present study's results showed that the protein content of composite flour increases with an increase in the proportion of germinated haricot bean flour. This is likely due to the high amount of protein in germinated haricot bean flour and teff flour. Germinated haricot bean flour is used for extrusion because germination improves the digestibility and bioavailability of macronutrients [17, 18].

3.1.3. Proximate Composition of Teff Flour

The proximate composition (dry weight basis) of teff flour was determined as 12.21% moisture, 6.33% total ash, 6.677% crude protein, 3.07% crude fat, 6.90% crude fiber, 64.88% carbohydrates, and 313.22 kcal/100 g. The protein and carbohydrate contents were in the range of the work reported by Satheesh and Fanta [38]. The ash result is greater than that of the results obtained from *kunco* teff, as reported by Araro et al. [17]. In addition, the ash, fiber, and fat contents of teff samples considered in this study showed greater values than the report of Yimer and Bultosa [7].

Overall, the study results suggest that germinated haricot bean flour and teff flour are good sources of essential macronutrients for complementary food formulations. However, it is important to note that the roots are not a preferential source of essential macronutrients and should be supplemented with legumes in complementary food formulations.

3.1.4. Proximate Composition of Composite Flour

The information on the composite flour proximate composite is presented in Table 2. As expected, protein quantity in the composite flour increased and ranged from 4.71% to 13.11% as the BR increased (germinated haricot bean flour). The higher protein content of composite flours might be accredited to biosynthesis during the germination of haricot beans [39].

The crude fat content of composite flour varied from 2.86% to 4.21%. The crude fat levels in the composite flours were linearly raised with the inclusion of haricot bean flour. This might be due to the inclusion of haricot beans, which have a higher fat content than teff flour.

Around 62.77–78.347% of the carbohydrate was determined in the composite flours considered in this study. However, as the level of BR increased, the carbohydrate quantity statistically ($p<0.05$) decreased in the composite flour (62.77–66.54%) compared to the control flour (78.347%). The energy values of the composite blends ranged from 345.37 to 350.61 kcal/100 g.

3.2. Minerals, Phytate, and Tannin Contents of Raw Materials and Composite Flours

The iron, calcium, zinc, phytate, and tannin of raw materials to produce bulla-teff: haricot bean complementary foods are shown in Table 2.

Minerals are important for human tissue function, and their existence in plants can have a positive impact as a source of essential nutrients or even active principles, or a negative impact due to the accumulation of potentially harmful elements at high concentrations. The Fe in the blended flour ranged from 6.11 mg/100g to 7.62 mg/100 g, which is in line with the recommended daily allowance (RDA) of Fe (7 mg) for children aged under 3 years [40]. As

the haricot bean ratio in the blended flour increased, the Fe content decreased significantly [41]. This trend may be ascertained by the fact that tef contains a higher amount of Fe than haricot bean (Table 2).

The Ca and Zn contents of the composite flours ranged between 77.16 to 78.23 mg/100 g and 3.60 to 3.99 mg/100 g, respectively. The Ca and Zn contents in the flour blends were relatively greater than the value obtained for the control. The rise in Ca and Zn of the blended flour may be attributed to the teff and haricot beans, which are excellent sources of Fe, Ca, and Zn [14, 17, 23]. Similarly, researchers also reported significant increases in the mineral compositions (especially Fe, Ca, and Zn) of complementary foods developed from teff flour blends [1, 7, 21, 29, 42].

The phytate quantity of the blended composite flour reduced from 199.25 ± 0.85 to 113.21 ± 0.99 mg/100 g as the percentage of germinated haricot bean ratio raised. This may be attributed to teff that contains a high amount of phytate, and germination of the beans also significantly reduces the phytate content [18]. The results trend of the present study corroborate with the findings of Mezgebo et al. [29], who reported the complementary food from red teff flour, malted soybean flour, and papaya fruit powder.

The tannin content of haricot bean flour was greater than teff and bulla flour. However, the germination of haricot beans reduced the tannin content significantly. The trends in the reduction of tannin content can be accredited to the activity of polyphenol oxidase produced by fermenting microflora [18].

Overall, the findings of this study suggested that the germinated haricot bean flour can be used to produce composite flours with improved mineral compositions and reduced phytate content. These composite flours can successfully be used to prepare value-added complementary foods, potentially contributing to satisfying the nutritional needs of young children.

3.3. Effect of Blending Ratio and Extrusion Operating Conditions on Extrudate Proximate Composition

The moisture content of the extruded complementary foods varied from 9.25% to 12.36% (Table 3), and this moisture content is in agreement with the suggested moisture content limits (<14%) for long-term storage and safety of food according to Marcel et al. [43] and Singh et al. [44]. The greater moisture contents of the prepared complementary food in the study may be attributed to the processing methods used in the study (soaking and germination), which can increase water retention and inclusion of starchy ingredients (*bulla*) [45]. A similar result was also reported by Adeoti and Osundahunsi [39], who developed complementary food from maize and fermented and germinated *Moringa oleifera* seed flour.

Table 3

Proximate, physical, and functional responses of the extruded complementary food samples.

Run	A: BR (%)	B: FMC (%)	C: BT (°C)	Mc (%)	Protein (%)	Ash (%)	C. fiber (%)	C. fat (%)	CHO (%)	Gross energy (kCal/100 g)	BD (g/ml)	WAI (g/g)	WSI (%)	OAC (g/g)	Viscosity (cP)
1	20	18	173.636	10.24	10.28	2.80	2.44	2.76	71.48	351.88	0.75	4.06	31.71	2.65	2633.00
2	20	18	106.364	10.77	13.05	2.48	2.06	1.76	69.89	347.55	0.68	3.79	30.57	1.65	2242.50
3	30	15	160	10.83	13.80	2.88	2.97	3.12	66.40	348.88	0.76	4.20	31.22	2.80	1986.00
4	20	18	140	10.62	12.84	2.78	2.02	1.95	69.80	348.10	0.74	3.98	25.36	1.75	2422.00

5	20	12.9546	140	10.28	12.99	2.80	1.76	2.24	69.93	351.88	0.63	3.75	19.97	1.70	2214.00
6	30	15	120	10.78	14.05	2.84	2.91	2.74	66.69	347.59	0.74	3.90	31.93	1.65	1976.00
7	20	18	140	9.95	12.01	2.74	2.38	2.16	70.77	350.49	0.71	4.04	29.45	1.80	2192.00
8	20	18	140	10.35	10.75	2.44	2.21	2.16	72.09	350.80	0.72	3.93	28.96	2.15	2312.00
9	10	21	160	9.96	9.09	2.47	1.85	1.42	75.21	349.98	0.63	4.58	19.97	1.60	2977.00
10	30	21	120	12.36	13.77	3.03	3.02	2.42	65.40	338.43	0.74	3.80	28.96	2.90	1882.00
11	20	18	140	10.31	10.55	2.67	1.91	1.99	72.57	350.41	0.73	4.26	19.90	1.95	2107.00
12	20	18	140	10.46	10.05	2.55	1.86	2.33	72.75	352.14	0.71	4.14	30.05	2.35	2192.50
13	36.8179	18	140	11.09	13.88	3.14	3.54	2.95	65.41	343.70	0.78	3.88	34.38	3.25	2007.00
14	10	21	120	10.06	9.80	2.46	1.85	1.83	74.00	351.65	0.72	4.45	10.06	1.55	2771.00
15	20	18	140	10.55	10.60	2.48	1.81	1.96	72.60	350.45	0.66	4.11	23.97	1.85	2112.00
16	10	15	160	9.25	8.00	2.48	1.69	1.45	77.13	353.59	0.63	4.44	19.97	1.50	3114.00
17	20	23.0454	140	10.75	10.17	2.47	2.38	1.32	72.91	344.19	0.66	4.81	11.02	2.45	2041.00
18	3.18207	18	140	9.45	7.82	2.17	1.14	1.17	78.26	354.86	0.70	4.79	10.02	1.40	3234.00
19	10	15	120	9.80	8.01	2.45	1.25	1.42	77.06	353.06	0.71	4.02	18.40	1.25	3016.00

20	30	21	160	10.97	13.48	3.28	3.93	2.50	65.84	339.76	0.75	3.90	30.61	3.15	1965.00

BR, blending ratio; FMC, feed moisture content; BT, barrel temperature; Mc, moisture content; C. fiber, crude fiber; C. fat, crude fat; CHO, carbohydrate; BD, bulk density; WAI, water absorption index; WSI, water solubility index; OAC, oil absorption capacity.

The ANOVA results indicate that the linear terms of BR, feed moisture content, and BT are statistically significant ($p < 0.0001$, $p=0.0068$, and $p=0.0206$, respectively) (Table 4). This implies that these factors have a significant linear relationship with the response variable. However, the interaction and quadratic terms of BR, FMC, and BT were not statistically significant ($p<0.05$). This implies that these factors do not have a significant nonlinear relationship with the response variable, or that the interaction between these factors is not significant.

Table 4

Regression coefficients and ANOVA of the quadratic model for proximate, physical, and functional attributes of complementary food.

Coefficient	Responses											
Mc	Protein	Ash	C. fiber	C. fat	CHO	Gross energy	BD	WAI	WSI	OAI	Viscosity	b0
10.37	11.14	2.61	2.02	2.09	71.77	350.43	0.7010	4.08	26.24	1.98	2221.14	*A*
0.6318	2.22	0.2791	0.074802	0.5600	−4.44	−3.84	0.0309	−0.2441	6.98	0.5646	−449.05	*B*
0.2548	−0.1804	0.0033	0.2111	−0.1554	−0.1335	−2.65	0.0037	0.1538	−1.98	0.2388	−57.70	*C*
−0.2110	−0.4335	0.0633	0.1501	0.1299	0.3012	0.6397	−0.0115	0.1111	1.05	0.2476	77.16	*AB*
0.0937	−0.4356	0.0750	0.0398	−0.1649	0.3920	−1.66	−0.0016	−0.1040	0.5959	0.1500	33.37	*AC*
−0.0863	0.0234	0.0335	0.0655	0.1044	−0.1405	0.4709	0.0241	−0.0360	−1.32	0.1375	−26.38	*BC*
−0.1238	−0.0924	0.0230	0.0518	−0.0931	0.2345	−0.2696	−0.0031	−0.0437	1.32	−0.1375	22.62	A2
−0.0222	−0.1271	0.0348	0.1507	0.0069	−0.0431	−0.6186	0.0125	0.0779	−1.20	0.0929	152.21	B2

0.0644	0.1314	0.0281	0.0568	−0.0923	−0.1884	−1.06	−0.0205	0.0593	−3.57	0.0046	−22.09	C2
0.0609	0.1607	0.0301	0.1211	0.0764	−0.4492	−0.4664	0.0146	−0.0432	1.96	0.0311	87.60	R2
0.8251	0.8479	0.7700	0.8773	0.8063	0.9102	0.9441	0.8639	0.8070	0.9148	0.9204	0.9519	Adj R2
0.7923	0.7111	0.7269	0.8543	0.7700	0.8933	0.8937	0.7414	0.6333	0.8381	0.8836	0.9086	Pred R2
0.7025	0.2450	0.6348	0.8125	0.6564	0.8555	0.7145	0.5734	0.4341	0.7699	0.8221	0.7667	CV (%)
2.91	10.18	5.41	12.16	12.95	1.81	0.4118	3.20	4.81	12.81	10.02	5.48	*F* value
25.16	6.20	17.85	16.30	10.83	54.03	18.75	7.05	10.96	11.93	25.04	21.99	Lack-of-fit
0.2463	0.3797	0.4888	0.3339	0.0771	0.4412	0.3518	0.8441	0.0773	0.9285	0.7054	0.4086	p value

b0, constant; *A*, blending ratio; *B*, feed moisture content; *C*, barrel temperature; *AB*, *AC*, and *BC*, interactions coefficient; A2, B2, and C2, quadratic coefficient; CV, coefficient of variation; R2, determination coefficient; Adj R2, adjusted R2; Pred R2, predicted R2. ⁎Significant at $p<0.05$.

From the regression analysis, it is revealed that the coefficient of BR and feed moisture and BT have significant positive and negative effects on the moisture content of the extrudate, respectively. The increasing blending ratio of haricot beans in complementary food formulation may be associated with a corresponding rise in moisture content. This phenomenon is likely attributable to the well-documented water retention properties associated with germinated and soaked haricot beans. Similarly, the positive correlation between feed moisture content and final product moisture content aligns with the findings of Adem et al. [25], who reported a rise in the moisture of extruded snacks as the feed moisture content of maize-lupine blends increased. Conversely, the negative correlation between barrel temperature (BT) and final product moisture content is consistent with the observations of Adem et al. [25], who found that increasing barrel temperature while decreasing feed moisture content resulted in lower moisture contents in the final maize-lupine-based extruded snacks. The results of the present study are in agreement with the study of Gemede et al. [46]. However, the result is greater than the recommendation of Codex moisture content (<5%). It may be due to the temperature difference that is used for cooking and the processing techniques used (soaking and germination of raw materials).

Proteins play a crucial role in the nutritional well-being of humans, particularly in the growth and development of the body, immune defense, energy provision, and metabolic regulation. Highlighting their significance, Klang et al. [47] and Tufa et al. [48] have emphasized the essential role of proteins in these key physiological processes, especially for humans and young children. In a study assessing the protein content of 20 different extruded complementary food samples, it was found that the protein content ranged from 7.821% to 14.05% (Table 3). It is noteworthy that this range is comparatively lower than complementary foods made from a combination of red teff flour, malted soybean flour, and papaya fruit powder, as observed by Mezgebo et al. [29]. However, the current findings align

more closely with the (7.0–14.9%) protein content obtained by Mezgebo et al. [6] for complementary foods (prepared with the teff, soybean, and papaya power) consumed by 6–24 months aged children in Jimma, southwest Ethiopia. Nevertheless, the protein content in all examined samples falls within the recommended range of 15%, as advocated by the World Health Organization [40] and the National Research Council (US) Subcommittee on the Tenth Edition of the Recommended Dietary Allowances (1989) for complementary foods.

A noteworthy finding is a significant ($p<0.0001$) (Table 4) increase in protein content corresponding to the escalating BRs. The highest protein content observed at the greatest percentage of haricot beans in the blend is likely attributed to the inherently high protein content of haricot beans and the inclusion of teff in the mixture, as indicated by Woldemariam et al. [10] and Yimer and Bultosa [7]. These findings also align with previous research demonstrating that blending of cereal and root-based foods with legumes improves their protein content [3, 45]. In addition, the germination process employed for the haricot bean might have contributed to an increasing crude protein. The increase in proteins may be due to loss of dry weight as some carbohydrates and fats are utilized during respiration while some amino acids are also synthesized during germination. Furthermore, germination can lead to the breakdown of complex proteins into simpler peptides, potentially improving their digestibility and contributing to the overall increase in crude protein bioavailability.

Interestingly, the level of feed moisture added and BT did not exhibit a statistically significant ($p<0.05$) effect. However, the regression analysis reveals negative coefficients for both feed moisture and barrel temperature, indicating an inverse relationship with the response variable. The barrel temperature rising from 120 to 160°C resulted in a significant reduction in the protein contents, from 14.04% to 7.82%. This is consistent with the observations of Adeleye et al. [49], who reported a decline in crude protein content of extruded African yam beans, Bambara groundnut, and pigeon pea as extrusion temperature increased from 100°C to 140°C. These observations could be related to the denaturation of protein by increase in heat, which leads to the loss of amino acids, especially lysine, which in turn reduces the protein content of the food [44, 49]. The interactions and quadratic effects of all independent variables were found to be nonsignificant ($p<0.05$).

The statistical analysis was performed according to the set of 20 treatments. Linear, interactive (2FI), and quadratic models were fitted to the experimental data to achieve regression models and the results are described in Table 4. The data presented in Table 4 indicate that the quadratic model was the most suitable model for representing experimental data. This is supported by the model's coefficient of determination (R2), the modified coefficient of determination (Adj R2), and the expected coefficient of determination (Pred R2) values. The response surface model demonstrated a significant ($p<0.05$) effect of BR and extrusion processing variables on most of the analyzed quality attributes of the extruded complementary food, as evidenced by a nonsignificant lack-of-fit ($p<0.05$) and high adequate precision values.

The overall ash content of a food item corresponds to the cumulative mineral content within the product. In the case of extruded complementary food samples, the total ash content varied between 2.166% and 3.284%, falling within the acceptable range recommended by WHO/FAO, [50] (<3 years). A parallel observation was noted in a prior study by Kindeya et al. [51], where the ash content demonstrated an upward trend in tandem with the inclusion of soybean flour in blended products. The ash content exhibited a significant increase ($p<0.0001$) with the escalating levels of haricot bean flour. Mezgebo et al. [29] and Fikiru et al. [5] reported similar trends where legumes such as malted soybean flour and roasted pea, respectively, are included in cereal-based complementary foods resulting in a rise in ash content. This increase in ash content could also be related to the utilization of *teff* [10, 52] and *bulla* as a base for the formulations, as these ingredients are naturally high in minerals. ANOVA indicated that feed moisture and BT did not exert significant linear, interaction, or quadratic effects on ash content ($p<0.05$). Similarly, the authors in [25] also reported insignificant effects of barrel temperature and feed moisture on the ash content of their maize-based extruded snacks. The *R*-square and adjusted *R*-square values were calculated as 0.7546 and 0.7168, respectively. The rise in total ash content in complementary food is ascribed to the elevated ash content of haricot bean and the utilization of teff and bulla.

The range of crude fiber content in extruded complementary food samples extended from 1.156% to 3.931%,

reaching its peak at a BR of 30%, with 21% feed moisture content, and a BT of 160°C. These findings align well with Araro et al.'s [17] findings, which observed an optimum level of crude fiber content in the formulation of orange-fleshed sweet potato, brown teff, and dark red kidney beans complementary food flour with different BR.

In comparison to Fikiru et al. [5], where complementary foods composed of maize, roasted peas, and malted barley exhibited higher fiber values ranging from 3.1% to 4.1%, the crude fiber content in our study was lower. Both linear and quadratic effects of BR ($p<0.0001$ and $p=0.0449$) and linear effect of FMC ($p<0.01$) significantly influenced crude fiber. The heightened crude fiber content in the complementary food can be attributed to the substantial fiber content of haricot bean and the ingredients employed in the formulation of the blends (teff and bulla flour). This observation aligns with [51], where the authors reported a similar increase in crude fiber content with increasing haricot bean blending ratio in complementary food produced from wheat, orange-fleshed sweet potato, and haricot bean flour. Importantly, all the crude fiber contents of complementary food samples remained within recommended ranges for complementary foods (<5%) [53].

Fat plays a vital role in the psychomotor growth, hormone production, cellular function control, mineral and fat-soluble vitamin absorption at the tissue level, sensory qualities, and concentrated energy supply in children [54]. However, the susceptibility of unsaturated oils to oxidative rancidity poses a challenge to the shelf life and stability of food products during storage. The crude fat content of the complementary food ranged from 1.173% to 3.12%, a range comparable to the fat content reported in complementary food developed from bulla, pumpkin, and germinated amaranth flours (1.9%–3.9%) as documented by Tadesse et al. [55].

The independent variables, specifically BR and FMC, significantly influenced the crude fat content of the complementary food ($p<0.0001$ and $p=0.0355$, respectively). Notably, the BR exhibited a positive effect on crude fat content, while feed moisture content had a negative impact. The elevated crude fat contents may be attributed to the high-fat content of the ingredients utilized in the formulation of the blends. Similar observations of increased fat content were reported by other researchers for wheat, orange-fleshed sweet potato, and haricot bean [51], as well as teff, soybean, and orange-fleshed sweet potato [55] complementary foods, which could possibly be attributed to the increased ratio of legumes (haricot and soybean) in the respective formulations. Other factors, such as barrel temperature, interaction effects, and quadratic effects, were not found to have a significant impact on crude fat content ($p<0.05$).

Carbohydrates serve as a crucial source of energy and are essential for the proper functioning of the brain, heart, nervous system, digestive system, and immune system [40]. The carbohydrate content in extruded complementary foods falls within the range of 65.34–78.23 g/100 g. The range observed satisfies the WHO/FAO recommendations (≥65 g/100 g) for complementary foods [53], highlighting the suitability of formulations to meet the energy demands of young children.

According to the results of the regression coefficients and ANOVA, the BR exerts the most significant ($p<0.0001$) effect on the carbohydrate content of the sample products. In the current study, there was a decrease in carbohydrate content with an increase in both BR and feed moisture content. This decline in carbohydrate content may be attributed to the inherently low carbohydrate content in haricot bean flour used to enrich the formulas. In addition, the germination process leads to a partial degradation of carbohydrate reserves, consequently increasing the respiration rate, which facilitates the release of energy through the breakdown of carbon compounds [29]. Furthermore, the reduction in the carbohydrate may be due to the caramelization or Maillard reaction that takes place in the extrusion process. The carbohydrate content observed in the developed samples aligns with values reported in fermented complementary food using a blend of orange-fleshed sweet potato, brown teff, and dark red kidney beans (68.23–83.96%) as documented by Araro et al. [17].

Proteins, fats, and carbohydrates are essential macronutrients that fuel the human body, and the energy content of a food matrix is determined by their composition. In the current study, the energy content of the extruded complementary food ranged from 338.43 to 354.86 kcal/100 g of dry matter (DM). These values are in line with those reported for complementary blends prepared from orange-fleshed sweet potato, brown teff, and dark red kidney beans (339.07–356.74 kcal/100 g) by Araro et al. [17]. The ANOVA indicated that BR, feed moisture content, the

interaction of BR and FMC, and the quadratic effect of FMC all significantly influenced gross energy content ($p < 0.0001$ and $p < 0.0001$, respectively). The negative influence of BR on energy content aligns with the observation that the carbohydrate content seems to have a greater influence on the overall energy value compared to the protein and lipid contents [56]. These findings underscore the potential of extrusion as a method for producing nutrient-rich and value-added complementary food products from a blend of protein-rich beans, iron-rich teff, and common staples. This is of significance as complementary foods play a crucial role in ensuring the nutritional status, health, and development of young children.

3.4. Effect of Blending Ratio and Extrusion Operating Conditions on the Physical and Functional Properties of the Extruded Complementary Food

Extrudates with different physical and functional properties (bulk density, water absorption index, water solubility index, oil absorption capacity, and viscosity) were obtained under different processing conditions (blending ratio, feed moisture content, and barrel temperature), as shown in Table 3.

The bulk density, accounting for expansion in all directions, ranged from 0.628l to 0.755 g/mL under various processing conditions. These findings coincide with those reported by Ocheme et al. [57], who observed bulk densities ranging from 0.66 to 0.70 g/mL for wheat and groundnut protein concentrate flour blends. In the development of complementary foods, a high nutrient density to low bulk is desired. The high bulk density can limit food consumption which potentially hinders their ability to consume enough food that hinders the required energy and nutrients [58]. However, the bulk densities in our study were slightly higher compared to those documented by Khatun et al. [59] and Suksomboon et al. [60]. A significant increase ($p < 0.05$) in bulk density was noted with an escalating BR, particularly with the incorporation of haricot bean in the formulations. This phenomenon can likely be attributed to two key factors, that is, high protein content and structural characteristics of haricot beans, which are known to bind water resulting in a dense product. This observation aligns with the findings of Awol et al. [61], who highlighted the increase of BD with an increase in the proportion of SB flour.

In development, complementary foods with high nutrient density to low bulk are desired, because high bulk density can limit the overall caloric and nutrient intake of children, potentially hindering their ability to consume enough to meet their energy and nutrient requirement.

ANOVA indicated that BR had the most significant effect on bulk density ($p < 0.05$). In addition, bulk density demonstrated a significant increase ($p < 0.05$) with a decreasing quadratic term for feed moisture content. This trend may be attributed to the fact that bulk density rises with increasing moisture content up to 18%, after which it decreases with further increases in moisture content, as observed by Hagenimana et al. [62]. BT exhibited a negative effect on bulk density ($p < 0.05$), signifying that an increase in BT resulted in a significant decrease in bulk density, which is also corroborated by the response surface plot (Figure 2(a)). Adem et al. [25] and Awol et al. [61] reported similar findings of a decrease in BD with an increase in barrel temperature in extruded snacks based on maize-lupin and teff-soybean, respectively.

[figure(s) omitted; refer to PDF]

In the formulation and packaging of complementary foods, a low bulk density is desirable, as it can lead to improved product handling and storage characteristics [63]. The quadratic model was fitted with a coefficient of determination (R2) of 0.8639, an adjusted R2 (Adj R2) of 0.7414, and a lack-of-fit of 0.8441 for BD, which indicates a strong fit of the model to the observed BD data. The regression model for bulk density as a function of the variables analyzed can be described by the following in terms of coded values:(7)$BD = 0.7096 + 0.0309 * A + 0.0037 * B - 0.0022 * C - 0.0016 * AB + 0.0241 * AC - 0.0031 * BC + 0.0115 * A2 - 0.0216 * B2 + 0.0040 * C2 R2 = 86.39$.

The WAI serves as a measure of the water absorbed by starch granules, providing an index for gelatinization and, to some extent, dextrinization [64]. In this study, the WAI ranged from 3.752 to 4.814 g/g, a range which is comparable to the results reported by Ayele et al. [45] for local complementary foods (2.5–5.92 g/g) but falls within the higher end of that spectrum. This suggests that the formulations in this study may offer enhanced water absorption capabilities, potentially leading to better nutrient delivery and digestive health benefits for young children.

The ANOVA indicated that BR and FMC had significant linear effects on WAI ($p = 0.0003$ and $p = 0.0115$,

respectively). The effects of independent variables on the WAI are indicated by response surface plots (Figure 2(b)). An increase in BR from 10% to 30% resulted in a notable decrease in WAI. This decrease is likely attributable to the relative reduction in starch content with the addition of haricot beans, impacting the extent of starch gelatinization in the barrel and leading to reduced water absorption. This trend is consistent with the findings of Filli et al. [65], who observed a similar decrease in WAI when soybean flour was added to millet-based extruded products for the Nigerian traditional food "*fura*." In fact, lower water absorption would be beneficial for complementary food formulation, as it allows for the preparation of thinner gruels with a higher caloric density per unit volume [58].

FMC also exhibited a significant effect on WAI, with WAI increasing as feed moisture content rose from 15% to 21%. This increment is likely due to water acting as a lubricant in the extrusion process, thus reducing friction between the screw, barrel wall, and starch molecules. In addition, an increase in BT led to a higher WAI (Figure 2(b)), likely due to elevated dextrinization at higher temperatures [66]. This trend is similar to the report of Awol et al. [61], who observed an increase in the WAI of teff-soybean-based extrudates as the feed moisture content and barrel temperature increased.

The quadratic model was significant ($p=0.0004$), indicating that the BR, FMC, and BT variables had a linear effect on the model. In addition, there is a strong fit of the model to the observed WAI data with an R2 of 0.8070, an adjusted R2 of 0.6333, and a lack-of-fit of 0.0773. The predicted model from regression analysis for WAI is shown in (7) (in terms of all independent variables in coded values) and was developed as follows:(8)WAI=4.07−0.234∗A+0.1437∗B+0.1042∗C−0.1212∗AB−0.0187∗AC−0.061∗BC+0.0866∗A2+0.0681∗B2−0.0588∗C2R2=80.70.

The WSI of the extruded complementary food varied from 10.024% to 34.38%, indicating a moderate to high level of starch conversion during the extrusion process [66]. These observations align with the results reported by Kharat et al. [67] and Wondimu and Admassu Emire [68] for pearl millet extrudates (12.72–19.39 g/cm^3) and extruded teff-based gluten-free snacks (13.17–22.21 g/cm^3), respectively. The fitted quadratic model for water solubility index (WSI) was statistically significant ($p=0.0003$), whereas the lack-of-fit was not significant ($p>0.05$), which suggests a good agreement between the model and the observed data (Table 4). A high coefficient of determination (R2) of 0.9148 and an adjusted R2 (Adj R2) of 0.8381 along with a nonsignificant lack-of-fit of 0.9285 for WSI demonstrate a strong fit of the model to the WSI data. The quadratic model obtained from regression analysis for the water solubility index in terms of coded levels of the variables is as follows:(9)WSI=26.24+6.98∗A−1.98∗B+1.05∗C+0.5959∗AB−1.32∗AC+1.34∗BC−1.20∗A2−3.57∗B2−1.96∗C2R2=91.48.

Analysis of the response surface model revealed that BR and BT exerted a significant positive linear impact on WSI, whereas feed moisture demonstrated a substantial negative linear and quadratic effect on WSI ($p<0.05$). These findings align with prior research indicating that elevating the BR and BT during extrusion can enhance starch conversion and subsequently increase WSI [69]. Higher extrusion temperatures likely promote a greater degree of starch degradation, resulting in the release of more soluble components, which explains the observed increase in WSI with increasing temperature (BT). Conversely, increasing FMC appears to reduce the WSI of the extrudates, potentially due to dilution effects or limitations on starch granules degradation at higher moisture levels [67].

The ANOVA results for the response surface model of WSI and the linear terms of BR ($p<0.0001$) and BT ($p<0.05$) were statistically significant ($p<0.05$), along with the quadratic terms of BT ($p<0.001$) and FMC ($p<0.05$). This signifies that elevating the BR or BT has a positive linear effect on WSI, while increasing FMC has a statistically significant negative linear and quadratic effect on WSI (all at $p<0.05$). The response surface plots (Figure 2(c)) show that the WSI of complementary food increased with increasing BR and BT, while it decreased with increasing feed moisture content. These findings are highly useful in optimizing the extrusion process to produce complementary foods with desired WSI values. The moderate to high WSI values obtained in this study suggest that the extrusion process was effective in converting starch to soluble polysaccharides. This is likely to enhance the digestibility and nutritional value of the extruded complementary food.

The OAI holds crucial significance in food processing, influencing the texture, flavor, and shelf life of food products. Fats, acting as flavor enhancers and lubricants, play a key role in shaping the mouth feel and taste of food [70, 71]. In the context of cereal-legume flours, the OAI is particularly vital for determining the textural and flavor

characteristics of food products.

In this investigation, OAI of complementary food ranged from 1.25 to 3.25 mL/g. This range surpasses the OAI values reported for complementary foods made with maize, sorghum, and mung bean malt by Onwurafor et al. [72], who documented that the OAI values fell within the narrower range of 1.42–1.76 mL/g. The observed variation in OAI values may stem from the differing ingredient combinations employed in the two studies. The response surface plots (Figure 2(d)) showed the effect of BR and extrusion parameters on the OAI of the complementary food. Notably, a gradual increase in OAI with the increase of haricot bean in the formulations might be explained by hydrolysis of protein during soaking and germination which potentially leads to enhanced oil retention capabilities [73]. As illustrated from response surface plots (Figure 2(d)), an increase in BT increases the OAI, which could be attributed to the solubilization of protein during extrusion cooking. As proteins become more soluble, their polarity increases, potentially contributing to the observed rise in OAI [56].

The ANOVA results revealed that the two-factor interaction (2FI) model significantly influenced the OAI of complementary food ($p<0.0001$), signifying that the selected model aptly represented the data. Notably, the significant terms of the model included the linear effects of blending ratio, feed moisture, and BT ($p<0.001$, $p<0.001$, and $p<0.001$, respectively) (Table 4). However, interaction terms involving blending ratio, feed moisture content, and BT had no significant effect ($p<0.05$) on OAI. These findings imply that each of these factors (blending ratio, feed moisture content, and barrel temperature) exhibits an independent and statistically significant ($p<0.0001$) linear effect on the OAI of complementary food. This implies that an increase in any of these factors is likely to result in a corresponding increase in OAI. The absence of significant interaction terms implies that the effects of these factors on OAI are additive, meaning that they act independently rather than influencing each other's impact.

The regression equation for the relationship between the dependent variable (OAI) and independent variables (BR, FMC, and BT) in terms of coded form can be shown by the following equation. The *R*-square value of 92.04% indicates a strong fit between the model and the data.(10)OAI=2.07+0.5646∗A+0.2388∗B+0.2476∗C+0.1500∗AB+0.1375∗AC−0.1375∗BCR2=92.04.

Viscosity serves as a critical quality parameter for complementary foods, influencing aspects such as bulk density, texture, and taste in the cooked product [57]. The current study reveals a viscosity range of 1882–3234 cP (MPa), surpassing the viscosity values reported for wheat and groundnut protein concentrate flour blends in the study by Ocheme et al. [57] (1178 to 751 $MPa{\cdot}s^{-1}$). This discrepancy is likely attributable to differing ingredient combinations and processing methods employed in the two studies. The response surface plots (Figure 2(e)) showed the effect of BR and extrusion parameters (BT) on the viscosity, revealing a decreasing trend with higher BR and barrel temperature. Similar observations were reported by Ocheme et al. [57], where increasing groundnut protein concentrate resulted in the reduction of peak viscosity. This effect can be attributed to a decrease in starch content as well as interactions between the starch, fat, and protein contents of the blends. In addition, the authors in [52] observed a decrease in viscosity during fermentation in their cereal-based complementary foods. This aligns with our findings, as the haricot bean used in our experiment underwent germination, a process known to reduce starch content and potentially alter protein-starch interactions. Furthermore, Borah et al. [74] observed a decrease in viscosity with increasing BT for breakfast cereal made from rice flour, seeded banana, and carambola pomace.

The best-fit quadratic (10) is as follows:(11)Viscosity=2221.14−449.05∗A−57.70∗B+77.16∗C+33.37∗AB−26.38∗AC+22.62∗BC+152.21∗A2−22.09∗B2+87.60∗C2R2=95.19.

The ANOVA results showed that the quadratic model was a good fit for the viscosity data ($p<0.0001$). The significant terms of the model included the linear and quadratic effects of BR ($p<0.0001$ and $p<0.0012$, respectively) and the quadratic term of feed moisture contents ($p<0.0283$). It also indicates that the BR and FMC had a negative significant effect, while BT had a positive effect on the viscosity of complementary food. The findings of this study are corroborated with previously reported studies [57]. Furthermore, the slight decrease in viscosity with an increase in feed moisture content is in agreement with the findings of previous studies [65].

3.5. Sensory Evaluation of Complementary Porridge from Teff: Bulla-Haricot Bean

A sensory assessment using a nine-point hedonic scale was conducted to evaluate the aroma, taste, texture, color,

and overall acceptability of the porridge samples. When developing new food products, the sensory properties of extruded complementary foods play a crucial role. The results of the sensory evaluation presented in Table 4 indicate a generally high overall acceptability for the porridge samples, with most ratings surpassing 6 on the 9-point hedonic scale. Nonetheless, there were variations in the ratings for specific sensory attributes, including aroma, taste, texture, and color.

The summarized sensory ratings for porridges derived from extruded blended flour of bulla, teff, and haricot bean exhibit a range from 5.111 ± 1.23 to 8.111 ± 0.75 for aroma, 5.056 ± 0.8 to 5 ± 0.68 for taste, 5 ± 0.76 to 8.389 ± 0.6 for texture, 5 ± 0.68 to 8.222 ± 0.54 for color, and 5 ± 0.68 to 8.222 ± 0.54 for overall acceptability (Table 4). The sensory scores for all the complementary porridge samples consistently exceeded the midpoint of the scale (approximately 5), which indicates that all products were well-accepted by the panelists. Table 5 further demonstrated that the extrusion process variables showed significant differences ($p < 0.05$) in all the sensory parameters evaluated across the extrusion process variable combinations for porridge samples. These results align with the findings of Araro et al. [17], who reported above-average overall acceptability for complementary processed food formulated from a blend of orange-fleshed sweet potato, brown teff, and dark red kidney beans.

Table 5

Sensory scores of porridges made from extruded composite flour of bulla, teff, and haricot bean.

BR (%)	FMC (%)	BT (°C)	Aroma	Taste	Texture	Color	Overall
20	18	140	7.11 ± 0.90^{cb}	7.22 ± 0.64^{bc}	7.28 ± 0.75^{bc}	6.67 ± 0.076^{bc}	7.00 ± 0.59^{bcd}
36.82	18	140	5.17 ± 0.85^{g}	5.00 ± 0.68^{f}	5.00 ± 0.76^{g}	5.06 ± 0.80^{c}	5.00 ± 0.68^{i}
30	21	160	6.67 ± 0.76^{bcdef}	6.67 ± 0.68^{cd}	6.72 ± 0.75^{cde}	6.72 ± 1.01^{bc}	6.67 ± 0.59^{def}
20	18	106.36	6.67 ± 1.23^{bcdef}	6.78 ± 1.47^{bcd}	6.83 ± 1.04^{cde}	6.78 ± 1.00^{bc}	6.72 ± 0.75^{cdef}
20	18	140	7.00 ± 0.97^{bcd}	7.22 ± 0.54^{bc}	7.28 ± 0.75^{bc}	6.67 ± 0.76^{bc}	7.00 ± 0.59^{bcd}
20	18	173.64	6.33 ± 0.90^{def}	6.00 ± 1.32^{e}	6.00 ± 1.02^{f}	5.94 ± 1.05^{bc}	5.78 ± 0.73^{h}
10	15	160	6.61 ± 0.69^{bcdef}	6.67 ± 0.59^{cd}	6.50 ± 0.98^{def}	6.22 ± 0.80^{bc}	6.39 ± 0.77^{g}
30	21	120	7.11 ± 1.00^{bc}	7.06 ± 0.93^{bc}	7.00 ± 1.10^{bcd}	7.00 ± 1.30^{bc}	6.83 ± 0.7^{cde}
20	18	140	7.00 ± 0.97^{bcd}	7.22 ± 0.54^{bc}	7.28 ± 0.75^{bc}	6.67 ± 0.76^{c}	7.00 ± 0.59^{cd}
20	23.05	140	6.67 ± 0.97^{bcdef}	6.33 ± 0.68^{de}	6.50 ± 0.51^{def}	6.56 ± 0.51^{bc}	6.44 ± 0.51^{gf}
20	12.95	140	6.44 ± 0.78^{cdef}	6.33 ± 0.68^{de}	6.28 ± 0.95^{ef}	5.94 ± 0.99^{bc}	6.166 ± 0.61^{h}
30	15	120	8.11 ± 0.75^{a}	8.61 ± 0.50^{e}	8.39 ± 0.60^{a}	7.78 ± 0.73^{bc}	8.22 ± 0.54^{a}
20	18	140	7.00 ± 1.02^{bcd}	7.22 ± 0.54^{bc}	7.28 ± 0.75^{bc}	6.67 ± 0.76^{ab}	7.00 ± 0.59^{bcd}

10	21	120	7.06 ± 1.21^{bc}	7.06 ± 1.05^{bc}	7.00 ± 1.10^{bcd}	6.89 ± 1.30^{bc}	6.83 ± 0.70^{cde}
10	21	160	6.00 ± 1.10^{f}	6.00 ± 1.30^{e}	6.00 ± 1.00^{f}	5.94 ± 1.00^{bc}	5.78 ± 0.73^{h}
10	15	120	6.89 ± 1.40^{bcde}	6.78 ± 1.40^{bcd}	6.83 ± 1.04^{deg}	6.78 ± 1.00^{bc}	6.72 ± 0.75^{cdef}
30	15	160	6.28 ± 1.30^{ef}	6.22 ± 1^{de}	6.00 ± 1.00^{f}	5.94 ± 1.00^{bc}	5.89 ± 0.67^{h}
20	18	140	7.06 ± 1.10^{bc}	7.28 ± 0.82^{b}	7.44 ± 0.85^{b}	6.33 ± 0.84^{bc}	7.11 ± 0.85^{bc}
3.15	18	140	5.11 ± 1.23^{d}	5.06 ± 0.8^{f}	5.00 ± 0.76^{g}	5.06 ± 0.8^{bc}	5.00 ± 0.68^{i}
20	18	140	7.28 ± 0.95^{b}	7.28 ± 0.82^{b}	7.56 ± 0.92^{b}	6.33 ± 0.97^{bc}	7.28 ± 0.57^{b}

The results were expressed as mean ± SD ($n = 15$). BR, blending ratio (%); FMC, feed moisture content (%); BT, barrel temperature (°C). Means with different superscripts within a column are significantly different ($p<0.05$).

The highest overall acceptability score was observed in the formulation with a 30% BR, 15% FMC, and 120°C BT. Conversely, the lowest overall acceptability score was noted in the porridge sample featuring a 36.82% BR, 18% FMC, and 140°C BT; this might be due to the beany flavor associated with the higher proportion of haricot bean (36.82%). Ayele et al. [45] reported similar observations in porridge containing red kidney beans (40%) for complementary food formulations based on maize, red kidney beans, kocho, and pumpkin fruit. On the other hand, the results from our current study suggest superior overall acceptability compared to those reported by the authors in [45] for complementary food made from maize, red kidney beans, kocho, and pumpkin fruit.

3.6. Optimum Condition for Teff-Bulla with Haricot Bean Supplemented Extruded Complementary Product

Utilizing Design-Expert v11, numerical optimization was undertaken to optimize five dependent (response) variables: WSI, WAI, viscosity, protein content, and energy content. Following the approach advocated by Bas and Boyaci [75], it is imperative to identify and prioritize key factors among numerous factors (physical, functional, proximate, and sensory) for the purpose of optimization.

Throughout the optimization process, the variables were constrained within predefined ranges. Specific objectives were assigned to each response parameter, aiming for maximum values for energy, WSI, and protein, while minimizing WAI and viscosity, ensuring they remained within acceptable ranges. Overlay plots were generated by superimposing contour graphs of all estimated responses using RSM.

The optimum predicted values of extrusion operation conditions were at BR of 28.2837%, feed moisture of 15.0007%, and BT of 120.001°C, with a desirability value of 0.856 (Figure 3). Under these conditions, the optimal responses were WSI of 32.4462 g/g, WAI of 3.69849%, viscosity of 2016.47 cP, protein of 14.046%, and energy of 348.034 kcal/100 g, respectively. This finding effectively demonstrates the potential of RSM and central composite design (CCD) to optimize multiple variables and achieve desired outcomes in various fields.

[figure(s) omitted; refer to PDF]

4. Conclusions

The results of this study demonstrate that a nutritious complementary food, derived from a blend of teff, bulla, and haricot bean flour, can serve as a cost-effective alternative for mothers to feed their infants and children during the complementary feeding phase, compared to commercial complementary foods. The complementary food produced from the combination of bulla-teff-haricot bean exhibited significant enhancements in various proximate compositions, minerals, and a reduction in antinutritional factors in the formulated complementary flour. Moreover, the complementary food generated from the specified blend offered a substantial quantity of essential nutrients (protein, iron, calcium, and zinc) as per the proximate composition. Porridge prepared from the extruded instant flour received above-average mean scores for all sensory attributes and gained acceptance from both

mothers and caregivers.

For optimization of blending and extrusion operations, the study employed the central composite rotatable design (CCRD) of response surface methodology (RSM), specifying BR, FMC, and BT at 28.2837%, 15.0007%, and 120.001°C, respectively, with a desirability level of 0.856. Overall, these findings underscore the potential of teff, bulla, and haricot bean composite flour for producing extrusion-cooked complementary foods with enhanced nutritional content [20, 22, 27, 55, 76–78].

Ethical Approval

This study was approved by Institutional Review Board of Bahir Dar Institute of Technology.

Authors' Contributions

Getu Weya Chewicha conceptualized the study, acquired the data, drafted the manuscript, and approved the study. Muhammed Adem Abdullahi designed the study, drafted the manuscript, and approved the study. Tadele Andargie Wudineh interpreted the data, drafted the manuscript, and approved the study. Tatek Tamiru Geletu designed the study, reviewed the manuscript, and approved the study. Neela Satheesh analysed and interpreted the data, critically reviewed the manuscript, and approved the study.

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DETAIL

Subjek:	Food security; Polyethylene; Variance analysis; Design of experiments; Moisture content; Nutritive value; Plastics; Agricultural research; Nutrition; Variables; Public health; Developing countries--LDCs; Food; Malnutrition; Dietary minerals; Beans; Proteins; Sensory perception; Nutritional status
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Lokasi:	Ethiopia
Judul:	Effect of Blending Ratio and Extrusion Operating Conditions on Nutritional Quality and Functional and Sensory Acceptability of Teff-Bulla-Based Complementary Food: A Response Surface Methodology Approach
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Study on a Moisture Ratio Curve Model for Refractance Window Drying Based on a D-Optimal Mixture Design

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ABSTRAK (ENGLISH)

Refractance window (RW) drying is a new thin-layer drying technology that can control well the heating temperature to effectively reduce the loss of heat-sensitive substances. Here, an experiment on tomato pulp drying was carried out to study the drying characteristics of RW drying based on a D-optimal mixture design. The fitting of the classical

model of thin-layer drying was studied, and SAS and 1stOpt calculation software were used to analyze the test data. The result showed that the RW drying equipment could dry 8 mm of tomato pulp in 120 min, and the maximum drying speed could reach 0.40 g/(g·min). Based on an effective diffusion coefficient under different conditions, the activation energy was 27.35 kJ/mol at an air speed of 3 m/s. When comparing the fitting of the moisture ratio curve in four classic thin-layer drying models, it was found that the R-square value of the modified Page model was 0.9960, which had better fitting properties. Then, the polynomial fitting model of thin-layer drying reflects the regression relationship between the coefficient of the classic model and drying conditions including temperature, wind speed, and time. After comparison with the classic model and validation experiment, the results showed that there is no significant difference between the polynomial fitting model and the validations under a confidence level of 0.95, which could well predict the change in the water content ratio over time under different conditions.

TEKS LENGKAP

1. Introduction

Most traditional industrial drying equipment used hot air as the medium to achieve drying [1]. However, during the heating process, some heat-sensitive substances, such as vitamins and volatile compounds, will be destroyed due to high temperature, resulting in material loss or deactivation, which could have a critical impact on food quality [2]. Refractance window (RW) drying was first proposed by Magoon in 1986, who applied for a patent. Generally, the RW drying method spread out the material evenly on Mylar film, and the heat was transferred to the material through a heated liquid under the film to achieve drying [3]. RW drying can control the temperature well by using liquid as the main heat transfer medium to avoid product overheating and finally restain heat-sensitive substances [4]. RW drying is a new thin-layer drying technology coupled with heat conduction and radiation. In the RW drying process, the liquid will be heated by the heat source and the heat energy will be transferred through the form of convection in the whole liquid. In general, the liquid will release energy through evaporation after the absorption of energy. However, in RW drying, the heat energy will be blocked and refracted back into the liquid under the influence of the film which is placed over the liquid. When the high wet materials are over the film, the materials act as a “window” that can permit heat transmission from liquid to the materials, and the moisture in the materials will be heated and evaporated. Once the drying process is accomplished and materials become dry products, the transfer of heat energy will be blocked again by the film. During the whole drying process, radiative heat transfer contributes to less than 5% of the total heat transmission [5, 6]. As it can be seen, there is a “window” in the RW drying process which can start and finish the drying process automatically based on the moisture content in materials. Thus, it can avoid high temperature at the end of drying to achieve the purpose of saving energy [7].

In recent years, research on RW drying has focused more on drying effect comparisons between different drying methods [8–12] and less on theoretical study. Ortiz Jerez and Ochoa-Martínez [13] of Columbia University studied pumpkin pieces based on heat transfer theory. They used temperature sensors to obtain the temperature in the upper, middle, and lower parts of the pumpkin as the data source and compared the different experimental conditions of natural convection and forced convection of the air above the material and whether the Mylar film was covered with aluminum foil. Based on an analysis of variance theory, the forced convection in the top air had significant effects on the temperature distribution of pumpkin pieces. Forced convection decreased the temperature of the pumpkin pieces. Meanwhile, the aluminum foil covering had a significant effect on the bottom temperature only. This shows that the radiation between the hot water and the pumpkin had positive effects on the bottom temperature increase of the pumpkin. Franco et al. [14] using salmon beef and apple as the research material to carry out the RW drying research studied the drying in the refraction window and found no improvements for lean beef and salmon compared with the traditional drying effect. Compared with the traditional drying method, the drying speed could be faster when drying fruits, and during the RW drying process, there was often a diffusion behavior that was different from that predicted by Fick’s second law, that is, abnormal diffusion behavior. Puente-Díaz et al.

[15] fitted moisture curves under different experimental conditions to take advantage of five classic models and found that the R-square value of each model was higher than 0.85, indicating a good fitting property. These studies did not establish a relationship between experimental conditions and model parameters and did not provide a reference for experiments under other experimental conditions.

This study used tomato slurry as the experimental material, and an RW drying bedstand built in a laboratory was used as an apparatus. The drying properties of RW drying were studied, a polynomial model was established based on classic models, and variance analysis of the model was carried out. The purpose of this paper was to predict the change in the moisture ratio in the drying process of RW drying under different experimental conditions and to provide a reference for other modelling studies.

2. Materials and Methods

2.1. Materials

Tomatoes used for the experiment were purchased from the Yimaisheng Supermarket in Jiayuan Square in Nanjing, China, and complied with the corresponding food safety regulations. Then, tomatoes were divided into 11 groups and the water content of each group was determined under pressure of 101.3 kPa and temperature of 105°C which confirms the direct drying method standardized in the National Food Safety Standard Determination of Moisture in Food [16]. The result shows that the average value of the moisture content was 96.2%.

2.2. Instruments and Apparatus

RW drying equipment was designed and assembled in a laboratory and mainly consisted of a constant temperature heating water bath, a 0.25 mm thick Mylar film, and a fan [17]. The length, width, and height of the tank are 180 mm × 180 mm × 140 mm, and the Mylar film is placed still on the water. There is a fan installed on the tank which can provide the required wind speed. The structure is shown in Figure 1. When the equipment starts working, water is added to the water tank and heated with a heater. The heater can work intermittently according to the temperature set so that the difference between the water temperature and the set temperature did not exceed 2°C. The moisture meter used in the experiment was the MB27 model of the American Ohaus, the electronic balance was the DT5000 model of the China G and G, and the blender was the MJ-WBL2501A model of the China Midea.

[figure(s) omitted; refer to PDF]

2.3. Experiment

2.3.1. Determination of Experimental Design

To improve the fitting properties of the polynomial fitting model, this experiment adopted a D-optimal mixture design by using SAS and 1stOpt software. In response to surface experiments, values such as *D*-value, *G*-value, and *A*-value could be used to evaluate the accuracy of the fitting equation. Among them, the use of *D*-value was the most common. The D-optimal design was an experimental design plan that pursued the optimal *D*-value in all experimental plans. The D-optimal design was based on Wald's determinant maximum criterion of the information matrix. A higher determinant value deduced a smaller variance of the predicted value of the regression coefficient as well as a smaller variance of the predicted value. The experimental design that reached the smallest variance was the best experimental design, thus named the D-optimal design [18]. Generally, the D-optimal design did not fit orthogonality and rotation. Therefore, based on the regression combination design, a D-optimal mixture design was proposed, which provided features of D-optimality, orthogonality, and rotation.

This experiment adopted the D-optimal mixture design scheme, assigned drying temperature, drying air speed, and drying time as variables, and took weight as the measured value for the experimental design. The R311 D-optimal experimental design table shown in Table 1 was used for the experiment. The first column of the design table was the experiment number, and the remaining tables described the coded value of different factors under each experiment number. In the table, different levels were represented by coded values, and the corresponding relationship between coded values and actual values is shown in Table 2.

Table 1

R311 experiment design table.

Number	Temperature	Air speed	Time
1	0	0	1.414
2	0	0	−1.414
3	−1	−1	0.707
4	1	−1	0.707
5	−1	1	0.707
6	1	1	0.707
7	1.414	0	−0.707
8	−1.414	0	−0.707
9	0	1.414	−0.707
10	0	−1.414	−0.707
11	0	0	0

Table 2

Comparison table of coding level and actual value.

	−1.414	−1.000	−0.707	0.000	0.707	1.000	1.417
Temperature (°C)	60	63.70		72.50		81.30	85
Air speed (m/s)	1	1.60		3		4.40	5
Time (min/min)	0		0.25τ	0.5τ	0.75τ		τ

Tontul et al. [19] held that to ensure the quality of fruits and vegetables, the drying temperature should not exceed 90°C. After pre-experimental results, when the surface temperature exceeded 85°C, the dried sample appeared obviously browning, so the highest temperature in this experiment was set to 85°C. Ochoa-Martínez et al. [20] indicated that the material temperature was approximately 10°C lower than that of a heat-transfer medium during an RW drying process. To ensure drying efficiency, the minimum temperature is selected as 60°C. To ensure that the experiment had reference in the maximum air speed range, the air speed range was set as 1–5 mm/s. As the total drying times under different temperatures and air speed are different, in order to make the time length consistent under different conditions, dimensionless time was adopted. Given that the total length of time was the unit length τ, the time range was 0 to τ. The comparison between the coding value and the actual value of each factor is shown in Table 2.

2.3.2. Experimental Process

An appropriate amount of water was added to the RW drying equipment to make the water surface and the Mylar film just contact, and then the equipment was preheated to the given temperature required by the experiment. The fan was turned on, and the fan speed was adjusted to meet the experimental requirements by monitoring the air speed in the middle of the Mylar film with an anemometer. After the temperature reached the experimental temperature, the tomatoes were washed, cut into pieces of approximately 20 mm in cubes, and placed in a small tray, and then, all the tomato pieces and juice were poured into a blender, which was run for 5 minutes to mix the tomatoes into a fine pulp and uniform slurry without visible particles. The Mylar film size was 180 mm × 180 mm and initially weighed before the experiment. Then, 200 g of tomato slurry was weighed and placed on the film and a steel sheet was used to scrape the tomato slurry evenly. A height vernier caliper was used to measure the thickness at 8 different points after leveling and the average thickness was approximately 8 mm. Then, every 15 minutes, the Mylar film and the tomato slurry were weighed as a whole and water on the bottom of the film was cleaned before weighing. The sample weight which is the difference between measured weight and the mass of the film was recorded in the experimental data until the weight no longer changed. Measuring weight can visually observe the water loss of the sample; furthermore, it can be used for calculating the drying speed of the sample. Eleven experiments were carried out according to the R311 design table.

2.4. Data Calculation Method

The moisture ratio (MR) refers to the residual moisture content of materials under certain drying conditions, which could indirectly reflect the drying speed under such conditions. The calculation method is shown in the following equations:(1)MR=Mt−MeM0−Me,(2)Mt=mt−mm,where MR is the moisture ratio; M0,Mt is the dry basis moisture content of tomato slurry at the initial time and *T* time, respectively, g/g; Me is the moisture content of the dry basis of the tomato slurry at the final moment of drying, g/g; mt is the weight of tomato slurry at time *t*, g; *m* is the absolute drying quantity of the tomato slurry, g. Generally, Me is relatively small to Mt,M0. Therefore, equation (2) could be simplified as the following equation [21]:(3)MR=MtM0.

The drying rate (DR), g/g·min, which reflected the dehydration rate of the tomato slurry, could be represented by the following equation [22]:(4)DR=−Mt+∆t−Mt∆t,where Mt+∆t,Mt referred to the moisture content ratio on a dry basis at the times of t+∆t and *t*, respectively, g/g.

The RW drying of the tomato slurry was a process of water transfer from inside to outside under the action of hot water. According to the analytical solution of Fick's second law and the experimental data, the effective diffusion coefficient (Deff) of moisture in the drying process of the tomato slurry could be calculated as the following equation [23, 24]:(5)lnMR=ln8π2−π2Defftl2,where Deff is the effective diffusion coefficient, m2/s; t is the drying time, *s*; and l is the half thickness of the tomato slurry, *m*.

According to equation (5), there was a linear relationship between LnMR and time. lnMR and t were fitted with SAS software to obtain the value of the slope *k*, and then Deff was calculated. The relationship between the activation energy and Deff could be set up according to the Arrhenius equation using the following equation [25, 26]:(6)Deff=D 0exp−EaRT,where D0 is the number of prefactors of the Arrhenius equation and it is a constant, m2/s; Ea is the activation energy, KJ/mol; R is the gas molecular constant, 8.314 J/mol·k; and T is the drying temperature, K. To simplify the calculation, the natural logarithm on both sides of the equal sign of equation (7) could be used to obtain the linear relationship between lnDeff and 1/T.(7)lnDeff=lnD0−EaRT.

In this experiment, four models commonly used in thin-layer drying were selected to study the drying process of tomato pulp by RW drying. The four models are, respectively, logarithmic, Page, modified Page, and Wang and Singh, and the expressions are, respectively, the following equations:(8)MR=ae−kτ+b,(9)MR=ae−τn,(10)MR=ae−kτn ,(11)MR=1−aτ−bτ2,where MR was the water ratio; *τ* was the dimensionless time; *a*, *b*, *k*, and *n* are the parameters to be fitted.

3. Results and Discussion

3.1. Experimental Data

According to the code value given in the R311 experimental design table, the experimental conditions were determined after calculation. We randomly assign values to eleven experimental groups and carry out eleven

experiments in order of the size of the assignment. According to the guidance of the R311 experimental design table, repeated in the sample center, which helped to improve the accuracy of the fitting equation. The experimental data are recorded in Table 3. Equation (4) was used to calculate the drying rate, and Figure 2 shows the relationship between MR and DR under 1–11 different experimental conditions of temperature and wind speed. In Figure 2, the DR of each line increases first and then decreases with the increase of the MR, which was similar to Yuda's results when exploring potatoes RW drying [12]. Most of these lines had a maximum value when the MR was 0.3. Studying these data, we found that the RW drying equipment could quickly dehydrate an approximately 8 mm tomato slurry within 120 minutes, with the moisture rate decreasing from 96.20% to 5.0%, and the fastest drying speed could reach 0.4035 g/g·min. Using equation (5), we calculated Deff under different test conditions and recorded the results in Table 4.

Table 3

Experiment datasheet.

Time (min)	60°C 3 m/s		63.7°C 1.6 m/s			72.5°C 1 m/s		72.5°C 3 m/s		72.5°C 3 m/s	
72.5°C 3 m/s			Weight (g)	MR (%)	Weight (g)		MR (%)	Weight (g)	MR (%)	Weight (g)	MR (%)
Weight (g)	MR (%)	Weight (g)		MR (%)	-						
0	200	100.00	200		100.00	200	100.00	200	100.00	200	100.00
200		100.00	15	188.0	93.76	184.0		91.68	186.0	92.72	174.0
86.49	176.0	87.53	176.0		87.53	30	176.0	87.53	170.0		84.41
170.0	84.41	154.0	76.09	150.0	74.01	150.0		74.01	45	156.0	77.13	156.0
77.13	154.0	76.09	128.0	62.58	124.0	60.50	124.0		60.50	60	146.0	71.93
142.0			69.85	136.0	66.74	96.0	45.95	96.0	45.95	96.0		45.95
75	130.0	63.62	118.0			57.38	116.0	56.34	70.0	32.43	70.0	32.43
70.0		32.43	90	112.0	54.26	104.0			50.10	98.0	46.99	48.0

21.00	48.0	21.00	48.0		21.00	105	94.0	44.91	86.0			40.75
76.0	35.55	30.0	11.64	32.0	12.68	32.0		12.68	120	76.0	35.55	66.0
30.35	56.0	25.16	18.0	5.41	18.0	5.41	18.0		5.41	135	58.0	26.20
48.0			21.00	40.0	16.84	12.0	2.29	12.0	2.29	12.0		2.29
150	42.0	17.88	32.0			12.68	28.0	10.60	8.0	0.21	8.0	0.21
8.0		0.21	165	28.0	10.60	18.0			5.41	22.0	7.48	
						180	18.0	5.41	14.0			3.33
18.0	5.41								195	12.0	2.29	12.0
2.29	12.0	2.29								210	10.0	1.25
10.0			1.25	8.0	0.21							
225	8.0	0.21	8.0			0.21						
			-									

Time (min)	72.5°C 5 m/s		63.7°C 4.4 m/s		81.3°C 1.6 m/s		85°C 3 m/s		81.3°C 4.4 m/s	
		Weight (g)	MR (%)	Weight (g)	MR (%)		Weight (g)	MR (%)	Weight (g)	MR (%)
Weight (g)	MR (%)		-							
0	20	100.00	200	100.00	200	100.00	200	100.00	200	100.00
		15	176.0	87.53	186.0	92.72		176.0	87.53	178.0
88.57	170.0	84.41			30	152.0	75.05		158.0	78.17

148.0	72.97	150.0	74.01	124.0	60.50			45	126.0	61.54	
136.0	66.74		118.0	57.38	118.0	57.38	96.0	45.95			60
96.0	45.95		110.0	53.22		84.0	39.71	86.0	40.75	64.0	29.31
		75	64.0	29.31		84.0	39.71		48.0	21.00	48.0	21.00
34.0	13.72				90	36.0	14.76		62.0	28.27	22.0
7.48	22.0	7.48	28.0	10.60				105	20.0	6.44	44.0
18.92		12.0	2.29	12.0	2.29	14.0	3.33			120	12.0
2.29		26.0	9.56		8.0	0.21	8.0	0.21	8.0	0.21	
135	10.0	1.25		16.0	4.37						
			150	8.0	0.21		10.0	1.25			
						165			8.0	0.21	

[figure(s) omitted; refer to PDF]

Table 4

Deff under different conditions.

Conditions	Slope	Deff	Conditions	Slope	Deff
72.5°C 3m/s	−0.0006967	4.5178×10−9	85°C 3m/s	−0.00091642	5.9426×10−9
72.5°C 3m/s	−0.00069334	4.4960×10−9	60°C 3m/s	−0.0004607	2.9874×10−9
63.7°C 1.6m/s	−0.00048007	3.1131×10−9	72.5°C 5m/s	−0.0007756	5.0294×10−9
81.3°C 1.6m/s	−0.00091502	5.9335×10−9	72.5°C 1m/s	−0.00046196	2.9956×10−9
63.7°C 4.4m/s	−0.0006528	4.2331×10−9	72.5°C 3m/s	−0.00069843	4.5290×10−9
81.3°C 4.4m/s	−0.00086818	5.6298×10−9			

In Table 4, the Deff increased with increasing temperature when the air speed remained the same. When the temperature was lower than 81.3°C, Deff increased with increasing air speed. However, the relationship between D eff and air speed still needed to be explored when the temperature was too high. In Table 4, the Deff at different temperatures when the air speed was 3m/s used SAS to fit lnDeff and 1/T. The experiment was repeated three

times at 72.5°C. These three experiments existed independently. The repetition at the center of the sample helped to improve the accuracy of the variance equation. In Figure 3, the ln Deff and 1/T had a linear relationship when the air speed was 3 m/s, and the linear equation could be expressed as ln Deff=−9.73−3289.191/T. According to equation (7), Ea was 27.346 kJ/mol.

[figure(s) omitted; refer to PDF]

3.2. Prediction of Total Drying Time

Excel software is used to visualize the change trend of average drying time under different drying conditions of wind speed and temperature as shown in Figures 4 and 5. It is shown that the drying time decreases with the increase in wind speed and drying temperature.

[figure(s) omitted; refer to PDF]

In order to better solve the relationship between temperature and air speed and total drying time, a three-dimensional scatter plot with total drying time as a function value and temperature and air speed as independent variables was drawn by Origin, and these points were connected as planes to form Figure 6.

[figure(s) omitted; refer to PDF]

In Figure 6, time was roughly distributed in a plane that was formed by temperature and air speed. In order to find the relationship between them, we used the least square method to calculate the estimated values of the following equation parameters:(12)θ=∑i=1nyi−y^i2,where yi was the actual value, y^i was the estimated value corresponding to yi, and θ was their difference. In order to ensure the good fit of the formula, it was necessary to constantly adjust the variance parameter to ensure that θ was the minimum value to obtain the parameters of the equation. This process was complicated, so we used SAS software to calculate. The experimental data referred to Table 3, and the variance analysis referred to Table 5. The analysis results showed that the P value of the model was 0.0014, the P value of the intercept was less than 0.001, the P value of the temperature regression coefficient was 0.0008, and the P value of the air speed regression coefficient was 0.0236. Given that the confidence coefficient was 95%, we could conclude that there was a linear relationship between the total drying time, the drying temperature, and the drying air speed. The R-square value was 0.8878, the RMSE (root mean square error) was 17.02, the mean value of the dependent variable was 166.67, and the coefficient of variation was 10.21%. It was generally considered that a coefficient of variation below 15% is acceptable, and the chi-square value was 5.01. In Figure 7, the drying time was similarly evenly distributed on both sides of the fitting curve. Combining the above values, it could be considered that the model had a good fit. The expression is shown in the following equation:(13)t=511.39−4.22T−12.84v,where t was the total drying time, min; T was the drying temperature, °C; and v was the drying air speed, m/s.

Table 5

Analysis of variance for prediction of total drying time.

Source	Analysis of variance						
DF	Sum of squares	Mean square	*F* value	Pr > *F*	RMSE	R-square value	Model
2	13759	6879.42878	23.33	0.0005	17.02	0.8878	Error
8	2359.32425	294.91553	—	—	—	—	Corrected total

[figure(s) omitted; refer to PDF]

3.3. Establishment of Classic Thin-Layer Drying Model

The thin-layer drying model was widely used in agricultural product drying [27]. Four commonly used models in thin-layer drying were selected for this experiment [28–30]. At the same time, based on the experimental data, the

parameters in the model were fitted with the software 1stOpt using the Marquardt method and the global optimization method [31]. The model selection and calculation results are shown in Table 6.

Table 6

Classic model fitting result table.

Temp (°C)	Air speed (m/s)	LogarithmicMR=ae−kτ+b				PageMR=ae−τn		
a1	k1	b1	R2	a2	n2	R2	72.5	3
0.031	2.51	3.58	0.9779	0.73	1	0.9828	63.7	1.6
2.35	2.53	17.69	0.942	0.73	1	0.9839	81.3	1.6
0.0058	2.32	5.25	0.9349	0.78	1	0.9829	63.7	4.4
51.51	2.509	−3.83	0.956	0.74	1	0.9909	81.3	4.4
2.99	2.65	10.49	0.9707	0.71	1	0.9855	85	3
0.0019	2.31	6.38	0.9321	0.78	1	0.9814	60	3
62.99	2.35	−4.02	0.9373	0.77	1	0.986	72.5	5
0.33	2.69	3.51	0.9472	0.71	1	0.9789	72.5	1
3.37	2.44	10.41	0.949	0.75	1	0.9934	·	
Temp (°C)	Air speed (m/s)	Modified pageMR=ae−kτn				Wang and SinghMR=1−aτ−bτ2		
a3	k3	n3	R2	a4	b4	R2	·	
72.5	3	0.96	3.9	1.79	0.9969	1.6	−0.6	0.9921
63.7	1.6	0.94	4.36	2.03	0.9945	1.52	−0.45	0.9855

81.3	1.6	0.96	4	2.05	0.9949	1.37	−0.31	0.9926
63.7	4.4	0.98	3.81	1.76	0.9974	1.54	−0.49	0.9924
81.3	4.4	0.99	3.68	1.52	0.9966	1.77	−0.76	0.9944
85	3	0.97	4.04	2.08	0.9955	1.34	−0.28	0.9839
60	3	0.95	3.88	2.06	0.9948	1.32	−0.25	0.9881
72.5	5	0.96	4.85	1.96	0.9958	1.71	−0.66	0.9853
72.5	1	0.96	3.92	1.92	0.9969	1.48	−0.43	0.9881

In the classic model, only time was an independent variable, which could only be fitted under known drying conditions and had no guiding role for other drying conditions without experimentation. For example, in Meric's study, although 11 drying model parameters were obtained, none of them could predict models under other experimental conditions [32]. To give the classic model a better applicability under other experimental conditions, a regression relationship between the coefficients obtained in the model and the experimental conditions was established. Based on the data in Table 6, a multiple linear regression model was established by SAS software with experimental conditions as variables and parameters as response values. If the linear model was not applicable, a nonlinear model was established. A relationship between the experimental parameters and the coefficients in the classic thin-layer drying model was obtained.

With a confidence coefficient of 0.95, the parameters in the logarithmic model could not establish a quadratic polynomial regression relationship with the experimental indices. Parameter a2 in the Page model could establish a nonlinear relationship as equation (14) with the experimental conditions at a confidence coefficient of 0.95.(14)a2= 1.68−0.031T+0.12v+0.00025T2−0.0016v×T−0.0014v2.

The parameters of the modified Page model could not establish a polynomial regression relationship with the experimental conditions. Parameter a4 in the Wang and Singh model could establish a nonlinear relationship with the experimental conditions as equation (15) at a confidence coefficient of 0.9.(15)a4=−4.60+0.19T−0.55v−0.0015T 2+0.0077v×T+0.0088v2.

A nonlinear relationship between parameter b4 and the experimental conditions could be established at a confidence coefficient like equation (16) at a confidence coefficient of 0.9.(16)b4=7.75−0.25T+0.52v+0.0019T2− 0.0083v×T.

3.4. Establishment of the Polynomial Regression Model

Among the four classic models used in this paper, only the parameters of the Wang and Singh model could establish a nonlinear relationship with the drying conditions, but the confidence coefficient was not good. At the same time, the establishment of a nonlinear relationship between each parameter and the drying conditions required the determination of the model parameters under different drying conditions, and the model could not be established directly and quickly. We found that the existing thin-layer drying models were all third-order derivative functions. For

such functions, we could write them in polynomial form by using Taylor expansion, so the thin-layer drying models were unified in form. Since they could be written in the form of polynomials, it was better to establish a polynomial model of thin-layer drying based on the original drying data. Time was used as the variable for regression analysis and a quadratic polynomial model was established in which moisture ratio as function and drying temperature, wind speed, and time as three independent variables. To make the prediction effect of the model more accurate, the D-optimal mixture design scheme was adopted in the actual experimental design, which is shown in Table 3. The total time of each drying was not the same due to different drying temperatures and air speeds. Measuring the time in minutes resulted in large errors and reduced the accuracy of the multiform model prediction. Therefore, dimensional time was used to establish a polynomial model applicable to different temperatures and air speeds. Then, according to the prediction of temperature and air speed for the total drying time, τ could be calculated.

The data selection of the regression model was mainly based on the guidance of the R311 experimental design table. However, the total drying time τ was not known before the experiment. If the drying sample was weighed frequently, the drying effect could be greatly affected. Therefore, the sample was weighed every 15 minutes during the experiment. However, the sampling points needed to process the data may not be the same as the actual sampling points. In order to calculate the weight of sampling points required for data analysis (required sampling points for short), the drying process is regarded as the uniform drying stage. When the required sampling point falls between two actual sampling points, the weight of the required sampling point is calculated according to the weight measured at the two actual sampling points before and after the required sampling point. The original weights of all drying samples were 200 g, and the dried weights of samples were 8 g, see Table 7 for detailed data.

Table 7

Polynomial model data table.

Temp (°C)	Air speed (m/s)	τ	Time (min)	MR	Temp (°C)	Air speed (m/s)	τ	Time (min)	MR
72.5	3.0	0.0	0.0	1.0000	81.3	4.4	0.0	0.0	1.0000
63.7	1.6	0.8	168.8	0.0541	85.0	3.0	0.0	0.0	1.0000
81.3	1.6	0.8	90.0	0.0748	60.0	3.0	0.0	0.0	1.0000
63.7	4.4	0.8	123.8	0.0852	72.5	5.0	0.0	0.0	1.0000
81.3	4.4	0.8	90.0	0.1060	72.5	1.0	0.0	0.0	1.0000
85.0	3.0	0.3	30.0	0.7401	63.7	1.6	1.0	225.0	0.0021
60.0	3.0	0.3	56.3	0.6881	81.3	1.6	1.0	120.0	0.0021
72.5	5.0	0.3	37.5	0.6830	63.7	4.4	1.0	165.0	0.0021
72.5	1.0	0.3	52.5	0.7401	81.3	4.4	1.0	120.0	0.0021
72.5	3.0	1.0	150.0	0.0021	85.0	3.0	1.0	120.0	0.0021

72.5	3.0	0.5	75.0	0.3243	60.0	3.0	1.0	225.0	0.0021
63.7	1.6	0.0	0.0	1.0000	72.5	5.0	1.0	150.0	0.0021
81.3	1.6	0.0	0.0	1.0000	72.5	1.0	1.0	210.0	0.0021
63.7	4.4	0.0	0.0	1.0000					

The data were imported into SAS software, and the model was established by regression analysis. According to Table 8, the P value of the model was less than 0.001, which indicates that the model had extreme prominence. The R-square value was 0.9932, which had good fitting properties.

Table 8

Significance table of the regression model.

Regression	DF	R-square	*F* value	Pr > *F*
Linear	3	0.9782	814.52	<0.0001
Quadratic	3	0.0149	12.4	0.0002
Cross product	3	0.0001	0.05	0.9863
Total model	9	0.9932	275.66	<0.0001

At the same time, according to the statistical results, we could obtain the relationship between the MR and the drying temperature and drying air speed and drying time, as shown in the following equation:(17)MR=1.41−0.011T−0.020v−1.61τ+0.000079T2+0.0029v2−0.0020τ∗T+0.0056τ∗v+0.58τ2.

3.5. Comparative Study of Models

The R-square average of the logarithmic model was 0.9449; the Page model was 0.9851; the modified Page model was 0.9960; the Wang and Singh model was 0.9892; and the multiple models were 0.9932. The R-square value of the polynomial model was second to that of the modified Page model, which had a better fitting property.

To better verify the fitting degree of each model, a validation experiment was carried out under a drying temperature of 70°C and at air speed of 4 m/s. At the same time, the model parameters were calculated by taking advantage of the relationship between the experimental conditions and the coefficients of the classic model. The Page model was MR=0.7446e−τ. The Wang and Singh model was MR=1−1.4468τ+0.6424τ2. Drawing Figure 8 to show the curve of the water ratio change with time, the polynomial model was basically equivalent to the measured value. The Page model transformation was smoother, the Wang and Singh model changed more dramatically, and a negative value will appear at an early stage. SAS software was used to calculate the variance of the three models and the measured values. The Duncan method was used to compare the averages, which are shown in Figure 9. The comparison shows that there was no significant difference between the polynomial model and the measured value at a confidence coefficient of 0.95, but there was a significant difference between the Page model and Wang and Singh model.

[figure(s) omitted; refer to PDF]

4. Conclusions

This experiment investigated the law of the moisture ratio change of tomato slurry during the RW drying process. With the guidance of a D-optimal mixture experimental design, this experiment adopted the R311 experimental design table to conduct RW drying experiments. Based on the experimental data and analysis, this study shows that

the drying speed of RW drying could reach 0.40 g/g·min, which could finish drying apace. The total drying time could establish a linear relationship with the drying conditions, which could be used to predict the drying time. Among the four classic models selected in this paper, the modified Page model had a good fit, but the equation parameters could not connect with the experiment conditions. At the same time, the parameters of the Page model and the Wang and Singh model could connect with the test conditions. The variance analysis of the verification experiment shows that the polynomial model could better predict the change of MR over time under other test conditions than the classic model.

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DETAIL

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Occurrence of *Salmonella* in Fresh Foods Sold in the City of Nampula, Northern Mozambique

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ABSTRAK (ENGLISH)

Salmonellosis, an infectious disease caused by the *Salmonella* species, encompasses a broad spectrum of clinical manifestations, ranging from mild self-limiting gastroenteritis to severe systemic infections. It affects millions of people annually, causing immense morbidity and economic losses worldwide. This study aims to evaluate the occurrence of *Salmonella* in water and raw foods, focusing on meat, fish, shellfish, and vegetables consumed in the city of Nampula, north of Mozambique. A total of 81 samples of meat, fish and shellfish, vegetables, and water were collected from nine of the ten municipal markets in Nampula City. *Salmonella* detections were performed according to ISO 6579-1. A chi-square test was performed in the Python programming language to detect associations between positive samples and market localization. The results showed a high frequency of *Salmonella*. From the total sample, 38.5% were *Salmonella* positive. Fresh vegetables were the most contaminated samples, followed by

fish, shellfish, raw meat, and water. The outcomes of this study did not find an association between the sample collection location and the test results for *Salmonella*. In this study, serovars of *Salmonella* were not identified which hinders the association of *Salmonella* occurrence with diseases. Consequently, we propose that the next study should focus on detecting the serotypes of *Salmonella* strains.

TEKS LENGKAP

1. Introduction

Salmonella presents a significant public health concern, being one of the most important foodborne pathogens globally [1]. Salmonellosis, the infectious disease caused by the *Salmonella* species, manifests across a wide spectrum, from mild gastroenteritis to severe systemic infections [2]. *Salmonella enterica* is the most common foodborne enteric pathogen affecting humans and animals [3]. Within the species of *Salmonella enterica*, there are important serovars associated with foodborne illnesses, namely, *S. typhimurium*, *S. enteritidis*, and *S. newport* [3, 4]. These serovars are notorious for their ability to produce toxins that contribute to the severity of infections, playing a significant role in the manifestation of symptoms and the overall impact on human health [5]. One of the primary toxins produced by the *Salmonella* species is an endotoxin known as lipopolysaccharide (LPS) [5, 6].

LPS is a component of the bacterial cell wall and is released when bacteria are destroyed or undergo lysis [6]. This toxin triggers a robust immune response in the host, leading to symptoms such as fever, inflammation, and septic shock [5, 6]. Another important class of toxins produced by pathogenic strains of *Salmonella* includes enterotoxins, proteins that target the gastrointestinal tract and lead to symptoms such as diarrhea, vomiting, and abdominal cramps [3–5]. These toxins disrupt normal gut function by altering ion transport and increasing fluid secretion, resulting in the characteristic watery diarrhea associated with salmonellosis [4]. In addition to endotoxins and enterotoxins, some *Salmonella* strains can produce other virulence factors such as cytotoxins and exotoxins, which contribute to tissue damage and systemic complications [7, 8]. These toxins can target various organs and tissues leading to a diverse array of clinical manifestations, ranging from localized inflammation to systemic dissemination and organ failure [8].

Most salmonellosis outbreaks are linked to the consumption of untreated water or contaminated foods, including animal-derived products such as eggs, pork, and poultry, as well as dairy, fish, shellfish, seafood, fruits, and vegetables [9, 10]. Annually, it directly affects millions of people causing substantial morbidity, mortality, and significant economic repercussions worldwide. Foods of animal origin, such as eggs, pork, and poultry meat, have long been recognized and well documented as prominent vehicles for *Salmonella* transmission [11, 12]. Poultry, such as chickens and turkeys, can harbor *Salmonella* asymptomatically, shedding the bacteria in their feces and thereby contaminating meat and eggs during food processing [13]. The consumption of undercooked or improperly handled poultry products remains a significant risk factor for salmonellosis.

Fish, shellfish, and seafood products are also recognized as common sources of *Salmonella* contamination [14, 15]. According to the Centers for Disease Control and Prevention of the United States, fish and shellfish contribute to 5% of individual cases and 10% of all foodborne illness outbreaks, with most outbreaks stemming from the consumption of raw molluscan shellfish [15]. Even fruit and fresh vegetables, typically perceived as low-risk foods, have been implicated in *Salmonella* outbreaks [10]. Although the *Salmonella* species do not naturally inhabit plant tissues, contamination can occur at various stages along the production and distribution chain [16, 17]. *Salmonella* can penetrate vegetables through contact with contaminated irrigation water, soil, or fertilizers [17–19]. Furthermore, inadequate handling practices during harvesting, packing, and transportation can introduce the pathogen to fresh vegetables, particularly those consumed as raw food [17, 20].

The ramifications of *Salmonella* contamination of fresh foods extend beyond the immediate health risks to consumers. Foodborne outbreaks often result in significant economic repercussions, impacting the agricultural and food industries, as well as public health systems [21, 22]. The cost associated with medical treatment, surveillance,

and outbreak investigations can be substantial, underscoring the urgent need for proactive measures to reduce the prevalence of *Salmonella* in fresh foods [21, 23]. Hence, this study aims to comprehensively assess the occurrence of *Salmonella* in water and fresh foods, with a particular focus on meat, fish, shellfish, and vegetables consumed in the city of Nampula, north of Mozambique.

2. Materials and Methods

2.1. Market and Neighborhood Sanitation Conditions

The hygiene and sanitation conditions in markets and neighborhoods were assessed using a checklist encompassing variables gauged through visual observation. These included overall cleanliness of the area, encompassing floors, walls, and ceilings, the presence of garbage, debris, or waste on the premises, signs of pest infestation, such as insects or rodents, proper exposure, handling, and storage of food, the availability of adequate and hygienic sanitation facilities, the existence of designated areas for proper waste disposal and confirmation of their correct utilization, the presence of an adequate sewage and waste treatment system, and the availability of a drainage system to prevent the accumulation of stagnant water.

2.2. Sample Collection

Fresh vegetables, raw meat, raw fish, shellfish, tap water, and well water sold in the municipal market of Nampula city were sampled and processed (Figure 1). In addition, water samples were collected from rivers.

[figure(s) omitted; refer to PDF]

A total of 81 samples were purchased from nine different municipal markets and six rivers (Table 1). Among the samples were 17 raw meat samples and 13 fish samples. The seafood category (six samples in total) included two octopi, three shrimp, and one squid. Regarding vegetables, 28 samples were collected, including seven lettuce, seven Portuguese cabbages, seven peppers, four pointed cabbage, and three amaranth samples. In addition, there were 17 water samples, five from the tap, five collected from the river, and seven from well water.

Table 1

Sample collection locations.

Neighborhood	Market names	River names
Matadouro	Matadouro	Matadouro river
Muhala	Belenenses	Muhala river
Nalokho	Namutequeliua	Namutequeliua
Napipine river and Nikutha river	Natiquiri	Waresta
Napipine river and Nikutha river	Mutauanha	Pinto Soares
Napipine river and Nikutha river	Murrapaniua	Trim-trim
Napipine river and Nikutha river	Gorongosa	Mutava
Muchinha memória	Karrupeia river	Marrere

Note. Tap and well water were collected from the markets.

2.3. Microbiological Analysis

All analyses were conducted in the microbiology laboratory of the Centre of Interdisciplinarity Studies of Lurio University (CEIL). *Salmonella* was detected according to ISO 6579-1 [24]. From each product, 25 g was aseptically transferred to a sterile BagFilter (INTERSCIENCE, France) containing 225 ml of buffered peptone water (Biokar Diagnostics, Beauvais, France). Subsequently, samples were homogenized in a paddle blender at 85 rpm for 2 minutes and then incubated at 37°C ± 1 for 24 hours. Following this pre-enrichment step, 0.1 ml of aliquots was added to 10 ml of Muller–Kauffmann Tetrathionate Novobiocin Broth (MKTTn) enrichment medium (Biokar Diagnostics, Beauvais, France), while 1 ml portions were, respectively, added to 10 ml of enrichment medium Rappaport Vassiliadis soya (RVS) (Biokar Diagnostics, Beauvais, France). These tubes were then incubated at 37 ± 1°C and 42°C for 24–48 hours, respectively. After the designated incubation period, aliquots of 0.1 ml were withdrawn from each enrichment medium and loop inoculated onto XLD Agar (Biokar Diagnostics, Beauvais, France) and Brilliant Green Agar (Biokar Diagnostics, Beauvais, France). Incubation continued at 37 ± 1°C for 24 hours to facilitate the detection of typical *Salmonella* colonies. These colonies typically exhibit a black centre and a slightly red-colored translucent zone on XLD agar, or pinkish-white or red colonies surrounded by a red halo on Brilliant Green Agar, owing to the indicator color change.

For water samples, Salmonella was performed by filtration of 1000 mL of water through four membranes with a 0.22 µm pore size (Cellulose Nitrate Filter, Sartorius, Germany). After filtration, the membrane of the same samples was considered as one sample of 25 g.

2.4. Data Analysis and Interpretation

A chi-square analysis was conducted to assess the relationship between *Salmonella* positivity and sanitation conditions within markets or neighborhoods. The statistical analysis was performed using the Python programming language with the significance level set at 5%.

3. Results

3.1. Market or Neighborhood Sanitation Conditions

All the markets surveyed lacked toilets equipped with running water. Only one market featured a public toilet accessible to vendors; however, this facility also lacked running water. Vendors frequently resorted to using toilets in nearby residences or engaging in open urination against building walls or tree trunks. Hand-washing practices were generally lacking with vendors often neglecting to wash their hands after toilet use or urination. Except for the central market, cleanliness standards were notably poor across all markets with scattered garbage evident on the ground in each instance. Due to the predominantly outdoor nature of the markets and their packed earth flooring, assessment of the cleanliness of floors and ceilings was not feasible in this study.

The presence of insect vector-borne diseases, including rodents, flies, and cockroaches, was observed in all markets surveyed. In these markets, food for sale is typically displayed on plastic surfaces (polyethylene) directly on the ground or on makeshift structures approximately 1 m tall, constructed from local materials. Proper waste disposal areas are nonexistent and an inadequate sewage and waste treatment system was noted to be absent.

3.2. Microbiological Analysis

Figure 2 illustrates the frequency of *Salmonella*-positive samples across different sample types. Out of the total samples analyzed, 38.5% tested positive for *Salmonella*. Fresh vegetables were the most contaminated samples, followed by fish, shellfish, and raw meat (Figure 3). The highest contamination rates were observed in cucumber pepper (85.7%), followed by amaranthus leaves (66.7%) and lettuce (42%). Among seafood products, fish showed a higher contamination rate (53.8%) than shellfish (33.3%). However, the contamination rate for raw meat was comparatively lower (30%). No *Salmonella* was detected in samples from river water, but positive samples were observed in the samples of well water (28.6%) and tap water (20%).

[figure(s) omitted; refer to PDF]

Regarding the markets, Nalokho and Namutequeliua showed 100% *Salmonella*-positive samples (Figure 3), relating to the contamination of raw meat and fish, respectively (Figure 4). In the Trim-trim market, 50% of the samples were

Salmonella positive, linked to four fresh vegetables, namely, lettuce, amaranthus leaves, cucumber, peppers, and pointed cabbage (Figure 4).

[figure(s) omitted; refer to PDF]

In the Belenenses and Pinto Soares markets, the occurrence of *Salmonella-positive* samples was 46.2% and 42.9%, respectively. In the Belenenses market, fish and raw meat were the most contaminated samples (33.3% for each), and in the Pinto Soares market, all contaminations were reported in fresh vegetables (lettuce, cucumber pepper, and pointed cabbage, 33.3% for each). Matador was the last neighborhood to be found *Salmonella* positive in both tap and well water samples.

The chi-square analysis did not find an association between the sample collection location and the test results for *Salmonella* positivity or negativity (P value >0.05) (Table 2).

Table 2

Association of the occurrence of *Salmonella*-positive samples with localization of markets/neighborhoods.

Case processing summary			
–			
Market*occurrence of *Salmonella*	Cases		
Positive results		Negative results	
N	Percentage	*N*	Percentage
32	39.5	49	60.5
–			
Chi-square test			
–			
	Value	d*f*	Asymp.sig (2-sided)
Pearson’s chi-square	13.949	13	0.377
Likelihood ratio	14.876	13	0.672
Linear-by-linear association	0.997	—	0.94
Number of valid cases	81		

4. Discussion

In the current study, out of the 81 samples analyzed, 38.5% tested positive for *Salmonella*. Specifically, 38.5% (15 out of 39) of the meat products showed *Salmonella* contamination. Among the meat products, fresh fish showed the highest contamination rate, with 53.8% of samples testing positive, followed by shellfish (33.3%) and raw meat (30%). Research on the occurrence of foodborne pathogens in northern Mozambique is limited. However, Kinyamba-Junior et al. [25] evaluated the hygiene practices and meat quality in butcheries of the city of Nampula, Mozambique, and concluded that most of the butcheries (71%) exhibited poor hygiene conditions, a factor which

could potentially contribute to the proliferation of pathogenic bacteria. In addition, a study on the microbial quality of ready-to-eat food [26] and water [27, 28] performed in Mozambique showed a high level of fecal contamination. A study in Hubei Province, China, found *Salmonella* present in 10.5% of retail raw meat products [29]. In Southern Nigeria, *Salmonella* was found in 15.4% of raw chicken meat [30].

Salmonella contamination in fish and seafood is prevalent, particularly in tropical low-incoming countries, due to factors such as sewage effluents, agricultural run-off, and direct fecal contamination from natural fauna. The aquatic environment, fishery equipment, and handling practices significantly impact the bacterial loads in harvested fish [31]. A study conducted in Ethiopia revealed the presence of *Salmonella* in 6.8% of fish samples [31].

In fresh vegetables, 50% of the samples tested positive for *Salmonella*. Cucumber peppers were the most contaminated fresh vegetable with 85.7% of samples testing positive for *Salmonella*. Amaranthus leaves (66.7%) and lettuce (42%) also showed a high percentage of *Salmonella*-positive samples. The results of this study are consistent with previous studies indicating that leafy vegetables are vulnerable to contamination by microorganisms due to their leaf area, which makes them more exposed than other types of fresh vegetables [32]. The most *Salmonella*-positive samples were those that were directly exposed on a tarpaulin on the floor during a sale, leaving them vulnerable to exposure to insects such as flies. This method of display may contribute significantly to the proliferation of *Salmonella* and other bacteria at the point of sale. Moreover, sellers were observed spraying water on fresh vegetables to prolong their freshness. If this water was contaminated, spraying it on fresh vegetables could potentially introduce pathogens to the vegetables [33].

The current rates of occurrence of *Salmonella* in fresh vegetables corroborate reports out of Malaysia where coriander and lettuce salads exhibited contamination rates of 52% and 32%, respectively [34]. However, these rates are higher than those observed in fresh vegetables sold in the United Arab Emirates, where only 5% of samples were reported to be contaminated [35]. *Salmonella* was not detected in fresh vegetable samples analyzed in several cities, such as São Paulo in Brazil [10], Abidjan in Côte d'Ivoire [36], and Maiduguri in the north of Nigeria [37]. Contamination of vegetables often stems from agronomic practices that entail the application of untreated manure, compost, or other organic fertilizers, as well as irrigation with contaminated water during the cultivation process [38–40]. Moreover, contamination can occur during processing and marketing due to several factors, including improper handling, transportation, storage, and the presence of vectors in the market environment [17].

Regarding the water samples, two out of seven samples tested positive for *Salmonella*. While the occurrence of *Salmonella* in tap water is not common, the presence of outdated pipelines in regularly waterlogged neighborhoods can contribute to the prevalence of *Salmonella* within water pipe systems [28, 41]. In well water, *Salmonella* contamination is prevalent, primarily resulting from the pollution of subterranean waters. This contamination often arises from the proximity of septic tanks (latrines) and wells. Research has shown that surface runoff plays a significant role as a driver of the *Salmonella* load in surface waters [26, 42–44]. In a study conducted in Ghana, 6.5% of water samples tested positive for *Salmonella* [45]. Similarly, in Anambra state, Nigeria, the *Salmonella* species was found to be the most prevalent (35%) in reservoir water samples and the least prevalent (7.45%) in borehole water samples examined [46]. The lack of access to safe water for human consumption, coupled with poor environmental sanitation, is a significant contributor to waterborne diarrheal diseases, including salmonellosis, in many developing countries [9, 27, 44].

While our study did not yield positive results regarding the occurrence of *Salmonella typhi*, it underscored the importance of monitoring the *Salmonella typhi* prevalence in markets. This is crucial due to the numerous sanitary conditions often found lacking, which result from inadequate hygiene practices stemming from person-to-person or person-to-material contact, ultimately posing risks to food safety. As our previous review highlighted [9], vigilance in monitoring *Salmonella typhi* occurrence in markets is imperative for safeguarding public health.

In our study, we did not collect data on hygiene and sanitation practices in markets through questionnaire applications to vendors, and we acknowledge this as a challenge for future studies. Nonetheless, our findings provided a snapshot of these practices in markets. Despite this limitation, our results revealed the presence of *Salmonella* in food, tap water, and well water in Nampula city, which is a significant outcome. However, we were

unable to isolate *Salmonella* to determine the predominant serotypes in food or water due to the lack of resources and equipment in our laboratory for serotype analysis. Nevertheless, we can conclude that we achieved our initial objective in this study.

Moving forward, we believe that further research to analyze the serovars of *Salmonella* associated with specific diseases, such as typhoid fever, is necessary. Currently, typhoid fever is a public health concern in Nampula city and our team is eager to contribute to the search for solutions to this pressing issue.

5. Conclusion

In this study, the occurrence of *Salmonella* in the total sample was 38.5%, and the greatest contributors were vegetables, fish and shellfish, and raw meat. However, the positive samples found in tap and well water remain important outcomes in this study as Nampula city faces challenges with water and sanitation facilities. On the other hand, this outcome showed deficiencies in the municipal market, namely, a lack of food conservation facilities and a lack of potable water. In future studies, it will be necessary to increase the number of samples from another type of food, and we need to improve our capacity to detect different serotypes of *Salmonella*. Nevertheless, we hope that the information presented in this paper can influence decision-making bodies regarding the need to improve water and sanitation facilities to reduce the incidence of foodborne illnesses, particularly salmonellosis, which has frequently been reported in the city of Nampula.

Authors' Contributions

A.M. and C.C. conceptualized the study and administrated the project. A.M., A.S., L.A., C.C., and B.M. developed the methodology. A.M., B.M., C.B., I.P., and A.S. investigated the study. A.M. C.C, A.S., and L.A. provided the resources and performed the formal analysis. A.M. curated the data. A.S., A.M., and L.A. reviewed and edited the manuscript and validated the study. A.M. and A. S. wrote the original draft and visualized and supervised the study. All authors have read and agreed to the published version of the manuscript.

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DETAIL

Subjek:	Pathogens; Plumbing fixtures; Shellfish; Food contamination &poisoning; Gastroenteritis; Salmonella; Vegetables; Sanitation; Waste disposal; Epidemics; Water; Meat; Public health; Toilet facilities; Seafood; Rivers; Neighborhoods; Poultry; Toxins; Infectious diseases; Urination; Fish; Infections; Clinical outcomes; Morbidity
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Assessing the Safety of Hotel Food: Knowledge, Attitude, and Practices of Food Handlers

Segbedzi, Cynthia Esinam; Edward Wilson Ansah; Apaak, Daniel.

ABSTRAK (ENGLISH)

Food safety is of global public health concern. Hotels and their restaurants provide hospitality like accommodation and food services and are spearheaded by food handlers who have varied experiences. This study was undertaken to examine knowledge, attitude, and practices (KAP) on food safety and explore knowledge-practice gaps among food handlers at hotels in Ghana. There were 233 participants, including 205 food handlers, 10 managers/chefs, and 18 regulatory officials, who were at post after the COVID-19 restrictions were eased on hotel and restaurant activities. Data were gathered through questionnaire with food handlers and face-to-face interview with managers/chefs and officials of the regulatory agencies. Frequencies and percentages, and Wilcoxon signed ranked test were employed to analyse the data. The knowledge on food safety was moderate, 58±0.169, and 82% of the food handlers portrayed positive attitude towards food safety, while only 42.8% demonstrated good food safety practices. There was significant variance between knowledge and practice such that food handlers' food safety knowledge did not reflect in their daily practices, yet most of the food handlers had training prior to being employed. Many people may be eating contaminated foods from hotels that may increase the rate of food-borne illnesses. We suggest continuous training in transmission of food-borne diseases and time and temperature control, and that the training should be obligatory to reinforce food handlers' KAP. In addition, managers need to intensify their collaborations with the regulators to improve monitoring and supervision activities at the hotels.

TEKS LENGKAP

1. Introduction

Globally, food safety has attained a significant public health concern, because many consumers now frequently eat outside their homes due to changes in lifestyles and work [1]. This is likely to expose many consumers to food-borne infections if care is not taken. For instance, the World Health Organization [2] estimates that 600 million people worldwide become ill, with 420,000 fatalities occurring annually from eating contaminated foods. Furthermore, 90% of cholera cases worldwide are recorded in Africa with 16% of deaths occurring in children under five years [3]. Evidence further showed that populations in Kenya, Senegal, and South Africa continue to experience a high burden of food-borne diseases because of inadequate knowledge of the specific causes [4]. Hence, there is a need to intensify food safety education and training of food handlers.

Evidence shows that at least one out of 40 people are affected with food-borne diseases resulting in an annual cost of 69 million dollars to the Government of Ghana [5]. For instance, a report from Food and Drugs Authority (FDA) Ghana showed that about fifty-three (53) persons were taken ill after consuming contaminated food from a chain restaurants in Accra. Samples of food tested at the laboratory from one of the outlets indicated high levels of microbial contaminants, which could plausibly be the source of the food contamination leading to the closure of some of the outlets [6]. In addition, analysis of typhoid fever surveillance data on the central and western regions, between 2016 and 2019, revealed 45,497 and 11,104 cases, respectively [7–9]. This was not a surprise since the two regions are major tourist sites with many hotel and restaurant facilities that attract visitors all year round [10].

Moreover, evidence showed that food contamination by food handlers accounts for about 10–20% of food-borne illnesses [2, 11–13]. Thus, the relevance of knowledge, attitude, and practices of commercial food handlers cannot be overemphasized [14].

Hotel restaurants are considered the highest category of food service establishments [15]. Therefore, to reduce the overwhelming effects of food-borne diseases, there is a need for continuous training of food handlers of such facilities to increase their knowledge. This could translate into safe food handling practices to protect customers and the general public. Besides, it is imperative to adopt appropriate and sustainable food safety management practices essential for the attainment of the Sustainable Development Goals (SDGs) 2 and 3 [3]. SDG 2 aims at creating a hunger-free world and improves nutrition, while SDG 3 guarantees health and well-being for all.

This study was anchored on the theory of planned behaviour [16]. The theory speculates that the best predictor of an individual's behaviour is the intention to perform the behaviour [17]. This is grounded on three antecedents: subjective norms, attitudes, and perceived behavioural control (PBC) [18]. Subjective norm represents the perceived pressure from significant individuals such as managers/supervisors, business owners, or colleagues whose words hold sway in the performance of a behaviour. Attitude, on the other hand, determines the level at which individuals have favourable or unfavourable assessment of a behaviour in question [19]. This is influenced by beliefs individuals hold regarding such behaviour and expectations from subjective norms. Consequently, a food service employee may develop favourable attitudes towards safe food handling practices, but if managers/supervisors think otherwise due to workplace challenges, such as time constraint, lack of equipment, motivation, or shortage of staff, it may result in unsafe food handling practices by such food handlers [20].

To facilitate the performance of safe food behaviours, there is a need to equip food service employees with knowledge, skills, equipment, and opportunities to build their confidence, deal with past behaviours, and increase monitoring and supervision [21]. Monitoring and supervision is key in the management of food safety practices since attitudes towards behaviour comprise individual's appraisal of how positive or negative performing a behaviour would be and how subjective norms reflect an individual's perceptions of social pressure to either perform or not to perform an action. Attitudes and subjective norms are based on beliefs, and individual's intentions serve as the mediation factor for behaviour to be performed. For instance, if food handlers believe that frequent hand washing is undesirable, they are less likely to engage in it. This attitude reflects a critical factor that determines food handlers' intentions as well as the successive likelihood to execute the behaviour. Therefore, if safe food handling practices are not properly monitored/supervised, it may be left to the discretion of the food handler. Thus, it is imperative to ensure an efficient regime of monitoring and supervision of food service employees by regulators, managers, and owners. Meanwhile, monitoring and supervision, which the theory seems to be silent on, is a gap the current study intends to bridge.

Although evidence exists on knowledge, attitude, and practices of food vendors on food safety practices [22–25], very little is known of food safety practices of food handlers at hotels and the impact on health of consumers. Given that most hotels are licensed, regulated, and operated in well-structured settings, data regarding the knowledge level, attitudes, and practices of these categories of food handlers in Ghana are essential to promote public health and safety. Therefore, this study aimed to assess knowledge, attitude, and practices of food handlers at hotels in the central and western regions of Ghana.

2. Materials and Methods

Due to COVID-19 restrictions, only 21 hotels were fully operational in the central and western regions at the time [10]. A mixed-method design was used to gather data from 233 participants, including 205 food handlers, 10 managers/chefs, and 18 officials from the regulatory agencies (Food and Drugs Authority [FDA], Environmental Health and Sanitation Unit [EHS], and GTA). The hotels comprised five 1star, nine 2star, and seven 3star hotels. We acquired ethical clearance (ID: UCCIRB/CES/2020/28) from the Institutional Review Board, University of Cape Coast. Moreover, the first author was medically screened and certified (Cert. No: 1,057) by EHS of Cape Coast, before commencing data collection. Permission was sought from the FDA, EHS, GTA, and the various hotel managers. The purpose of the study was explained to the respondents, and anonymity and confidentiality of their

information were also assured. Also, they signed informed consent forms to participate in the study [26].
The instruments were then distributed and collected two weeks after administration. The interviews were conducted for the food and beverage managers/chefs and the regulatory officials using audio recording and note-taking. The interviews were conducted at the offices of the regulatory officials, and each session lasted about 40 minutes [27].

2.1. Instruments

Data collection commenced from January 25, 2021, to May 20, 2021. The instruments employed included a questionnaire, an interview guide, and an observation checklist. The instruments were adapted from related literature [25, 28–31]. The questionnaire on food handlers was divided into four segments (A, B, C, and D). The items were structured such that the same questions were employed to measure knowledge, attitude, and practices, but were framed to suit their different domains. This was to enable a fair comparison of the variables [32, 33]. Section "A" explored the demographic attributes of the respondents, such as sex, age, level of education, training, and work experience and medical examination. Section "B" consisted of 32 items, in which responses ranged from "Yes," "No," and "I don't know." This section dealt with participants' knowledge on transmission of food-borne diseases, personal hygiene, cross-contamination, and time and temperature control. Section "C" consisted 30 items on 4-point scale, strongly disagree (1) to strongly agree (4), measuring attitude of the workers to food safety. Section "D" evaluated the regularity of appropriate food safety behaviours exhibited by the employees. It comprised 35 questions, on a 3-point scale "always" (1), "sometimes" (2), to "never" (3).
Using an observation checklist based on the FDA's code for food service establishments [34], on-site observations were conducted at all 21 hotels to ascertain conformity of the facilities and kitchen environment to the FDA's code of practice. This covered areas like ventilation, lighting, wall/floors/ceilings, sanitary facility conditions, kitchen equipment and their location, and storage facilities. Interviews were also conducted for the regulatory officials to explore their role in ensuring compliance with food safety standards and whether food safety was a threat within the two regions. To ascertain the validity and reliability, the instrument was reviewed by experts in food safety from the Department of VOTEC UCC and FDA and pretested using four fast-food restaurants in Cape Coast and Elmina [27].

2.2. Data Analysis

We classified knowledge, attitude, and practice questions using the frequency and percentage tallies and categorized the responses into low, moderate, and high knowledge. A score of "1" was given for every correct answer and "0" for every incorrect answer or an unanswered question [25, 28]. Hence, the score range between 0 and 32 and was converted to 100 percentage points. A score of 0–10 was considered low, 11–20 moderate, and 21–32 good. Therefore, a score <50% of food safety knowledge was rated low knowledge, between 50 and 59% moderate, while >60% is good. The scores for attitude of each worker were determined by adding the weights obtained by each worker for the 32 items. A correct answer to less than 16 statements signalled "negative or poor" attitude towards food safety practices, whereas more than 16 indicated "positive or good" attitude [25]. Food safety practices were established by calculating the proportions of the right responses using a three-point ordinal scale. A score <50% signalled "negative" and ≥70% "positive" food safety practices.
To determine the gap between knowledge and practice of the food handlers, the Wilcoxon signed rank test was utilized, because the data are not normally distributed, and it is ordered and had many outliers [35, 36]. The interviews and FGDs we transcribed verbatim and coded into manageable categories of varied themes focused on specific word patterns that were indicative of the research questions [27]. Repetitions, filler words, and hesitations were eliminated in the reporting, as they did not add value to the content.

3. Results

A total of 205 food handlers responded to the questionnaire. The knowledge level on food safety of the food handlers was assessed across four domains that are personal hygiene, cross-contamination, transmission of food-borne diseases, and time and temperature control. The same variables were used to examine attitude and practices of the food handlers. Most of the food handlers were females (57.6%). Majority were between 21 and 30 years (68.8%), with a greater percentage (81.5%) receiving training in food safety. Also, 45.4% of the food handlers had 2 to 4 years of working experience, while 69.0% had undergone medical examination as required by the EHS of the

Ministry of Local Government and Rural Development (MLGRD). The rest of the demographic information is in Table 1.

Table 1

Levels of education, knowledge, and attitude of food handlers at hotels in central and western regions.

Levels of education, knowledge, and attitude of food handlers	Frequency (*f*)	Percentage (%)
Below secondary	12	5.9
SHS	62	30.2
Vocational	44	21.5
Tertiary	87	42.4
Knowledge score		
Good knowledge	46	22
Moderate knowledge	119	58
Low knowledge	40	20
Attitude		
Positive attitude	168	82
Negative attitude	18	37
Total	205	100

3.1. Food Safety Knowledge of Food Handlers

The food safety knowledge of food handlers was measured across four domains, including knowledge on transmission of food-borne diseases, knowledge on personal hygiene, knowledge on cross-contamination, and knowledge on time and temperature control. Of the four domains of food safety assessed, respondents demonstrated high level of knowledge in personal hygiene (71.4%), cross-contamination (67.7%), and average (58.1%) in transmission of food-borne diseases, and time and temperature control (57.4%) (Tables 1–9). The level of knowledge on food safety among food handlers across the four domains of food safety examined was found to be moderate (58%; mean score 0.619 ± 0.169). However, 20% of the participants were classified as low in their food safety knowledge (Figure 1).

Table 2

Knowledge on transmission of food-borne diseases of food handlers at hotels in central and western regions.

Questions on food safety knowledge about transmission of food-borne disease	Correct response	Incorrect response

f (%)	*f* (%)	*Transmission of food-borne diseases*
(1) Canned foods can contain harmful microbes	138 (67.3)	67 (32.7)
(2) The first-in-first-out principle of food storage relates to only perishable foods	77 (37.6)	128 (62.4)
(3) Preparing food in advance reduces the risk of contamination	113 (55.1)	92 (44.9)
(4) Canned foods that are dented and rusty can still be used to cook or for direct consumption	147 (71.7)	58 (28.3)
(5) It is more important to serve safe food than tasty food	48 (23.4)	157 (66.6)
(6) Using food one day past its expiration date poses risk to health	131 (63.9)	74 (36.1)
(7) Food additives can cause food contamination	95 (46.3)	110 (53.7)
(8) Fresh vegetables from farms can cause food-borne diseases	68 (33.2)	137 (66.8)
(9) Change in colour of food is a symptom of food contamination	158 (77.1)	47 (22.9)
(10) Refrigerating and freezing kills all the bacteria/microbes that contribute to food-borne illness	108 (52.7)	97 (47.3)
–		
Overall knowledge on transmission of food-borne diseases	58.1	

Table 3

Personal hygiene knowledge of food handlers at hotels in central and western regions.

Questions on food safety knowledge on personal hygiene	Correct response	Incorrect response
f (%)	*f* (%)	*Personal hygiene*
(1) It is safe to wash hands with water only after handling raw meat	171 (83.4)	34 (16.6)
(2) The use of hand sanitizers is a substitute for hand washing	139 (67.8)	66 (32.2)
(3) When wearing gloves, you can handle cooked foods after handling raw meat	158 (77.1)	47 (22.9)

(4) Food handlers can keep long and painted fingernails provided they are clean	161 (78.5)	44 (21.5)
(5) Wearing of nose mask during meal preparation and service reduces the risk of disease infection and not food contamination	127 (62.0)	78 (38.0)
(6) It is safe to wash hands with only water after using the urinary	169 (82.4)	36 (17.6)
(7) It is only essential for food handlers to wash hands with soap and water after touching someone else and not their own body parts	173 (84.4)	32 (15.6)
(8) It is safe for food handlers who have short hair not wear cap while handling food	146 (71.2)	59 (28.8)
–		
Overall knowledge on personal hygiene	71.4	

Table 4

Knowledge on cross-contamination of food handlers at hotels in central and western regions.

Questions on food safety knowledge on food contamination and cross-contamination	Correct response	Incorrect response
f (%)	*f* (%)	*Contamination and cross-contamination*
(1) Food handlers should be medically examined	142 (69.3)	63 (30.7)
(2) It is safe to use one cutting board to process ready-to-eat foods and raw meat/fish	167 (81.5)	38 (18.5)
(3) After using the bathroom, hands can be washed in the kitchen sink	175 (85.4)	30 (14.6)
(4) A healthy food handler can be a carrier of infectious food-borne diseases	73 (35.6)	132 (64.4)
(5) After washing hands, hands may be dried with a kitchen towel	131 (63.9)	74 (36.1)
(6) Cooked and uncooked foods can be stored on the same shelf in the refrigerator	158 (77.1)	47 (22.9)
(7) It is safe for food handlers with cuts/wounds on hands to handle food provided, the wound is covered	138 (67.3)	67 (32.7)

(8) Food preparation surfaces can contaminate foods	117 (57.1)	88 (42.9)
(9) Eating and drinking when preparing or serving food increase the risk of food contamination	118 (57.6)	87 (42.4)
(10) It is safe to shred/cut vegetables before washing	169 (82.4)	36 (17.6)
–		
Overall food safety knowledge on food contamination and cross-contamination	67.7	

Table 5

Knowledge on time and temperature control of food handlers at hotels in central and western regions.

Questions on food safety knowledge on time and temperature control	Correct response	Incorrect response
f (%)	*f* (%)	*Time and temperature control*
(1) The frozen foods can be left outside the refrigerator as long as there is ice in them	104 (50.7)	101 (49.3)
(2) It is safe to touch food with the fingers to see if it is cooked	104 (50.7)	101 (49.3)
(3) The ideal place to store raw meat in the refrigerator is on the bottom shelf	115 (56.1)	90 (43.9)
(4) It is safe for defrosted unused food to be refrozen	76 (37.1)	129 (62.9)
(5) Checking the temperature of refrigerators periodically reduces the risk of food contamination	126 (61.5)	79 (38.5)
(6) It is safe to thaw/defrost raw meat/fish in a bowl of water	145 (70.7)	60 (29.3)
(7) Fresh vegetables and other perishable foods can be stored on the shelves in the kitchen	154 (75.1)	51 (24.9)
–		
Overall food safety knowledge on time and temperature control	57.4	

Table 6

Attitude of food handlers towards transmission of food-borne diseases at hotels in central and western regions.

Questions on food safety attitude towards transmission of food-borne diseases	Positive attitude	Negative attitude
f (%)	*f* (%)	*Transmission of food-borne diseases*
(1) Canned foods do not contain microbes because they are processed	115 (56.1)	90 (43.9)
(2) Canned foods that are dented and rusty can still be used in cooking or for direct consumption	137 (66.8)	68 (33.2)
(3) I think foods prepared in advance have reduced risk of contamination	94 (45.8)	111 (54.2)
(4) I believe tasty food is more important than safe food	115 (56.1)	90 (43.9)
(5) I think change in colour of food is a symptom/sign of food contamination	145 (70.7)	60 (29.3)
(6) I think food additives can cause food contamination	169 (82.4)	36 (17.6)
(7) I think eating or using food a day past its expiration date poses risk to health	88 (42.9)	117 (57.1)
(8) Refrigerating and freezing destroy/kills all the bacteria/microbes that contribute to food-borne illness	121 (59.0)	84 (41.0)
(9) Fresh vegetables from farms can cause food-borne diseases	102 (49.8)	103 (50.2)
(10) The first-in-first-out principle of food storage relates to only perishable foods	100 (48.8)	105 (51.2)
–		
Overall food safety attitude towards transmission of food-borne diseases	57.8	

Table 7

Attitude of food handlers towards personal hygiene at hotels in central and western regions.

Questions on food safety attitude towards personal hygiene	Positive attitude	Negative attitude
f (%)	*f* (%)	*Personal hygiene*
(1) When wearing gloves, you can handle cooked foods after handling raw meat	177 (86.3)	28 (13.7)

(2) I think food handlers can keep long and painted fingernails provided they are clean	184 (89.8)	21 (10.2)
(3) I can use hand sanitizers as a substitute for hand washing	111 (54.2)	94 (45.8)
(4) It is not necessary for food handlers with short hair to wear a cap	162 (79.0)	43 (21.0)
(5) It is not necessary to wash hands before handling food that is already cooked	168 (82.0)	37 (19.0)
(6) I think wearing of nose mask only reduces the risk of disease infection and not food contamination	148 (72.2)	57 (27.8)
(7) I think it is safe to wash my hands with only water after using the urinary	179 (87.3)	26 (12.7)
(8) I think it is essential for food handlers to wash hands with soap and water after touching someone else and not their own body parts	171 (83.4)	34 (16.6)
–		
Overall food safety attitude towards personal hygiene	79.3	

Table 8

Attitude towards cross-contamination of food handlers at hotels in central and western regions.

Questions on food safety attitude towards food contamination and cross-contamination	Positive attitude	Negative attitude
f (%)	*f* (%)	*Food contamination and cross-contamination*
(1) I think hands can be washed in the kitchen sink after using the bathroom	178 (86.8)	27 (13.2)
(2) I can prepare food if, I have wounds or cuts on my hands provided it is covered with bandage	134 (65.4)	71 (34.6)
(3) It is safe to use one cutting board to process ready-to-eat foods (e.g., vegetables and fruits) raw meat/fish	162 (79.0)	43 (21.0)
(4) Cooked and uncooked foods can be stored on the same shelf in the refrigerator	161 (78.5)	44 (21.5)
(5) A healthy food handler cannot be a carrier of infectious food-borne diseases	98 (47.8)	107 (52.2)

(6) I think evaluating the health status of food handlers every six months is very important	157 (76.6)	48 (23.4)
(7) Hands can be dried with a kitchen towel after washing	117 (57.1)	88 (42.9)
(8) Food preparation surfaces can contaminate foods	112 (54.6)	93 (45.4)
(9) It is appropriate to eat and drink when preparing or serving food	155 (75.6)	50 (24.4)
(10) It is safe to shred/cut vegetables before washing	160 (78.1)	45 (21.9)
–		
Overall food safety attitude towards food contamination and cross-contamination	70.0	

Table 9

Time and temperature control of food handlers at hotels in central and western regions.

Questions on food safety attitude towards time and temperature control	Positive attitude	Negative attitude
f (%)	*f* (%)	*Attitude towards time and temperature control*
(1) Freezing kills the microbes that may cause spoilage of food	92 (44.9)	113 (55.1)
(2) It is safe to touch food with the fingers to see if it is cooked	120 (58.5)	85 (41.5)
(3) I think defrosted food can be refrozen	89 (43.4)	116 (56.6)
(4) I think frozen foods can be left outside the refrigerator as long as there is ice in them	102 (49.8)	103 (50.2)
(5) I think it is safe to thaw/defrost raw meat/fish in a bowl of water	156 (76.1)	49 (23.9)
(6) I think vegetables can be store on the shelves of the kitchen	161 (78.5)	44 (21.5)
(7) I believe periodic checks on the temperature settings of refrigerators/freezers reduce the risk of food contamination	146 (71.2)	59 (28.8)
–		
Overall food safety attitude towards time and temperature control	60.4	

[figure(s) omitted; refer to PDF]

3.2. Food Safety Practices of Food Handlers

The results of the food safety practices of the food handlers were evaluated in four areas, including food safety practices on transmission of food-borne diseases, practices on personal hygiene, practices on cross-contamination, and practices on time and temperature control. Meanwhile, of the four areas of food safety behaviours examined, respondents demonstrated good practice in personal hygiene (68.1%) and cross-contamination (52.6%), whereas they were poor in time and temperature control (39.6%) and practices related to the transmission of food-borne diseases (37.7%) (Table 10–13). On the whole, the result indicated that 42.8% ($n=87$) of the food handlers had good food safety practices (≤50), while 57.8% ($n=118$) recorded poor food safety practices (≤50) (Figure 1).

Table 10

Transmission of food-borne diseases practices of food handlers at hotels in central and western regions.

Questions on food safety practices on transmission of food-borne diseases	Good practice	Poor practice
f (%)	*f* (%)	*Transmission of food-borne diseases*
(1) I used food that has changed in colour	105 (51.2)	100 (48.8)
(2) I prepare food in advance to reduce the risk of contamination	73 (35.6)	132 (64.4)
(3) I use canned foods that are dented and rusty in cooking or for direct consumption	130 (63.4)	75 (36.6)
(4) It is more important to serve safe food than tasty	55 (26.8)	150 (73.2)
(5) I use only stored perishable food first-in-first-out basis, e.g., vegetables, fruits, and meat/fish	70 (34.2)	135 (65.8)
(6) Use of food additives can cause food contamination	22 (10.7)	183 (89.3)
(7) I use food one day past its expiration date	105 (51.2)	100 (48.8)
(8) I do not wear cap when I cut my hair or wear short wig while handling food	122 (59.5)	83 (40.5)
(9) Fresh vegetables from farms can cause food-borne diseases	30 (14.6)	175 (85.4)
(10) I refrigerate and freeze food to destroy/kill all the bacteria/microbes that contribute to food-borne illness	65 (31.7)	140 (68.3)
–		
Overall practice score on transmission of food-borne diseases	37.9	

Table 11

Personal hygiene practices of food handlers at hotels in central and western regions.

Questions on food safety practices on personal hygiene	Good practice	Bad practice
f (%)	*f* (%)	*Personal hygiene*
(1) I wash my hands before handling food that is already cooked	151 (73.7)	54 (26.3)
(2) I handle cooked food and raw meat at the same time when wearing gloves	149 (72.7)	56 (27.3)
(3) I use hand sanitizers as a substitute for hand washing	70 (34.2)	135 (65.8)
(4) I keep long and painted fingernails provided they are clean	170 (82.9)	35 (17.1)
(5) It is safe for food handlers who have short hair not to wear cap	146 (71.2)	59 (28.8)
(6) I wash my hands with soap and water after touching my own body parts and others	120 (58.5)	85 (41.5)
(7) I wear nose mask to prevent diseases infection and food contamination	134 (65.4)	71 (34.5)
(8) I wash hands with soap and water after using the urinary	177 (86.3)	28 (13.7)
–		
Overall food safety practice score on personal hygiene	68.1	

Table 12

Cross-contamination practices of food handlers at hotels in central and western regions.

Questions on food safety practices on food contamination and cross-contamination	Good practice	Bad practice
f (%)	*f* (%)	*Food contamination and cross-contamination*
(1) I store raw and cooked food items together in the same place in the refrigerator	131 (63.9)	74 (36.1)
(2) If I have a wound on the hands, I can prepare food provided the wound is covered with a bandage	107 (52.2)	98 (47.8)
(3) I pay attention to expiration dates on food and do not use foods that have passed the expiry date	114 (55.6)	91 (44.4)

(4) I wash my hands in the kitchen sink after using the bathroom	146 (71.2)	59 (28.8)
(5) I am medically examined every six months	114 (55.6)	91 (44.4)
(6) I dry my hands with a kitchen towel after I washing hands	97 (47.3)	108 (52.7)
(7) A healthy food handler cannot be a carrier of infectious food-borne diseases	23 (11.2)	182 (88.8)
(8) I eat and drink when preparing or serving food	132 (64.4)	73 (35.6)
(9) I shred/cut vegetables before washing	155 (75.6)	50 (24.4)
(10) I use the same cutting board for handling raw and ready-to-eat foods	59 (28.8)	146 (71.2)
–		
Overall food safety practice score on food contamination and cross-contamination	52.6	

Table 13

Time and temperature control practices of food handlers at hotels in central and western regions.

Questions on food safety practices on time and temperature control	Good practice	Bad practice
f (%)	*f* (%)	*Time and temperature control*
(1) I check the temperature of refrigerators/freezers periodically to reduce the risk of food contamination	89 (43.4)	116 (56.6)
(2) I refreeze defrosted foods	158 (77.1)	47 (22.9)
(3) I touch food with the fingers to check if it is cooked	75 (36.6)	130 (63.4)
(4) I freeze foods to kill the microbes that may cause spoilage of food	51 (24.9)	154 (75.1)
(5) I keep frozen food items out of the refrigerator provided there is ice in them	59 (28.8)	146 (71.2)
(6) I thaw/defrost raw meat/fish in a bowl of water	85 (41.5)	120 (58.5)
(7) I store vegetables on the shelves in the kitchen	43 (21.0)	162 (79.0)
–		
Overall food safety practice score on time and temperature control	39.6	

3.3. Knowledge-Practice Gap among Food Handlers

The results of the Wilcoxon signed rank test showed a significant variance between knowledge and practice (9% = 0.04) where average knowledge = 64.0% and practice = 54.6%)). That is food handlers' knowledge at hotels in central and western regions on food safety did not translate into their practices (Tables 14–17).

Table 14

Paired differences between food safety knowledge and practices on transmission of food-borne diseases among food handlers at hotels in central and western regions.

Transmission of food-borne diseases	Knowledge	Practice	Gap
Canned foods can contain harmful microbes	67.3	63.4	3.9
The first-in-first-out principle of food storage relates to only perishable foods	38	34.2	3.8
Preparing food in advance reduces the risk of contamination	55.1	35.6	19.5**
Canned foods that are dented and rusty can still be used to cook or for direct consumption	71.7	63.4	8.3*
Using food one day past its expiration date poses risk to health	63.9	51.1	12.7**
Refrigerating and freezing kills all the bacteria/microbes that contribute to food-borne illness	52.7	31.7	21**
Average	58.1	46.6	11.5**

*P<0.05; **P<0.01.

Table 15

Paired differences between food safety knowledge and practices on personal hygiene among food handlers at hotels in central and western regions.

Personal hygiene	Knowledge	Practice	Gap
It is safe to wash hands with water only after handling raw meat	83.4	73.7	9.7*
The use of hand sanitizers is a substitute for hand washing	32.2	34.2	−2
When wearing gloves, you can handle cooked foods after handling raw meat	77.1	72.7	4.4
Food handlers can keep long and painted fingernails provided they are clean	78.5	82.9	−4.4
Wearing of nose mask during meal preparation and service reduces the risk of disease infection and not food contamination	62	65.4	3.4

It is safe to wash hands with only water after using the urinary	53.2	73.7	20.5**
It is only essential for food handlers to wash hands with soap and water after touching someone else and not their own body parts	55.1	58.5	−3.4
It is safe for food handlers who have short hair not wear cap while handling food	71.2	71.2	0.0
Average	64.1	66.5	−2.4

*P<0.05; **P<0.01.

Table 16

Paired differences between food safety knowledge and practices on cross-contamination among food handlers at hotels in central and western regions.

Cross-contamination	Knowledge	Practice	Gap
Food handlers should be medically examined	69.3	55.6	13.7**
It is safe to use one cutting board to process ready-to-eat foods and raw meat/fish	81.5	28	53.5**
After using the bathroom, hands can be washed in the kitchen sink	85.4	71.2	14.2**
After washing hands, hands may be dried with a kitchen towel	63.9	47.3	16.6**
Cooked and uncooked foods can be stored on the same shelf in the refrigerator	77.1	63.9	13.2**
It is safe for food handlers with cuts/wounds on hands to handle food provided, the wound is covered	67.3	52.2	15.1**
Eating and drinking when preparing or serving food increase the risk of food contamination	57.6	64.4	−6.8
It is safe to shred/cut vegetables before washing	82.4	75.6	6.8
Average	73.1	57.3	15.8**

*P<0.05; **P<0.01.

Table 17

Paired differences between food safety knowledge and practices on time and temperature control among food handlers at hotels in central and western regions.

Time and temperature control	Knowledge	Practice	Gap

The frozen foods can be left outside the refrigerator as long as there is ice in them	50.7	28.8	21.9**
It is safe to touch food with the fingers to see if it is cooked	50.7	36.6	14.1**
It is safe for defrosted unused food to be refrozen	37.1	22.9	14.2**
Checking the temperature of refrigerators periodically reduces the risk of food contamination	61.5	43.4	18.1**
It is safe to thaw/defrost raw meat/fish in a bowl of water	70.7	41.5	29.2**
Fresh vegetables and other perishable foods can be stored on the shelves of the kitchen	75.1	79	−3.9
Average	57.6	42.0	15.6**

*P<0.05; **P<0.001.

4. Discussion

The purpose of this study was to assess the level of knowledge, attitude, and practices of food handlers at hotels in central and western regions of Ghana and to appraise their knowledge-practice gap on food safety. The findings show that more than half of the food handlers had moderate knowledge on food safety, a large percentage portrayed positive attitude towards food safety, while only a few demonstrated good food safety practices. In addition, there was significant variance between knowledge and practice such that food handlers' food safety knowledge did not reflect in their daily practices.

4.1. Knowledge of Food Handlers on Food Safety

The findings show that even though close to half of the food handlers had higher education and trained in food safety, this did not reflect in their level of knowledge on food safety. To this, a participant RDC1FG1 confirmed: *…hmmm!!! It is not easy oo!!! When we go on inspection and detect an anomaly in the way food is being handled or there is an emerging issue like the current corona virus pandemic, we organize training for both managers and food handlers.*

This affirms a previous study [37] conducted in Malaysia reporting similar findings. However, findings from the current study are inconsistent with previous studies in Kuwait [32], Zimbabwe [38], and Northern Ghana [30] reporting higher knowledge among food handlers in hotels.

Our findings revealed that generally, the respondents had average knowledge on the transmission of food-borne diseases (58.1%), but their knowledge on indicators such as "expiration dates on canned foods, foods prepared in advance for service can become contaminated, refrigeration kills microbes in food, and a symptom of food spoilage is change colour" was good. Meanwhile, the food handler's knowledge on the "first-in-first-out principle, tasty food is better than safe food, food additives causing food contamination and use of dented and rusty canned foods" was poor. Giving that knowledge sustains compliance, the poor knowledge of these food handlers is likely to impact their ability to prevent contamination of food and spread of food-borne diseases and calls for effective monitoring and control to safeguard public health. This finding corroborates the findings from previous studies [30, 39–42].

Knowledge on poor personal hygiene and cross-contamination are major contributory factors to the outbreak of food-borne infections. Our finding indicated that the majority of the food handlers had good knowledge on personal hygiene (71.4%) and cross-contamination (67.7%). For instance, they recorded good knowledge on "hand washing, the occasions for washing hands and its importance in reducing the risk of food contamination, wearing of hair restraints and nose masks, storage of both cooked and uncooked foods, checking their health status, abstinence

from work when sick, and the use of same cutting board for processing meat/fish and ready-to-eat food." This is very good because the food handlers are conversant with public health regulations on hand washing before, during and after preparing food, regular medical examination, and abstinence from work when sick. This will help to reduce the risk of food-borne illnesses and increase public health safety. For instance, studies [43, 44] show that a wide range of microorganisms can be found on the human hand, which can be easily transmitted to food if hands are not frequently washed. Hence, regular hand washing with soap and under running water while preparing food is key in preventing cross-contamination. This concurs with the results of previous studies [2, 32, 45–49]. Unfortunately, it is worrying that a large percentage of the participants believed that the usage of hand sanitizer is a substitute for hand washing. The food handlers' lack of knowledge in this area could be due to inadequate training on the appropriate use of hand sanitizers and the surge in sanitizer usage during the peak of the COVID-19 pandemic. Although the use of sanitizers has been proven to kill bacteria and viruses, the use of soap and water is more effective against pathogens [50]. Evidence showed that hand sanitizers may not be effective on soiled or greasy hands and may in some cases contaminate food if the hands are not properly dried before touching food [51, 52]. Also, only a few (35.6%) of the food handlers have good knowledge concerning whether a healthy food handler can transmit infectious food-borne pathogens into foods. This could result in asymptomatic food handlers transmitting infectious diseases into food that may endanger public health. Therefore, it is key to prevent cross-contamination at any level of food preparation and service to guarantee the safety of foods for human consumption. The current finding is consistent with the findings of Yimam et al. [53] and Wainaina et al. [54] who found that the majority of food handlers in their study were asymptomatic for norovirus and intestinal parasitic infections.

On the issue of time and temperature control, most (57.4%) of the respondents in the present study possess average knowledge on time and temperature control. For instance, the respondents portrayed good knowledge on the parameters such as "storing cooked and uncooked foods separately, storage of meat on the bottom shelf of the refrigerator, and checking refrigerator temperatures regularly reduce food contamination and spoilage." This agrees with earlier studies [55, 56], which reported adequate knowledge of food handlers on the appropriate method of storing cooked and uncooked foods in the refrigerator and regular checks on refrigerator temperatures. Meanwhile, the food handlers recorded poor knowledge on indicators such as "defrosting frozen food in a bowl of water, refreezing defrosted foods, and leaving frozen foods outside the refrigerator if it has ice in it." This is quite worrying because thawing/freezing cyclically increases the microbial loads and decreases the nutritional quality of food, which may render it unsafe for consumption. This demands for a proper training and monitoring/supervision of food handlers to improve their knowledge and practices to prevent cross-contamination, and food poisoning to protect public health. This finding is similar to previous studies [41, 57, 58].

4.2. Attitude of Food Handlers towards Food Safety

The findings revealed a positive attitude of the respondents towards food safety as a little above half of the respondents disagreed with the statements that "canned foods do not contain microbes because they are processed" and "canned foods which are dented and rusty can still be used in cooking or for direct consumption and refrigeration/freezing kills all bacteria or microbes that contribute to foodborne illness." This suggests that the food handlers perceived the hazards linked with using canned foods, which are dented, bulging, cracked, or leaking and those that are not properly processed and packaged to health as well as their comprehension of the critical function of food safety temperatures in protecting the health of consumers. The finding of the current study concurs with those of previous studies [37, 59]. For instance, Li et al. [60] identified the growth of *Salmonella paratyphi A* at minimum temperature of 8.9°C in roasted chicken. This is an indication of some microorganisms being able to proliferate under substandard refrigeration temperatures; hence, the critical function of adequate refrigeration temperatures for the storage of both cooked/uncooked foods to ensure the safety of food served to consumers is vital.

Additionally, on personal hygiene and cross-contamination, many of the food handlers recorded positive attitude in areas, such as glove usage, keeping long and painted fingernails, wearing nose masks, hair restraint, washing hands with soap and water prior to handling cooked food, and not handling food with wounds or cuts even if such is

covered with bandage. Also, the majority of these food handlers believed that wearing of hair restraints/nose mask and checking their health regularly were a vital practice to prevent contamination of food. To this, a manager THC1 confirmed:

Oh! For us in this hotel we do not joke with the issue of the health examination results of our food handlers. It is one of the key requirements for employment and also for being fired. There have been situations where those who were due for the examination but failed to do so were suspended.

A regulator RMS3FG2 retorted during:

Yes! The hotels are very responsive with the regular examination of their employees. In the past, the employees used to do it individually on their own. However, to guarantee that all the food handlers are examined and the results are authentic, the Environmental Health Directorate in collaboration with the hotel managers have decided to undertake routine health examination and within a specific period. Any employee who is not able to have the examination done within the stipulated period will bear the full cost of the examination.

This positive attitude could be due to education and training of the general public on the use of protective clothing during the COVID-19 pandemic [32, 41, 55, 61].

Although the food handlers exhibited a generally positive attitude towards time and temperature control, many of them portrayed negative attitude in areas, such as “food spoilage microbes being destroyed by freezing and refreezing defrosted foods and frozen foods can be kept out of the refrigerator as long as it contains ice.” This indicates that food handlers do not pay much attention when food is left in the temperature danger zone for a prolonged period, which could increase the microbial load leading to spoilage of food and thereby making it unsuitable for human use. Hence, there is a need to provide adequate training to improve the proper storage and defrosting of perishable foods. The finding of this study is consistent with studies [25, 32, 39]. Furthermore, the findings revealed that a greater percentage of the food handlers believed periodic checks on the temperature of refrigerators and freezers reduce the dangers of food contamination. The knowledge of the respondents will help in the proper maintenance of refrigerator/freezer temperature to sustain the microbiological and sensory quality of foods and prolong its life span and safety. The finding of the current study agrees with past studies [62, 63].

4.3. Food Safety Practices of Food Handlers

The findings revealed a generally poor (42.8%) food safety practices of the respondents. The respondents exhibited poor practices on the following indicators: refrigeration/freezing food to destroy or kill the microbes that contribute to food-borne illness, serving tasty food is important than safe food, food additives can cause food contamination, use of stored perishable food on first-in-first-out basis, and preparing food in advance reduces the risk of contamination. This can pose health risks because microbes in food become reactivated when food is defrosted as refrigeration or freezing only slows down their growth. The finding of this current study is in line with several earlier studies [30, 48], which reported similar findings. For instance, the WHO’s five keys and golden rules to safer foods recommend keeping foods at appropriate temperatures to prevent spoilage but not kill or destroy microorganisms [64, 65]. However, in relation to safety practices on the transmission of food-borne diseases, our finding showed that the food handlers performed well in areas such as “never used food that has changed colour, use of expired foods, and wearing of caps/hair restraints even for those with short hair or wigs.” This is a good practice because food can serve as a conducive avenue for the growth of varied microbes that may endanger public health. This finding is in consonant with the study of Ref. [66].

We also found that the respondents maintained good practices on personal hygiene and cross-contamination when handling food. This was exhibited in areas like “wash hands with soap and water after touching my own body parts and others, wear hair net/caps and nose masks, wash hands with soap and water after using the urinary and never handled food with long and painted fingernails, pay attention to expiration dates on food, do not use foods that have passed the expiry date, and get medically examined every six months.” This indicates that the food handlers understand the need to handle food safely to protect public safety, but their efforts may be impeded due to work pressure, inadequate resources/training, poor monitoring and supervision, and environmental conditions. For instance, although the self-reported practices showed that the respondents largely wear cap and nose masks, our

observation showed that otherwise even though they reported always to wear hair net/caps and nose masks, many of the food handlers, especially the males, were found not to have worn cap a practice at variance with FDA code and WHO COVID-19 guidelines for food handlers on the use of protective clothing and hair restraints at work [34, 65]. To this, a chef RFFC1 lamented:

It's not easy to wear the cap, though it is a standard practice to ensure the safety of food. We work for long hours and sometimes the heat becomes unbearable.

The finding of this study concurs with the study of Ref. [28, 30, 67, 68] who reported similar findings.

However, on the contrary, the food handlers had poor practices in areas, such as not washing their hands frequently before and during food preparation, use of hand sanitizers as a substitute for hand washing with soap and water, eat or drink during food preparation and service, use the same dish towels used for handling food to dry hands after hand washing, washing hands in the kitchen sink after using the bathroom, store raw and cooked food items together in the same place in the refrigerator, and prepare food with wound on hands provided the wound is covered with a bandage. However, none of the respondents were observed using sanitizers in place of handwashing with soap under running water, a practice contrary to proper hand hygiene for food handlers. The poor practices demonstrated by the food handlers highlight a possible need for risk-based food safety training, monitoring, and supervision to improve food handling practices and safeguard the safety of food served to the public. The finding is in consonant with several previous studies [32, 41, 65, 69, 70].

Furthermore, the general practices of the food handlers on time and temperature control practices were poor (39.6%), and these were exhibited ion indicators, such as not checking temperatures of refrigerators/freezers to minimize the dangers of food contamination and spoilage, thaw/defrost raw meat/fish in a bowl of water, refreeze defrosted foods, and freeze foods to kill the microbes that may cause spoilage of food and keep frozen food items out of the refrigerator provided there is ice in them. These are poor practices, which also matches observation data and could be due to inadequate training that has the likelihood of exposing consumers to health hazards. A manager THC2 responded:

You know, some of the employees come with very high academic qualifications but are not very good in the practical, so, from time to time, we organize training for them to help improve their level of practice. But some are also lazy and not ready to learn…. you have to correct the same mistake over and over again before they do the right thing.

This is affirmed by the findings from the studies of Ref. [38, 71] and Osaili et al. [58]. For instance, a study conducted to find out the outcome of the recurrent freeze-thaw cycles on beef quality and safety revealed adverse effects on taste, colour, and texture of beef resulting in its deterioration with resultant increased in microbial load. It is suggested that meat should be defrosted or thawed in the refrigerator instead in a bowl of cold or warm water or meat apportioned into manageable quantities needed for each cooking session to prevent the practice of defrosting and refreezing the rest [38, 71]. Meanwhile, Keaney et al. [72] and Wang et al. [73] observed that it is imperative to ensure the safety of consumers and hence recommended the adoption and implementation of measures, such as HACCP system, standard operating procedures and good hygiene practices, and strict monitoring/supervision to curb the poor food handling practices of food handlers to forestall the risk of food poisoning and safeguard the health of consumers.

4.4. Knowledge-Practice Gap of Food Handlers

The finding further indicated a knowledge-practice gap among food handlers at hotels in central and western regions. This may be ascribed to the condition that training of food handlers was purely centred on the acquisition of knowledge, something which is less likely to be translated into practice. Also, there may be inadequate monitoring/supervision by managers and regulators. However, in areas where practice exceeded knowledge, it is plausible that some of the food handling practices have become routine and undertaken without much thought and effort. Also, it could be due to workplace culture, which makes it obligatory for food handlers to engage in practices without necessarily understanding their implications. This confirms the assertion of Akabanda et al. [39] and da Vitoria et al. [74]. Besides, several past studies [46, 75, 76] showed that the value of training to produce an

anticipated behavioural change in food safety environments is inhibited by factors, such as inadequate number of training and exposure rates, work pressure, intellectual overloads, and deficits in implementers' competences. Consequently, food service establishments need to be equipped with adequate resources, conducive environment, opportunities to reinforce training to develop the needed skills, confidence, motivation, and strict monitoring/supervision to enhance practice to serve healthy food that protects the health of consumers [30].

5. Limitations

The study was conducted at the time when restrictions were imposed on movement due to COVID-19, and this affected patronage at the hotels. As a result, only hotels in operation with their food handlers at after COVID-19 restriction was eased were used for this study. Consequently, the findings may not be generalized to the entire population. Furthermore, all the food handlers who responded to the questionnaire could not be observed because of the COVID-19 restrictions; therefore, the observed data could not be compared with the self-reported practices. In addition, the study did not create room for comparison of findings between the hotels involved in the two regions. Regardless of limitations on the generalizability of the findings, the overall public health relevance of the study remains valid.

6. Conclusions

This study concludes that food handlers at hotels in the central and western regions have moderate level of knowledge on food safety. Many of the food handlers had tertiary education, but this did not impact their level of food safety knowledge. Although the food handlers had positive attitude towards safe food practices, most of them displayed poor food safety practices. Consequently, it is plausible for the food handlers to violate food safety measures, thereby exposing consumers to health risks. The study also provided valuable information to guide regulators in developing innovative strategies to boost the safety of food served at hotels. Nonetheless, we believe that the improvement in food handlers' knowledge will enhance their food safety practices. Therefore, it is important to intensify monitoring and supervision by managers and regulatory authorities, organize regular training/workshops to reinforce food handlers' food safety knowledge, and also elevate their food safety practices [77–82].

Ethical Approval

Ethical approval for the study was obtained from the Institutional Review Board (IRB) at the University of Cape Coast, Ghana (UCCIRB//CES/2020/28), and medical screening and certification of the first author by the Environmental Health and Sanitation Unit of Cape Coast, Ghana (Cert. No: 1,057).

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Nutritional Indices, Phytochemistry, and the In Vitro Antioxidant Activity of Carrot Fortified Tomato Concentrate

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ABSTRAK (ENGLISH)

Researchers are constantly looking at the development of functional foods from local materials that offer both nutritional and therapeutic potentials. The study investigated the proximate composition, antioxidant capacity, and phytochemical profile of different compositions of formulations produced from tomato and carrot. The plant materials were sourced locally, dried to a uniform weight, and milled using a mechanical blender. Nutritional indices, viz., proximate analysis, were analyzed using a standard protocol. The phytocompounds present in the formulations and their *in vitro* antioxidant activities were analyzed using spectrophotometric methods. The results for proximate composition showed low moisture content in formulations (0.44 ± 0.015–0.54 ± 0.021%). The protein content of the formulations (16.51 ± 0.217–17.94 ± 0.134%) was significantly higher than that of carrot alone (8.41 ± 0.154%). Similarly, the crude fat was elevated in the formulations (0.31 ± 0.008–1.63 ± 0.017%) compared to tomato alone (0.10 ± 0.399). However, these values were lower than the values obtained for carrot alone (8.72 ± 0.009%). The energy value for the formulations ranged from 87.01 to 93.30 kcal, which was low compared to carrot alone (136.89 kcal). Phytochemical screening showed the presence of terpenoids, cardiac glycosides, saponins, phenols, flavonoids, and alkaloids in the studied formulations. Furthermore, the incorporation of carrot led to an increase in TPC, TFC, and alkaloid concentration. The pH values observed were around the neutral range, while lactic acid concentrations reduced following the incorporation of carrot. Trace element analysis showed improved iron and manganese concentrations in the formulation. Similarly, increased antioxidant ability was observed in the formulation. For sensory evaluation, reporters indicated good sensory parameters. Concluding, this study has shown that formulating concentrate from tomato and carrot showed improved nutritional potential and enormous antioxidant capacity that can be attributed to the presence of elevated total phenolics and flavonoid concentrations. Therefore,

this formulation is warranted for improving the health of mankind.

TEKS LENGKAP

1. Introduction

The crave for a change from pharmaceuticals to nutraceuticals due to the high cost, side effects, and sometimes scarce availability of the former has led to aggressive research into low-cost and ever-available food-based nutraceuticals. Food is regarded as a nutraceutical as it contains excellent physiological benefits beyond its normal nutritional benefits. The physiological benefits of food-based nutraceuticals are triggered by the food's antioxidant potential, which finds practical expression in the mediation of diseases, especially in the treatment of cancers and cardiovascular diseases [1].

Tomatoes are a vital crop species, a fruit berry with an ovary filled with seeds. This fruit is recognized globally, with an annual production of $41.52 million tons in the year 2019 and a growth projection from $165.72 billion in 2022 to $186.46 billion in 2024 at a compound annual growth rate (CAGR) of 6.7% [2]. Tomatoes are eaten raw as a fruit, processed into juice, salads, and ingredients for savoury meals, and can also be processed into paste, especially for commercial sales. Tomatoes are known to be a rich source of water, minerals, vitamin C, folate, vitamin K, potassium, insoluble fibers such as hemicellulose, cellulose, and lignin, and a rich source of a potent antioxidant, lycopene [3]. Lycopene is the primary antioxidant in tomatoes, and it is responsible for the bright red colouration of the fruit. It is a highly unsaturated tetraterpene with molecular formular of $C_{40}H_{56,}$ an acyclic open chain structure consisting of 13 double bonds (eleven (11) conjugated double bonds and two (2) unconjugated double bonds) [4]. The multiple unconjugated double bonds are responsible for the strong antioxidant potential of lycopene, which invariably makes it a very strong free radical quencher. Its intense colour and nontoxic nature have increased its usage in food colouring [5]. Several studies have shown the relationship between the consumption of tomatoes and tomato-based products which also include their phytochemicals, and a reduced hazard of diverse chronic diseases such as certain types of cancer particularly prostate cancer and cardiovascular diseases [6, 7]. This is mainly due to its antioxidant content which differs in chemical nature thereby providing a wide variety of dietary lipophilic and hydrophilic antioxidants such as lycopene and beta-carotene, tocopherol, ascorbic acid, and phenols [8].

Carrot is a member of the *Umbelliferae* family, specifically belonging to the genus *Daucus* and species *Carota*. It is also a vital vegetable root crop with potent bioactive compounds and global recognition due to its fleshy edible roots and numerous health benefits. It is a good source of vitamins, fibre, potassium, and beta-carotene. It is tasty, crunchy, and highly nutritious. Research has recorded the antihypertensive, renoprotective, wound healing, cardiac and cholesterol disease reducing, and antioxidant properties [9]. The seed of carrots have been found to possess antifungal, antibacterial, anti-inflammatory, analgesic, and cardio-hepatoprotective properties [9]. The diverse health benefits of carrots have been attributed to its high consumption rate because of its antioxidant and anticancer properties.

The addition of one or more essential nutrients to food, regardless of whether or not it is contained in the parent food, is known as food fortification. The essence of food fortification is to prevent or correct a demonstrated deficiency in one or more nutrients in the main food. It has also helped reduce micronutrient deficiencies (MNDS) in children and older age groups. Some researchers have worked on the fortification of tomato concentrate. Biofortification of tomatoes with magnesium and iodine was found to enhance the nutritional value of the tomatoes and also prevent diseases caused by iodine deficiency in diets [10, 11]. Food fortification with tomatoes is safe for pregnant and lactating women because lycopene increases in human milk with increased consumption of tomatoes [12].

This present study is aimed at developing a functional food-mix from tomatoes and carrots, tomatoes being the parent food. The formulation was investigated for its nutritional indices, phytochemicals, antioxidant, and mineral profile for a probable nutritional and functional food-mix which can be included as food condiments as to enhance

the nutritional benefits of foods.

2. Materials and Methods

2.1. Plant Materials Sampling

Tomato (*Solanum lycopersicum*) and carrot (*Daucus carota*) fruits were purchased from a market in Ota, Ogun State, Nigeria. Ota. The plant materials were identified by Dr. J. O. Popoola, a taxonomist at Bowen University, Nigeria. Herbarium numbers with voucher numbers Dc/Bio/H849 and SI/Bio/H850 were assigned, and a sample was deposited at the herbarium of the university.

2.2. Production of Tomatoes Powder

Exactly twenty kilograms (20 kg) of matured tomatoes were thoroughly washed in clean water to remove dirt and debris. The tomatoes were cut into smaller pieces using a kitchen knife to facilitate drying. The samples were oven (SM9050, Surgifriend Medicals, England) dried at a temperature of 60°C for 9 hr till a uniform weight was attained. The dried samples were milled to powdered form using a mechanical blender (700 g Electric Grains Spices, Cereal Dry Food Grinding Machine Blender −220V EU plug) and stored at room temperature in a clean sterile glass container until further use.

2.3. Production of Carrot Powder

About ten kilograms (10 kg) of carrots were washed in clean water to remove dirt and debris. The outer layer of the carrots was peeled and cut into small pieces using a kitchen knife to facilitate drying. The samples were oven dried at a temperature of 40°C for 6 hr. The dried samples were milled to powdered form using a mechanical blender and stored at room temperature in a clean sterile container until further use.

2.4. Batch Composition of Tomato-Carrot Powder

The preparation of the formulation is presented in Table 1 below.

Table 1

Batch composition of tomato-carrot powder.

Samples	A	B	C	D	E	F	G
Tomatoes powder (%)	100	90	80	70	60	50	0
Carrots powder (%)	0	10	20	30	40	50	100

The mixtures were mixed thoroughly to ensure homogeneity.

2.5. Proximate Analysis

The percentage of moisture, ash, protein, fibre, carbohydrate, and fat content were determined in the formulations using the method described by AOAC [13]. Briefly, ash content was measured by incinerating 1 g of the sample in a muffle furnace (1500°C (2730°F) Muffle Box Lab Furnace) at 550°C for 5 hr [14]. The crude fat was determined by complete extraction of 5 g of the sample using petroleum ether as solvent in a Soxhlet extractor set at 50°C [14]. Using the Micro-Kjeldahl method as described by Okalebo et al. [15]; the protein content was determined % total nitrogen multiplied by 6.25. For crude fibre determination, 5 g of milled samples were digested in 100 mL of 1.25% H_2SO_4 30 min. This was quickly followed by filtration, and the residue obtained was further digested with 100 mL of 1.25% NaOH. The mixture was filtered again, and residue collected was oven dried at 100°C. The dried residue was then incinerated in the muffle furnace at 550°C for 5 hr. The crude fibre was calculated as (loss of weight on ignition/weight of sample used) multiplied by 100%. The energy value (kcal) was calculated using the method described by AOAC [13]. Briefly, the % values gotten for protein, fat, and carbohydrate were multiplied by 4, 9, and 4, respectively. Energy in kcal = (protein × 4) + (fat × 9) + (carbohydrate × 4). One gram of each sample was weighed and dissolved in 10 mL of distilled water. The solution was left to settle for 24 h. Standard buffer of 4.0 and 9.1 was used to get the pH of the samples using a pH metre. Titratable acidity of all the samples were determined according to the method described by [16]. The electrode was rinsed with de-ionized water and wipe dry. The electrode was

calibrated using a buffer of pH 7 and pH 4. 10 g of the sample was weighed into a 250 mL beaker, 90 mL of distilled H_2O was added and mixed thoroughly. The mixture was left for 30 min at room temperature after which 3 drops of phenolphthalein indicator were added to the mixture. The resulting mixture was titrated with 0.1 M NaOH till a pH of 8.1 was attained. The result was expressed as grams of citric acid per 100 g of dry samples. Sensory evaluation was determined by expert who were trained in the assignment from Clifford University, Warrrianta, Abia state using a 7-point Hedonic index of colour, aroma, texture, flavour, mouthfeel, and acceptability. This scale ranges from 1 (dislike extremely) to 7 (like extremely), allowing for a comprehensive assessment of the food product using a questionnaire, higher scores on the hedonic scale indicate greater acceptability. Ethical approval for this study was obtained from the Research Ethics Unit, Clifford University, Warrrianta, Abia state with ethical clearance code (CLU/FOS/CHM/ETC/22/81).

2.6. Preparation of Hydroethanolic Extract of Formulations

The milled samples (50 g) were soaked in 500 mL of 70% ethanol or water for 48 h with constant agitation, and the resulting mixture was filtered through cheesecloth. The lipophilic nature of tomatoes also contributed to the use of 70% ethanol for extraction. The residues were soaked again in fresh solvents to enable complete extraction. The filtrates were then pooled and filtered using a filter paper (Whatman No. 1). The filtrates were concentrated using a rotary evaporator (Rotary evaporator PE-8910), and the resulting residues were properly labelled and stored in a refrigerator at 4°C for future use.

2.7. Phytochemical Analysis

2.7.1. Qualitative Phytochemistry

The qualitative phytochemical screening for oxalate, quinone, terpenoid, cardiac glycoside, saponin, tannin, flavonoid, and alkaloid was determined using the method described by Varadharajan et al. [17]. Briefly, 2 mL of ferric chloride (5%) was added to 1 mL of the extract. The presence of tannin was indicated by the formation of greenish black colouration. For oxalate determination, a few drops of glacial ethanoic acid were applied to a 3 mL section of the extract. Presence of a greenish black colouration indicated the presence of oxalate. About 1 mL of the extracts was treated with 1 mL of concentrated sulphuric acid, and the presence of quinones was indicated by the formation of red colouration. To 1 mL of the extract, 2 mL of chloroform was added afterwards, and concentrated sulphuric acid was carefully added. The formation of brown colour at the interface indicated the presence of terpenoids. For phenol determination, 1 mL of the extracts was added to 2 mL of distilled water. After mixing, 10% of ferric chloride was added to the mixture dropwise. The presence of green colouration indicated the formation of phenols. To 2 mL of the extract, 2 mL of concentrated hydrochloric acid was added, after which a few drops of Mayer's reagent were added. The presence of alkaloids in the extracts was indicated by the formation of a green colouration. A mixture of 2 mL of glacial acetic acid and a few drops of ferric chloride (5%) was added to 500 μL of the extracts, after which 1 mL of concentrated H_2SO_4 was added to underlay the mixture. The formation of a brown ring at the interface indicated the presence of cardiac glycosides. Exactly 2 mL of the extract and 2 mL of distilled water were added to a tube and shaken for 15 mins. The formation of 1 cm layer of foam indicated the presence of saponins in the extract. To 10 mL of sample, 5 mL of diluted ammonia solution was initially added before 5 mL of concentrated sulphuric acid was added. The presence of flavonoids in the sample was indicated by the formation of yellow colouration.

2.7.2. Quantitative Phytochemistry

The total phenolic content (TPC) was quanitifed using the method described by Paśko et al. [18]. Briefly, 2.7 mL of de-ionized water, 0.3 mL of extracts, 0.3 mL of 7 g/100 g Na_2CO_3, and 0.15 mL of Folin–Ciocalteu reagent (with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide) were mixed thoroughly. The absorbance of the mixture was measured at 725 nm. A gallic acid standard curve was prepared, and results were expressed as gallic acid equivalents (GAE) [19]. For the quantification of flavonoids in the extracts, the aluminium chloride colorimetric method described by Chang et al. [20] was employed. Briefly, 0.25 mL of the sample was diluted to 1.25 mL with distilled water. Exactly 75 μL of 5% sodium nitrite was 0.15 mL of aluminium chloride solution were. After 5 minutes, 0.5 mL of 0.1 M NaOH was added and made up to 2.5 ml with distilled water. The solution was thoroughly mixed, and the absorbance of the mixture was measured at 510 nm. The total flavonoid content was

calculated from a quercetin calibration curve, and the result was expressed as mg quercetin equivalents per gram dry weight. Lycopene content was quantified as described by Okonkwo and Ofodum [21]. Extraction of crude lycopene was done using ethyl acetate as solvent. The crude lycopene was purified using benzene (2 mL) and methanol (1 mL). The quantification of lycopene was done using a spectrophotometer. Beta-carotene was quantified as described by Hasan et al. [22]. Briefly, the samples were mixed with ethanol and heated for 5 mins. The mixture was filtered, and DCM was added with further heating for 4 min. Standard solution of beta-carotene was prepared and used to estimate the beta-carotene in the sample spectrophotometrically. Total alkaloid was determined using the method described by Li et al. [23].

2.7.3. Determination of pH and Titratability of Formulations

Titratable acidity of the samples was determined using the method of [24]. The pH of the samples was measured using the pH meter (Sartorius, USA), which was calibrated using pH 4.0 and 9.0 [13].

2.7.4. Determination of Elements Present in the Formulation

The minerals and elements present in the formulations were quantified using an atomic absorption spectroscopy as described by Uroko et al. [25]. The formulations were digested for minerals using a solution of strong acids containing HNO_3 and $HCLO_4$ in a ratio of 5 : 1, until a transparent solution is seen. The minerals presented in the formulations were quantified using atomic absorption spectroscopy.

2.7.5. *In Vitro* Antioxidant Capacity of Formulation

The free radical scavenging activity of the formulations was investigated by the DPPH assay [26] using a spectrophotometer. The extracts from the formulation were mixed with 1 mL of 0.5 mM DPPH (in methanol) in a cuvette. The absorbance at 517 nm was taken after 30 minutes of incubation in the dark at room temperature. Ascorbic acid (vitamin C) was used as a reference standard. The ferric reducing antioxidant power of the formulations was investigated using the protocol described by Benzie and Strain [27]. To 100 μL of sample solution, 3 mL of FRAP reagent was added, and the mixture allowed to stand for 4 min. The absorbance was recorded at 593 nm, at 37°C. Total antioxidant activity (TAC) was determined according to the method described by Munteanu and Apetrei [28].

2.8. Statistical Analysis

The present data were statistically analyzed using SPSS Version 22. The mean values were separated using the Duncan multiple tests. The different levels of significance within the groups were examined using one-way analysis of variance (ANOVA) for the proximate analysis and *in vitro* antioxidant assessment. The data were expressed as mean ± SD and considered significant at $p<0.05$.

3. Results and Discussion

3.1. Proximate Composition of Formulations

The protein content was significantly $p<0.05$ lower in the 100% carrot group (G) (8.41 ± 0.154), compared to other experimental group in the study. For crude fat, carbohydrate, and total energy (8.72 ± 0.099, 6.193 ± 0.022, and 136.892) the values obtained for group G were significantly $p<0.05$ higher that those of the other experimental groups.

3.1.1. pH and Titratable Acidity of the Formulations

The pH of the formulation was seen to be near the neutral range, while titratable acidity value was high in formulation A (23.00 ± 2.022) and least in formulation G (1.44 ± 2.045).

3.1.2. Qualitative Phytochemical Analysis

The preliminary phytochemical analysis showed that oxalate, quinones, and tannins were not present in the ethanol and aqueous extracts of the formulations.

3.1.3. Quantitative Phytochemistry of Formulations

The total phenolic, flavonoids, and alkaloid concentrations were significantly $p<0.05$ higher in groups B–F compared to other groups in the study. However, the beta-carotene content was higher in Group G (0.581 ± 0.14) compared to other groups in the study.

3.1.4. Mineral Estimation of Formulations

The result showed that iron is the most prominent mineral in the formulation, with formulation F having the highest concentration (19.5080).

3.1.5. *In Vitro* Antioxidant Potentials of the Ethanol Extract of the Formulation

In Vitro Antioxidant Potentials of the Ethanol Extract of the Formulation are presented in Tables 2–4.

Table 2

DPPH radical scavenging ability of ethanol extract of the formulations.

Samples	IC_{50}	R2
A	0.2 ± 0.10^{a}	0.8498
B	0.2 ± 0.01^{b}	0.8378
C	0.2 ± 0.10^{a}	0.9807
D	0.2 ± 0.15^{c}	0.9475
E	0.2 ± 0.11^{d}	0.9141
F	0.2 ± 0.13^{e}	0.8101
G	0.2 ± 0.10^{b}	0.8664
Standard (AA)	0.1 ± 0.00^{f}	0.9784

A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot. AA-ascorbic acid. Data are mean ± SD ($n=3$). Values with different superscripts across the same row are significantly different ($p<0.05$).

Table 3

Ferric reducing antioxidant power of the ethanol extract of formulations.

Conc (mg/mL)	A (mgQE/g)	B (mgQE/g)	C (mgQE/g)	D (mgQE/g)	E (mgQE/g)	F (mgQE/g)	G (mgQE/g)
1	4.63 ± 0.31^{a}	4.32 ± 2.46^{a}	3.83 ± 1.11^{a}	3.99 ± 0.91^{a}	4.14 ± 0.34^{a}	3.47 ± 2.34^{a}	3.86 ± 2.34^{a}
2	4.40 ± 0.51^{a}	4.34 ± 2.11^{a}	4.77 ± 3.60^{a}	3.94 ± 2.30^{a}	4.00 ± 1.74^{a}	3.94 ± 0.16^{a}	3.80 ± 0.77^{a}
3	4.50 ± 0.54^{a}	4.31 ± 0.77^{a}	4.83 ± 2.60^{a}	3.66 ± 3.45^{a}	3.86 ± 3.44^{a}	3.34 ± 0.34^{a}	3.93 ± 3.22^{a}
4	4.30 ± 1.84^{a}	4.41 ± 0.98^{a}	4.70 ± 4.50^{a}	3.93 ± 1.23^{a}	4.58 ± 2.45^{a}	2.64 ± 2.43^{a}	3.06 ± 4.22^{a}
5	4.37 ± 0.14^{a}	4.68 ± 0.55^{a}	4.96 ± 2.98^{a}	3.73 ± 1.22^{a}	3.76 ± 7.21^{a}	3.34 ± 0.52^{a}	3.65 ± 2.23^{a}

A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot. “FRAP” and “mg QE/g” means

ferric reducing antioxidant power and mg quercetin equivalents per gram extract, respectively. Data represented are the mean ± SD (n = 3). Values with different superscripts across the same row are significantly different ($p<0.05$).

Table 4

Total antioxidant capacity power of tomatoes-carrots mix ethanol extracts.

Conc (mg/mL)	A (μg AAE/g)	B (μg AAE/g)	C (μg AAE/g)	D (μg AAE/g)	E (μg AAE/g)	F (μg AAE/g)	G (μg AAE/g)
1	4.78 ± 0.02^{a}	4.82 ± 0.41^{a}	4.98 ± 1.38^{a}	5.04 ± 0.12^{a}	5.23 ± 0.22^{a}	5.80 ± 0.12^{a}	4.56 ± 0.45^{a}
2	8.53 ± 0.16^{b}	7.89 ± 0.30^{ab}	8.99 ± 2.11^{b}	9.01 ± 1.44^{a}	10.45 ± 2.13^{a}	11.04 ± 0.23^{a}	7.89 ± 0.78^{a}
3	10.26 ± 1.81^{bc}	11.78 ± 2.23^{abc}	12.43 ± 2.23^{abc}	13.44 ± 0.21^{abc}	14.56 ± 2.34^{ab}	15.09 ± 0.34^{a}	9.89 ± 0.23^{c}
4	15.08 ± 0.23^{a}	15.99 ± 5.63^{a}	16.78 ± 1.32^{a}	17.04 ± 1.45^{a}	17.97 ± 0.12^{a}	18.01 ± 0.12^{a}	15.10 ± 1.23^{a}
5	20.76 ± 1.54^{a}	21.45 ± 0.24^{a}	22.67 ± 0.94^{a}	23.76 ± 2.34^{a}	24.11 ± 0.44^{a}	25.01 ± 0.34^{a}	21.33 ± 1.24^{a}

A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot. “TAC” and “μg AAE/g” means total reducing antioxidant power and μg ascorbic acid equivalent per gram, respectively. Data represented are the mean ± SD (n = 3). ^{a}significantly lower than other groups in the same row; ^{b}significantly higher than other groups in the same row.

3.1.6. Sensory Evaluation of the Formulations

Sensory Evaluation of the Formulations is presented in Table 5.

Table 5

Sensory evaluation of tomato-carrots powder.

Samples	Colour	Texture	Aroma	Flavour	Mouthfeel	Acceptability
A	3	1	1	4	4	2
B	3	1	1	4	4	2
C	3	1	1	4	4	2
D	3	1	1	4	4	2
E	3	1	1	4	4	3
F	2	1	1	4	4	3

G	2	1	1	3	4	3

Colour: 1-gritty, 2-orange, 3-red, 4-caking, 5-artificial colour. Texture: 1-gritty, 2-hard, 3-dry, 4-soft, 5-caking. Aroma: 1-tomato, 2-nutty, 3-savoury, 4-corn flour. Flavour: 1-woody, 2-salty, 3-sweet. Aroma, 4-tomato, 5-no smell. Mouthfeel: 1-creamy, 2-smooth, 3-slightly smooth, 4-residual particles 5-nauseating. Acceptability: 1-very good, 2-good, 3-fair, 4-barely acceptable, 5-not acceptable.

4. Discussion

The nutritional quality of any food sample is evaluated through its proximate composition. This is very crucial to evaluate its effect on the final product after being labelled as a novel ingredient [29]. In this study, the proximate composition of the formulations is presented in Table 6. The moisture content of the food blends ranged from 0.44 to 0.54%. Moisture content is an important parameter that must be considered in the analysis of food samples because it helps to determine the food's shelf life, durability, and storage conditions. Food samples with the lowest moisture content can have a longer shelf life by inhibiting the growth of microorganisms compared to food samples with higher moisture content. In this study, sample D (70 : 30 tomato-carrot powder) was found to have the lowest moisture content the proportion of carrot powder in the 70 : 30 formulation might have contributed to the lower moisture content compared to other formulations with different ingredient ratios, which reflects that the formulation may be able to withstand spoilage and have a longer shelf-life. The moisture content of these formulation disagreed with the reports of Srivastava and Kulshreshtha [30], Srivalli et al. [31], Opadotun et al. [32], and Oladipupo et al. [33] for tomatoes. The result obtained for moisture content in this study suggests that the formulation consisting of 70% dried tomatoes and 30% carrots will have a longer shelf life than their fresh counterparts. The ash content of the formulations was high which is indicative of high mineral content of the two ingredients used in the formulation. Minerals are of utmost importance in biological systems. They are required for growth and development of the body. Furthermore, they are used by enzymes to catalyse metabolic reactions. Fruits have been reported to be rich sources of minerals, vitamins, and nutrients [34]. There was an observed increase in the fat content of the formulation as the carrot concentration increased. Fats from plants have been reported to contain unsaturated fats that are heart friendly [14]. The result obtained in this study for fat composition agrees with the report of Kasale et al. [35]. Crude fibre was seen to be high in formulation E and F compared to tomato and carrot alone. Crude fibre which refers to the indigestible material in food obtained after acidic and alkaline digestion helps to improve the proper digestion and absorption of food; also, it helps to improve waste elimination in the gastrointestinal tract due to its water-absorbing ability. The result for crude fibre in this study suggested that the formulation will increase the fibre intake of consumers. In this study, the protein content was high in all the formulation compared to carrot alone. Among the formulation, F showed the highest protein content. This indicates that the consumption of the formulation will support the protein demand of consumers. The value reported for protein content in the tomato was higher than the values reported by Oladipupo et al. [33]. The difference in protein content can be labelled to several factors such as planting season, geographical location, climate, and age [36]. Carbohydrates are energy giving food and are required in high quantity in diets. Formulation F showed the least carbohydrate composition, indicating its inefficiency to support consumer's energy demand. A strange trend was observed for carbohydrate composition, which suggests that the combination of both materials (carrots and tomatoes) led to a reduction in carbohydrate composition. The significant findings in the proximate composition analysis provide valuable insights into the nutritional quality of the formulations. The differences observed in moisture content, ash content, fat content, crude fiber, protein content, and carbohydrate composition can be attributed to the intrinsic properties of the different composition of the individual fruits. These findings have implications for the shelf life, mineral content, heart-friendly fats, fiber intake, protein demand, and energy provision of the formulations. Further research can explore the underlying factors contributing to these differences and their potential impact on the final product.

Table 6

Showing % composition of carbohydrates, fats, proteins, fibre, mineral, water, and energy value of formulations.

	A	B	C	D	E	F	G
Moisture (%)	0.51 ± 0.006^{ab}	0.47 ± 0.012^{bc}	0.49 ± 0.015^{abc}	0.44 ± 0.015^{c}	0.54 ± 0.021^{a}	0.50 ± 0.032^{ab}	0.47 ± 0.021^{bc}
Ash (%)	72.58 ± 0.055^{bc}	72.98 ± 0.035^{abc}	71.82 ± 0.119^{d}	73.52 ± 0.015^{a}	73.12 ± 0.261^{ab}	72.90 ± 0.451^{bc}	72.42 ± 0.234^{cd}
Protein (%)	18.10 ± 0.350^{a}	17.63 ± 0.072^{a}	17.44 ± 0.248^{ab}	16.51 ± 0.809^{b}	16.51 ± 0.217^{b}	17.92 ± 0.134^{a}	08.41 ± 0.154^{c}
Crude fiber (%)	4.556 ± 0.089^{a}	4.254 ± 1.024^{a}	5.438 ± 0.089^{a}	5.432 ± 0.435^{a}	6.457 ± 0.029^{a}	6.547 ± 2.221^{a}	3.787 ± 1.250^{a}
Crude fat (%)	0.10 ± 0.039^{f}	0.31 ± 0.008^{e}	0.86 ± 0.012^{d}	1.63 ± 0.017^{b}	1.49 ± 0.048^{c}	1.51 ± 0.008^{bc}	8.72 ± 0.099^{b}
Carbohydrate (%)	4.154 ± 0.010^{c}	4.356 ± 0.025^{b}	3.952 ± 0.046^{d}	2.468 ± 0.003^{e}	1.883 ± 0.013^{f}	0.628 ± 0.001^{a}	6.193 ± 0.022^{b}
Total energy (kcal)	89.916	90.734	93.308	90.582	87.012	87.782	136.892

Data are presented as mean ± SD of triplicate determination; ^{a}significantly lower than other groups in the same row; ^{b} significantly higher than other groups in the same row. A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot.

The result for pH and titratability is documented in Table 7. Result showed that pH increased as the quantity of carrot was increased in the formulation. However, the observed pH values were around the neutral range. The near neutral pH of the formulations in this study further justifies it suitability for consumption. The values for pH in this study were lower compared to the values reported by Ajayi and Olasehinde, [37] and Samuel and Orji [38]. A low pH value is known to prevent spoilage of food, and prevents microbial deterioration of foods [39]. The titratable acidity measures the amount of lactic acid (%) in a food sample or solution. It measures the total acidity but does not account for the strength of the acids. Interesting, the titratable acidity of the formulations in this study reduced as the concentration of carrot increased. This suggests that carrot contains more bases than acid, and this was able to neutralize the acid content coming from the tomatoes, making the formulations more palatable. The organic acids present in food samples affect the flavour, color, and microorganism growth retardation and help in the minimization of lipid oxidation by providing a proper environment for metal ion chelation [40]. The results for titratable acidity corroborated the values obtained for pH.

Table 7

pH and titratable acidity of the various formulations.

Samples	pH	Titratable acidity (lactic acid %)
A	6.6	23.00 ± 2.022^{a}

B	6.7	16.39 ± 1.145^{ab}
C	6.9	11.89 ± 7.166^{bc}
D	7.0	7.92 ± 2.034^{cd}
E	7.0	5.76 ± 0.001^{cd}
F	7.1	4.50 ± 0.032^{cd}
G	7.2	1.44 ± 0.045^{d}

Data are presented as mean ± SD of triplicate determination; [a]significantly lower than other groups in the same column. A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot.

The result for qualitative phytochemistry is presented in Table 8. Phytocompounds present in the various extracts were terpenoids, cardiac glycosides, saponins, phenols, flavonoids, and alkaloids. Cardiac glycosides are known to have a massive impact in medicine, especially in the treatment and management of several heart conditions, this was reported in our previous work [41]. Saponins are chemical compounds found in herbs, seeds, and some vegetables. They are known to have foaming properties which also boost their antibacterial activity which has made them very effective in the production of soaps, shampoos, and some household cleaning agents. Biologically, saponins help to reduce cholesterol levels, curb oxidative stress, inhibit tumor growth, and improve lipid metabolism, which aids in the prevention and treatment of obesity [42]. Terpenoids are used in the prevention and treatment of certain types of cancers; studies have also shown they possess antifungal, antiparasitic, antiallergenic, antihyperglycemic, antiviral, and anti-inflammatory properties [43]. Alkaloids are known to contain anesthetics, cardioprotective, and anti-inflammatory properties. They are very useful in diet formulation and pharmaceuticals for the effective management and treatment of pain [44]. The presence of the phytochemicals gives a useful insight into the therapeutic properties of tomatoes and carrots, as reported by [45]. These findings agree with the report of Akilu et al. [46] on the phytochemicals present in tomato and carrot extracts. The result for quantitative phytochemistry of the 70% ethanol extract of the formulations is presented in Table 9. The formulations showed greater number of total phenolics, flavonoids, and alkaloids. For beta-carotene and lycopene concentration, the formulation showed a low value. The values reported for beta-carotene, lycopene, and phenolics in this study for tomato disagree with the report of Oboulbiga et al. [47]. Phenolics, flavonoids, lycopene, and beta-carotene have been reported to have antioxidant, anticancer, and cardioprotective properties [48–51].

Table 8

Qualitative phytochemical constituents of the various formulations.

Samples	Oxa.	Qui.	Terp.	Car. Gly.	Sap.	Tan.	Phen.	Flav.	Alk.
A_1	—	—	+ +	+ +	+ +	—	+	+	+
A_2	—	—	+	+	++	—	+	+	+
B_1	—	—	+ +	+ +	+ +	—	+	+	+ +

B_2	—	—	+	+	++	—	+	+	+
C_1	—	—	+ +	+ +	+ +	—	+	+	+ +
C_2	—	—	+	+	++	—	+	+	+
D_1	—	—	+ +	+ +	+ +	—	+	+	+ +
D_2	—	—	+	+	++	—	+	+	+
E_1	—	—	+ +	+ +	+ +	—	+	+	+ +
E_2	—	—	+	+	++	—	+	+	+
F_1	—	—	+ +	+ +	+ +	—	+	+	+ +
F_2	—	—	+ +	+ +	+ +	—	+	+	+ +
G_1	—	—	+ +	+ +	+ +	—	+	+	+
G_2	—	—	+	+	++	—	+	+	+

Notes. —: not detected, +: moderately detected, ++: Detected. Subscript 1: 70% ethanol extract, Subscript 2: aqueous extract. Oxa-oxalate, Qui-quinones, Terp-terpenoids, Car. Gly-cardiac glycosides, Tan-tannins, Phen-phenol, Flav-flavonoids, Alk-alkaloids. A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot.

Table 9

Total phenolic, total alkaloid, total flavonoid, lycopene, and *β*-carotene concentration of formulations.

	A	B	C	D	E	F	G
TPC (mg GAE/g)	256.88 ± 0.11^{a}	278.11 ± 4.34^{a}	285.08 ± 1.34^{b}	287.14 ± 2.34^{b}	290.45 ± 3.77^{a}	294.10 ± 2.12^{a}	135.8 ± 2.1124^{a}
Total alkaloid (pg/g)	181.89 ± 0.34^{a}	198.88 ± 0.66^{b}	201.34 ± 5.43^{ab}	215.43 ± 2.22^{b}	230.33 ± 1.45^{a}	241.34 ± 3.12^{a}	128.45 ± 0.99^{a}
TFC (mg RE/g)	9.16 ± 0.98^{b}	9.05 ± 2.11^{abc}	9.81 ± 1.01^{e}	10.01 ± 0.14^{c}	9.78 ± 3.23^{a}	10.28 ± 0.92^{b}	7.23 ± 0.11^{a}
Lycopene (mgCE/g)	0.182 ± 1.25^{b}	0.167 ± 0.23^{abc}	0.131 ± 1.99^{f}	0.139 ± 5.77^{bc}	0.119 ± 0.21^{a}	0.071 ± 3.44^{ab}	0.051 ± 3.44^{a}

β-carotene (mgCE/g)	0.461 ± 0.77[bc]	0.379 ± 1.24[a]	0.281 ± 1.09[c]	0.234 ± 2.11[e]	0.159 ± 4.34[b]	0.421 ± 0.907[c]	0.581 ± 0.14[b]

Data are presented as mean ± SD of triplicate determination; [a]significantly lower than other groups in the same row; [b] significantly higher than other groups in the same row. A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot. TPC and TFC means total phenolic content and total flavonoid content, respectively. “mg GAE/g” mg gallic acid equivalents per gram extracts” and “mg RE/g” mg carotenoid equivalents per gram extracts, respectively.

The result for mineral estimation in this study is presented in Table 10. Lead and cadmium are heavy metals always available in tomato paste sold in markets [25]. They are heavy metals that are threat to lives. They are carcinogenic and, in some cases, affect energy metabolism and respiration in biological systems [52]. The values reported for lead and cadmium in this study were insignificant and do not pose a threat to consumers. Copper and iron are required for proper formation of red blood cells in the body, improves bone health, ensures proper functioning of the blood vessels and several nerves, augments defensive mechanism of the immune system again diseases, and helps in proper absorption of iron in the body. The formulation showed a good concentration of copper and iron. Generally, the minerals present in the formulation will support the attainment of the RDA for these minerals.

Table 10

Trace metals analysis of tomato-carrot powder.

Samples	Cu (mg/L)	Fe (mg/L)	Mn (mg/L)	Pb (mg/L)	Cd (mg/L)
A	0.1731	11.5077	0.4967	0.0040	0.0152
B	0.2593	13.7913	0.5323	−0.0206	0.0565
C	0.3264	16.2089	0.5258	0.0004	0.0099
D	0.2776	10.9283	0.4503	−0.0290	0.1430
E	0.4421	10.6142	0.4599	−0.0136	0.0341
F	0.2667	19.5080	0.5226	−0.0073	0.0066
G	0.2488	12.1097	0.4132	−0.0128	0.0225

A-100% tomato powder; B-90% tomato and 10% carrot; C-80% tomato and 20% carrot; D-70% tomato and 30% carrot; E-60% tomato and 40% carrot; F-50% tomato and 50% carrot; G-100% carrot. Lead (Pb), Cadmium (Cd), Manganese (Mn), Copper (Cu), and Iron (Fe).

The results for *in vitro* antioxidant activity are presented in Tables 2–4. Generally, to determine the antioxidant activity of a plant material, free radicals’ generators are employed, and the ability of the plant material to prevent the generation of free radicals is considered as antioxidant ability [53]. The extracts showed strong DPPH inhibition capacity, and this can be linked to the presence of phytochemicals, some of which are phenolics, flavonoids, lycopene, and beta-carotenes that are known to inhibit the generation of free radicals. Antioxidants play a vital role in protecting cells from damage caused by harmful molecules called free radicals. Excessive free radicals in the body can lead to oxidative stress, which is associated with various chronic diseases such as cardiovascular diseases, cancer, and neurodegenerative disorders. Therefore, our formulated food-mix with significant antioxidant activity

may provide health benefits by reducing oxidative stress and potentially lowering the risk of these diseases. The data reported (IC_{50}) for DPPH scavenging activity of the extracts was higher than the values reported by Borguini et al. [54] on the DPPH radical scavenging activity of alcohol extract of tomatoes. Another parameter used to assess the antioxidant potential of a plant material is the ferric reducing antioxidant power (FRAP). As shown by the result, the extracts from the formulations showed great FRAP activity which was similar to ascorbic acid used as standard antioxidant agent. The total antioxidant activity (TAC) is used to evaluate the antioxidant potential of any compound through the phosphomolybdenum complex formation [55]. The result for TAC followed a dose-dependent approach, and further indicates that the formulations had stronger antioxidant capacity. This activity can be linked to the presence of phenolics and flavonoids.

Mean sensory evaluation of this study is shown in Table 5. All the batches were acceptable by the food panelist. Color is an essential sensory attribute because it affects the acceptability of food products. Color is a critical sensory attribute that can impact consumer acceptability and marketability. Different colors can evoke different emotions and perceptions in consumers. For example, research has shown that the color red can stimulate appetite and increase perceived sweetness, while the color green can be associated with healthiness and freshness [56]. A study by Park et al. [57] found that the color of a product can also influence consumer expectations, with a product's color affecting perceived taste, aroma, and overall liking. Texture is another important sensory attribute that can impact consumer acceptability and marketability. The texture of a product can influence how much consumers enjoy eating it, as well as their perception of its quality. For example, a study by Kim et al. [58] found that a crunchy texture can enhance the perception of freshness and quality in snack foods. Research by also showed that the texture of a product can affect consumer expectations, with a product's texture influencing perceived taste, aroma, and overall liking. Aroma is a critical sensory attribute that can impact consumer acceptability and marketability. Aroma can influence a consumer's initial perception of a product and can affect their willingness to try it. Research by found that the aroma of a product can also influence consumer expectations, with a product's aroma affecting perceived taste, quality, and overall liking. Flavor is a crucial sensory attribute that can impact consumer acceptability and marketability. Flavor can influence a consumer's liking and enjoyment of a product, as well as their perception of its quality. For example, a study by Lawless et al. [59] found that a product's flavor can affect consumer expectations, with a product's flavor influencing perceived taste, aroma, and overall liking. Mouthfeel is the sensation of a product's texture, temperature, and viscosity in the mouth. Mouthfeel can also impact consumer acceptability and marketability. For example, research by Kim et al. [58] found that a product's mouthfeel can influence how much consumers enjoy eating it, as well as their perception of its quality. A study by Yoon et al. (2019) also showed that the mouthfeel of a product can affect consumer expectations, with a product's mouthfeel influencing perceived taste, aroma, and overall liking. Fortification of tomato powder with carrots powder slightly affected the color and flavor as fortification increased while other parameters remained unchanged. The general acceptability of the samples is due to the fact that judges were already familiarized with the two fruits.

In conclusion, sensory attributes like color, texture, aroma, flavor, and mouthfeel can significantly impact consumer acceptability and the marketability of food products. These attributes play a crucial role in attracting consumers, creating positive sensory experiences, meeting consumer expectations, and influencing purchasing decisions. Understanding and optimizing these sensory attributes are key factors in developing successful and appealing food products. Therefore, it is important for companies to carefully consider these sensory attributes when designing and marketing their products.

5. Conclusion

The study has shown that the formulation (tomato and carrot mix) has improved nutritional profile. The development of a food product with a mixture of tomatoes and carrots has significant implications for the nutrition and food science field. Its unique nutritional profile, increased consumer acceptance, expanded market opportunities, potential for reduced food waste, new culinary possibilities, scientific research opportunities, potential for sustainable agriculture, educational opportunities, potential for addressing nutritional deficiencies, and contribution to food security all contribute to its importance and relevance in the field. Furthermore, the major antioxidant molecules *viz*

phenolics, flavonoids, lycopene, and beta-carotene were significantly increased, and this improved the antioxidant capacity of the formulations. The formulations in this study can be considered a functional food with numerous nutritional and pharmacological benefits. The formulations can be exploited and promoted for consumption following the results for sensory evaluation [32, 42, 46, 59–64].

Disclosure

The research was performed as part of employment and Doctoral graduation requirement for the corresponding author, Olabisi Theresa Ademosun.

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DETAIL

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Enhancing Physicochemical Characteristics of Donkey Meat through High-Voltage Electrostatic Stimulation during Acid Excretion

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ABSTRAK (ENGLISH)

To investigate the impact of high-voltage electrostatic stimulation on the quality of donkey meat, ten 30-month-old Dezhou donkeys (each weighing 250 ± 50 kg) were selected. Following slaughter, longissimus dorsi (LD) samples from both left and right carcasses were collected. The control group refrigerated the right muscle in a 0–4°C refrigerator for normal acid excretion. Meanwhile, the high-voltage electrostatic group subjected the left muscle to high-voltage electrostatic stimulation (30000 V) for 60 s before refrigerating it in the same conditions for acid excretion. The study showed that at the 1st, 6th, and 24th h of acid excretion, the high-voltage electrostatic group exhibited significantly lower pH levels than the control group (P<0.05). Furthermore, a* values in the high-voltage electrostatic group showed an increasing trend relative to the control group at the 6th and 24th h of acid excretion (P =0.0751, P=0.0860). With increasing acid excretion time, both treatment groups displayed increased PV and TBARS values. At 48 and 72 h, the PV values in the high-voltage electrostatic group were significantly lower than those in the control group (P<0.05). As acid excretion time increased, T-AOC, SOD, and GSH-Px in both groups decreased. However, the high-voltage electrostatic group consistently exhibited higher antioxidant enzyme activity than the control group at all time points. SOD and GSH-Px activity in the high-voltage electrostatic group showed an increasing trend at 48 h of acid excretion (P=0.0838, P=0.0860). At 72nd h of acid excretion, the shear force of donkey meat in the high-voltage electrostatic group was significantly lower than that in the ordinary treatment group (P<0.05). With increasing acid excretion time, the MFI of both treatment groups gradually increased. In particular, at the 1st and 48th h of acid excretion, the MFI of the high-voltage electrostatic group was significantly higher than that of the ordinary treatment group (P<0.05). Following high-voltage electrostatic stimulation, ultrastructure images of donkey meat at 6 and 72 h revealed numerous contractions and stretching bands. Therefore, this stimulation significantly improved the tenderness of donkey meat, reduced the oxidation rate of donkey meat, and improved the quality of donkey meat.

TEKS LENGKAP

1. Introduction

Donkeys have played an important role in Chinese agriculture and transportation. However, as agricultural production and mechanization have advanced, their utilitarian function has diminished. In response to improving living standards, people are rediscovering the value of donkey meat. It boasts lean meat and exquisite flavor and serves as a premium protein source with abundant essential amino acids and unsaturated fatty acids. Furthermore,

it is low in fat, cholesterol, and calories [1]. Consequently, Chinese specialty donkey meat snacks, including Hejian donkey meat barbecue, spiced donkey meat, donkey meat jerky, and donkey meat sausage, enjoy widespread consumer popularity.

Currently, China lacks specialized meat varieties, leading to the slaughter of older donkeys, resulting in high shear strength and challenging processing of donkey meat. Consequently, its relatively high market price constrains its growth. Donkey meat exhibits high myoglobin content, with myoglobin's iron atoms prone to oxidation, causing a shift in meat color from red to bright red, dark red, or brown, thereby impacting shelf life. Although donkey meat is rich in healthy unsaturated fatty acids, these are susceptible to oxidation. Therefore, enhancing the antioxidant properties of donkey meat, maintaining meat color stability, and safeguarding unsaturated fatty acids represent pressing concerns.

Electrical stimulation, as a key means of postslaughter treatment, plays a significant role in improving meat quality using stimulation at different voltages. High-voltage stimulation is excellent for optimizing meat quality due to its high-intensity characteristics, but safety concerns should be taken into account. In contrast, low-voltage stimulation, although safe, is often accompanied by the disadvantage of significant water loss [2–4]. High-voltage electrostatic stimulation, however, skillfully combines the multiple advantages of strong stimulation, low current, low power, and high safety, providing a novel solution for meat treatment. Compared with the problem of water loss that may be caused by low-voltage stimulation, high-voltage electrostatic stimulation is more effective in reducing microbial contamination while maintaining meat quality. Several studies have confirmed that both total microbial counts and drip losses were significantly reduced when thawed pork was exposed to high-voltage electrostatic fields (4, 6, 8 kV, and 10/15 kV), as reported by He et al. [5, 6]. This highlights not only the advantages of high-voltage electrostatic stimulation in enhancing meat quality but also its important role in ensuring food safety.

Despite the potential of high-voltage electrostatic stimulation in meat treatment, its application in donkey meat treatment has rarely been investigated. Therefore, the present study was devoted to the external stimulation of Dezhou donkeys using high-voltage electrostatic stimulation to investigate its specific effects on the quality changes of donkey meat. This study not only helps to fill the research gap of high-voltage electrostatic stimulation in donkey meat treatment but also provides strong theoretical support to improve the tenderness and economic benefits of donkey meat.

2. Materials and Methods

2.1. Sample Collection

Ten healthy Dezhou donkeys (30 months old, 250 ± 50 kg) from the Donkey Breeding Base of Dong'e Ass Hide Glue Co., Ltd., were selected and subjected to a 24 h fast before slaughter. The procedures for slaughter and sample collection followed the Animal Welfare Committee guidelines of Inner Mongolia Agricultural University (Approval Code: 2020102).

2.2. Experimental Design

Following slaughter, the 6−12th LD samples (length: 20 cm; width: 10 cm; height: 6 cm) from both sides of the carcass were divided into two treatment groups. The left sample received 30,000 V high-voltage electrostatic stimulation for 60 s, followed by acid excretion at 4°C. The right sample served as the control group, undergoing acid excretion at 4°C without electrical stimulation. pH and meat color were measured at the 1st, 6th, 24th, 48th, and 72nd h of acid excretion. Meat samples were collected and frozen in liquid nitrogen to assess antioxidant indicators. Muscle fiber ultrastructure samples were prepared at 1 h, 6 h, and 72 h of acid excretion, fixed in 2.5% glutaraldehyde phosphate buffer, and examined for muscle fiber structure. The shear strength of meat samples was measured after 72 h of acid excretion in both treatment groups.

2.3. Test Indicators and Methods

2.3.1. Meat pH

The pH of the LD muscle was determined using a Testo 205 portable pH meter, randomly inserted into the muscle site at a depth of 2 cm, and the mean value was calculated from two consecutive measurements.

2.3.2. Meat Color

Meat color (L*, a*, b*) was measured using a Minolta CR-400 colorimeter (Konica Minolta Sensing Inc., Osaka, Japan) with the D65 light source. Three different positions were measured for each sample randomly, and the average of the three values determined the flesh color. L* represents brightness, a* indicates redness, and b* indicates yellowness [7] (fresh cuts should not be exposed to air for >5 min).

2.3.3. Shear Force

The LD muscle samples, each measuring 5 cm × 5 cm × 5 cm (L × W × H), underwent different treatments and maturation durations. After removing fat, fascia, and connective tissues from the muscle surface, they were placed in transparent self-sealing bags and the openings were secured with leather bands. These bags were then immersed in a constant temperature water bath at 80°C. The center temperature of the muscle was measured with a thermometer, and when the center temperature of the muscle reached 70°C, it was removed and cooled to room temperature. Subsequently, meat columns were turned out along the direction of the muscle fibers by a 1-cm-diameter hollow sampler, and four meat columns were taken out from each sample. The shear forces of these samples were measured with a C-LM tenderizer, with the shear force value being the average of the four measurements.

2.3.4. Determination of PV

PV was determined as per Richards' method [8]. A 100 μM isopropyl benzene peroxide standard solution was prepared. In separate volumetric flasks, 0, 0.5, 1, 2, and 3 ml of the 100 μM solution were added, followed by the addition of chloroform to reach a total volume of 100 ml. Then, 2 ml was pipetted out; another 1.3 ml of chloroform-methanol solution, 25 μl of ammonia thiocyanate, and 25 μl of solution C (2 ml of solution A (0.02 g of barium chloride + 2.5 ml of 0.4 M hydrochloric acid) + 2 ml of solution B (0.04 g of ferrous sulphate heptahydrate + 2 ml of water)) were added; and then incubated at room temperature for 20 min, and absorbance was measured at 500 nm.

For meat samples stored at −80°C, PV was determined for 2 treatments at 0, 1, 6, 24, 48, and 72 h. A 0.3 g sample was homogenized in 10 ml of chloroform-methanol solution (1 : 1) using a high-speed dispersion homogenizer. Subsequently, 3.08 ml of 0.5% NaCl solution was added, and the mixture was centrifuged at 4°C, 4000 rpm for 6 min. After careful separation using a long-needle syringe, the mixture was carefully passed through the upper (methanol + water) and intermediate muscle layers; then 2 ml of the lower chloroform layer was removed; and another 1.3 ml of chloroform-methanol solution, 25 μl of ammonia thiocyanate, and 25 μl of solution C were added. Then, the mixture was incubated at room temperature for 20 min and absorbance was measured at 500 nm.

2.3.5. Determination of TBARS

The determination of TBARS, following Richards' method [8], involved the preparation of a 10 μmol/ml tetraethoxypropane (TEP) standard solution. Successively, 0, 0.5, 1, 1.5, and 2 ml of the standard solution were added to five separate 100 ml volumetric flasks, and the volume was adjusted to the scale line. After aspirating 0.1 ml from each, 1.2 ml of TCA-TBA solution was added. The solution was then incubated at 65°C for 60 min in a water bath at 250 rpm, cooled to 4°C for 60 min, and then centrifuged at 1600 rpm for 5 min. Finally, the absorbance was measured at 532 nm.

Then, 0.12 g sample was treated with 1.2 ml of TCA-TBA solution. After incubating in a 65°C water bath for 60 min at 250 rpm, the mixture was cooled at 4°C for 60 min and then centrifuged at 1600 rpm for 5 min. The extracted supernatant's absorbance was measured at 532 nm.

2.3.6. Transmission Electron Microscopy Observation of Muscle Microstructure

Li et al. method [9] was followed with slight modifications. Samples were collected at 1 h, 6 h, and 72 h postslaughter and cut into 3 cm × 0.5 cm × 0.5 cm meat strips. They were fixed in 2.5% glutaraldehyde and subsequently processed in the laboratory. Sample processing involved the following steps: rinsing, fixation, dehydration, and permeation. First, the sample was rinsed thrice with 1.1 M PB, for 10 min each. Then, 1% starvation acid was quickly added to it in a ventilated area, and the lid was closed and was mixed slowly to fix the sample well for 1 h 30 min. Finally, it was rinsed thrice with water for 5 min each. Then, a series of alcohol dehydration steps at 30%, 50%, 70%, 80%, 90%, and 95% were carried out, each for 15 min. Subsequently, propylene oxide was applied twice for 15 min each, followed by sequential permeabilization using propylene oxide: resin (2 : 1) for 30 min, propylene oxide: resin (1 : 2) for

30 min, and overnight permeabilization in pure resin. The samples were then polymerized at 60°C for three days after embedding. Tissue block trimming involved securing the embedded block to the sample holder and shaping it into a four-sided cone. Afterwards, the top surface of the block was further trimmed to a smooth, flat surface using a double-sided blade under a dissecting microscope. Sectioning was performed using a Leica Ultracut R ultrathin sectioning machine, followed by staining with 1% uranyl acetate for 12 h. Finally, observation and photography were carried out using a Hitachi H-7650B transmission electron microscope.

2.3.7. Determination of Myofiber Fragmentation Index

Culler et al.'s method [10] was referenced and slightly modified. A 1 g muscle sample from the longest section of the donkey's back, subjected to various treatments and time points, was used. The experiments were conducted on ice. To a centrifuge tube, 10 ml of precooled MFI buffer (100 mM KCl, 11.2 mM K_2HPO_4, 8.8 mM KH_2PO_4, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3) was added. The sample was homogenized at high speed using a high-speed homogenizing and dispersing machine and then centrifuged for 15 min at 4°C at 1000×g. The supernatant was discarded. The remaining precipitate was treated with another 10 ml of precooled MFI buffer and centrifuged at 4°C for 15 min at 1000×g. The resulting supernatant was utilized as the buffer for the remaining precipitate. This precipitate was fully suspended in 2.5 ml of MFI buffer and filtered through a 150 mesh cloth to eliminate connective tissue, and the centrifuge tube was rinsed with 2.5 ml of MFI buffer. The cleaning solution was filtered again, and the protein concentration was determined by the bisulfite method. The protein concentration was then adjusted to 0.5 mg/ml using MFI buffer, and the absorbance was measured at 540 nm. The final value was obtained by multiplying the result by 200, representing the MFI value.

2.4. Statistical Analysis

Preliminary statistics were generated with Excel (2007) software, and the GLM model in SAS 9.2 statistical software was conducted for donkey meat pH and color (L*, a*, b*), lipid oxidation products, antioxidant properties, MFI, and shear force over 72 h of acid excretion across various treatment groups. Duncan's multiple comparisons were used among the different treatments. $P>0.1$ indicates insignificant differences, $0.05<P<0.1$ suggests a trend, and $P<0.05$ signifies significant differences.

3. Results

3.1. Effect of High-Voltage Electrostatic Stimulation on the pH of Donkey Meat

Figure 1 illustrates the impact of high-voltage electrostatic and control groups on postslaughter pH in donkey meat (Table 1). Both treatment groups exhibited a decreasing pH trend. Electrical stimulation significantly accelerated pH decline early in the postslaughter period, with the electrical stimulation group reaching the pH limit at 24 h, while the control group reached it at 48 h. After 72 h of acid excretion, the pH in the high-voltage electrostatic group was significantly lower than that in the control group at 1, 6, and 24 h ($P<0.05$). Subsequently, over 24 h, the pH values in both groups gradually converged, with no significant difference ($P>0.05$).

[figure(s) omitted; refer to PDF]

Table 1

Effect of high-voltage electrostatic stimulation on the antioxidant properties of donkey meat.

Items	Time	Control	Stimulated	SEM	P
T-AOC (U/g)	1	3.49	3.67	0.18	0.5203
6	3.26	3.42	0.09	0.2023	24
3.23	3.42	0.08	0.1077	48	3.14
3.19	0.29	0.9024	72	2.98	3.04

0.16	0.7898	-			
SOD (U/g)	1	212.56	241.38	22.56	0.3726
6	154.06	190.93	36.78	0.4793	24
120.41	174.94	37.94	0.3395	48	85.38
156.76	25.56	0.0838	72	88.56	126.66
23.09	0.2770	-			
GSH-Px (U/g)	1	640.52	671.07	38.86	0.5763
6	614.61	646.77	36.79	0.5700	24
428.80	576.20	50.79	0.0860	48	368.05
399.32	44.75	0.6200	72	233.16	240.13

3.2. Effect of High-Voltage Electrostatic Stimulation on the Color of Donkey Meat

The impact of high-voltage electrostatic stimulation on the color of donkey LD muscles is detailed in Table 2. High-voltage electrostatic stimulation had no significant effect on meat color ($P>0.07$). At 6 h and 24 h postslaughter, the a^* value tended to be higher in the high-voltage electrostatic group than in the control group ($P=0.0751$, $P=0.0860$). With increasing acid drainage time, the L^* value gradually increased, while a^* and b^* initially increased and then decreased.

Table 2

Effect of high-voltage electrostatic stimulation on meat color of donkey muscle.

Items	Time	Control	Stimulated	SEM	P
L^*	1	26.58	27.01	2.90	0.7431
6	29.06	30.16	2.58	0.3517	24
33.7	33.67	0.82	0.9790	48	37.1
35.56	0.68	0.1291	72	35.83	35.13
0.44	0.3504	-			
a^*	1	12.21	12.2	0.65	0.9932
6	13.65	14.44	0.29	0.0751	24

12.57	13.50	0.36	0.0860	48	12.47
12.60	0.48	0.8527	72	11.66	11.32
0.49	0.6414	-			
b*	1	7.91	7.62	0.62	0.7474
6	8.00	8.17	0.42	0.7730	24
7.78	8.83	0.70	0.5861	48	9.66
8.47	0.48	0.1969	72	7.52	7.40

3.3. Effect of High-Voltage Electrostatic Stimulation on the Lipid Oxidation Products of Donkey LD Muscle

Table 3 illustrates the impact of high-voltage electrostatic stimulation on PV and TBARS in LD muscle during acid drainage. As the acid excretion time increased, PV values for both the control and high-voltage electrostatic groups exhibited a rising trend. Notably, at 48 and 72 h, the high-voltage electrostatic group demonstrated significantly lower PV values than the control group ($P<0.05$). While the effects of high-voltage electrostatic stimulation on TBARS in donkey meat were not statistically significant ($P>0.10$), both groups showed an increasing trend in TBARS values, with the high-voltage electrostatic group consistently displaying lower TBARS values than the control group across all time points.

Table 3

Effect of high-voltage electrostatic stimulation on lipid oxidation products of donkey meat.

Items	Time	Control	Stimulated	SEM	P
PV (μmol/kg)	1	286.81	231.93	20.20	0.1271
6	303.21	311.44	21.14	0.7798	24
337.68	292.84	22.11	0.2249	48	419.77[a]
343.04[b]	20.48	0.0252	72	446.12[a]	383.2[b]
12.93	0.0263	-			
TBARS (μmol/kg)	1	17.32	16.86	3.19	0.9217
6	18.94	17.35	2.85	0.7034	24
19.25	17.18	1.77	0.4308	48	19.72
18.73	2.53	0.7896	72	22.28	19.22

Different letters in the same row indicate significant differences (a, b: P<0.05).

3.4. Effect of High-Voltage Electrostatic Stimulation on the Antioxidant Properties of Donkey Meat

Table 1 outlines the impact of high-voltage electrostatic stimulation on antioxidant enzyme activities in donkey meat during acid drainage. The influence of high-voltage electrostatic stimulation on T-AOC in donkey meat did not yield significant differences (P>0.10). However, a decreasing trend was observed in both the high-voltage electrostatic group and the control group, with the T-AOC of the high-voltage electrostatic group surpassing that of the control group at all time points.

At 48 h, high-voltage electrostatic stimulation significantly increased SOD levels in donkey meat (P=0.0838) compared with the control group. However, at all other time points, high-voltage electrostatic stimulation had no significant effect on SOD levels (P>0.10). In both groups, a decreasing trend in SOD levels was observed, with the high-voltage electrostatic group consistently exhibiting higher SOD levels than the control group.

At 24 h, there were significantly higher GSH-Px levels in donkey meat subjected to high-voltage electrostatic stimulation compared with the control group (P=0.0860). However, at the remaining time points, high-voltage electrostatic stimulation did not have a significant impact on GSH-Px levels (P>0.10). Both groups demonstrated a decreasing trend in GSH-Px levels, with the high-voltage electrostatic group consistently displaying higher GSH-Px levels than the control group.

3.5. The Effect of High-Voltage Electrostatic Stimulation on Shear Force of Donkey Meat

The impact of high-voltage electrostatic stimulation on donkey meat shear force at 72nd of acid excretion is shown in Figure 2. The shear force was significantly reduced in the high-voltage electrostatic stimulation group compared with the control group (P<0.05). This indicates that electrical stimulation treatment accelerated the rate of glycolysis, myogenic fiber degradation, and skeletal protein breakdown in postslaughter meat. Additionally, it disrupted the myofiber structure, ultimately enhancing tenderness [11, 12].

[figure(s) omitted; refer to PDF]

3.6. Effect of High-Voltage Electrostatic Stimulation on the Myofibril Fragmentation Index of Donkey Meat

Figure 3 illustrates the effect of high-voltage electrostatic stimulation on the MFI of donkey meat. The MFI of myogenic fibers progressively increased in both the high-voltage electrostatic and control groups as the acid drainage duration increased. At 1st and 48th h, the high-voltage electrostatic group exhibited a significantly higher MFI than the control group (P<0.05). This can be attributed to electrical stimulation, which disrupted the sarcoplasmic reticulum and accelerated myofibrillar protein degradation, leading to increased MFI. Beyond these time points, the MFI of the high-voltage electrostatic group remained higher than that of the control group, with no significant difference, and the MFI of both groups gradually converged at 48 h until the late stage.

[figure(s) omitted; refer to PDF]

3.7. Effect of High-Voltage Electrostatic Stimulation on the Structure of Muscle Fibers of Donkey Meat

The electrical stimulation treatment resulted in physical damage to myogenic fibers, leading to an increased number of contracture and stretch bands. Figure 4 illustrates the impact of high-voltage electrostatic stimulation on myofibril structure during acid drainage of donkey meat. In the control group, the ultrastructure of donkey muscle fibers showed a clear, normal, and orderly arrangement, with distinct bright bands, dark bands, H bands, Z lines, and M lines. However, after 1 h of high-voltage electrostatic stimulation, donkey muscle fibers displayed spasmodic bands, wider gaps between muscle fibers, and a “single thin at both ends and thick in the middle” phenomenon. The filaments on both sides of the Z line in the bright band and the filaments in the dark band remained intact and clear.

[figure(s) omitted; refer to PDF]

After 6 h, the control group’s donkey muscle fibers showed minor degradation of the Z line due to protease activity. Near the contracture band, the Z line was partially lysed, with some Z-line remnants. The boundaries of the H band became less distinct, the M line blurred, and the I band gradually faded. In contrast, following 6 h of high-voltage electrostatic stimulation, the ultrastructure of donkey muscle fibers exhibited further degradation, including substantial Z-line disruption and degradation by a small amount of protease. The boundaries of the bright bands of adjacent myogenic fibers became indistinct, and the muscle node length contracted noticeably, disrupting the normal

fiber arrangement and leading to irregularly shaped nodes.

The ultrastructure of donkey muscle fibers in the control group after 72 h revealed dissolved and broken Z lines. In contrast, those exposed to high-voltage electrostatic stimulation for the same duration exhibited damaged ultrastructure, characterized by blurred M and H bands, extensive Z-line degradation, and disintegration. This led to I-band muscle fiber degradation, more Z-line debris, and smaller fiber fragments.

4. Discussion

pH is a crucial parameter in assessing meat quality, directly impacting meat palatability, shelf life, and color [13]. Variations were observed in the time taken to reach the limiting pH value following electrical stimulation across different livestock breeds. Some studies concluded that electrical stimulation accelerated the rate of beef pH decline within the initial 24 h postslaughter, reaching the limiting pH value at this point, with no significant difference compared with the control group after 24 h [11, 12]. Channon [14] noted that pig pH values were significantly lower than those of the control group from 40 min to 8 h postslaughter. The electrically stimulated group's pH values were also significantly lower, reaching the critical pH value for the pig's longest dorsal muscle at 8 h postslaughter. Our study aligns with these findings, demonstrating that high-voltage electrostatic stimulation significantly expedites early postslaughter pH decline, with the high-voltage electrostatic stimulation group reaching the critical pH value at 24 h, compared with the control group at 48 h. Electrical stimulation consistently leads to rapid pH reduction in the early postslaughter period in livestock and poultry [15]. In this experiment, the pH of the high-voltage electrostatic stimulation group quickly reached the lowest value, indicating accelerated acid excretion and shorter acid excretion time. This acceleration is primarily attributed to electrical stimulation's facilitation of lactic acid production from glycogen anaerobic fermentation and hydrogen ion production from ATP degradation within the muscle [16]. Additionally, it mitigated meat darkening and the loss of unsaturated fatty acids due to donkey meat oxidation during acid drainage, thus improving meat color and nutritional value. Beyond 72 h, the pH difference between the two treatments in this experiment was not statistically significant, falling within the normal range [17, 18], suggesting both methods effectively optimized donkey meat pH.

Meat color is a vital indicator of meat quality, influencing consumer sensory evaluation and purchasing decisions [19]. In our experiment, the L^* value progressively increased with increased acid drainage time. During rigor mortis, protein hydrolysis increased the free water content within the muscle, leading to the loss of juices and brighter meat. Conversely, the a^* values initially increased and then decreased over time. This pattern resulted from prolonged maturation causing juice loss and myoglobin concentration, subsequently elevating the a^* value. However, as the myoglobin content gradually decreased, the a^* value followed suit. Flesh color closely correlated with myoglobin pigment amount and state. Myoglobin exists in a reduced form under anoxic conditions, giving meat a purplish-red hue. When exposed to oxygen, myoglobin combines with it, producing oxygenated myoglobin and a bright-red appearance. With prolonged oxygen exposure, myoglobin oxidizes into methemoglobin, causing meat to appear dull and brownish, affecting consumer appeal. Furthermore, b^* values showed an initial increase followed by a decrease over time, likely due to the initial formation of oxygen and hemoglobin in donkey meat exposed to air, followed by gradual oxidation into methemoglobin. Brewer [20] concluded that L^* values were significantly correlated with visual meat color and that the combination of L^* and a^* values contributed up to 69% to pink color variability. Generally, b^* values have limited influence on flesh color and are sensitive to intramuscular fat and hemoglobin's oxidative status. a^* values are associated with hemoglobin content and oxidative status, while L^* values have weak correlations with these factors [19, 20]. The pH of electrically stimulated donkey meat and control meat decreased at different rates, but the results of this study showed no significant difference in meat color between the two treatments. This may be due to differences in myoglobin content and lipid oxidation.

Lipids are susceptible to oxidation during extended storage, undergoing peroxidation in the presence of free radicals or lipoxygenase. Peroxides, the intermediate products of this reaction, act as mediators for ongoing oxidation. These unstable peroxides further oxidize, yielding end products such as aldehydes, ketones, and alcohols [8], ultimately compromising meat quality. Our study revealed that both PV and TBARS values increased as acid drainage time extended, starting at 24 h and 1 h, respectively. The control group exhibited higher values than the PV group. This

could be attributed to the hydrolysis of donkey meat fat by endogenous lipases or microorganisms, yielding free fatty acids and resulting in higher PVs. While peroxides, as intermediate products of lipid oxidation, do not impact meat flavor directly, they serve as an important prerequisite for flavor, with elevated PVs indicating decreased freshness. Different acid removal methods significantly affected PV values in donkey meat ($P<0.05$), with the high-voltage electrostatic group exhibiting significantly lower values than the control group. This suggests that high-voltage electrostatic stimulation slowed the rate of freshness decline. In contrast, the impact of different acid removal methods on TBARS values was not significant ($P>0.05$). However, the high-voltage electrostatic group consistently showed lower TBARS values than the control group at all time points, indicating that high-voltage electrostatic stimulation slowed meat rancidity and extended product shelf life.

In meat, rancidity is linked to the T-AOC and its interaction with free radicals. As free radicals combine with ROS, they decompose into peroxides and eliminate catalytic metal ions. The determination of T-AOC indirectly reflects meat product oxidation [21–23]. In our study, both treatment groups showed a gradual decline in T-AOC over time, indicating free radical reactions with atmospheric oxygen. Notably, the electrically stimulated group consistently exhibited higher T-AOC, signifying enhanced antioxidant capacity. Superoxide dismutase (SOD) is pivotal in maintaining oxidative balance. It neutralizes superoxide anion free radicals (O_2^-) and safeguards cells from damage [24]. Over time, both treatment groups experienced a decrease in overall SOD activity. However, the electrically stimulated group consistently displayed higher SOD activity, demonstrating superior SOD activity. GSH-Px, a crucial intracellular antioxidant, plays a role in mitigating lipid peroxidation. It is a small-molecule peptide comprising methionine, glycine, and cysteine, which, together with antioxidant enzymes, such as SOD, catalyzes the decomposition of lipid peroxides into nontoxic alcohols by scavenging O_2 and H_2O_2 to attenuate and block the first-order triggering of lipid peroxidation [25]. The overall decrease in GSH-Px activity over time in the two treatment groups suggests that continuous lipid peroxidation is occurring in stored donkey meat during storage. The electrically stimulated group consistently exhibited higher GSH-Px activity, suggesting its ability to slow down lipid oxidation and extend the shelf life of donkey meat.

Shear force serves as a prominent indicator for meat tenderness evaluation. Key factors influencing tenderness encompass myofiber characteristics, particularly myogenic fibers within myofibers, as pivotal determinants of muscle tenderness. Myogenic fibers consist of various proteins, and existing evidence links meat tenderness closely to the degradation of skeletal proteins, including myosin, actin, troponin, and intermuscular myosin [26]. In this experiment, the high-voltage electrostatic group had significantly lower shear force than the control group, thereby suggesting that high-voltage electrostatic stimulation accelerated the tenderization process of donkey meat. Devine [4, 26] found that electrical stimulation significantly reduced the μ-calpain and calpain inhibitor activities with electrical stimulation, thus expediting the activation of the calpain inhibitor system, a critical factor in tenderization. This was attributed to the swift pH decrease, thereby affecting the activation and inactivation of calcium-dependent proteases. In the process of muscle maturation, myofibrils break from the Z line, leading to MFI. MFI serves as a critical indicator for meat tenderness assessment. The MFI value directly reflects the degree of myofibrillar degradation and destruction. A higher value signifies more severe myofibrillar fragmentation, resulting in lower shear values, indicating superior tenderness. Therefore, MFI is closely related to meat tenderness. The mechanism behind meat tenderization via electrical stimulation is generally attributed to two factors [27]. However, electrical stimulation accelerates the release of calcium ions from the sarcoplasmic reticulum, leading to myofibril contraction and alterations in myofibrillar ultrastructure. Furthermore, the postslaughter carcasses experience higher temperatures and a lower pH due to electrical stimulation, which enhances enzyme activity and speeds up protein degradation. Our observations of myofiber ultrastructure align with these mechanisms. Figure 4 depicts slight myofiber fragmentation in the control group at 72 h. Enzymes in the donkey muscle are released upon slaughter, hydrolyzing skeletal proteins and other relevant proteins in myogenic fibers. This hydrolysis leads to myogenic fiber breakage and an increase in the MFI in our study. George et al. also noted that electrical stimulation induces an excessive release of calcium ions from the beef sarcoplasmic reticulum, resulting in the formation of contracture bands that disrupt the myofibrillar structure and enhance tenderness [28].

5. Conclusion

High-voltage electrostatic stimulation significantly reduced the shear force and improved the tenderness of donkey meat by promoting the biochemical reactions of meat tenderization and disrupting the myofiber structure. At the same time, electrical stimulation was able to influence the fragmentation process of myogenic fibers, which had a significant effect on MFI, especially in the early stages of acid excretion. Therefore, high-voltage electrical stimulation has great potential for the industrial processing of donkey meat.

Authors' Contributions

Xinzhuang Zhang and Manglai Dugarjaviin conceptualized the study; Xinzhuang Zhang designed the methodology; Shuqi Gong wrote the original draft and prepared the manuscript; Xinzhuang Zhang wrote, reviewed, and edited the manuscript; and Xinzhuang Zhang and Manglai Dugarjaviin acquired funding. All authors have read and agreed to the published version of the manuscript. Xinzhuang Zhang and Shuqi Gong contributed equally to this work.

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DETAIL

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Microencapsulated Oregano Essential Oil as a Natural Preservative of Beef Burger during Refrigerated Storage

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ABSTRAK (ENGLISH)

Meat products are highly perishable and prone to deterioration, which poses potential health hazards. This research aims to assess the efficacy of encapsulated oregano essential oil (EOEO) as a natural preservative agent to maintain the chemical and microbiological quality of beef burgers during refrigerated storage. Hydrodistillation was used to extract OEO from oregano. The encapsulation of OEO using a combination of biopolymers (maltodextrin, gum arabic, and whey protein) provides high encapsulation efficiency (89.1%). Both crude and encapsulated OEOs were analyzed for their chemical constituents and antimicrobial activity. Encapsulated OEO, at levels of 0.25%, 0.5%, 0.75%, and 1%, was incorporated during the beef burger processing. Microbiological and chemical parameters were assessed every 4 days over a 16-day storage period. GC-MS results revealed that carvacrol (70 and 79.31%) and p-cymene (11.56% and 9.05%) dominated the crude and encapsulated OEOs, respectively. Both forms of OEO exhibited potent antimicrobial activity, with encapsulation further enhancing this property. The incorporation of EOEO into burger samples reduced the total microbial count. Subsequently, it decreased the formation of total volatile nitrogen (TVN), trimethylamine (TMA), thiobarbituric acid (TBA), and biogenic amines (BAs) during storage. Addition of EOEO at the level of 1% retarded the formation of BAs, TVN, TMA, and TBA in burger samples by 72.8%, 43.23, 42.07, and 44.44%, respectively, compared to the control sample after 16 days of

storage. Principal component analysis (PCA) was applied to establish correlations between microbiological and biochemical markers of beef burgers. The PCA results show that PC1 (89.81%) and PC2 (7.25%) can explain more than 97% of the variability in the dataset. The results support the potency of EOEO as an effective and safe preservative agent to maintain the safety and quality of beef burgers during storage.

TEKS LENGKAP

1. Introduction

Meat and meat products are considered good sources of essential nutrients like B-complex vitamins, iron, and proteins containing essential amino acids [1]. Although meat and meat products generally maintain a good safety record [2], the WHO reported 582 million cases suspected to food-borne diseases in 2010, resulting from the consumption of foods contaminated with pathogenic microorganisms [3]. Furthermore, food-borne pathogens are contributing to approximately 5000 annual deaths, with one-third linked to meat and poultry products.

Despite the high nutritional value of meat, its high moisture content renders it a highly perishable food and shortens its shelf life. Even under refrigerated storage, microbial growth, proteolysis, and lipid peroxidation are the key contributors to the spoilage and deterioration of meat products [4]. These deteriorations lead to produce harmful substances such as biogenic amines (BAs), total volatile nitrogen (TVN), trimethylamine (TMA), and peroxides. The EU regulation (2073/2005) set the acceptable limits for TMA, TVN, and TBA in meat and meat products as 10 mg/100 g, 25 mg/100 g, and 5 mg of malonaldehyde/kg, respectively.

In attempts to control the microbial activity and prevent the proteolysis and lipid peroxidation, chemical preservatives such as nitrates and nitrites were extensively employed. However, the concerns about health hazards associated with synthetic antimicrobial and antioxidant agents prompted a shift toward the use of safe alternatives to ensure the quality and safety of such products [5, 6]. In this context, herbal extracts, particularly essential oils (EOs), have been explored for preserving meat, fish, and their products [7–10]. Essential oils, such as oregano essential oil (OEO), have demonstrated antioxidant activity and broad-spectrum antimicrobial potency against food-borne pathogens, making it a promising choice for this purpose [11].

Although the in vitro antimicrobial activity of OEO has been extensively studied, limited research studies have been conducted on its efficacy in food matrix. The antimicrobial activity of EOs in food systems is lower than that observed in vitro due to the effect of food matrix. The application of EOs in food products faces several challenges regarding their chemical properties including hydrophobicity, volatility, thermal lability, oxidation, and flavor [12]. To overcome these challenges, food-grade delivery systems such as encapsulation techniques have been employed to harness the advantages of EOs and mitigate their drawbacks [13]. This study aims to evaluate the potency of encapsulated OEO as a preservative agent in vitro and a food system. The efficiency of encapsulated OEO at different concentrations in maintaining the microbiological and chemical quality of beef burgers was assessed by monitoring total microbial counts, proteolysis products (TVN, TMA, and BAs), and lipid peroxidation (TBA reactive substances) during refrigerated storage.

2. Materials and Methods

2.1. Materials

All of the chemicals and reagents used to determine microbiological and chemical parameters were of analytical grade. Biogenic amines (cadaverine, spermine, spermidine, *β*-phenylethylamine, histamine, and putrescine), 1,1,3,3-tetramethoxypropane, and thiobarbituric acid were obtained from Sigma-Aldrich.

2.2. Beef Meat and Other Ingredients

Fresh beef meat, fat, salt, and black pepper were purchased from local market at Giza, Egypt. Soy protein was purchased from the Agricultural Research Center, Giza, Egypt.

2.3. Oregano Plant Materials

Origanum vulgare subsp. *hirtum* L. (*Lamiaceae*) seeds were obtained from the Agriculture Research Center in Giza,

Egypt. The seeds were sown in the farm of the National Research Center in El-Nubaria Province, Beheira Governorate, Egypt, and mature plants were collected at the blossoming phase. The *Origanum vulgare* subsp. *hirtum* L. plant was identified and classified by Mrs. Therese Labib, consultant on plant taxonomy, Agriculture Ministry, Giza, Egypt.

2.4. Extraction of Oregano Essential Oil

Aerial part of oregano plants was extracted using the hydrodistillation technique in Clevenger-type apparatus with an extraction ratio of 1 part plant to 5 parts water for 4–6 hours. The obtained OEO was dried over sodium sulphate anhydrous and preserved in amber glass vial with Teflon stoppers at −20°C until use.

2.5. Preparation of Encapsulated Oregano Essential Oil

Microcapsules of OEO were prepared according to Rodea-Gonzalez et al. [14], and the microcapsule wall materials were composed of maltodextrin, gum arabic, and whey protein isolate in a proportion of 2 : 1 : 1. The polymer solution was prepared at a concentration of 20% in distilled water and stirred overnight for full hydration at ambient temperature. Oregano essential oil was emulsified in Tween 80 and slowly dropped into the polymer solution with high-speed homogenization at 20,000 rpm. The amount of OEO used was 10% of the polymer mass. The encapsulation process of emulsion was performed using spray drier (B-290, Buchi) as described by Mohammad et al. [15]. Encapsulated oregano essential oil (EOEO) was stored at 4°C until use.

2.6. Particle Size (PS), Zeta Potential (ZP), and Polydispersity Index (PDI) Measurement

The PS diameter, ZP, and PDI of the microcapsules were determined in triplicate using particle size analyzer (Malvern Instruments Ltd., UK). The EOEO sample was immediately diluted fivefold with deionized water before measurement [16].

2.7. Total Surface Oil and Encapsulation Efficiency

The surface OEO content was measured using a UV-VIS spectrophotometer (Agilent, USA) according to Soottitantawat et al. [17] with slight modification. One gram of EOEO was extracted twice with 10 mL of hexane and shaken for 10 minutes, and the filtrates were combined. The content of OEO in n-hexane fraction was then determined using a spectrophotometer at 275 nm. Serial dilutions of OEO (10–100 μg/mL) were used to prepare a standard curve for the calculation of surface OEO in the microcapsules.

The total OEO content of the microcapsules was determined by suspending 1 g of EOEO in 10 mL of distilled water, sonicated for 5 min and then partitioned with n-hexane. The extracted OEO was determined using a spectrophotometer at 275 nm and calculated as previously described. Encapsulation efficiency was calculated according to the following equation.(1)Encapsulation efficiency=total oil−surface oiltotal oil×100.

2.8. GC-MS Analysis of OEO

Chemical profile of OEO was determined using an Agilent/HP GC-MS system model 5973-6890 in electron ionization mode at 70 eV. The separation was conducted using a HP-5MS capillary column (30 mm × 0.25 mm; 0.25 μm) with a thermal gradient elution initially set at 60°C and gradually increased at 3°C/min to 280°C. Helium served as the carrier gas at a flow rate of 1 ml/min, and the injection volume was 1 μL with a splitless ratio of 1 : 10. The identification of separated peaks was done by matching it with the NIST/NBS Wiley library of mass spectra.

2.9. In Vitro Tests of Antimicrobial Activity

Antimicrobial activity of crude and encapsulated OEO was estimated using disc diffusion assay. Three concentrations of crude OEO (10, 25, and 50 mg/ml) and their equivalent concentrations of encapsulated OEO (50, 100, and 150 mg/ml) were prepared in DMSO and used for antibacterial and antifungal estimations.

Antibacterial activity of the tested concentrations was carried out according to Ahmed et al. [18] and EUCAST [19] against Gram-positive MRSA (methicillin-resistant *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Shigella* sp., and *Salmonella typhimurium*) pathogenic bacteria. Bacterial species were grown on tryptic soy broth (TSB) tubes and incubated at 35°C for 4 h. Discs (6 mm) were impregnated in different tested concentrations and applied on the surface of bacterial film developed on nutrient agar plates and then incubated at 35°C for about 18 h. Oxytetracycline (1 mg·ml^{-1}) was used as positive control.

Eight fungal species: *Aspergillus flavus NRRL 3357*, *A. niger*, *A. parasiticus SSWT 2999*, *A. westerdijikiae* CCT

6795, *A. ochraceus ITAL 14*, *A. carbonarius ITAL 204*, *Fusarium verticillioides ITEM 10027*, and *F. proliferatum MPVP 328*, were used for antifungal assay. Potato dextrose agar (PDA) was used as the assay media. The impregnated discs with tested concentrations were applied on the surface of PDA plates streaked with 100 μL of fungal spore suspension. The plates were incubated at 28°C, and the diameters of inhibition zones were recorded at 24 and 48 h.

2.10. Beef Burger Preparation

The beef meat and fat were minced in a Moulinex meat mincer immediately before burger manufacturing. Burger samples were prepared as described by the Egyptian standard specifications [20] using a mixture of 65% minced beef, 20% fat, 5% soy protein, 0.3% black pepper, 1.7% salt, and 8% water. The ingredients were mixed well to achieve uniform dough and divided into five portions. The first portion was prepared without EOEO (control) and the four portions received 0.25, 0.5%, 0.75, and 1.0% EOEO. The treatments were chosen regarding the acceptable sensorial limits of OEO in meat product reported by Hernández et al. [21]. Each portion was separately formed using manual burger press maker, then packaged in foam plates, and stored at 4°C for 16 days. Over the storage period, samples were taken at intervals of 0, 4, 8, 12, and 16 days according to Ghanbarinia et al. [22].

2.11. Microbiological Analysis of Burger Samples during Storage

Burger samples were evaluated for their total viable count and mold and yeast count using the serial dilution and pour plate technique. In aseptic conditions, 25 g of each sample was mixed with 225 ml of 0.1% peptone water solution. Then, serial decimal dilutions from 10^{-2} to 10^{-7} were plated on nutrient agar (NA) and PDA to determine total viable bacteria and mold and yeast counts, respectively. The plates were incubated at 35 ± 2°C for 48 hours for determination of total viable bacteria, and at 28 ± 2°C for 5 days for mold and yeast counts. After incubation, the counts were calculated in logarithmic scales (log CFU/g).

3. Biochemical Marker Analysis of Burger Samples

3.1. Determination of TVN

Burger samples were extracted for 5 min by the Kjeldahl steam distillation unit (Gerhardt Vapodest 30, Germany) [23]. The extract was titrated with 0.01 N HCL, and the TVN content was represented as mg N/100 g.

3.2. Determination of Trimethylamine

Trimethylamine of burger samples was extracted as TVN method after appropriate modifications. Twenty milliliters of formaldehyde solution (20%) was added to sample for blocking the primary and secondary amines [23].

3.3. Determination of Thiobarbituric Acid Value

Thiobarbituric acid value of burger samples were determined by a distillation method according to Pikul et al. [24]. Ten grams of burger was distillated with 4 M hydrochloric acid. Then, 5 mL extract and 5 mL TBA reagent were combined in test tube with screw cap, shaken, and incubated at 100°C in water bath for 35 min. The absorbance was measured at 538 nm using spectrophotometer against distilled water. Serial dilutions of malonaldehyde (1–10 μg/mL) were used as standard curve, and the results were calculated and represented as mg MDA per kg sample.

3.4. Determination of Biogenic Amines

Burger sample (25 g) of beef was homogenized with 125 mL of 5% trichloroacetic acid for 3 minutes using a Waring blender and filtered using filter paper (Whatman No. 1). Ten milliliters of the filtrate, along with 4 g of NaCL and 1 ml of 50% NaOH, was extracted with 5 ml of 1 : 1 mixture of n-butanol and chloroform. The extract was shaken for 3 minutes and centrifuged at 3000 rpm for 5 minutes. The upper solvent layer was separated, and then 15 mL of n-heptane was added and partitioned with 1 mL of 0.2 N HCL. Serial concentrations of BAs (10–100 μg/mL) were prepared in 0.1 N HCL. The extracts and standard solutions were dried using a rotary evaporator at 50°C [25]. Derivatization of BA standards and sample extracts was performed by adding 1 mL of dansyl chloride solution (0.5 g/100 ml acetone) to the dried extracts in the presence of saturated $NaHCO_3$ solution. The reaction was completed by incubating the mixture at 55°C for 45 minutes. The BA derivatives were extracted twice using 10 ml of diethyl ether and evaporated using a rotary evaporator at 35°C. The dry film was reconstituted in 1 ml of methanol and filtered through a Millipore filter (0.45 μm) prior to injection into the HPLC system (Agilent 1100 series, Germany). The BAs were monitored by DAD at 254 nm, and the separation was performed on an Eclipse XDB-C18 column

(150 mm × 4.6 μm; 5 μm) with a mobile phase and gradient elution as described by Ayesh et al. [26].

3.5. Statistical Analysis

The data of antimicrobial activity and biogenic amines were analyzed using one-way ANOVA and Tukey test to compare the means at $p<0.05$, while the effect of treatments, storage periods and their interaction on the microbiological analysis, and biochemical markers were analyzed using two-way ANOVA and Tukey test at $p<0.05$ using Assistat Software Version 7.7.

The principal component analysis was conducted by OriginPro software version 2019b (Origin Lab corporation, Northampton, MA, USA) to investigate the correlation between the application of EOEO at varying levels and quality indicators (chemical and microbiological) during the refrigerated storage period of beef burgers.

4. Results and Discussion

4.1. Chemical Composition of OEO

The chemical constituents of raw and encapsulated OEO were analyzed using gas chromatography-mass spectrometry, and the results are illustrated in Table 1. The total number of identified components was 20 for crude essential oil and 14 for encapsulated oil, constituting 98.65% and 99.63% of the total essential oil components, respectively. In both crude and encapsulated OEOs, aromatic oxygenated monoterpenes, including carvacrol (70% and 79.31%, respectively) and p-cymene (11.56% and 9.05%, respectively), were the dominant constituents. Additionally, γ-terpinene (4.31% and 1.95%), trans-caryophyllene (2.95% and 3.83%), and caryophyllene oxide (1.13% and 1.93%) were reported in crude and encapsulated oils, respectively. The increase in the relative percentage of carvacrol in EOEO is attributed to its chemical nature. The hydroxyl group in carvacrol, which is located in the orthoposition to a methyl group, can easily interact via hydrogen bonding with carboxylic and amino groups in whey protein and gum arabic [27].

Table 1

Chemical profile of crude and encapsulated OEO (relative %).

Compound	KI	Crude oil	Encapsulated oil
(*R* %)	(*R* %)	Monoterpenes	
α-Thujene	930	0.21	ND
α-Pinene	939	0.87	0.52
Camphene	954	0.3	0.18
α-myrcene	991	0.99	0.36
Phellandrene	1003	0.33	0.16
α-terpinene	1017	1.74	0.75
p-Cymene	1026	11.56	9.06
b-Phellandrene	1030	0.27	ND
γ-Terpinene	1060	4.31	1.95

α-terpinolene	1089	0.15	ND
Oxygenated monoterpenes			
L-Linalool	1097	0.67	0.26
endo-Borneol	1169	0.82	0.65
Terpin-4-ol	1177	0.82	0.37
a-Terpineol	1189	0.12	ND
Thymol	1266	1.13	0.3
Carvacrol	1299	70	79.31
Carvacrol acetate	1373	0.15	ND
Sesquiterpenes			
trans-Caryophyllen	1419	2.95	3.83
Humulene	1455	0.1	ND
Oxygenated sesquiterpene hydrocarbons			
Caryophyllene oxide	1466	1.13	1.93
Total identified		98.62	99.63
Total monoterpenes		20.73	12.98
Total oxygenated monoterpenes		73.71	80.89
Total sesquiterpenes		3.05	3.83
Total oxygenated sesquiterpenes		1.13	1.93

ND = not detected.

Our results align with those reported by Tsitsos et al. [28] for essential oil of oregano (*Origanum vulgare*), revealing carvacrol (53.76–76.1%), thymol (2.32–5.66%), *β*-caryophyllene (3.83–6.32%), *γ*-terpinene (3.6–5.25%), and p-cymene (3.82–5.55%) as the prominent components. Weglarz et al. [29] also reported varying amounts of carvacrol in the essential oils of Greek oregano plants (*O. vulgare* ssp. *hirtum*) grown in different locations in Greece, ranging from 1.7% to 69.6%. Furthermore, Hao et al. [11] obtained a carvacrol-rich essential oil (84.38%) from *O. vulgare* “Hot and Spicy.”

It is well-established that the chemical constituents of essential oils differ based on cultivation area, harvesting time,

and climate conditions [30]. Bejaoui et al. [31] preformed a study to detect the variation in the essential oil of wild *O. vulgare* at various growth stages. They observed that carvacrol increased from 61.08% at the vegetative stage to 83.37% at the flowering phase, while p-cymene and c-terpinene decreased from 9.87 to 3.02% and 6.34 to 4.13%, respectively.

4.2. Encapsulation Efficiency and Particle Size of EOEO

Encapsulation efficiency (EE) represents the amount of OEO trapped in the dried microcapsules. In this context, the combination of biopolymers (maltodextrin, gum arabic, and whey protein) provides high encapsulation efficiency (89.1%) for OEO. This is attributed to physical interactions such as van der Waals forces and hydrogen bonding, which significantly enhance the entrapment of essential oil within the microcapsules [32]. The emulsifying properties of nonionic Tween 80, anionic gum arabic, and whey protein provide low particle size (215 ± 7.7 nm) with excellent entrapment of essential oil during encapsulation by spray dryer [33]. Furthermore, the low value of polydispersity index (0.352 ± 0.06) as well as negative zeta potential (−5.61 ± 0.05 mV) indicates the homogeneity and stability of microcapsules during application in food systems.

4.3. Antimicrobial Activity of OEO

The antimicrobial activity of both crude and encapsulated OEO against various pathogens is detailed in Table 2. The disc diffusion technique was applied to evaluate three concentrations of crude OEO (10, 25, and 50 mg/mL) and three concentrations of EOEO (50, 100, and 150 mg/mL). Bacteriological analysis revealed comparable antibacterial activity across all tested concentrations, with varying effects. The resulting inhibition zones increased as the concentration of OEO increased. It ranged from 8.0 mm for 10 mg/mL to 16.3 mm for 50 mg/mL crude OEO. Among the tested bacteria, *Escherichia coli, Shigella, and MRSA* were more sensitive to OEO doses. Crude OEO at 50 mg/mL exhibited significant larger inhibition zones (16.0, 16.3, and 16.3 mm, respectively) against them, compared to the positive control oxytetracycline (11.3–12.0 mm). However, *S. typhi* and *S. typhimurium* were more resistant, representing smaller inhibition zones (14.3 and 15.3 mm, respectively) at the concentration of 50 mg/mL compared to oxytetracycline (16.3 and 17.3 mm, respectively).

Table 2

Antimicrobial activity of crude and encapsulated OEO at different concentrations.

Microorganisms	Inhibition zones (mm)						
	Crude oil (mg/ml)		Encapsulated oil (mg/ml)			Positive control*	10
25	50	50	100	150	Bacterial strains		
S. typhi	9.3 ± 0.33^{d}	11.3 ± 0.67^{c}	14.3 ± 0.67^{b}	0.0 ± 0.0^{f}	7.7 ± 0.33^{e}	9.3 ± 0.33^{d}	16.3 ± 0.67^{a}
E. coli	8.0 ± 0.0^{e}	9.7 ± 0.33^{c}	16.0 ± 0.55^{a}	8.0 ± 0.0^{e}	8.3 ± 0.33^{de}	9.3 ± 0.33^{dc}	11.3 ± 0.67^{b}
Shigella	9.3 ± 0.67^{cd}	9.3 ± 0.67^{cd}	16.3 ± 0.83^{a}	8.0 ± 0.0^{d}	8.7 ± 0.67^{d}	10.7 ± 0.33^{bc}	11.3 ± 0.67^{b}
S. typhimurium	9.0 ± 0.55^{d}	11.3 ± 0.33^{c}	15.3 ± 0.67^{b}	6.7 ± 0.67^{e}	8.3 ± 0.33^{de}	10.0 ± 0.55^{dc}	17.3 ± 0.67^{a}

MRSA	8.7±0.33^{ed}	10.7±0.67^{bc}	16.3±0.33^{a}	8.3±0.33^{e}	8.7±0.67^{ed}	10.0±0.55^{cd}	12.0±0.0^{b}
Fungal strains							
A. flavus	10.3±0.33^{d}	13.3±0.33^{b}	20.7±0.67^{a}	8.0±0.0^{e}	8.0±0.0^{e}	10.3±0.33^{d}	12.0±0.0^{c}
A. parasiticus	12.0±0.0^{cd}	14.7±0.33^{b}	20.0±0.0^{a}	8.0±0.0^{f}	9.7±0.33^{e}	11.3±0.33^{d}	12.3±0.33^{c}
A. niger	10.0±0.0^{c}	11.3±0.33^{b}	16.3±0.66^{a}	8.0±0.00^{d}	8.7±0.33^{d}	10.0±0.0^{c}	12.0±0.0^{b}
A. ochraceus	12.7±0.33^{d}	14.7±0.33^{c}	21.3±0.66^{a}	9.0±0.00^{e}	10.3±0.33^{e}	12.7±0.67^{d}	18.7±0.67^{b}
A. westerdijikia	10.0±0.0^{d}	12.3±0.33^{c}	22.0±0.00^{a}	8.7±0.33^{e}	10.7±0.67^{d}	13.3±0.33^{c}	20.0±0.0^{b}
A. carbonarius	10.3±0.33^{d}	13.7±0.33^{b}	20.7±0.67^{a}	8.0±0.00^{e}	9.0±0.0^{e}	11.7±0.33^{c}	14.0±0.0^{b}
F. proliferatum	10.7±0.33^{c}	13.0±0.57^{b}	22.7±0.67^{a}	9.3±0.33^{d}	10.3±0.33^{d}	12.0±0.0^{b}	12.0±0.0^{b}
F. verticillioides	10.3±0.33^{d}	13.0±0.00^{b}	20.3±0.33^{a}	8.0±0.00^{f}	9.0±0.0^{e}	10.3±0.33^{d}	12.0±0.0^{c}

*Oxytetracycline (1 mg/ml) and nystatin (1000 Unit/ml) were used as the positive antibacterial control and positive antifungal control, respectively. MRSA = methicillin-resistant *Staphylococcus aureus*. Data in the same row with different letters are statistically significant ($p<0.05$).

Table 2 also illustrates the antifungal activity of both crude and microencapsulated OEO against mycotoxigenic fungi. Crude OEO displayed robust antifungal activity, increasing with OEO concentration from 10 to 50 mg/mL. Inhibition zones at 50 mg/ml ranged from 16.3 mm for *A. niger* to 22.7 mm for *F. proliferatum*. In comparison, the inhibition zones of positive control nystatin at 1000 Unit/mL ranged from 12.0 mm for *A. niger* to 20.0 mm for *A. westerdijikiae*. Notably, inhibition zones for all tested fungi at 50 mg/ml crude oregano oil were significantly larger than those of nystatin.

The robust antimicrobial activity of OEO is evidenced by larger inhibition zones at the concentrations of 25 and 50 mg/ml compared to control antibiotics. Except *S. Typhi*, EOEO at the concentration of 150 mg/mL (containing 15 mg OEO) demonstrated statistical ($p<0.05$) comparable antibacterial activity to crude OEO at the concentration of 25 mg/mL. This may be attributed to the chemical constituents of OEO and their synergistic effect. Furthermore, EOEO at 150 mg/mL exhibited comparable antifungal activity to crude OEO at 10 mg/mL against all tested fungi except *A. westerdijikiae and F. proliferatum*, which were comparable to 25 mg/mL crude oil. The obtained results are consistent with previous findings of Yoncheva et al. [34] for encapsulated oregano essential oil. They stated that encapsulation process enhances the OEO solubility, thermal stability, antioxidant activity, and consequently the antimicrobial activity, suggesting its potential as a delivery system for OEO in food processing.

The antimicrobial activity action of essential oils against microorganisms is attributed to their major components and their synergistic effect with minor components. Carvacrol, the predominant component in OEO, exhibits antimicrobial activity against a broad spectrum of microorganisms. Its mode of action includes damaging cell membranes, inducing cellular morphological changes, and inhibiting intracellular ATP synthesis, thereby suppressing energy-dependent cellular processes such as enzyme and toxin synthesis [28, 35]. Moreover, minor constituents like c-terpinene may contribute to antimicrobial activity through synergistic or antagonistic effects [36].

4.4. Microbiological Analysis of Burger Samples during Cold Storage

Microbiological analysis results of burger samples incorporated with EOEO are elucidated in Table 3. The obtained data revealed initial total bacterial counts for burger samples ranging from 5.53 to 5.79 log CFU/g. During the initial eight days of storage, the total bacterial count of the control sample dramatically increased ($p<0.05$) to 7.66 log CFU/g. In contrast, burger samples mixed with EOEO exhibited insignificant microbial growth.

Table 3

Microbiological analysis of burger samples mixed with encapsulated OEO during refrigerated storage.

EOEO level (%)	Storage period (day)				
0	4	8	12	16	Total bacterial count (log CFU/g)
0.0	5.58 ± 0.02^{aE}	6.60 ± 0.04^{aD}	7.66 ± 0.25^{aC}	8.85 ± 0.21^{aB}	9.83 ± 0.22^{aA}
0.25	5.53 ± 0.08^{aD}	6.42 ± 0.04^{aC}	6.94 ± 0.11^{bC}	8.78 ± 0.18^{aB}	9.67 ± 0.21^{aA}
0.50	5.79 ± 0.08^{aD}	6.59 ± 0.20^{aC}	6.71 ± 0.17^{bC}	7.61 ± 0.19^{bB}	8.48 ± 0.23^{bA}
0.75	5.58 ± 0.02^{aD}	6.29 ± 0.11^{aC}	6.63 ± 0.19^{bC}	7.51 ± 0.24^{bB}	8.20 ± 0.11^{bA}
1.0	5.55 ± 0.04^{aD}	6.12 ± 0.06^{aC}	6.60 ± 0.18^{bC}	7.33 ± 0.19^{bB}	8.07 ± 0.11^{bA}
Mold and yeast count (log CFU/g)					
0.0	4.03 ± 0.15^{aC}	4.82 ± 0.28^{aBC}	4.89 ± 0.26^{aBC}	5.60 ± 0.12^{aAB}	6.75 ± 0.35^{aA}
0.25	3.95 ± 0.05^{aC}	4.53 ± 0.32^{aBC}	4.54 ± 0.11^{aBC}	5.56 ± 0.10^{aAB}	6.65 ± 0.36^{aA}
0.50	4.12 ± 0.28^{aB}	4.43 ± 0.35^{aB}	4.60 ± 0.33^{aB}	5.44 ± 0.08^{abAB}	6.50 ± 0.41^{aA}
0.75	4.07 ± 0.26^{aB}	4.32 ± 0.42^{aAB}	4.55 ± 0.26^{aAB}	5.31 ± 0.07^{abAB}	5.74 ± 0.34^{aA}
1.0	4.04 ± 0.1^{aB}	4.06 ± 0.25^{aB}	4.42 ± 0.28^{aAB}	4.96 ± 0.02^{bAB}	5.22 ± 0.06^{aA}

In column, data with different small letters indicate statistical significance ($p<0.05$). In row, data with different capital letters indicate statistical significance ($p<0.05$).

In adherence to the ICMSF [2] specifications and guidelines, a total viable count of 7 log CFU/g represents the

maximum allowable limit for meat products. On the 8th day, the control burger sample had total bacterial count higher than this limit, while burger samples incorporated with EOEO remained within acceptable limits. For instance, the bacterial count in burgers mixed with 1% EOEO increased by 1.05 log on the 8th day, compared to 2.08 log for the control sample.

A significant increase ($p<0.05$) in total bacterial count of burger samples mixed with EOEO was observed on the 12th and 16th days of storage. The addition of EOEO at 1% significantly reduced the bacterial count until the 12th day compared to other EOEO levels. However, by the 16th day, there were no significant differences in the bacterial count among burger samples incorporated with EOEO at levels of 0.5%, 0.75%, and 1%. Overall, EOEO exhibited a bacteriostatic effect, delaying bacterial growth during the storage of burger samples.

Mold and yeast analysis revealed a slight (nonsignificant) increase in mold and yeast counts up to eight days of storage. This proliferation was reduced by increasing the addition of EOEO from 0 to 1%. At the end of the sixteenth day, mold and yeast counts were 6.75 log CFU/g and 5.22 log CFU/g for additions of 0% and 1% EOEO, respectively. The 1% EOEO addition showed the lowest increase in the total count from the initial time to the end of the 16 days, approximately 1.18 log cycles, resembling the trend observed in bacteria.

The application of OEO as an antimicrobial agent in the food system against food-borne pathogenic bacteria such as *L. monocytogenes*, *S. typhimurium, E. coli* O157 : H7, *S. aureus*, and *B. cereus* has been confirmed in earlier studies [37, 38]. In this concern, Leonelli Pires de Campos et al. [38] reported the effective antimicrobial activity of OEO against *E. coli*, multidrug-resistant *S. aureus*, and various fungi including *A. flavus*, *Penicillium citrinum*, and *Fusarium oxysporum*. Moreover, Javadian et al. [39] reported the preservative and antibacterial effects of encapsulated thyme extract against *Escherichia coli* O157 : H7.

Also, the antimicrobial activity of OEO has been reported in various meat products including minced meat at 0.5 and 1% v/w [40], sausage at 0.69, 1.725, and 3.45 mg/g [41], and samarella (traditional cured meat of Cyprus) at 1 and 5% [42]. Furthermore, Tsitsos et al. [28] found that oregano essential oil maintains the microbiological quality and prolongs the shelf life as well as enhance the organoleptic properties of mutton meat during storage. The authors attribute the antimicrobial effect of OEO to carvacrol, which disrupts cell membrane permeability, causing the release of cytoplasmic contents like lactate dehydrogenase enzymes and nucleic acids [28, 35, 43].

4.5. Biochemical Indices of Burger Samples

Total volatile nitrogen, TMA, TBA, and BAs are commonly used markers of meat freshness and quality. They serve as general measurements for spoilage microorganisms, autolytic enzymes, and oxidation processes during storage [7]. Table 4 shows the changes in biochemical markers of control and EOEO-incorporated burger samples during 16 days of refrigerated storage.

Table 4

Effect of EOEO on TVN, TMA, and TBA contents (mg/100 g) of burger samples during refrigerated storage.

Treatment	Storage time (days)				
0	4	8	12	16	Total volatile nitrogen (mg/100 g)
0.0	13.61 ± 0.34^{aE}	21.32 ± 0.13^{aD}	25.32 ± 0.32^{aC}	33.75 ± 0.62^{aB}	74.91 ± 0.47^{aA}
0.25	13.58 ± 0.35^{aE}	18.08 ± 0.09^{bD}	20.03 ± 0.61^{bC}	29.40 ± 0.33^{bB}	54.39 ± 0.27^{bA}

0.50	13.92 ± 0.03^{aE}	16.58 ± 0.02^{cD}	19.07 ± 0.31^{bcC}	27.49 ± 0.26^{cB}	50.66 ± 0.4^{cA}
0.75	13.58 ± 0.31^{aE}	16.09 ± 0.09^{cD}	18.46 ± 0.19^{cdC}	24.48 ± 0.21^{dB}	46.21 ± 0.11^{dA}
1.0	13.21 ± 0.02^{aE}	15.42 ± 0.29^{cD}	17.68 ± 0.27^{dC}	23.26 ± 0.07^{dB}	42.52 ± 0.31^{eA}
Trimethylamine (mg/100 g)					
0.0	1.25 ± 0.12^{aD}	4.02 ± 0.13^{aC}	4.32 ± 0.14^{aBC}	4.73 ± 0.01^{aB}	5.68 ± 0.13^{aA}
0.25	1.25 ± 0.13^{aC}	3.89 ± 0.01^{abB}	3.90 ± 0.01^{abB}	4.01 ± 0.12^{bcB}	4.68 ± 0.26^{bA}
0.50	1.12 ± 0.01^{aC}	3.49 ± 0.13^{abcB}	3.49 ± 0.13^{bcB}	4.16 ± 0.11^{abA}	4.16 ± 0.28^{cAB}
0.75	1.12 ± 0.01^{aB}	3.34 ± 0.01^{bcA}	3.46 ± 0.13^{bcA}	3.46 ± 0.15^{cdA}	3.70 ± 0.12^{cdA}
1.0	1.12 ± 0.11^{aB}	3.05 ± 0.25^{cA}	3.17 ± 0.13^{cA}	3.18 ± 0.12^{dA}	3.29 ± 0.26^{dA}
Thiobarbituric acid (mg malonaldehyde/kg)					
0.0	0.16 ± 0.03^{aE}	0.49 ± 0.01^{aD}	1.32 ± 0.04^{aC}	3.02 ± 0.07^{aB}	5.91 ± 0.14^{aA}
0.25	0.09 ± 0.01^{aE}	0.36 ± 0.04^{abD}	1.27 ± 0.03^{aC}	2.87 ± 0.06^{aB}	3.99 ± 0.10^{bA}
0.50	0.14 ± 0.01^{aD}	0.28 ± 0.01^{abcCD}	0.46 ± 0.06^{bC}	2.31 ± 0.05^{bB}	3.52 ± 0.09^{cA}
0.75	0.13 ± 0.01^{aC}	0.22 ± 0.03^{bcC}	0.28 ± 0.04^{bcC}	2.11 ± 0.07^{bcB}	3.33 ± 0.08^{cA}
1.0	0.13 ± 0.01^{aC}	0.09 ± 0.01^{cC}	0.14 ± 0.02^{cC}	1.96 ± 0.05^{cB}	3.05 ± 0.08^{dA}

In column, data with different small letters indicate statistical significance ($p<0.05$). In row, data with different capital letters indicate statistical significance ($p<0.05$).

The data reveal that the initial TVN values of the burger samples ranged narrowly from 13.21 to 13.92 mg/100 g at zero time and progressively increased during storage for all samples. The TVN values were significantly influenced by both the duration of cold storage and the concentration of EOEO. As the storage period extended, the TVN levels in all burger samples increased significantly ($p<0.05$). The control burger sample exhibited higher TVN values compared to the burger samples incorporated with EOEO at any storage period. The degradation of protein by microbiological proliferation and its autolytic endogenous enzymes led to generate ammonia, biogenic amines, and TVN through the deamination and decarboxylation of amino acids [44, 45].

According to the Egyptian Organization for Standardization and Quality, TVN levels below 20 mg/100 g indicate the freshness of chilled meat and meat products, while levels exceeding 30 mg/100 g render the meat noncompliant with health and food safety standards and unsuitable for human consumption [46]. The control burger exceeded the permissible TVN limits on the 12th day of refrigerated storage, whereas the burger samples mixed with EOEO remained within the acceptable range until the 12th day. However, all burger samples exceeded the permissible limits by the 16th day under the same storage conditions. The TVN values were positively correlated with the results of the microbiological analysis of burger samples.

These findings align with the reported TVN values for sea bream fillets and red porgy [7, 47]. On the contrary, Aa Moham et al. [48] found that camel meat burger samples treated with OEO at different concentrations (10, 25, and 50 μL/100 g) had increased TVN values compared to the control samples.

The initial TMA values ranged from 1.12 to 1.25 mg·N/100 g and increased progressively during the storage period, following similar patterns as TVN. Burger samples containing EOEO exhibited lower TMA values compared to the control sample at all storage periods. Notably, during the initial days of analysis, all burger samples showed a faster evolution of TMA. For instance, the TMA value of the control sample peaked to 4.02 mg/100 g on the 4th day, while the burger sample incorporated with 1% EOEO slightly increased to 3.05 mg/100 g. Subsequently, the EOEO-treated burger samples showed slower TMA formation. The burger sample mixed with 1% EOEO recorded 3.29 mg/100 g, compared to 5.68 mg/100 g for the control (Table 4).

The acceptable limit for TMA varies in the literature: 5 mg/100 g [49], 5–10 mg/100 g [50], and 10–15 mg/100 g [51]. Considering 4-5 mg/100 g as the maximum limit of TMA appears more realistic based on the microbiological and TVN analysis results. Thus, the addition of EOEO at levels of 0.75% and 1% extended the shelf life of the burger samples to the 12th day. Similar results were reported by Vatavali et al. [47] for red porgy fish treated with encapsulated OEO with chitosan. Additionally, Giatrakou et al. [52] reported significantly lower TMA values for swordfish samples treated with OEO (0.1% v/w) compared to the control sample.

Thiobarbituric acid (TBA) values of the burger samples were monitored during the 16 days of cold storage and are presented in Table 4. The results indicated that TBA values were significantly affected ($p<0.05$) by EOEO addition and storage time. The initial TBA values ranged from 0.09 to 0.16 mg MDA/kg and thus indicate the good quality of raw meat. Throughout the cold storage period, the TBA values of all burger samples significantly ($p\leq0.05$) increased. The control sample recorded TBA values of 0.49, 1.32, 3.01, and 5.91 mg MDA/kg on the 4th, 8th, 12th, and 16th day of storage, respectively. However, the burger samples incorporated with 0.75% and 1% EOEO showed a significant increase in TBA values on the 12th day of storage, recording 2.11 and 1.96 mg MDA/kg, respectively.

These results are in agreement with previous research that highlights the potential use of OEO to preserve meat products from lipid peroxidation and protein proteolysis [53, 54]. The progressive increase in TBA values during meat product storage may be attributed to the formation of oxidized byproducts such as aldehydes and ketones during the lipid autooxidation process [55]. A limit of 2 mg MDA/kg has been recommended by Campo et al. [56] as a criterion for the rejection of meat and meat products. All burger samples, except those containing 0.75% and 1% EOEO, exceeded this limit on the 12th day of storage. This may be attributed to the antioxidants present in OEO, which act as hydrogen donors and scavenge free radicals [57]. The antioxidant protective effect of carvacrol and other oxygenated terpenes promotes their ability to scavenge oxygen molecules and form cross-links with proteins, contributing to the retardation of lipid and hydroxyl radical-mediated protein oxidation processes [43, 58].

4.6. Biogenic Amines (BAs)

The biogenic amines are important biochemical markers for monitoring the freshness and quality of meat products. The changes in BA contents in burger samples during cold storage periods were tracked and are presented in Table 5. Among the investigated biogenic amines, β-phenylethylamine was not detected at any storage duration. At zero time, cadaverine, spermine, and spermidine were the dominant biogenic amines representing 0.398, 0.353, and 0.324 mg/100 g, respectively. Putrescine and histamine represented lower concentrations (0.165 and 0.022 mg/100 g, respectively), while tryptamine and tyramine were not detected. The data revealed that the concentrations of all BAs gradually increased in parallel with increasing storage period.

Table 5

Effect of EOEO on biogenic amine (mg/100g) formation in burger samples during refrigerated storage.

Storage period	EOEO	*Biogenic amines*							Total
Tryptamine	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	Zero time	Control	0.000^{n}
0.165^{h}	0.399^{m}	0.022^{hi}	0.000^{i}	0.324^{j}	0.354^{l}	1.266°	·		
4 days	Control	0.573^{ijl}	0.360^{h}	4.648^{bc}	0.112^{g}	0.357^{fg}	0.857^{cde}	1.922^{gh}	8.832^{hij}
0.25	0.522^{jl}	0.309^{h}	3.389^{fgh}	0.047^{h}	0.228^{h}	0.651^{hi}	1.753^{h}	6.901^{lm}	0.50
0.426^{l}	0.250^{h}	3.192^{ghi}	0.040^{hi}	0.222^{h}	0.568^{i}	1.322^{i}	6.022^{mn}	0.75	0.211^{m}
0.230^{h}	2.779^{hij}	0.026^{hi}	0.180^{h}	0.431^{j}	0.603^{jl}	4.462^{n}	1.00	0.097^{mn}	0.219^{h}
1.205^{l}	0.020^{i}	0.042^{i}	0.353^{j}	0.465^{l}	2.403°	-			
8 days	Control	0.978^{ef}	1.568^{ef}	4.560^{bcd}	0.188^{bcd}	0.376^{ef}	0.889^{cd}	2.133^{efg}	10.694^{fg}
0.25	0.901^{efg}	1.159^{fg}	3.792^{efg}	0.192^{bcd}	0.375^{ef}	0.795^{defg}	2.002^{fgh}	9.218^{ghi}	0.50
0.835^{efgh}	1.145^{fg}	3.740^{efg}	0.181^{cd}	0.301^{g}	0.765^{defgh}	1.922^{gh}	8.892^{hij}	0.75	0.755^{gh}
0.978^{g}	3.590^{efg}	0.136^{fg}	0.300^{g}	0.683^{ghi}	0.812^{j}	7.258^{jlm}	1.00	0.690^{hij}	0.290^{h}
2.401^{j}	0.134^{fg}	0.191^{h}	0.638^{hi}	0.673^{jl}	5.020^{n}	-			
12 days	Control	1.823^{b}	5.243^{b}	4.799^{b}	0.219^{a}	0.496^{bcd}	1.044^{ab}	2.608^{bc}	16.23^{b}
0.25	1.673^{b}	1.666^{e}	4.114^{cde}	0.188^{bcd}	0.439^{cde}	0.877^{cd}	2.544^{bcd}	11.504^{ef}	0.50
1.009^{e}	1.554^{ef}	3.299^{ghi}	0.184^{bcd}	0.426d^{ef}	0.769^{defgh}	2.386^{cde}	9.630^{gh}	0.75	0.811^{fgh}
0.962^{g}	2.772^{hij}	0.172^{de}	0.425^{ef}	0.722^{fgh}	2.304^{cdef}	8.171^{hijl}	1.00	0.750^{ghi}	0.511^{h}
2.748^{ij}	0.140^{f}	0.427^{def}	0.692^{ghi}	2.242$^{def}_{g}$	7.513^{ijlm}	-			
16 days	Control	2.034^{a}	6.756^{a}	5.612^{a}	0.221^{a}	0.691^{a}	1.165^{a}	3.041^{a}	19.521^{a}
0.25	1.455^{c}	4.144^{c}	4.744^{b}	0.208^{ab}	0.639^{a}	0.944^{bc}	2.739^{ab}	14.875^{bc}	0.50

1.297^{cd}	4.055^{c}	3.985^{def}	0.199^{abc}	0.646^{a}	0.846^{cdef}	2.442^{bcde}	13.472^{cd}	0.75	1.205^{d}
3.868^{c}	3.753^{efg}	0.181^{cd}	0.546^{b}	0.746^{efg}	2.373^{cde}	12.675^{de}	1.00	1.202^{d}	3.020^{d}

In column, means with different letters indicate statistical significance ($p \leq 0.05$).

It was noteworthy that cadaverine increased in the first duration (4 days) of cold storage to be the dominant compound representing 4.63 mg/100 g in the control sample. Then the concentration of cadaverine slowly increased to reach 5.59 mg/100 g in the control burger on the 16th day. Moreover, putrescine compound progressively increased during storage periods repressing the highest concentration on the 16th day of storage (6.73 mg/100 g) in the control sample. The other biogenic amine compounds progressively increased during storage representing lower concentrations being 3.03, 2.03, 1.16, 0.69, and 0.22 mg/100 g for spermine, tryptamine, spermidine, tyramine, and histamine, respectively.

In general, the obtained results align with those of Triki et al. [59] and Muñoz-Esparza et al. [60] as they reported that polyamines such as spermine and spermidine are the first biogenic amines that naturally occurred in fresh meat at levels of 20–60 and 10 mg/kg, respectively. Also, the formation of BAs including histamine, cadaverine, putrescine, and tyramine compounds during chilled storage of meat was reported by Durak-Dados et al. [61]. BAs are formed in protein-rich foods during microbial spoilage activities (>7 log CFU/g) via decarboxylation of amino acids. Gram-negative and positive bacteria and some fungi are involved in biogenic amine formation [62, 63].

Regarding the protective effect of EOEO, control burger showed the highest content of BAs, whereas the formation of BAs in burger samples containing EOEO was significantly reduced in proportion to its concentrations. Biogenic amines of burger samples incorporated with EOEO showed the same trend as in the control sample, but with lower concentrations. Totally, addition of EOEO at the levels of 0.75 and 1% retarded the biogenic amine formation in burger samples by 49.5 and 72.8%, respectively, compared to the control sample.

From the toxicological point of view, the maximum allowed limit of biogenic amines, reported to be 100 mg/kg [64], was exceeded by the control burger at the 8th day of storage. However, burger samples containing EOEO at 0.5, 0.75 and 1% remained below this limit until 12th day, indicating its efficacy as a preservative agent in maintaining the safety and quality of the burger samples during storage. In this context, Özogul et al. [65] illustrated the effect of carvacrol on the formation of biogenic amines in histidine decarboxylase broth by different pathogens at the levels of 0.1, 0.5, and 1 ml/100 ml. The results reported the ability of carvacrol to decrease the production of biogenic amines. The same result was obtained by the addition of carvacrol-rich *Zataria multiflora* essential oil to Gouda cheese [66].

The multivariate principal component analysis (PCA) was used to study the correlation between the addition levels of EOEO and microbiological and biochemical parameters during refrigerated storage of beef burgers, as illustrated in Figure 1. The results of PCA showed that the biochemical markers (BA, TBA, TMA, and TVN) were highly positively correlated with total viable counts of microorganisms over the storage period of beef burger samples. Furthermore, it was found that these parameters can be explained by PC1 (89.81%) and PC2 (7.25%) with cumulative values greater than 97%. This percent indicates the strong correlation between the tested variables. As shown in Figure 1, the biochemical and microbiological vectors are very close and located on the right-hand side of PC1, indicating a strong positive correlation between these variables.

[figure(s) omitted; refer to PDF]

In addition, the biochemical indicators (TMA, BAs, TVN, TMA, and TBA) and microbiological viable count were found to be positively correlated with PC1. However, these parameters were negatively correlated with the storage period until the eighth day in all samples except the control samples. These findings align with the reported total viable microbial and chemical results of beef slices containing tarragon essential oil [67]. In general, the high levels of EOEO retarded the putrefaction degree of beef burger samples, confirming its antimicrobial activity and its ability to maintain the safety and quality of beef burger under storage condition.

5. Conclusion

In conclusion, the study confirmed the efficacy of EOEO as a safe preservative agent to retard the microbiological and chemical deteriorations of beef burgers during refrigerated storage. The incorporation of EOEO exhibits a significant reduction in the biochemical markers and total microbial count in proportion to its concentrations, thereby prolonging the shelf life and maintaining the safety of burger samples. This protective effect, even if it diminishes over time, underscores the potential of EOEO as an efficient biopreservative agent.

Authors' Contributions

Fathy M. Mehaya was responsible for writing, reviewing, and editing original draft, methodology, investigation, interpretation of data, statistical analysis, manuscript curation, and conceptualization. Ayman A. Mohammad was responsible for writing and editing original draft, methodology, investigation, statistical analysis, and data curation. Salah H. Salem was responsible for writing and editing original draft, methodology, investigation, and data curation. Heba M. Amer was responsible for writing original draft, methodology, plant cultivation, essential oil extraction, and oil constituent identification. Wenyi Kang was responsible for supervision and reviewing and editing original and final draft.

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DETAIL

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Characterization of the Proximate Composition, Lipid Oxidation Status, and Mineral Content of Mature Tree Nuts from Nine Hazelnut Cultivars Grown in the United States

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ABSTRAK (ENGLISH)

Hazelnuts are the most popular tree nuts in the world, and regions adjacent the Black and Mediterranean seas are the historic production centers. Characterization of hazelnut cultivars grown in these regions is well reported but is lacking for cultivars grown in the United States. The aim of our study was to characterize nine cultivars selected from the USDA National Germplasm Collection for their proximate composition, lipid oxidation status, and minerals, as well as by NIR spectroscopy. Except for ash content, proximate composition varied across the cultivars and lipids were the predominant component. NIR spectra were similar in pattern and differences in intensity could be accounted for by differences in proximate composition, including lipid, moisture, and protein. Cultivars with the highest moisture content and water activity levels were also those with highest levels of lipid oxidation. Carbon and sulfur content on a fresh weight basis varied from 44.82 g/100 g to 63.82 g/100 g and 96.56 mg/100 g to 164.79 mg/100 g, respectively. The K, P, Ca, Mg, Cu, Fe, Mn, Zn, and B contents were determined by MP-AES. Potassium followed by phosphorus was the most abundant elements. Hazelnuts appear to be a good source of dietary copper and manganese providing up 60.5% and 60.4%, respectively, of the recommended daily value while contributing no more than 0.03% of the daily value for sodium. Characterization results were in ranges like those reported for hazelnuts from Asian and European growing regions. However, each cultivar possessed a unique profile.

TEKS LENGKAP

1. Introduction

The European hazelnut (*Corylus avellana* L.) is among the world's most popular tree nuts, second only to almonds [1]. Hazelnut is a deciduous tree cultivated in temperate climates close to large bodies of water [2]. Worldwide production has increased from 0.38 mil tons in 1970 to 1.07 mil tons by 2020, with Turkey accounting for more than 50% of the total, followed by Italy, the United States, Azerbaijan, and Chile [3].

Tree nuts are a major U.S. crop, with total production of $9.7 billion in 2021, comprising mostly almonds ($5.0 billion), pistachios ($2.9 billion), and walnuts ($1.0 billion). California dominates the U.S. market with over 90 percent of production. At $167 million, hazelnuts are currently a relatively minor crop exceeding only macadamia ($63 million) in commercial production. U.S. hazelnut exports were valued at $144 million, with top markets including Canada ($62 million), China ($53 million), and Mexico ($13 million), while imports amounted to $39 million, almost all Turkish. Historical reasons for low U.S. hazelnut production include low consumer demand and the lack of suitable cultivars for widespread commercial production.

Current domestic production is mostly in Oregon and Washington. Rising domestic and international demand for hazelnuts along with recent successes cultivating hazelnuts in nontraditional climates (e.g., South Africa and Australia) [4] and a multiyear field trail that identified hazelnut cultivars Lewis and Ennis as potentially suitable for commercial production in California's San Joaquin Valley [5] suggests a strong potential for additional growth in U.S. production. In 2021/2022, the unit export value of $2.22 per pound for fresh or dried hazelnuts was nearly identical to almonds or walnuts, although significantly less than pistachios ($3.53 per pound) [6].

Given the recent extreme drought situation throughout the western United States, including California, agricultural water use is a rising concern and the source of political and cultural stress. The water footprints of almonds and pistachios are very high as compared to walnuts and hazelnuts, as much as triple depending on the data source. For instance, according to Waterfootprint.org, water consumption for almonds is 17,700 L/kg and for pistachios 12,500 L/kg compared to 5,783 L/kg for hazelnuts [7]. However, [8] distinguishes between blue water use (rivers, lakes, and reservoirs) and green water use (precipitation and groundwater) and reports blue water use (most relevant to California) of pistachios with the highest water footprints (7602 L/kg), followed by almonds (3816 L/kg), cashew nuts (3070 L/kg), walnuts (2451 L/kg), and hazelnuts (2180 L/kg). While it is apparent that water consumption by nut species has different interpretations, it is also clear that hazelnut requires substantially less than almonds or pistachios. Thus, given their popularity, comparative value, suitability for growth in California, and lower water footprint, hazelnuts seem poised to explode in future production.

Hazelnuts are consumed raw (with skin) or roasted (without skin) and used as an ingredient in a variety of foods, including chocolate, confectionery, and bakery products. Hazelnut oil is also used as a cooking oil. Shell and skin by-products from hazelnut production and processing have shown promise as feedstocks for value-added products with health-promoting properties [1]. Consumer appreciation for hazelnuts is derived from the organoleptic properties and nutritional composition of the nuts. Lipids are a chief contributor to the organoleptic properties and typically account for 50–60% of the total mass of a nut [9–11]. Phenolics are the most abundant phytochemicals in both kernels and skins, and a summary of the potential associated health benefits may be found in a review by Bottone et al. [12]. Growing evidence from clinical studies indicates that consumption of hazelnuts protects against oxidative stress and inflammation [13] and leads to lower low-density lipoprotein (LDL) and total cholesterol levels [14] without weight gain.

Hazelnuts are not self-pollinating, and orchards need several varieties of trees to produce nuts. Certain varieties produce few nuts and are planted specifically as pollinizers. Certain pollinizer varieties are compatible with certain nut-producing cultivars. Hazelnut trees in Oregon were massively infected in the 1960s by the fungal disease eastern filbert blight (EFB), and many varieties were either wiped out or significantly diminished. Researchers at Oregon State University discovered that a particular pollinizer variety (Gasaway) possessed resistance to EFB and began a program of controlled crosses to develop resistant varieties. Since 2002, 15 of these new varieties have been released, including seven main crops and eight pollinizers [8]. Thus, hazelnut varieties are in transition, with some gone, others disappearing, and still others being created. The USDA's hazelnut germplasm collection is

located in Corvallis, Oregon, and is part of larger National Plant Germplasm System (NPGS) with locations throughout the United States. NPGS is charged with the evaluation, characterization, and preservation of genetic resources. In support of this mission, nine commercially important hazelnut cultivars were selected from the collection for characterization, including proximate contents, degree of lipid oxidation, elemental analysis, and near-infrared spectroscopy (NIRS).

2. Materials and Methods

2.1. Chemicals and Reagents

Petroleum ether and hexanes (certified ACS), chloroform and methanol (HPLC grade), nitric acid (67%, Optima grade), and water ultratrace (elemental analysis grade) were purchased from Fisher Scientific Ltd. (Fair Lawn, NJ). Ferrous chloride tetrahydrate (EM Science, Darmstadt, Germany), ferric chloride hexahydrate (Fisher Scientific, Fair Lawn, NJ), and ammonium thiocyanate (LabChem Inc., Zelienople, PA) were used to determine peroxide value. Multi-element ICP calibrator IV-STOCK-8 with 24 elements at the concentration of 100 mg/L diluted in 5% nitric acid (Inorganic Ventures, Christiansburg, VA) and phosphorus of 1000 mg/L diluted in water (SPEX CertiPrep, Metuchen, NJ) were used for the preparation of standard solutions, and a custom solution of 10 elements (P, 80 mg/L; K, 200 mg/L; Ca, 50 mg/L; Mg, 40 mg/L; Na, 1.25 mg/L; Fe, 500 μg/L; Cu, 300 μg/L; Mn, 600 μg/L; Zn, 300 μg/L; and B, 300 μg/L) diluted in 20% nitric acid (Inorganic Ventures, Christiansburg, VA) was used as a control. Water was purified and deionized to ≥18.1 MΩ/cm resistance using a Barnstead GenPure xCAD Plus Ultrapure Water Purification System (Thermo Scientific, Waltham, MA) and filtered through a 0.22 μm type HA membrane filter (Millipore, Billerica, MA) before use.

2.2. Sample Preparation

Hazelnut samples harvested in 2021 were obtained from the USDA National Germplasm Repository in Corvallis OR. Upon receipt samples were store protected from light at ambient temperature. Approximately 90 g of in-shell hazelnuts was cracked using a mortar and pestle, damaged kernels were discarded, and the remaining kernels ground using a coffee grinder. The ground material was immediately analyzed for proximate composition (ash, lipid, and moisture), water activity, and NIR spectra collected. A portion of the ground material was vacuum packed, placed at −20°C, and later sent to a commercial lab (Ward Laboratories, Inc., Kearney, NE) for %Carbon and %Nitrogen determinations.

2.3. Proximate Composition

2.3.1. Total Ash Determination

Ash content in ground hazelnut was determined using a Lindberg Blue M furnace (Thermo Fisher Scientific, Waltham, MA). 2.000 + 0.001 g of ground material was weighed in a porcelain crucible and heated on an infrared radiator in a fume hood until smoke was no longer visible. The crucibles were then placed in a furnace at 550°C for 17 h. The sample was transferred to a desiccator after heating, cooled, and weighed. Ash determination in the sample was conducted in triplicate. The ash content was calculated using the following formula:(1)%Ash=Ash Weight g×100Sample Weight g.

2.3.2. Total Lipid Content

Lipids were extracted by accelerated solvent extraction using the Dionex AE350 instrument (Dionex Corp., USA). A stainless-steel extraction cell was loaded with 1.000 + 0.001 g of ground material mixed with sand and filled up to 98% of the cell capacity. The extraction was accomplished using petroleum ether at 125°C and 1500 psi for 30 min, and the extraction collected in a 60-mL amber vial. The vial was placed in a water bath at 50°C and stream of nitrogen applied for 30 min to evaporate the solvent. The resulting oil was stored at 4°C until analyses. Extraction of each sample was conducted in triplicate. Percentage of total lipid extraction was calculated by the following formula: (2)%Total Lipid=Extracted Oil Weight g×100Sample Weight g.

2.3.3. Moisture Content

Moisture content was determined gravimetrically using a convection oven Model F750 (Fisher Scientific, USA). 2.000 + 0.001 g of ground material was weighed into a previously dried and weighed aluminum cup containing approximately 5.0 g of sand and a glass rod. The ground sample was dried at 105°C for 48 h and cooled in a

desiccator then weighed. Moisture content in sample was determined in triplicate. Wet basis moisture was calculated by the following formula:(3)%MCwet=WaterWeightg×100SampleWeightg

2.3.4. Protein and Carbohydrate Contents

The protein content (N 6.25 for hazelnuts) was calculated using the %Nitrogen values obtained from Ward Laboratories, Inc. Total carbohydrate content was calculated by difference using the formula: total carbohydrates $(g/100\,g) = 100 - (g_{lipid} + g_{ash} + g_{protein} + g_{moisture})$.

2.4. Water Activity

Water activity measurements were determined using a AquaLab 4TE (Decagon Devices, USA) and conducted in triplicate.

2.5. Lipid Oxidation

Primary and secondary oxidation was determined spectrophotometrically following the procedure of Pannico et al., 2015. In a 10-mL volumetric flask, oil extracted from 1.000 + 0.002 g of ground hazelnut was diluted with and brought up to 10 mL with hexane. The resulting solution was further diluted 1 : 5 with hexane, and the absorbance measured at 232, 262, 268, 270, and 274 nm using a Molecular Devices SpectraMax 384-Plus plate reader (Sunnyvale, CA). Lipid oxidation in terms of specific extinction coefficients was calculated using the following formulas:(4)Kλ=Eλc×s, where $K\lambda$ is the extinction coefficient at λ wavelength, $E\lambda$ is the absorbance, c is the concentration (wt/vol%), and s is the length of the cuvette (1 cm).

Lipid primary oxidation: K_{232}

Lipid secondary oxidation: K_{270}

Also, for lipid secondary oxidation:(5)ΔK=K268−K262+K2742.

Lipid oxidation for each sample was determined in triplicate from three independent extractions.

2.6. Peroxide Value

Peroxide value was analyzed following the procedure of Ribeiro [16]. In a 10-mL volumetric flask, oil extracted from 1.000 + 0.002 g of ground hazelnut was diluted and brought to 10 mL with chloroform: methanol (7 : 3 v/v). 2.0 mL of extract solution was transferred to a 16 × 150 mm glass disposable culture tube and combined with 7.9 mL chloroform: methanol (7 : 3 v/v). Then, 50 μL of 30% (w/v) ammonium thiocyanide in water was added to the solution, followed by 50 μL of 0.06 M ferrous chloride. The solution was mixed by vortexing after the addition of each reagent. For the quantification of peroxide value, a ferric chloride standard curve (0–0.0150 M) was prepared from a 0.025 M ferric chloride stock solution. Briefly, 100 μL of each calibrator was combined with 9.8 mL of chloroform-methanol solution. Then, 50 μL of ammonium thiocyanide and 50 μL of filtered water were added and the resulting solution mixed by vortexing. Calibrator solutions were measured before and after reading the samples.

Ferric chloride (0.0080 M) was used as a positive control, and the blank solution consisted of a 200 μL of filtered water dissolved in 9.8 mL of the chloroform-methanol solution. The sample background was obtained by combining 9.9 mL of chloroform-methanol with 50 μL of ammonium thiocyanide and 50 μL of ferrous chloride. Solutions were incubated for 5 min protected from light, and the absorbance was read at 500 nm on a Molecular Devices SpectraMax 384-Plus plate reader (Sunnyvale, CA). Peroxide value was determined in triplicate using independent extracts and reported as ferric chloride milligram equivalents per gram oil.

2.7. Elemental Analysis

Inorganic residue obtained during ash determination of 2.000 g + 0.001 of ground hazelnuts was used for the analysis of macro- and microelements. Ashes contained in a porcelain crucible were dissolved in 20% nitric acid. The resulting solution was transferred to a 50-mL volumetric flask and brought up to volume. The ash acid solution was filtered through a 0.45 μm FlipMate PES/PTFE filter (Environmental Express, Charleston, SC) and collected into a 50-mL free metal centrifuge tube (Labcon, Petaluma, CA). The neat solution was used for the determination of iron, copper, manganese, boron, and sodium. The neat solution was diluted 1 : 4 with filtered water for zinc and phosphorus determination and the 1 : 4 dilution further diluted to 1 : 100 with 5% nitric acid for the potassium, calcium, and magnesium determinations. Analyses were conducted using an Agilent 4200 Microwave Plasma Atomic Emission Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). A custom multi-element solution

(Inorganic Ventures, Christiansburg, VA) in 20% nitric acid was used as a positive control and 5% nitric acid was used as a blank. Quantification of elements was obtained using calibrators prepared from a multi-element standard solution (IV-STOCK-8, Inorganic Ventures, Christiansburg, VA) and a phosphorus standard solution (SPEX CertiPrep, Metuchen, NJ) in 5% nitric acid. Concentrations were reported in milligrams of element per a hundred grams of sample (mg/100 g sample).

2.8. NIR Measurements

Ground samples were placed in a glass vial. The mean diffuse reflectance spectra in the long-wavelength NIR regions (900 nm to 2600 nm) were measured using a benchtop FT-NIR spectrophotometer (MPA; Bruker Optics, Billerica, MA) linked to a personal computer running the OPUS software (Version 7.5; Bruker Optics, Billerica, MA). The spectrophotometer has an internal gold reference for the reflectance measurements, and the background spectrum was taken hourly. All spectra were output as absorbance.

The optical measurements were nondestructive, and the reflectance was collected from 128 individual scans in 64 cm^{-1} (every 4 to 50 nm) increments. Three measurements were taken per sample, and an average spectrum was calculated.

2.9. Statistical Analysis

Tukey's multiple comparison was used to test the difference between means of different cultivars for each organic and inorganic compound and property analyzed. Principal component analysis (PCA) was used to explore and visualize the differences between all cultivars based on all chemical analysis results.

2.9.1. NIR Spectra Pretreatment

A 5-point Savitzky–Golay first derivative and standard normal variate spectral processing were applied to all spectra to remove additive and multiplicative effects and improve the signal-to-noise ratio [17, 18]. All statistical analyses and spectral processing were performed using JMP (Pro 16; Cary, NC).

3. Results and Discussion

3.1. Proximate Contents

Proximate contents, water activity, and elemental carbon and sulfur levels found for the nine hazelnut cultivars evaluated are shown in Table 1. The highest moisture content was found in Fitzgerald (23.84%), followed by Ennis (22.07%) and Hall's Giant (16.72%). Moisture content of the remaining six cultivars was more than four times less and ranged from 6.35% (Tonda di Giffoni) to 3.09% (Giresun). Moisture contents reported in the literature span from 3.13% for Tonda Gentile Trilobata [19] to as high as 26.59% for Ordu Levant [20]. The moisture content we obtained here for Tonda di Giffoni (6.35%) was close to the 5.98% reported by [10], whereas the 3.80% measured here for Tombul was much lower than the 26.13% reported by [20]. Water activity levels in the samples ranged from 0.965 (Ennis) to 0.438 (Giresun Ordu) with those samples exhibiting the highest moisture contents also possessing the highest water activity levels. Ferrão [10] in their study of unprocessed hazelnuts from nine cultivars reported water activity levels from 0.59 to 0.80, whereas Belviso et al. [20] reported a range of 0.60 to 0.65 for dried nuts from three cultivars. Three samples in this study had water activity levels below the range reported by Ferrão et al. [10], and this was likely due to the moisture content of these samples being lower than the lowest moisture content (4.77%) measured by Ferrão et al. [10].

Table 1

Proximate contents, water activity, and C and S levels determined for nine hazelnut cultivars collected from the USDA Germplasm collection.

	Moisture (%)	Ash (%)	Protein (%)	Oil (%)	Carbohydrate (%)	Water activity	Carbon (g/100 g)	Sulfur (mg/100 g)
Hall's Giant	16.72 ± 0.29	2.56 ± 0.05[a]	17.25	32.62 ± 1.27[e]	30.85 ± 1.31	0.922 ± 0.001	57.37	146.54

Ennis	22.08 ± 0.10	2.06 ± 0.08^{f}	11.98	36.13 ± 2.28^{d}	27.76 ± 2.28	0.965 ± 0.003^{a}	55.54	96.56
Fitzgerald	23.84 ± 0.15	2.17 ± 0.03^{d,e}	12.06	34.39 ± 1.44^{d,e}	27.53 ± 1.45	0.956 ± 0.005^{a}	44.82	124.70
Tonda di Giffoni	6.35 ± 0.09^{a}	2.40 ± 0.02^{b,c}	11.20	61.90 ± 2.29^{c}	18.15 ± 2.29	0.801 ± 0.003	60.01	135.68
Tombul	3.80 ± 0.09^{b,c}	2.34 ± 0.01^{c}	14.15	65.76 ± 1.36^{a}	13.95 ± 1.36	0.513 ± 0.001^{b}	63.06	151.05
Yamhill	4.05 ± 0.31^{b}	2.48 ± 0.03^{a,b}	13.36	62.88 ± 0.83^{b,c}	17.23 ± 0.88	0.497 ± 0.012^{b}	61.50	153.11
Nixon	6.41 ± 0.17^{a}	2.58 ± 0.03^{a}	12.43	62.66 ± 1.50^{b,c}	15.91 ± 1.52	0.656 ± 0.007	45.47	164.79
Jefferson	5.48 ± 0.61	2.07 ± 0.03^{e,f}	9.21	65.88 ± 1.96^{a}	17.37 ± 2.06	0.749 ± 0.004	57.71	109.02
Giresun	3.09 ± 0.10^{c}	2.21 ± 0.03^{d}	16.94	64.93 ± 1.23^{a,b}	12.83 ± 1.23	0.438 ± 0.010	63.82	159.49
Minimum	3.09	2.06	9.21	32.62	12.83	0.438	44.82	96.56
Maximum	23.84	2.58	17.25	65.88	30.85	0.965	63.82	164.79
Mean	10.20	2.32	13.18	54.13	20.17	0.722	56.59	137.88
SD	7.82	0.19	2.46	14.05	6.30	0.194	6.63	22.09

Mean reported with standard deviation for $n = 3$ for moisture, ash, oil, and water activity. Standard deviation for carbohydrate calculated from standard deviation of moisture, ash, and oil. Data sharing the same letter (a, b, c, d) in the same column are not significantly different, $p > 0.05$, Tukey's multiple range tests.

Ash contents for the cultivars did not show large variability and were in a narrow band ranging from 2.06% (Ennis) to 2.58% (Nixon). Ash content results obtained in the present study are within the 1.75% to 3.80% range found in the literature [9, 10, 20], and our results for Ennis (2.06%) and Hall's Giant (2.56%) are similar to those reported by Müller et al. [9] for same cultivars, 2.2% and 2.5%, respectively.

For crude protein content, Jefferson (9.21%) and Hall's Giant (17.25%) were the cultivars with the lowest and highest contents, respectively. Crude protein contents reported in the literature [9, 10, 21] ranged from 10.02% to 22.1%. In contrast to the ash results, the protein content reported by Müller et al. [9] for Ennis (12.4%) and Hall's Giant (18.4%) was slightly higher than the values we found.

Lipid content of the cultivars tested in this study ranged from 32.62% (Hall's Giant) to 65.88% (Jefferson) and is within the range of the lipid contents reported within the literature. Müller et al. [9] reported a range of 47.9% to 64.8% for 15 cultivars grown in Germany. For seven cultivars grown in Portugal [10], the lipid content ranged from 46.0% to 72.5%, and for four cultivars grown in Turkey [11], the range was 8.1% to 38.0%. Turan [20] using the

cultivar Mortarella evaluated the influence of harvest year and region (2015) and canopy location (2017) on lipid content and reported ranges of 54.8% to 64.6% and 58.5.0% to 61.8%, respectively. Taken into consideration these reports and others [22 and references therein] describing lipid content over multiple cultivars, geographical regions, and harvest years, the typical lipid concentration for hazelnuts may be near to 60% but is still highly variable and dependent on multiple factors. Six of the nine cultivars evaluated fell near this value, but those cultivars with much lower lipid contents (Hall's Giant, Fitzgerald (34.39%), and Ennis (36.13%)) were also those with the highest moisture content. Thus, it may be speculated these three cultivars may be more resistant to drying during storage or that their lipid content would have been higher had the samples been subjected to a formal drying treatment. Carbohydrate content was calculated as the residual mass after accounting for moisture, oil, ash, and protein contents. Carbohydrate content for the samples varied from 12.83% (Giresun) to 30.85% (Hall's Giant). Carbohydrate contents reported by others have ranged from 5.3% to 22.2% [9, 10, 21]. A combination of high moisture content and lower lipid content is the reason for the elevated levels of carbohydrates in Hall's Giant (30.85%), Ennis (27.76%), and Fitzgerald (27.53%).

Total carbon and sulfur analyses are routinely performed on leafy materials to assess plant health, but results for analyses performed on nuts are not widely reported. Carbon content of the cultivars ranged from 44.82 g/100 g FW (Fitzgerald) to 63.82 g/100 g FW (Giresun). Multiple compounds, including lipids, proteins, and carbohydrates, all broadly contribute to the overall carbon content making a direct comparison to a specific class of compounds difficult, whereas most of the sulfur content may be attributed to the amino acids, methionine and cysteine, and trace-level sulfur-containing secondary metabolites [23]. For comparison purposes, the methionine and cysteine concentrations reported by Burdack-Freitag and Schieberle [23] and Alasalvar et al. [24] were used to estimate total sulfur content values of 171 mg/100 g and 121 mg/100 g, respectively. Sulfur contents determined for the samples spanned from 96.56 mg/100 g FW (Ennis) to 164.79 mg/100 g FW (Nixon) and are in the same range as the estimated values.

3.2. NIR Spectroscopy

The average raw spectra and preprocessed spectra of all nine cultivars are shown in Figures 1 and 2, respectively. The most prominent wavebands existing in the NIR region are the strong overtone and combination absorptions of hydrogen-containing bonds O–H (found in water), C–H (found in carbohydrates and oil), and N–H (found in protein) [26]. The three cultivars, Hall's Giant, Ennis, and Fitzgerald, that have the highest moisture content and lowest lipid content can be seen separated in the processed spectra at water bands around 1500 nm and 1900 nm, as well as wavebands influenced by fats around 1400 nm, 2000 nm, and 2300 nm. Especially at 1390 nm, which is a known lipid band, the raw spectra of the three cultivars did not exhibit a peak, whereas the other six all have small peaks. Good separation at 1400 nm and 2070 nm regions also confirms with other literature that indicates these regions are the key wavelengths used for measuring lipid peroxide [27, 28]. Jefferson cultivar can be seen separated from the other cultivars at around 1700 nm and 2250 nm, which are wavebands influenced by protein [29]. This is likely due to the low protein content of Jefferson cultivar.

[figure(s) omitted; refer to PDF]

3.3. Lipid Oxidation Status

Peroxide values (PVs) are a measure of hydroperoxide content and are directly associated with lipid degradation. PVs in the literature range from 0.01 [19] to 7.46 meq O_2/kg oil [21]. PV results (Table 2) for the samples varied from 0.92 (Yamhill) to 7.16 (Hall's Giant) meq O_2/kg oil. Samples with the highest PVs (Hall's Giant, Ennis (6.11 meq O_2/kg oil), Fitzgerald (5.15 meq O_2/kg oil)) were also those samples with the highest moisture contents.

Table 2

Lipid oxidation determined for nine hazelnut cultivars collected from the USDA germplasm collection.

	K323	K270	Delta K	Peroxide (mmol eq O_2/kg extracted oil)

Hall's Giant	1.630 ± 0.105[a]	0.28 ± 0.02[a]	0.014 ± 0.001	7.16 ± 0.10[a]
Ennis	1.489 ± 0.090[a,b]	0.25 ± 0.07[a]	0.006 ± 0.000[a,b,c]	6.11 ± 0.21[a,b]
Fitzgerald	1.506 ± 0.053[a,b]	0.24 ± 0.01[a]	0.007 ± 0.001[a,b]	5.15 ± 0.21[b]
Tonda di Giffoni	1.255 ± 0.017[d]	0.13 ± 0.01[b]	0.006 ± 0.000[a,b,c]	2.27 ± 0.29[c]
Tombul	1.416 ± 0.021[b,c]	0.10 ± 0.00[b]	0.005 ± 0.000[a,b,c]	2.45 ± 0.19[c]
Yamhill	1.329 ± 0.017[c,d]	0.09 ± 0.01[b]	0.005 ± 0.001[b,c]	0.92 ± 0.31[d]
Nixon	1.322 ± 0.038[c,d]	0.10 ± 0.01[b]	0.005 ± 0.001[a,b,c]	2.31 ± 0.10[c]
Jefferson	1.277 ± 0.025[c,d]	0.11 ± 0.01[b]	0.007 ± 0.001[a]	2.00 ± 0.08[c,d]
Giresun	1.342 ± 0.014[c,d]	0.09 ± 0.01[b]	0.004 ± 0.000[c]	1.61 ± 0.15[c,d]
Minimum	1.255	0.09	0.004	0.92
Maximum	1.630	0.28	0.014	7.16
Mean	1.396	0.15	0.007	3.33
SD	0.117	0.07	0.003	2.08

Mean reported with standard deviation for $n = 3$. Data sharing the same letter (a, b, c, d) in the same column are not significantly different, $p > 0.05$, Tukey's multiple range tests.

Spectrophotometric measurements at specific wavelengths between 232 and 274 nm are another method to assess lipid oxidation. Primary oxidation (K_{232}) values (Table 2) ranged from 1.25 (Tonda di Giffoni) to 1.63 (Hall's Giant), and Hall's Giant was followed by Fitzgerald (1.51) and Ennis (1.49). All the samples exhibited K_{232} values lower than 2, suggesting that lipid oxidation levels were below those associated with off-flavors or rancidity [30, 31]. For secondary oxidation (K_{270}), the values (Table 2) ranged from 0.0901 (Giresun Ordu) to 0.2827 (Hall's Giant) and as might be expected Hall's Giant, Ennis (0.2499), and Fitzgerald (0.2400) also possessed the highest values for K_{270}. For ΔK (Table 2), which is another measurement of secondary oxidation, the samples with the lowest and highest values were again Giresun Ordu (0.0043) and Hall's Giant (0.0138). Primary and secondary oxidation values for the nine cultivars were comparable to the values reported in the literature [10, 21, 30].

3.4. Mineral, Macro-, and Microelements

The K, P, Ca, Mg, Cu, Fe, Mn, Zn, and B contents were determined by MP-AES using the ashed samples taken up in nitric acid, and results for Na and Mo were obtained by ICP-MS by a commercial lab (Table 3a). Mean concentrations from highest to lowest were K > P > Mg > Ca >> Fe > Mn > Zn > Na > Cu > B >> Mo. For potassium, the most abundant element of the eleven elements measured, and contents ranged from 566 mg/100 g FW (Jefferson) to 800 mg/100 g FW (Nixon) with a calculated mean of 667 mg/100 g FW. Phosphorus followed potassium with an observed range of 244 mg/100 g FW (Ennis) to 347 mg/100 g FW (Giresun) and a mean of 291 mg/100 g FW. Magnesium concentrations ranged from 139 mg/100 g FW (Ennis) to 194 mg/100 g FW (Hall's Giant) with a mean of 165 mg/100 g FW. The mean for calcium was 140 mg/100 g FW, and concentrations ranged from 97 mg/100 g FW

(Fitzgerald) to 206 mg/100 g FW (Hall's Giant). For the microelement iron concentrations ranged from 2.19 mg/100 g FW (Jefferson) to 4.36 mg/100 g FW (Nixon) with a mean of 3.51 mg/100 g FW. Manganese concentrations ranged from 1.61 mg/100 g FW (Fitzgerald) to 4.90 mg/100 g FW (Hall's Giant) with a mean of 2.49 mg/100 g FW. Zinc concentrations ranged from 1.83 mg/100 g FW (Jefferson) to 3.23 mg/100 g FW (Hall's Giant) with a mean of 2.21 mg/100 g FW. Sodium concentrations ranged from 1.44 mg/100 g FW (Tonda Giffoni) to 2.24 mg/100 g FW (Nixon) with a mean of 1.72 mg/100 g FW. Boron concentrations ranged from 0.90 mg/100 g FW (Giresun) to 1.43 mg/100 g FW (Nixon) with a mean of 1.11 mg/100 g FW. For the trace element, molybdenum concentrations ranged from 1.9 μg/100 g FW (Nixon) to 25.3 μg/100 g FW (Fitzgerald) with a mean of 13.1 μg/100 g FW. There was not a single cultivar that uniformly possessed either the highest or lowest concentrations, although the cultivar Hall's Giant exhibited the highest contents of five of the elements measured (Ca, Mg, Cu, Mn, and Zn). Correlation analysis suggested that Fe contents are correlated (>0.70) with P, Ca, and Cu, that Zn contents are correlated (>0.70) with Cu and Mn, and that B contents are correlated (>0.70) with K.

Table 3

(a) Fresh weight composition of minerals, trace, and ultratrace elements found in nine hazelnut cultivars collected from the USDA germplasm collection, Corvallis, Oregon. (b) US FDA recommended daily value (DV) and minimum and maximum percentages of DV provided by single servings (28.35 g) of raw hazelnuts for the nine cultivars tested. (c) Summary of minerals, trace, and ultratrace element composition found in the literature for hazelnuts adjusted to fresh weight basis.

(a)											
	K (mg/100 g)	P (mg/100 g)	Ca (mg/100 g)	Mg (mg/100 g)	Cu (mg/100 g)	Fe (mg/100 g)	Mn (mg/100 g)	Zn (mg/100 g)	B (mg/100 g)	Na (mg/100 g)	Mo (μg/100 g)
–											
Hall's Giant	671 ± 12	293 ± 5	206 ± 9	194 ± 2	1.92 ± 0.05	4.05 ± 0.04	4.90 ± 0.08	3.23 ± 0.68	1.23 ± 0.07	1.53	4.3
Ennis	628 ± 18	244 ± 2	98 ± 0.4	139 ± 1	0.94 ± 0.02	2.65 ± 0.10	2.16 ± 0.05	1.91 ± 0.02	1.23 ± 0.03	1.77	4.2
Fitzgerald	687 ± 13	259 ± 2	97 ± 2	140 ± 3	1.12 ± 0.01	3.02 ± 0.03	1.61 ± 0.02	2.00 ± 0.02	0.91 ± 0.02	1.52	25.3
Tonda di Giffoni	734 ± 8	304 ± 3	134 ± 0.4	156 ± 1	1.86 ± 0.03	3.57 ± 0.01	2.98 ± 0.03	2.08 ± 0.03	1.28 ± 0.01	1.44	23.1
Tombul	651 ± 4	299 ± 1	160 ± 6	164 ± 1	1.44 ± 0.03	3.97 ± 0.20	1.88 ± 0.02	2.17 ± 0.03	1.09 ± 0.03	1.72	16.2
Yamhill	699 ± 16	291 ± 1	177 ± 2	181 ± 2	1.33 ± 0.01	3.92 ± 0.13	2.18 ± 0.03	1.88 ± 0.02	0.97 ± 0.03	1.68	13.3
Nixon	800 ± 7	310 ± 1	118 ± 3	170 ± 1	1.74 ± 0.01	4.36 ± 0.005	2.08 ± 0.01	2.76 ± 0.02	1.43 ± 0.02	2.24	1.9

Jefferson	566 ± 14	268 ± 3	98 ± 2	188 ± 2	1.03 ± 0.02	2.19 ± 0.03	2.15 ± 0.03	1.83 ± 0.01	0.92 ± 0.02	1.68	12.3
Giresun	567 ± 2	347 ± 1	171 ± 1	155 ± 1	1.34 ± 0.01	3.82 ± 0.01	2.43 ± 0.001	2.00 ± 0.02	0.90 ± 0.01	1.90	17.2
Minimum	566	244	97	139	0.94	2.19	1.61	1.83	0.90	1.44	1.9
Maximum	800	347	206	194	1.92	4.36	4.90	3.23	1.43	2.24	25.3
Mean	667	291	140	165	1.41	3.51	2.49	2.21	1.11	1.72	13.1
SD	71	29	38	19	0.34	0.68	0.92	0.45	0.18	0.23	7.9

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(b)

	K (mg)	P (mg)	Ca (mg)	Mg (mg)	Cu (mg)	Fe (mg)	Mn (mg)	Zn (mg)	B (mg)	Na (mg)	Mo (μg)
–											
DV	4700	1250	1300	420	0.9	18	2.3	11	N/A	2300	45
Minimum as % DV	3.4%	5.5%	2.1%	9.4%	29.6%	3.4%	19.8%	4.7%	—	0.02%	1.2%
Maximum as % DV	4.8%	7.9%	4.5%	13.1%	60.5%	6.9%	60.4%	8.3%	—	0.03%	15.9%

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(c)

N (# values)	46	46	61	61	74	77	58	77	30	31	35
K (mg/100 g)	P (mg/100 g)	Ca (mg/100 g)	Mg (mg/100 g)	Cu (mg/100 g)	Fe (mg/100 g)	Mn (mg/100 g)	Zn (mg/100 g)	B (mg/100 g)	Na (mg/100 g)	Mo (μg/100 g)	
Minimum	382	192	65	54	0.76	2.36	0.68	0.29	1.36	2.04	9
Maximum	1470	412	328	224	5.07	5.16	15.22	4.40	2.99	14.55	515
Mean	749	282	168	145	2.21	3.72	5.61	2.68	1.98	5.30	138

SD	186	55	47	45	0.80	0.64	3.02	0.63	0.36	3.76	157

Table 3c summarizes the minimum, maximum, and mean contents found in the literature for recent reports (2006–2020) on hazelnuts. The results obtained for K, P, Ca, Mg, Cu, Fe, Mn, and Zn contents for the nine cultivars tested fall within the minimum to maximum ranges, and the means observed are within one standard deviation of the values calculated from the literature results. Although not numerically large, the B, Na, and Mo contents found in some of the cultivars evaluated were lower than the values reported in the literature, and this is more likely due to the soil and cultivation conditions than the cultivars evaluated. For example, Müller et al. [9] in their evaluation of the Mo contents of hazelnuts grown in Germany found a range of 109 μg/100 g FW to 515 μg/100 g FW, whereas Ozkutlu et al. [32] found a range of 9 μg/100 g FW to 31 μg/100 g FW for hazelnuts from the Black Sea Region of Turkey and Ozenc [33] in their evaluation of the influence of nitrogen, phosphorus, and potassium fertilizer applications on hazelnuts also from the Black Sea Region of Turkey reported a range of 40 μg/100 g FW to 51 μg/100 g FW for untreated trees and that the application of the fertilizers significantly decreased Mo contents as much as 80%. Minerals, trace, and ultratrace elements are broadly categorized as beneficial or detrimental depending on their concentrations and biological functions. Greater than trace levels, concentrations of potassium [34], phosphorous [35], calcium [36], and magnesium [37] are required for homeostasis and good health, whereas elevated levels of sodium [38] are associated with disease. The World Health Organization has further classified Cu, Zn, and Mo as essential elements and Mn and B as probably essential elements [39] Table 3b lists the minimum and maximum percent of US FDA daily value (DV) provided by a single serving (28.35 g, 1 U.S. ounce) of hazelnuts for the nine cultivars evaluated. Our results suggest that a single serving of raw hazelnuts (28.35 g, 1 U.S. ounce) will provide 29.6% to 60.5% and 19.8% to 60.4% of the recommended DV of copper and manganese, respectively, while maintaining a low sodium diet by contributing no more than 0.03% of the DV.

3.5. Correlation among Proximate Contents, Lipid Oxidation, Mineral, Macro-, Microelements, and Principle Component Analysis (PCA)

Review of Person correlations (data not shown) calculated for the properties measured revealed that moisture content was strongly correlated with peroxide value (PV), K_{232}, and K_{270}. Lipid oxidation measures (PV, K_{232}, K_{270}, and Delta K) were also strongly correlated with each other as expected. Aliasgharpour and Rahnamaye [39] in their storability study of hazelnuts found that lipid oxidation was reduced by removing moisture, and Jung et al. [40] hypothesized that increases in lipid oxidation in samples with higher moisture content were not due to water alone, but the result of increased levels of solubilized metal ions that in turn accelerated lipid oxidation. In contrast to the positive correlations noted above, strong negative correlations were found between lipid content and both moisture and water activity, and lipid content and lipid oxidation measures (PV, K_{232}, K_{270}). Higher lipid contents leading decreased lipid oxidation may seem counterintuitive; however, this outcome might be attributable to a greater concentration of natural minor components that prevent oxidation existing in the oils [42].

Results for the PCA are displayed in Figure 3 as a biplot. One of the most apparent features is the division between the low lipid/high moisture cultivars (Hall’s Giant and Ennis Fitzgerald) and the remaining cultivars. A second apparent feature is the distance between Hall’s Giant and Ennis and Fitzgerald on the Component 2 axis, which can be accounted for the stark differences in ash and mineral contents between the cultivars. Likewise, similarly lower ash and mineral contents account for the separation between Jefferson and the more lipid-rich cultivars.

[figure(s) omitted; refer to PDF]

4. Conclusions

Hazelnuts are the most popular tree nuts in the world, and the majority of world production occurs in regions in mild climates adjacent to the large bodies of water. In the United States, almost all commercial production is limited to a single area because the cultivars used are of European and Asian descent. Only thru the introduction of new cultivars or improved cultivation practices can U.S. and worldwide commercial production be expanded to meet the growing consumer demand accelerated by increased interest in plantbased diets and efforts to substitute nut materials for the traditional animal products in processed foods and culinary dishes [43], and overcome the

pressures caused by diseaseand climate change [44]. The availability of germplasm collections is essential to developing new cultivars, and the NPGS contributes to these efforts through maintaining and characterizing these collections. Nine commercially important hazelnut cultivars were selected from the NPGS collection for characterization, including proximate contents, degree of lipid oxidation, elemental analysis, and near-infrared spectroscopy (NIRS). Results from the characterization demonstrated that nuts grown in the United States possess characteristics similar to hazelnuts from Asian and European growing regions, but each cultivar possessed a unique profile. Hazelnuts may contribute to nutrition as a low sodium source of lipids and dietary copper and manganese.

Additional Points

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Microwave-Assisted “One-Pot” Acidolysis and Extraction for the Rapid Determination of Mancozeb in Fruit and Vegetable Samples

Tian, Qiaoxia; Li, Hongxing; Chen, Lixia; Han, Bingjun.

ABSTRAK (ENGLISH)

Mancozeb is an extensively consumed fungicide, which often leaves high residue levels on agricultural products. The conventional method for detecting mancozeb involves a time-consuming process using gas chromatography (GC) after a 2-hour water-bath acidolysis, resulting in low efficiency and recovery rates. This study developed a rapid method for detecting mancozeb in fruits and vegetables using microwave-assisted acidolysis and extraction coupled with GC analysis. Mancozeb underwent “one-pot” acidolysis to generate CS_2 gas and was subsequently extracted from samples using microwave treatment, requiring only 50 seconds of pretreatment time. The average recoveries of mancozeb ranged from 81% to 112%. The limit of detection and limit of quantification were 0.003 and 0.01 mg kg^{-1}, respectively. The scanning electron microscope imaging showed that strong cell crumpling after microwave treatment improved the acidolysis rate significantly, where the acidolysis rate was 91.8% for mancozeb. In addition, this method is rapid, simple, and precise for detecting residues of mancozeb and other dithiocarbamate fungicides.

TEKS LENGKAP

1. Introduction

Mancozeb is a dithiocarbamate (DTC) fungicide that is extensively consumed in agriculture, with a proportion of more than 20% and an annual consumption of approximately 30,000 tons and 1 billion dollars [1]. Mancozeb is widely used because it has a good effect in preventing fungal diseases of many crops [2, 3] and other aspects [4]. However, mancozeb exposure inhibits mitochondrial complexes in HT-29 cells [5], cardiotoxic effects in zebrafish [6], and neurodegenerative conditions such as Parkinson’s disease [7]. Even though mancozeb is presently banned in the EU, both the EU and USA have established maximum residue limits (MRLs) for it. This is because there’s a concern that it could still be used illegally within the EU or legally in other countries whose products may be imported. The current MRLs for mancozeb in the EU and USA are based on the content of dithiocarbamates, which include mancozeb, maneb, metiram, propineb, thiram, and ziram. These MRLs are expressed as CS_2 equivalents. The lowest MRLs for mancozeb in the EU and USA are 0.05 and 0.06 mg kg^{-1}, respectively, which are equivalent to 0.089 and 0.11 mg kg^{-1} of mancozeb. Therefore, a reliable and sensitive determination method for mancozeb and other DTCs is of great practical importance for safeguarding human health, protecting the environment, and strengthening pesticide residue monitoring.

Rapidly and accurately determining mancozeb and other DTCs residue in food samples has always been a big challenge. Various classical methods have been established for determining pesticide residues, such as methylated derivatization high-performance liquid chromatography-mass spectrometry (HPLC-MS) [8, 9], gas chromatography (GC) coupled with a flame photometric detector (FPD) with a sulfur filter [10, 11], a surface-enhanced Raman scattering (SERS) [12, 13], atomic emission spectrometry [14], atomic absorption spectrometry [15], and electron

capture detector (ECD) [16]. However, the determination of mancozeb using the HPLC-MS method requires a complicated methylation derivatization step [17, 18]. Furthermore, HPLC-MS is expensive to be accepted in all laboratories [19]. GC is another standard method for determining mancozeb, which is a more convenient and feasible instrument in the laboratory, with high sensitivity and good selectivity. Generally, before the determination of mancozeb by GC, classical water bath heating acidolysis is required to generate CS_2 gas for a long time (90–120 min) under 90°C. Subsequently, the generated CS_2 gas was absorbed by the hexane solvent, and the solvent was injected into the GC for CS_2 determination [20]. Considering the molecular weights of mancozeb and CS_2, one mole of mancozeb will generate two moles CS_2. The conversion factor μg of mancozeb × 0.564 equaled μg of CS_2 was established. The entire process usually requires at least 2 h with a traditional water-bath heater and sometimes even longer. Moreover, the acidolysis efficiency of this water-bath heater method is usually limited to 50−60% for mancozeb. The determination of mancozeb required additional correction according to the acidolysis efficiency, which reduced the accuracy and efficiency of the method [21].

Microwaves have high energy to heat solvents in contact with a sample to improve the efficiency of the chemical reaction or extraction of analytes from the sample matrix into the solvent [22]. As an alternative to conventional heating [23], microwave-assisted detection has been applied in various analyses [24]. Paiga et al. [25] developed a method for determining carbamate and urea pesticide residues in fresh vegetables using microwave-assisted extraction−liquid chromatography. Wu et al. [26] also determined organophosphorus pesticides in fruits by gas chromatography−mass spectrometry (GC−MS) with the aid of microwave-assisted extraction. Recently, microwave-assisted extraction also applied for simultaneous determination of mycotoxins and pesticide residues in soil and other samples [27, 28]. This indicates that the use of microwave-assisted sample pretreatment for the determination of pesticide residues has great application prospects [29–31]. In our previous study, we used the microwave-assisted hydrolysis reactor coupled molecular emission spectrometer (MES) to determination the mancozeb and other DTCs fungicide successfully [14]. However, the MES detector has poor sensitivity for the mancozeb at 0.5 mg kg^{-1} only, and the mechanism and influence of the microwave acidolysis were also not investigated.

Therefore, in this study, a “one-pot” acidolysis and extraction method with microwave was established for the conversion of mancozeb to CS_2 gas in fruit and vegetable samples, and the classical GC-ECD method was used for further analysis to ensure the high sensitivity. The acidolysis time of the traditional water-bath heater will shorten significantly from 2 h to 50 s. The change in the microstructural morphology of the sample was also observed using a scanning electron microscope (SEM) to prove the efficiency of the microwave. The microwave-assisted acidolysis method is high efficiency, short time consumption, and low cost for the rapid determination of DTCs residues in fruits and vegetables.

2. Materials and Methods

2.1. Instrumentations and Equipment

A gas chromatograph (Agilent Technologies, USA) consisting of a 7890B GC system coupled with an ECD detector was used for the extracted pesticides, standard samples, and test samples in this study. A MARS microwave accelerated solvent extraction (CEM Corporation, USA) was used for the microwave-assisted acidolysis of DTCs to improve the CS_2 conversion efficiency. A high-speed refrigerated centrifuge (CR22N/21N, Hitachi Koki Co., Ltd., Tokyo, Japan) was used to rapidly separate the supernatant and the hypolimnion. A thermostatic water-bath (B-260, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) was used for traditional sample pretreatment water-bath heating.

A field-emission scanning electron microscope (ZEISS ∑IGMA, Germany) was used to observe the microstructure of the fruit and vegetable samples before and after microwave treatment. A vacuum freeze dryer LGJ-10D (Beijing Sihuanqihang Technology Co. Ltd., China) was used to dry the samples for SEM observation.

2.2. Reagents and Sample

All reagents used in this experiment were of analytical grade. Deionized water (DIW, 18.2 MΩ cm), prepared using a Milli-Q water purification system (Millipore, USA), was used in all trials. The main reagents, $SnCl_2$ and HCl, used in this study were procured from the Shanghai Sui-Test Company (Shanghai, China). Ascorbic acid was purchased

from Shandong West Asia Chemical Engineering Co., Ltd. (Shandong, China), and hexane was purchased from Huate Gas Co., Ltd. (Guangzhou, China). Mancozeb, metiram, thiram, and propineb standards were purchased from Sichuan Lier Crop Science Co. Ltd. (Sichuan, China). High-purity chemicals ethylenediaminetetraacetic acid (EDTA) and NaOH were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). L-cysteine (L-cys) and CS_2 gas were purchased from Aladdin Shanghai Biochemical Technology Co., Ltd. (Shanghai, China).

Fruit and vegetable samples, including banana, mango, pineapple, cowpea, dragon fruit, lychee, apple, eggplant, and peanuts, were collected from the experimental base of the Analysis and Test Center of the Chinese Academy of Agricultural Sciences in Hainan Province, China. The required samples were broken up using a wall-breaker and stored at −20°C for further analysis.

2.3. Analysis Procedure

The mancozeb was dissolved with a mixed solution prepared using 12.5 g EDTA and 12.5 g L-cysteine (L-cys) in 800 mL of ultrapure water, where the pH value was adjusted to 9.0–10.0 with a NaOH solution. Standard stock solutions of 50 μg mL^{-1} mancozeb were prepared by weighing and dissolving 0.0025 g mancozeb standard in 50 mL mixed solution and then diluting the stock solution to prepare standard working solutions with different concentrations. The solution was prepared on the day of the experiment and was stored in a refrigerator at 4°C. 0.100 g CS_2 was dissolved in hexane (100 mL) to obtain a mother solution with a mass concentration of 1000 μg mL^{-1}. The standard solution was gradually diluted with hexane to 0.2 μg mL^{-1}, which was used for the assessment of converted efficiency of mancozeb and other DTCs.

10 g of $SnCl_2$ was dissolved in 250 mL (5 mol L^{-1}) hydrochloric acid (HCl) solution and a 40 mg L^{-1} $SnCl_2$–HCl solution was obtained when $SnCl_2$ was completely dissolved into a colorless transparent liquid.

2.4. Chromatographic Conditions

A GC chromatographic column (GS-Gas Pro, 30 m × 0.32 mm) with nitrogen gas (>99.999%) at a flow rate of 2.0 mL min^{-1} was used as the carrier gas for the separation of pesticides. The inlet temperature was set to 130°C, and the detector temperature was set to 240°C. The injection volume was 2 μL with split mode and a split ratio of 2 : 1. The oven temperature was programmed as follows: the initial column temperature was 40°C, held for 4 min, increased at 25°C min^{-1} to 90°C, fixed for 4 min, then at 30°C min^{-1} to 240°C, and held for 3 min.

2.5. Sample Pretreatment

This procedure is schematically shown in Figure 1. Briefly, 2 g of each fruit and vegetable samples were accurately weighed and added into PTFE (polytetrafluoroethylene) the microwave digestion tubes. The mancozeb standard solution was added to the samples for further study. Also, 0.2 g of ascorbic acid was added separately. Then, 20 mL of the $SnCl_2$–HCl solution and 4 mL of hexane were added to the tubes separately. The mouths of the PTFE tubes were sealed with PFA (perfluoroalkoxy) cover seal to ensure no air leakage, followed by heating in a microwave oven at 720 W for 50 s. Subsequently, the reacted solution was cooled to room temperature and transferred to a plastic centrifugal tube, and the mixture was centrifuged for 5 min at 4000 r min^{-1}. The solution was stratified, and the upper hexane layer supernatant was aspirated into the injection vial for further analysis.

[figure(s) omitted; refer to PDF]

2.6. Microstructure Observation with SEM

To verify the reason for the high efficiency of microwave-assisted acidolysis, banana and mango samples were selected as typical samples through the SEM images for the investigation. Three kinds of samples, untreated, treated with bath, and treated with microwave, were prepared for the observation of microstructural morphology using SEM. To enhance the clarity of the SEM images, the samples were dried in a frozen vacuum and coated with gold before observation.

3. Results and Discussion

3.1. Feasibility of the Efficiency Using Microwave-Assisted Acidolysis

The traditional acidolysis from mancozeb to CS_2 used the water-bath heating method. Ultrasound and microwaves have been reported to enhance the efficiency of some chemical reactions such as the extraction of active compounds [21, 32]. Therefore, the initial experiment investigated the feasibility of microwave-assisted acidolysis.

The water-bath acidolysis and ultrasound-assisted acidolysis methods were selected to compare the acidolysis efficiency with that of the microwave method, and the results are summarized in Figure 2. The samples were treated using water-bath heating at 60°C for 60, 120, and 180 min. The ultrasound method was used to assist the acidolysis method at 10, 30, and 60 min, and the microwave method was used to assist the acidolysis method at 10, 30, and 50 s. Surprisingly, the 50 s microwave treatment at 720 W of microwave power could completely convert mancozeb to CS_2, while the acidolysis efficiencies after 180 min of water-bath heating at 60°C and 60 min of ultrasonic-assisted water-bath heating at 60°C were only 50% and 61%, respectively, which is similar to the earlier report [33]. Hence, the microwave method is considered a high-efficiency method to improve the acidolysis efficiency of mancozeb, and the processing time is only 50 s.

[figure(s) omitted; refer to PDF]

3.2. Microstructure of the Sample Treated with Microwave

To explore the reason for the improvement in acidolysis efficiency, the microstructures of the banana and mango samples were observed using SEM before and after water-bath heating and microwave treatment, and the results are presented in Figure 3. As shown in Figures 3(a) and 3(d), untreated mango and banana cell granules were closely arranged and the cell structure was well preserved. Figures 3(b) and 3(e) show that after 2 h of water-bath heating, mango and banana granule cells were loosely arranged, and the cell structure was slightly damaged. Compared with the untreated sample, there were no significant changes after treatment with water-bath heating.

[figure(s) omitted; refer to PDF]

The microwave system has a strong radiation ability, which can cause rapid internal warming or destruction of the sample tissue (cell) structure, increasing the solubility of the target compounds in the sample in the extraction solvent [34]. Moreover, the rapid warming ability of the electromagnetic field generated by microwaves increases the diffusion rate of the target compounds, and high-frequency electromagnetic waves penetrate the solvent to reach the vascular bundle and glandular cell system in the fruit and vegetable tissue. Figures 3(c) and 3(f) depict that after 50 s of microwave treatment, mango and banana cell walls were strongly ruptured, and cell crumpling was evident. Although both Figures 3(c) and 3(f), were microwave treated, mango cell rupture in Figure 3(c) is more evident, and wrinkling is also more obvious owing to the high-water content in mango. Because water-containing materials have deep transient heating characteristics to microwaves, mango is more likely to have cell rupture during this process, whereas the starch content in a banana is high and the starch structure is less affected by the microwaves [35], resulting in a weaker degree of banana cell rupture.

3.3. Optimization of Operating Parameters

Microwave power is an important factor in the acidolysis of mancozeb into CS_2. The microwave power in the range of 80 to 800 W was then tested for the best converted efficiency, and the results are presented in Figure 4(a). A mancozeb concentration of 10 μg mL^{-1} was used to test the acidolysis efficiency. When the concentration of the $SnCl_2$–HCl solution was set at 40 mg L^{-1} and the microwave time was adjusted to 50 s, the response of the mancozeb standard increased with the microwave power and reached a plateau at 720 W. This may be because, with the increase in microwave heating power, mancozeb and $SnCl_2$–HCl solution undergo cohesive acidolysis to completely generate CS_2 gas. Therefore, 720 W microwave power was selected for further study.

[figure(s) omitted; refer to PDF]

The effect of microwave time on the acidolysis efficiency was investigated between 10 and 70 s. As shown in Figure 4(b), the response of the mancozeb standard solution significantly increases with the increasing microwave heating time until 50 s and reached a plateau. Which indicated microwave heating time affects the CS_2 generation, and it is completely converted to CS_2 at 50 s. Therefore, optimum microwave time was set as 50 s for this study.

Further experiments were performed to determine the effects of HCl concentration on acidolysis by ranging it from 1 mol L^{-1} to 6 mol L^{-1} and constant amount of $SnCl_2$ (0.8 g) was then added. As summarized in Figure 4(c), the response increased with the increase in HCl concentration and reached a plateau after 5 mol L^{-1}. Hence, the concentration of HCl affects the dissolution of $SnCl_2$ crystals, which in return affects the acidolysis of mancozeb in the $SnCl_2$–HCl solution. When the concentration of HCl reached 5 mol L^{-1}, $SnCl_2$ was entirely dissolved and

mancozeb acidolysis completely generated CS_2 gas. Therefore, the concentration of 5 mol L^{-1} was selected for further experiments.

The effects of the concentration of $SnCl_2$–HCl from 10 to 80 mg L^{-1} were studied, and the results are shown in Figure 4(d). The best results were obtained by choosing 40 mg L^{-1} of $SnCl_2$−HCl solution. Since the concentration of the $SnCl_2$–HCl solution affects the acidolysis of mancozeb to produce CS_2; the acidolysis rate of stannous chloride hydrochloric acid solution with different concentrations is different, and a more suitable acid digestion concentration was obtained when the concentration of $SnCl_2$−HCl was 40 mg L^{-1}, which ensured the accuracy of the test.

3.4. Analysis Characteristics

The analytical performance was evaluated by directly injecting of converted CS_2 in hexane solution with different concentrations of mancozeb standard under optimal conditions. The typical chromatograms of blank, standard solution, and spiked samples in banana, mango, pineapple, and cowpea are shown in Figure 5. The results were evaluated following the criteria outlined in the standard SANTE 11312/2021, which provides guidelines for analytical quality control and method validation procedures for analyzing pesticide residues in food and feed. No interfering peaks were detected at the retention time of 5.64 minutes for mancozeb in blank tested samples, which were extracted and analyzed under the same conditions. The retention time falls within the standard requirement with a deviation of ±0.1 minute. The concentrations of mancozeb standard solution were in the range from 0.005 to 5.0 μg mL^{-1}, and the test was repeated for 6 times, indicating that the peak area exhibited a clear linear response (R2 = 0.9995). The obtained regression equation was $Y = 4.05 \times 10^4$ $X = 3.76 \times 10^2$. And the deviation of back-calculated concentration from true concentration ≤ ±20%.

[figure(s) omitted; refer to PDF]

The limit of detection (LOD) of the method was 0.003 mg kg^{-1} at three times the signal-to-noise ratio of analytes (3 signal/noise). The limit of quantification (LOQ) of the method was 0.01 mg kg^{-1} at the content corresponding to 10 signal/noise. The LOQ of 0.01 mg kg^{-1} met the requirement for accurate determination, considering that the lowest MRLs for mancozeb in the EU and USA are 0.089 and 0.11 mg kg^{-1}, respectively.

Mancozeb was spiked at 0.01, 1.0, and 10 mg kg^{-1} into the banana, mango, pineapple, and cowpea samples to test the method accuracy, and the analytical results are presented in Table 1. Overall recovery rates of mancozeb in the fruit and vegetable test samples spiked at 3 fortification levels ranged from 81% to 112% with relative standard deviations was 1.4% to 8.1%. The method satisfied the criteria with a recovery range falling within 70–120% and precision achieving a relative standard deviation (RSD) of ≤20%. Moreover, the proposed method is faster than traditional GC methods, as summarized in Table 2. Microwave-assisted acidolysis significantly improved the pretreatment time and conversion efficiency, with only 50 s required for conversion and absorption.

Table 1

The results for precision and recoveries of mancozeb (n=6).

Samples	Spiked (mg kg^{-1})	Found (mg kg^{-1})	Recovery (%)	RSD (%)
Banana	0.01	0.112 ± 0.0063	112 ± 6.3	3.8
1.0	0.832 ± 0.041	83 ± 4.1	2.8	10
9.41 ± 0.20	94 ± 2.0	1.4	-	
Mango	0.01	0.108 ± 0.0092	108 ± 9.2	7.3
1.0	0.844 ± 0.040	84 ± 4.0	3.0	10

9.73±0.70	97±7.0	5.1	-	
Pineapple	0.01	0.0851±0.0070	85±7.0	4.6
1.0	9.86±0.065	99±6.5	3.4	10
8.46±0.38	85±3.8	2.0	-	
Cowpea	0.01	0.0811±0.0020	81±2.0	8.1
1.0	8.28±0.013	83±1.3	3.1	10

Table 2

Comparison of the pretreatment time and LOD with those reported in references (n=6).

Methods	Time of pretreatment	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	References
LC–MS/MS	20 min	0.015	0.05	[17]
GC-FPD	2 h	0.026	0.089	[11]
SERS	15 min	0.1	—	[12]
LC-DBD-MES	10 min	0.3	1	[14]
Water bath heating acidolysis GC-ECD	2 h	0.053	0.18	[16]
Microwave assisted acidolysis GC-ECD	50 s	0.003	0.01	This method

Note. "—"means not mentioned.

3.5. Acidolysis Efficiency of Microwave Treatment

The efficiency of microwave-assisted acidolysis is the most important factor affecting accuracy. Therefore, 0.25 μg mancozeb was selected to add the $SnCl_2$–HCl solution and hexane, which were then moved into the microwave oven for 50 s acidolysis. The generated CS_2 was absorbed by hexane and injected into the GC instrument to determine its CS_2 content. Pure CS_2 was also injected as a standard to quantify the yield of mancozeb acidolysis. The theoretical yield of CS_2 from mancozeb was calculated using the molar mass of CS_2 in mancozeb. Then, an acidolysis efficiency of 91.4% was calculated relative to the measured yield for the theoretical yield (set as 100%). The results are shown in Figure 4, suggesting that the acidolysis efficiency is high.

Furthermore, to confirm the feasibility of this microwave-assisted acidolysis method for other types of DTCs fungicides, it was used to determine the residues of other dithiocarbamate pesticides, including metiram, thiram, and propineb. The principle behind the acidolysis of mancozeb and other DTCs to CS_2 involved a chemical reaction where mancozeb underwent hydrolysis in acidic conditions to yield CS_2 as one of the reaction products. This reaction typically involved the cleavage of the carbon-sulfur bonds present in the mancozeb molecule, resulting in the formation of CS_2 along with other byproducts. The specific mechanism and intermediates were not clear yet, which involved in this acidolysis process may vary depending on the reaction conditions and the presence of catalysts or other factors. Considering that one mole of mancozeb produces two moles of CS_2, a conversion factor of

μg of mancozeb multiplied by 0.564 is established as equivalent to μg of CS_2. As shown in Table 3, under optimal conditions, all tested DTCs exhibited acidolysis efficiencies exceeding 72.2% of CS_2, with mancozeb achieving a conversion rate of 91.5%.

Table 3

CS_2 conversion of mancozeb and other DCTs fungicides (n=6).

Compounds	Added of DTCs (μg)	Theoretical content of CS_2 (μg)	Actual measured content of CS_2 (μg)	Conversion rate (%)
Mancozeb	0.25	0.141	0.129±0.0041	91.5±2.9
Metiram	0.25	0.140	0.128±0.0060	91.4±4.3
Thiram	0.25	0.158	0.114±0.011	72.2±7.0
Propineb	0.25	0.131	0.108±0.0072	82.4±5.5

Note. The theoretical conversion rate is based on molar mass. 1 g of mancozeb produces 0.562 g of CS_2, 1 g of metiram generated 0.559 g CS_2, 1 g of thiram generated 0.632 g CS_2, and 1 g of propineb generated 0.525 g CS_2.

3.6. Analysis of Real Samples

Fifty fruit and vegetable samples purchased from a local market were analyzed to preliminarily demonstrate the potential application of the proposed method. The results showed no DTCs residues in the tested fifty samples (data not shown). Mancozeb was spiked at 0.25 μg into nine different real fruit, vegetable, and rice samples (banana, mango, dragon fruit, lychee, apple, eggplant, cowpea, and peanuts) to test the accuracy, and the results are presented in Table 4. The results produced by the proposed method are not significantly different from those obtained by traditional water bath heating acidolysis method (90°C for 120 min) at a confidence level of 95% through the t-test. This indicates that this method (microwave-assisted acidolysis) has good precision and accuracy, and it is suitable for the analysis of different types of matrix samples.

Table 4

Results of 10 kinds of mancozeb spiked positive samples (n=6).

Sample	Added (μg)	Found (μg)	
Water-bath acidolysis method	This method	Banana	0.25
0.20±0.014	0.21±0.0063	Mango	0.25
0.20±0.025	0.21±0.0030	Dragon fruit	0.25
0.21±0.017	0.23±0.0075	Lychee	0.25
0.18±0.0087	0.19±0.012	Apple	0.25
0.20±0.019	0.21±0.012	Eggplant	0.25

0.21 ± 0.022	0.23 ± 0.0025	Cowpea	0.25
0.21 ± 0.019	0.24 ± 0.0053	Peanuts	0.25

4. Conclusion

In this study, a rapid method for mancozeb determination was established using microwave-assisted acidolysis coupled with GC-ECD. Compared to traditional water-bath heating acidolysis, microwave-assisted acidolysis had a high conversion efficiency, where the acidolysis time was directly reduced from 2 h of the original water-bath to 50 s. Moreover, microwave-assisted acidolysis greatly shortens the pretreatment time to reduce the probable gas leakage during the heating process, which improves the recovery of the method. Finally, rapid and high-efficiency acidolysis coupled with the high sensitivity of the GC-ECD method, mancozeb, and other DTCs fungicides in the fruit and vegetable samples could be determined with high sensitivity and accuracy. The proposed method is fast and accurate to operate and possibly applied as a standard method for the determination of mancozeb and other DTCs residues. Further exploration into an online and continuous flow microwave-assisted acidolysis coupled with the GC method could be beneficial for determining mancozeb and other DTCs in food.

Authors' Contributions

Qiaoxia Tian performed the experiments and wrote the original draft of the manuscript. Hongxing Li performed the experiments. Lixia Chen had discussions on the experimental design and performed the experiments. Bingjun Han conceived, designed, and wrote the manuscript. All authors have reviewed the manuscript.

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DETAIL

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Application of Hyperspectral Imaging for Watermelon Seed Classification Using Deep Learning and Scoring Mechanism

Hengnian Qi; He, Mengbo; Huang, Zihong; Yan, Jianfang; Chu, Zhang.

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ABSTRAK (ENGLISH)

Watermelon seeds are a significant source of nutrition in the diet. To assess the potential of near-infrared hyperspectral imaging technology for swift and nondestructive identification of watermelon seed varieties, near-infrared hyperspectral imaging (NIR-HSI) technology was used. The Savitzky–Golay (SG) smoothing algorithm and standard normal variable (SNV) algorithm were combined to preprocess the extracted spectral data. The successive projections algorithm (SPA) was used to reduce the dimensionality of the spectral data. Subsequently, three deep learning models (LeNet, GoogLeNet, and ResNet) were used to classify 10 common watermelon seeds. SPA was used to reduce the dimensionality of hyperspectral data. In terms of full band, the ResNet model achieved a classification accuracy of 86.77% on the test set. By using characteristic bands, the GoogLeNet model achieved a

classification accuracy of 83.85% on the test set. The ensemble fusion model based on a scoring mechanism achieved accuracy rates of 99.56%, 90.88%, and 87.97% on the training, validation, and test sets, respectively. The results indicated that the ensemble fusion model based on a scoring mechanism can enhance accuracy. Combining deep learning with NIR-HSI can effectively distinguish different varieties of watermelon seeds.

TEKS LENGKAP

1. Introduction

The main uses of watermelon seeds are as seeds for planting and as edible kernels. As seeds, they can be planted to grow watermelons. Watermelon ranks among the key economic crops cultivated in 122 countries globally [1]. It is also a popular, delicious, and refreshing fruit, serving as a significant source of vitamins and minerals [2]. As food, they serve as a type of snack. Watermelon seeds represent a significant source of nutrients in the diet [3], and it may offer health and economic advantages owing to their fiber, mineral, phenolic content, and antioxidant activity. Therefore, it is very popular among everyone. The factors affecting watermelon quality include watermelon varieties, seed quality, environmental factors, and nutritional factors. Selecting suitable and high-quality seeds is particularly important [4]. In the actual market, the price of each watermelon seed varies, and they are often quite expensive. Due to the difficulty of distinguishing watermelon seeds by eyes, market transactions frequently encounter situations of substandard or adulterated seeds, causing harm to the interests of farmers. For watermelon seeds, the purity of the seeds (varieties) is crucial. Because impurities in seeds can cause unhealthy and uneven plant populations, leading to elevated production expenses and decreased yields [5]. With the increasing variety of watermelon seeds, there is an urgent need for a detection technology that can identify multiple varieties quickly and without causing damage.

Traditional approaches for seed variety detection encompass morphological methods [6], electrophoretic identification methods, fluorescence-based identification methods, and chemical identification methods, among others. However, these traditional detection approaches are time-consuming and labor-intensive and are not conducive to the efficient detection of smart agriculture. RGB cameras had been used for discriminating watermelon ploidy seeds [5]. When species have similar morphological features and colors, it is difficult to classify them using visual methods [7]. Machine vision only acquires image information within the visible light range. As the variety of seeds increases, their seed characteristics overlap severely, and a single technique cannot provide sufficient information for their classification. Hyperspectral imaging has the advantages of machine vision and visible infrared spectroscopy [8]. Near-infrared spectroscopy analysis technology had been widely applied in seed quality detection due to its inherent advantages [9]. It had been used for quality assessment in seeds such as wheat seeds [10, 11], peanut seeds [12], maize seeds [13], radish seeds [14], pine seeds [15], etc. However, traditional near-infrared spectroscopy analysis technology also had limitations. This technique could only analyze a single point on a seed and cannot comprehensively cover the entire seed. Therefore, for individual watermelon seeds, achieving the desired detection accuracy was challenging.

Near-infrared hyperspectral imaging (NIR-HSI) technology is a combination of imaging and spectroscopic detection techniques. Hyperspectral imaging captures spatial images of samples across various wavelengths in the electromagnetic spectrum, creating a three-dimensional hypercube. Due to the spectral and spatial information provided by hyperspectral imaging, it had gained popularity in various fields [16]. In hyperspectral image data, each band is a grayscale image and each pixel has a spectrum. Typically, the spectral average of the regions of interest in each band is utilized. NIR-HIS is an advanced technology engineered to swiftly and nondestructively assess the quality and safety of diverse agricultural products [17]. More and more scholars have been applying NIR-HSI technology to seed variety detection in recent years. In the field of watermelon varieties, Zhang et al. successfully classified watermelon seed varieties using BPNN and ELM models based on NIR-HIS [4]. Additionally, researchers had applied NIR-HSI to other seeds. Singh et al. successfully identified barley seed varieties by combining NIR-HSI

with CNN [18]. Feng et al. used PCA-based SVM and RBFNN models to identify grape varieties in raisins [19]. Han et al. successfully classified licorice seeds by applying NIR-HSI combined with an SVM discriminant model based on feature bands [20]. Zhou et al. achieved the recognition of wheat grain varieties using a large near-infrared spectral dataset and a novel feature selection method based on deep learning [21]. In the field of cotton seed classification, a combination of NIR-HSI and partial least squares discriminant analysis (PLS-DA) were used to successfully classify variety [22].

Deep learning (DL) [23] stands as a pivotal artificial intelligence technique, empowering machines to autonomously glean knowledge from data. The application of DL is progressively expanding into the domain of spectral analysis [24]. Bai et al. utilized the combination of NIR-HSI and ResNet to achieve classification of coix seeds [25]. Barrio-Conde et al. utilized NIR-HSI combined with AlexNet to achieve classification of high oleic sunflower seed [26]. Previous studies had used a single model. This study would use multiple models to integrate to improve the learning ability of features. The studies mentioned above all used a single network architecture and a single-scale feature extraction capability. When encountering more complex problems, the accuracy of a single network may be limited. In practice, near-infrared hyperspectral data exhibited characteristics such as large data volume, high noise, and high dimensionality. Therefore, there would be a considerable amount of redundant information in the modeling process, posing challenges for model classification. Typical techniques for dimensionality reduction encompass principal component analysis (PCA), successive projections algorithm (SPA), and various other approaches. In recent years, some scholars have employed a combination of PCA and SPA dimensionality reduction algorithms with hyperspectral imaging systems to classify three different degrees of freeze damage in corn seeds [27]. Soybean classification had been achieved using PCA and artificial neural network (ANN) classifiers [28]. The construction of spectral feature vectors using SPA had been applied to the classification of waxy corn seed varieties [7]. The SPA had been utilized to select the optimal wavelength for corn seed variety classification [29]. In summary, combining hyperspectral data with deep learning models for dimensionality reduction and accuracy improvement is very meaningful.

Maintaining variety purity is crucial throughout the agricultural process due to its significant benefits for seed storage and economic efficiency [30]. This study explored a model suitable for classifying 10 watermelon varieties. Additionally, confusion matrices were utilized for visualizing prediction results, and a weighted scoring mechanism was employed to improve the accuracy of the deep learning models. The primary objectives of this study are (1) to classify 10 watermelon seed varieties using NIR-HSI technology combined with deep learning models; (2) to explore the enhancement of model accuracy through a weighted scoring mechanism; (3) to explore the optimal combination of initial points and the number of bands in the SPA.

2. Materials and Methods

2.1. Material Preparation and Data Division

There were 10 kinds of watermelon varieties, respectively, Quanmei2k, Quanmei4k, Quanmei8k, Xinxin, Caihongguazhibao, Heishuai, Mingyu, Zaojia, Yuyiguazhibaojiuhao, and Yuyijinxiabahao (Figure 1). A total of 2283 watermelon seed samples were used in this study. The detailed distribution of samples is shown in Table 1. The dataset was divided in a 6 : 1 : 1 ratio. The watermelon seeds used in this study were provided by Zhejiang Provincial Seed Management Station.

[figure(s) omitted; refer to PDF]

Table 1

Dataset partitioning of watermelon seeds.

Variety	Training	Validation	Test	Total
Caihongguazhibao	87	14	15	116

Heishuai	226	38	38	302
Mingyu	226	38	38	302
Quanmei2k	150	25	26	201
Quanmei4k	152	25	26	203
Quanmei8k	147	25	25	197
Xinxin	258	43	44	345
Yuyijinxiabahao	85	14	15	114
Yuyiguazhibaojiuhao	96	16	17	129
Zaojia	280	47	47	374
Total	1707	285	291	2283

2.2. Acquisition and Correction of Near-Infrared Hyperspectral Images

The hyperspectral imaging platform (Specim, Spectral Imaging Ltd., Oulu, Finland) used in this study belongs to the LSCA-0720-148 series. The scanning speed of the equipment was set at 24.7 mm/s, with a frame rate of 70 Hz. Due to the influence of dark current, white reference images and dark reference images should be used to correct the acquired raw hyperspectral images to reflective hyperspectral images. A white reference board was placed in front of the object to obtain a white reference image for light intensity calibration, while a dark reference image was obtained using a black opaque lens cover for dark current removal. The corrected image can be obtained by the formula as follows:(1)R=I−DW−D,where I represents the original image, R is the corrected image, W denotes the white reference image, and D is the dark reference image.

Due to the presence of noise before and after the bands, and the 1140−1350 nm wavelength range being utilized for detecting the C-H second overtone [6], this study adopted a wavelength range of 1053.43 to 1680.87 nm. In this research, every seed within the hyperspectral image was regarded as a region of interest (ROI), where the spectrum of each pixel within the ROI was averaged to derive a spectral vector representing the seed sample.

2.3. Spectral Preprocess

The spectral reflectance data obtained from the experiments were coarse and contained noise caused by factors such as equipment, experimental environment, and sample impurities. To reduce the impact of noise on the results, the Savitzky–Golay (SG) smoothing algorithm and the standard normal variate (SNV) algorithm were employed in this study. The working principle of the SG smoothing algorithm [31] is to slide a window over the data and, at each position, fit a polynomial to the points within the window. The center point of the window is replaced by the value estimated by the polynomial fit. This process is repeated for each point in the data, effectively smoothing and reducing noise. In this study, the SG cubic polynomial 7-point smoothing method was used to process the spectral data. The SNV (formula (2)) centralized and standardized the spectral data, making the mean of the data equal to 0 and the variance equal to 1. The use of SNV could effectively eliminate unwanted variations, such as baseline shifts and drifts [32]. SNV could enhance the stability and reliability of spectral data, improving data comparability and interpretability. The successive projections algorithm (SPA) is a commonly used method for selecting feature wavelengths [33]. SPA is a variable selection algorithm designed to choose wavelengths with minimal redundant

information [34]. Therefore, SPA was selected as the wavelength selection algorithm.(2)xSNV=x−x¯∑k=1mxk−x¯2/m−1,where x¯=∑k=1mxk/m, m is the number of bands, and k=1,2,3,…,m.

2.4. Classification Model and Scoring Mechanism

2.4.1. XGBoost and SVM

The extreme gradient boosting (XGBoost) model is currently the fastest and most effective ensemble decision tree algorithm and has been successfully applied in many fields [35]. He et al. utilized hyperspectral imaging (HSI) technology combined with the XGBoost model to accurately differentiate between naturally ripened and artificially ripened bananas [36]. Support vector machine (SVM) is one of the most efficient supervised classification methods [37]. The combination of HSI technology and support vector machine (SVM) has been widely used in various applications such as variety identification [25]. Zhang et al. utilized traditional machine learning and deep learning to identify different levels of freezing damage in corn seeds [38]. The results showed that deep learning performed the best.

2.4.2. LeNet Model

The LeNet model stood out as one of the pioneering convolutional neural networks [39]. In its architectural composition, the LeNet model comprised two integral components: a convolutional encoder and a densely connected block. The LeNet structure implemented in this study encompassed 3 convolutional layers, 3 max-pooling layers, 3 activation functions, 3 batch normalization layers, and 1 fully connected layer (Figure 2(a)). The convolutional layers utilized 1 × 4 convolutional kernels with channel numbers 8, 16, and 32. Feature extraction was facilitated through max-pooling layers with a kernel size of 1 × 4 and a stride of 1. The ReLU function was employed as the activation function.

[figure(s) omitted; refer to PDF]

2.4.3. GoogLeNet Model

Inception was a deep convolutional neural network architecture [40]. The Inception module can obtain information at multiple scales. The main characteristic of this architecture was the improvement of internal computational resource utilization within the network. While focusing on deepening the network structure, GoogLeNet introduced a new fundamental structure called the Inception module to increase the network's width. The Inception module in the GoogLeNet model utilized different convolutional kernels to extract information at various levels, employed 1 × 1 convolutional kernels for dimension reduction and computational efficiency, increased the model depth, and enhanced nonlinear expression capability. The GoogLeNet model (Figure 2(b)) employed in this study consists of two convolutional layers, two Inception modules, 2 max-pooling layers, 2 activation functions, 2 batch normalization layers, and 1 fully connected layer. The convolutional layers had kernels of size 1 × 5 with channel numbers of 10 and 20, respectively. The Inception module (Figure 2(d)) consists of four parallel pathways. Different paths can obtain different feature information, which is more conducive to model analysis. Finally, all four paths used appropriate padding to ensure consistency in height and width between input and output.

2.4.4. ResNet Model

He et al. introduced the residual learning framework (ResNet) to simplify the training of deeper networks compared to previous approaches [41]. ResNet had become the most cited neural network of the 21st century.

For a stacked-layer structure (composed of several stacked layers), when the input is x and the learned features are denoted as $H(x)$, the goal is to learn the residual $F(x) = H(x) - x$. This means that the original learned features are essentially $F(x) + x$. The reason for using this approach is that learning the residual is easier compared to directly learning the original features. When the residual is 0, the stacked layers essentially perform an identity mapping, ensuring that the network's performance does not degrade. In practice, the residual is not exactly 0, allowing the stacked layers to learn new features on top of the input features, leading to improved performance. The structure of the ResNet model in this study is depicted in Figure 2(c). The ResNet model employed in this study consisted of two convolutional layers, two residual modules, 2 max-pooling layers, 2 activation functions, 2 batch normalization layers, and 1 fully connected layer. The convolutional layers had kernels of size 1 × 5 with channel numbers of 16 and 32, respectively. The residual module is shown in Figure 2(e). The residual module directly transmits feature

information through a short-circuit-like connection method.

2.4.5. Scoring Mechanism Model

Ensemble learning was a method that helps improve the predictive accuracy of machine learning [42]. The objective of ensemble methods was to amalgamate various classifiers into a metaclassifier possessing superior generalization capabilities when compared to individual classifiers within it.

The approach studied in this study was the multimodel weighted scoring model. It combined the predicted results of models through a series of weighted summations. It selected the highest score for output and thereby improved overall accuracy. In the first step, the output values of each model were transformed using the formula (3), ensuring nonnegativity of the output. In the second step, the weighted formula (formula (4)) was employed to output the highest score. The workflow diagram of the score-based ensemble fusion model in this study is shown in Figure 3.

(3)yj=expoj∑kexp ok,where oj represents the output value indicating the classification as the *j*-th category and yj represents the output value indicating the classification as the *j*-th category after nonnegative transformation.(4)y_ hat=∑i=1nai×y_hati,where y_hat represents the prediction result of the ensemble model, ai represents the weight coefficient corresponding to each model, and y_hati represents the prediction result of each model.

[figure(s) omitted; refer to PDF]

2.5. Model Evaluation and Implementation Details

Figure 4 represents the schematic structure of the entire experiment. The evaluation of model performance in this study employed the classification accuracy metric. Classification accuracy is defined as the ratio of correct predictions to the total number of predictions made. Deep learning models were built using the PyTorch (version 1.12.1) framework. Data analyses were carried out on a computer equipped with an NVIDIA GeForce RTX 4060 Laptop GPU.

[figure(s) omitted; refer to PDF]

3. Results and Discussion

3.1. Spectral Analysis

The spectral feature of each seed was derived by computing the average value of all pixels within the corresponding region of interest (ROI) across effective bands. Figure 5(a) shows the original spectral data of watermelon seeds, revealing discrete data that were not conducive to data analysis. Therefore, it could apply Savitzky–Golay (SG) and standard normal variate (SNV) for denoising (Figure 5(b)). Figure 5(c) shows the average spectra for each watermelon seed variety. Differences were noted in the average spectra across various types of watermelon seed varieties. The average spectrum of Mingyu differed significantly from the other nine varieties. As different watermelon seeds contained varying chemical components such as proteins, the wavelength range of 1140–1350 nm was utilized for detecting the C-H second overtone [6]. Distinct disparities were noted in the average spectra of different watermelon varieties within the range of 1100–1300 nm (Figure 5(c)). In the remaining wavelength ranges, differences between some varieties were less pronounced. Overall, there were variations in the average spectra of samples from different watermelon varieties, indicating the feasibility of classifying watermelon seed varieties.

[figure(s) omitted; refer to PDF]

3.2. Dimensionality Reduction Results' Analysis

3.2.1. Loss Values in Different Band Numbers

Figure 6 shows the values of the loss function on the validation sets for various models under different numbers of spectral bands. Figure 6(a) shows loss function values of LeNet. Figure 6(b) shows loss function of GoogLeNet. Figure 6(c) shows loss function of ResNet. While computing the loss function, the model's hyperparameters remain consistent, with only the number of spectral bands varying. The cross-entropy loss function was used as the loss function. This study defined effective dimensionality reduction as reducing the number of bands to within 100 dimensions. In the models, lower loss function values indicated a closer proximity to the true classification results. The loss function values for all three models were decreasing, primarily due to the increase in spectral information. Around 80–100 bands, the loss function values for all three models were stabilized. It indicated that the added spectral information no longer significantly contributes to the differentiation of the classification models (Figure 6).

[figure(s) omitted; refer to PDF]

3.2.2. Specific Bands

Table 2 shows the wavelength ranges corresponding to the input features for each best model after dimensionality reduction. Due to variations in chemical components such as proteins among different watermelon seeds, the wavelength range of 1140–1350 nm was essential for detecting the second overtone of C-H bonds [6]. In this research, the initial point selected by the successive projections algorithm (SPA) was the 26th band at a wavelength of 1140.47 nm. As the loss values decrease, the band range essentially covered 1140–1350 nm. The LeNet model took 90 bands determined by SPA as input, the GoogLeNet model took 90 bands determined by SPA as input, and the ResNet model took 87 bands determined by SPA as input. The determination of the number of bands for each model was based on extensive experimentation (Figure 6). The selection aimed to achieve maximum dimensionality reduction while maintaining accuracy.

Table 2

Model specific bands.

Model	Wavelength (nm)
LeNet	1053.43, 1056.9, 1060.38, 1063.85, 1067.33, 1070.81, 1074.29, 1077.76, 1081.24, 1140.47, 1203.37, 1206.87, 1210.37, 1213.87, 1217.37, 1269.95, 1273.46, 1276.97, 1280.48, 1283.99, 1287.51, 1291.02, 1294.53, 1298.05, 1301.56, 1305.08, 1308.59, 1312.11, 1315.62, 1319.14, 1322.66, 1326.18, 1329.7, 1333.22, 1336.74, 1340.26, 1343.78, 1396.67, 1400.2, 1403.73, 1407.26, 1410.79, 1414.32, 1417.86, 1421.39, 1424.92, 1428.46, 1531.21, 1534.76, 1538.32, 1541.87, 1545.42, 1548.98, 1552.53, 1556.09, 1559.64, 1563.2, 1566.75, 1570.31, 1573.87, 1577.43, 1580.99, 1584.55, 1588.11, 1591.67, 1595.23, 1598.79, 1602.35, 1605.92, 1609.48, 1613.04, 1616.61, 1620.17, 1623.74, 1627.31, 1630.87, 1634.44, 1638.01, 1641.58, 1645.15, 1648.71, 1652.29, 1655.86, 1659.43, 1663, 1666.57, 1670.14, 1673.72, 1677.29, 1680.87
–	
GoogLeNet	1053.43, 1056.9, 1060.38, 1063.85, 1067.33, 1070.81, 1074.29, 1077.76, 1081.24, 1140.47, 1203.37, 1206.87, 1210.37, 1213.87, 1217.37, 1269.95, 1273.46, 1276.97, 1280.48, 1283.99, 1287.51, 1291.02, 1294.53, 1298.05, 1301.56, 1305.08, 1308.59, 1312.11, 1315.62, 1319.14, 1322.66, 1326.18, 1329.7, 1333.22, 1336.74, 1340.26, 1343.78, 1396.67, 1400.2, 1403.73, 1407.26, 1410.79, 1414.32, 1417.86, 1421.39, 1424.92, 1428.46, 1531.21, 1534.76, 1538.32, 1541.87, 1545.42, 1548.98, 1552.53, 1556.09, 1559.64, 1563.2, 1566.75, 1570.31, 1573.87, 1577.43, 1580.99, 1584.55, 1588.11, 1591.67, 1595.23, 1598.79, 1602.35, 1605.92, 1609.48, 1613.04, 1616.61, 1620.17, 1623.74, 1627.31, 1630.87, 1634.44, 1638.01, 1641.58, 1645.15, 1648.71, 1652.29, 1655.86, 1659.43, 1663, 1666.57, 1670.14, 1673.72, 1677.29, 1680.87
–	

ResNet	1053.43, 1056.9, 1060.38, 1063.85, 1067.33, 1070.81, 1074.29, 1077.76, 1081.24, 1140.47, 1206.87, 1210.37, 1213.87, 1217.37, 1269.95, 1273.46, 1276.97, 1280.48, 1283.99, 1287.51, 1291.02, 1294.53, 1298.05, 1301.56, 1305.08, 1308.59, 1312.11, 1315.62, 1319.14, 1322.66, 1326.18, 1329.7, 1333.22, 1336.74, 1340.26, 1343.78, 1396.67, 1400.2, 1403.73, 1407.26, 1410.79, 1414.32, 1417.86, 1421.39, 1424.92, 1428.46, 1538.32, 1541.87, 1545.42, 1548.98, 1552.53, 1556.09, 1559.64, 1563.2, 1566.75, 1570.31, 1573.87, 1577.43, 1580.99, 1584.55, 1588.11, 1591.67, 1595.23, 1598.79, 1602.35, 1605.92, 1609.48, 1613.04, 1616.61, 1620.17, 1623.74, 1627.31, 1630.87, 1634.44, 1638.01, 1641.58, 1645.15, 1648.71, 1652.29, 1655.86, 1659.43, 1663, 1666.57, 1670.14, 1673.72, 1677.29, 1680.87

3.3. Classification Results' Analysis

3.3.1. Confusion Matrix Results

Figure 7 displays the confusion matrices illustrating the classification results of the three best deep learning models on the validation set. Figure 7(d) shows the variety label. This matrix facilitated the observation of correct predictions and misclassifications for each category. The visualization of model prediction results through confusion matrices is beneficial for the analysis of scoring mechanisms. In Figure 7(a), it could be observed that the LeNet model was prone to misclassify Quanmei8k as Zaojia, Xinxin as Zaojia, and Zaojia as Xinxin. In Figure 7(b), the GoogLeNet model tended to misclassify Quanmei8k as Zaojia and Zaojia tended to be misclassified as Quanmei8k. In Figure 7(c), the ResNet model tended to misclassify Quanmei8k as Quanmei4k and Xinxin as Quanmei4k. By observing the confusion matrix, this study found that different models exhibited inconsistent accurate classification results for different seed varieties. There were cases where two models had accurate classifications, but another model had misclassifications. Therefore, this study proposed a scoring mechanism model to enhance accuracy.

[figure(s) omitted; refer to PDF]

3.3.2. Model Classification Results

Table 3 displays the average accuracy and variance of each model. The results indicated that the classification performance of traditional machine learning (XGBoost, SVM) was not as good as that of deep learning models. The reason may be that deep learning had a stronger feature representation ability. Deep learning can typically automatically extract features through end-to-end learning, giving it an advantage in feature representation. Deep learning models usually have stronger generalization ability, enabling them to better adapt to new data and unseen situations. On the test set, the scoring mechanism model has a higher average accuracy than the other models, but its variance is higher compared to the other models. In the full band, the ResNet model showed the best classification performance, achieving an accuracy of 86.77% on the test set. By using characteristic bands, the GoogLeNet model performed best, with a classification accuracy of 83.85% on the test set. In the full band, the model based on the scoring mechanism achieved accuracies of 99.56%, 90.88%, and 87.97% on the training, validation, and test sets, respectively. By using characteristic bands, the scoring mechanism model achieved accuracies of 93.94%, 90.00%, and 84.71% on the training, validation, and test sets, respectively (Table 3). The scoring mechanism model scoring mechanism showed an improvement in accuracy compared to single models. This improvement was attributed to the enhanced generalization of the models through the scoring mechanism, leading to higher accuracy. After applying the successive projections algorithm (SPA), the LeNet model used 90 bands from the SPA result, the GoogLeNet model used 90 bands from the SPA result, and the ResNet model used 87 bands from the SPA result (Table 2). The initial band was selected at the wavelength of 1140.47 nm. It could achieve not only dimensionality reduction but also demonstrate a relatively good classification performance. Compared to models without using the successive projections algorithm, there was a slight decrease in accuracy. This decrease was due to the reduction in data dimensions, leading to information loss. However, utilizing SPA resulted in a reduction in data complexity and an improvement in computational speed.

Table 3

Model classification results.

Model	Training (%)		Validation (%)		Test (%)	
Accuracy	s2	Accuracy	s2	Accuracy	s2	XGBoost
88.49	0.04	62.64	0.28	65.46	3.57	SVM
92.94	0.00*	79.58	0.28	78.01	0.12	LeNet
97.28	0.82	87.20	0.03	85.22	1.06	GoogLeNet
98.42	0.01	89.65	0.28	84.71	0.27	ResNet
99.94	0.00*	88.60	0.28	86.77	0.27	Scoring mechanism model
99.56	0.00*	90.88	4.43	87.97	2.96	XGBoost+SPA
85.15	0.62	61.58	0.28	67.01	26.25	SVM+SPA
85.18	0.01	79.13	0.77	77.15	1.44	LeNet+SPA†
90.89	0.01	88.78	6.03	83.51	1.89	GoogLeNet+SPA†
91.74	0.58	86.32	0.50	83.85	1.89	ResNet+SPA†
93.03	16.81	85.24	1.70	82.99	1.44	Scoring mechanism model+SPA†

†: SPA is the successive projections algorithm. ∗: it is approximately equal to zero after rounding, and s2: s2 is variance.

3.4. Discussion

The identification of watermelon varieties was highly significant for reducing the adulteration of watermelon seeds and minimizing losses for farmers. In this study, near-infrared hyperspectral imaging technology was employed for watermelon seed variety classification. In summary, the scoring mechanism model achieved the best results in terms of accuracy, indicating the superiority of the scoring mechanism model. It was an effective approach. The integration of ensemble techniques enhanced the model's generalization ability, subsequently improving accuracy. Within this study, the successive projections algorithm (SPA) was utilized for data dimensionality reduction, resulting in an enhanced computational speed. However, by using SPA, the accuracy of models decreased. The main reason for that was the decrease in data information. Based on the overall results, the model constructed in this study may exhibit signs of overfitting. We believe that the primary reason for this may be the insufficient coverage of the entire data distribution by the training data, leading to the model's inability to generalize well to new data. In the future, we propose collecting more diverse and abundant watermelon seed data or using techniques like generative adversarial networks (GANs) to generate more varied data. This aims to enhance the model's adaptability to different scenarios and variations. From the perspective of parameter quantity, integrating multiple models into an ensemble model resulted in a higher number of parameters compared to a single model, leading to increased computational workload during model computation. From the perspective of accuracy, ensemble models had the ability to capture features at different scales and demonstrated improved accuracy. In previous studies on watermelon variety classification using near-infrared spectroscopy (NIR), Deák et al. utilized a more traditional qualitative evaluation polar qualification system (PQS) with automatic wavelength range optimization for analysis [43]. Near-infrared hyperspectral imaging (NIR-HSI) combines the advantages of machine vision and near-infrared spectroscopy. Although NIR spectroscopy technology is cheaper than NIR-HSI, the data information obtained is limited. Compared to NIR spectroscopy, NIR-HSI technology can capture the entire seed's spectral information. It makes the analysis results more accurate. In the future, NIR-HSI technology can be applied to the detection of watermelon vitality. Compared to the study by Mukasa et al. [5], we successfully classified 10 watermelon seed varieties, achieving good classification results. Compared to the watermelon variety classification by Zhang et al. [4], this study utilized the popular convolutional neural network (CNN) for modeling. Compared to previous studies that combined NIR-HSI with DL [25], this study integrated a single network using a scoring mechanism, further improving the effectiveness. The sample size in this study was relatively limited and may be expanded in future research. For our model, transferring this model and research results to new varieties is a challenging task. At present, we believe that transfer learning and incremental learning may help address this issue.

4. Conclusion

In the research, a near-infrared hyperspectral imaging technology system and deep learning were successfully applied to the classification of watermelon seeds. Experimental results suggested that the best deep learning models to classify watermelon seeds was the ResNet model with a model accuracy of 86.77% on the test set. In contrast to prior research, this study facilitated the enhanced utilization of deep learning models in seed classification, resulting in improved efficiency. For this purpose, the scoring mechanism was used for the integration of three deep learning models, and the accuracy of the scoring mechanism model on the test set is 87.97%. In addition, the accuracy of deep learning models using the SPA was lower than that of models using full spectra on the test set. For future exploration, the study will delve into classifying a broader range of watermelon seed varieties. The watermelon seed classification model was used as the foundation for the classification of other seeds to save the time of model training by transfer learning.

Authors' Contributions

Hengnian Qi handled software, carried out methodology, and collected resources; Mengbo He handled software and carried out methodology and writing of the original draft; Zihong Huang collected resources and performed data curation; Jianfang Yan collected resources and conducted investigation. Chu Zhang was involved in

conceptualization, methodology, and software. The authors Hengnian Qi and Mengbo He contributed equally to this manuscript.

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DETAIL

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Effects of Storage Temperature and Spices Incorporation on the Stability and Antibacterial Properties of Fontitrygon margarita (Günther, 1870) Liver Oil

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ABSTRAK (ENGLISH)

Fontitrygon margarita liver oil, rich in unsaturated fatty acids, is susceptible to oxidation during storage, which can diminish its antibacterial qualities. This study examines the effects of storage temperature and the addition of spices on the stability and antibacterial properties of *F. margarita* liver oil. Oils with added spices were stored in opaque bottles at room temperature (28 ± 2°C) and in a refrigerator (4°C) and were periodically analyzed over a six-month period. Standard methods were used to determine oil quality indices; the Fourier transform infrared (FTIR) profile was assessed by spectroscopy; and antibacterial activities were measured using the broth microdilution method. The quality indices, FTIR profile, and antibacterial activities of the oil were evaluated and compared based on the incorporation of spices. The quality indices of oil extracted without a stabilizer and stored at room temperature significantly increased over time. The antibacterial activity of these oils gradually decreased during storage, with the minimal inhibitory concentrations (MICs) on bacterial strains of *Escherichia coli* (EC 137), *Enterobacter cloacae* (ENT 119 and ENT 51), and *Yersinia enterocolitica* (YERB 1) increasing from 16 to 128 mg/ml. Regardless of oil quality indices, oils stored in a refrigerator had lower values and better antibacterial activities than those stored at room temperature ((16 ≤ MIC ≤ 64 mg/ml on the strains of EC 137, YERB 1, ENT 51, and *Klebsiella pneumoniae* (KL 11)). The inclusion of spices significantly reduced the oxidative reaction in the oils and maintained the antibacterial activities of the tested oils. Given its antibacterial properties, *F. margarita* liver oil holds significant potential for the nutraceutical industry and could be used as a dietary supplement. This research underscores the importance of proper storage conditions and the use of natural stabilizers in maintaining the quality of such valuable natural resources.

TEKS LENGKAP

1. Introduction

Omega-3 fatty acids, especially long-chain polyunsaturated fatty acids (PUFAs) from fish oil, have been the focus of much research in recent years. The global fish oil market was projected to reach a value of USD 4.08 billion by 2022, spurred by a rise in aquaculture activities and increased consumer awareness of the health benefits of omega-3 PUFAs, including eicosapentaenoic acid (EPA; C20 : 5*n* − 3) and docosahexaenoic acid (DHA; 22 : 6*n* − 3).

EPA and DHA are associated with potential health benefits such as the prevention of coronary artery diseases, hypertension, diabetes, arthritis, autoimmune disorders, neurological diseases, cancer, improved brain development, and eye function in infants [1]. They exhibit a range of biological activities, including anti-inflammatory, antioxidant, anti-obesity, neuroprotective, and antimicrobial activities [2, 3]. PUFAs possess a unique capability to integrate with microbial cell membranes, thereby substantially increasing membrane fluidity. This alteration can trigger the opening of permeability channels, disrupt crucial concentration gradients, and ultimately lead to the death of microbial cells [4, 5]. In addition to influencing membrane fluidity, PUFAs demonstrate a range of antimicrobial activities. They have the ability to hinder microbial metabolism, inhibit growth, and thwart the formation of biofilms, which serve as a microorganism's shield against external threats. PUFAs also bolster the host's immune defenses and interrupt microbial communication, a process known as quorum sensing. Furthermore, PUFAs can induce oxidative stress in microorganisms, causing cellular damage and death. These varied mechanisms underscore the potential of PUFAs as antimicrobial agents. Current research is exploring these effects in depth, with the aim of harnessing PUFAs to combat microbial infections [5–7].

Numerous research studies conducted in Cameroon have uncovered that certain fish species, particularly those inhabiting the regions of Yabassi and Youpwe, Douala, are capable of producing oils rich in long-chain PUFAs, such as EPA and DHA. These oils have also been found to possess antibacterial properties. A recent review by Dongho et al. [8] highlighted that, as of March 2023, numerous studies have confirmed the antibacterial efficacy of oils derived from four Cameroonian fish species: *Chrysichthys nigrodigitatus*, *Fontitrygon margarita*, *Hepsetus odoe*, and *Lutjanus dentatus*. Furthermore, a subsequent study by Zokou et al. [9] has showcased the antibacterial potential of oil from *Oreochromis niloticus*. Among these, the oil obtained from the liver of *F. margarita*, a common species in Youpwe, Douala, stands out due to its high oil extraction yield of 14.49 to 16.90% as shown by Noutsa et al. [2]. They also noted that this oil is rich in PUFAs, accounting for 28.52% of its composition, and includes EPA, DHA, linolenic acid (C18 : 3), arachidonic acid (C20 : 4), and oleic acid (C18 : 1). Moreover, it has been found to be effective against both Gram-positive and Gram-negative bacteria, which are often responsible for food poisoning. Interestingly, oils extracted through exudation have shown higher activity than those obtained by cooking and pressing.

Indeed, the high content of unsaturated fatty acids in fish oil makes it susceptible to oxidation. This process can lead to the formation of various compounds such as peroxides, aldehydes, and ketones [10]. Lipid oxidation is a complex series of reactions that occur in the presence of oxygen, resulting in a state commonly referred to as rancidity. This is one of the primary factors contributing to the deterioration of oil quality. The consequences of lipid oxidation are manifold. It can decrease the nutritional and market value of the oil, alter its taste, and modify its texture and appearance [11]. Furthermore, lipid oxidation can reduce the shelf life of the oil and limit its therapeutic benefits. This presents a significant challenge for the use of ω-3 PUFAs in food and pharmaceutical applications. Therefore, it is crucial to find effective ways to prevent or slow down the oxidation process to preserve the quality and benefits of these oils.

To ensure the sustainable use of *F. margarita* liver oil, it is essential to establish effective storage strategies that reduce oxidation and improve the longevity and quality of the oil [12]. Synthetic antioxidants such as butylhydroxytoluene (BHT) and citric acid have traditionally been the industry standard due to their proven effectiveness [12, 13]. However, the trend towards natural products has led to an increased demand for natural antioxidants such as tocopherols, which are found in a variety of foods [13, 14]. The high metal content in fish oil can potentially compromise the effectiveness of these antioxidants [15], prompting the investigation of alternative plant extracts such as rosemary, oregano, and tea. These extracts are rich in phenolic compounds and act as potent antioxidants that neutralize free radicals, chelate metals, or counteract singlet oxygen [12, 16]. The application of these antioxidants, either directly or through marination, requires careful management of concentrations to maintain the oil's flavor and aroma [12, 13]. It is crucial to maintain low temperatures during extraction and process quickly. After extraction, the oil's resistance to oxidation can be enhanced by promptly adding antioxidants, cooling rapidly, and employing techniques such as emulsification and encapsulation [12, 14]. Nano-emulsion has been

demonstrated to enhance the preservation and antibacterial properties of *F. margarita* oil [2].

The stability of fish oil is influenced by storage duration and temperature. Appropriate storage postprocessing is vital, with unopened oils lasting 1–2 years and opened oils remaining fresh for 3–8 months if refrigerated [17, 18]. Storing away from heat, moisture, and light, particularly in a refrigerator (0–4°C), extends shelf life and slows down spoilage [12]. These practices ensure the maintenance of high-quality fish oil, thereby preserving its health benefits. Low-temperature storage enhances the fatty acid profile and physical properties of fish lipids, and the addition of antioxidants and plant extracts at low temperatures further improves lipid quality [12]. However, to the best of our knowledge, no existing studies have yet investigated the impact of incorporating antioxidants and varying storage conditions on the biological attributes of fish oils, especially their antimicrobial properties.

To further enhance the biological properties, particularly the antimicrobial properties, of oil extracted from the liver of *F. margarita* and, more generally, fish oils in Cameroon, it is crucial to devise strategies for their sustainable use. While refrigeration is the optimal preservation method, it is not a viable option for many in Cameroon due to its high cost and frequent power outages, especially in rural areas lacking electricity [19]. This underscores the need for supplementary methods that can augment refrigeration, such as the incorporation of natural antioxidants prior to extraction. This strategy would shield the oil from oxidation during the extraction process and subsequent storage at room temperatures, and it would boost protection during storage at low temperatures. When identifying potential sources of natural antioxidants, dietary spices and herbs stand out for their rich phytocompounds with antioxidant properties, including phenolic compounds [20, 21]. In fact, Cameroon is renowned for its rich variety of herbs and spices, notably *Allium sativum*, *Piper nigrum*, and *Monodora myristica*. These species are not only prevalent but also extensively used [22, 23]. They are distinguished for their abundant phenolic compounds and flavonoids. Research has revealed that these spices contain phenolic compounds known for their antioxidant and antimicrobial properties. This includes phenolic acids such as caffeic, chlorogenic, cinnamic, p-coumaric, and syringic acids, along with flavonoids such as catechin, kaempferol, and quercetin. Not to be overlooked are other phenolic compounds with beneficial properties, such as elemicin in *M. myristica* and piperine in *P. nigrum* [24, 25]. Additionally, *A. sativum*, like other members of the Liliaceae family, contains sulfur compounds such as allyl trisulfide, allicin, diallyl disulfide, and diallyl sulfide, which are recognized for their antioxidant and antimicrobial activities [26]. The antioxidant and antimicrobial properties of these spices are well-established in Cameroon [23, 27]. Furthermore, their extracts and powders have proven to be potent antioxidants, stabilizing crude soybean oil during extraction and throughout accelerated storage [27]. However, the potential of these spices to mitigate lipid oxidation in fish oil and their impact on the antibacterial activities of fish oil warrant further investigation.

The primary aim of this study is to investigate the impact of storage temperature and the addition of *A. sativum*, *P. nigrum*, and *M. myristica* on the stability and antibacterial properties of *F. margarita* liver oil. The study will specifically target bacteria that cause food-borne illnesses.

2. Materials and Methods

2.1. Materials

2.1.1. Collection of Livers of *Fontitrygon margarita*

The livers were sourced from freshly caught samples of *F. margarita* fish at the Youpwe market, Douala, in April 2020. Throughout the month, we conducted three distinct collection drives. Skilled fish cleaners meticulously removed the livers from the fish's abdominal region. To maintain optimal freshness and quality, the livers were promptly placed in iceboxes for transport to the Institute of Fisheries and Aquatic Sciences' Valorization and Quality Control Laboratory at the University of Douala in Yabassi, Cameroon. The time from the market to the laboratory was 2 to 3 hours. This procedure was consistently replicated across all three collection campaigns, thereby ensuring three replicates for each experimental condition.

2.1.2. Spices

The bulbs of *A. sativum*, seeds of *P. nigrum*, and hulls of *M. myristica* used in this research were sourced from the local Youpwe market. These raw materials underwent a meticulous drying process before being finely ground into powder using a traditional stone grinder. A small portion of these powders was then subjected to aqueous extraction

to quantify the total phenolic and favonoid contents, which are key constituents contributing to the spices' potent antioxidant properties. The remaining powder was carefully stored for subsequent extraction procedures.

2.1.3. Bacteria

The study used several bacteria, including two reference strains of *Escherichia coli* (ATCC 10536) and *Salmonella enterica serovar typhi* (ATCC 28579). These were sourced from the Laboratory of Microbiology and Antimicrobial Substances at the University of Dschang in Cameroon. Additionally, clinical strains of *Escherichia coli* (EC 137), *Enterobacter cloacae* (ENT 119 and ENT 51), *Klebsiella pneumoniae* (KL 11), *Salmonella enterica serovar typhi* (SAL 9), *Citrobacter freundii* (CITB 81), *Yersinia enterocolitica* (YERB 1), and *Staphylococcus aureus* (ST 120) were obtained from the Laboratory of Biochemistry at the University of Douala.

2.2. Methods

2.2.1. Aqueous Extraction of Spices and Determination of Total Phenolic and Flavonoid Contents

Approximately 25 g of each spice powder was combined with sterilized distilled water to yield a 100 ml solution, thus creating an aqueous extract with a concentration of 25% w/v. This concoction was allowed to stand at room temperature (28 ± 2°C) for 48 hours in a sterile flask, with regular shaking. The solution was then centrifuged (3000 rpm, 10 min) and filtered through a Whatman No. 1 filter paper. The filtrate was concentrated using a rotary evaporator set to a temperature range of 55–60°C.

The extracts were subsequently evaluated for their total phenolic, flavonoid, and tannin contents. The total phenolic content (TPC) was determined using the Folin–Ciocalteu method, as described by Womeni et al. [27]. In this procedure, 20 μL of the appropriately diluted spice extract (100 mg/ml) was combined with 0.2 mL of Folin–Ciocalteu reagent and 2 mL of distilled water in a test tube. This mixture was incubated at room temperature for 3 minutes. Subsequently, 1 mL of a 20% sodium carbonate solution was added, and the mixture was re-incubated at room temperature for an additional 2 hours. A blank was prepared using 400 μL of distilled water in place of the sample. The absorbance of the resultant blue mixture was measured at 765 nm using a quartz cuvette. Gallic acid was used as the standard, and the TPC was quantified as mg of gallic acid equivalents (mgGAE/g).

The total flavonoid content of the extracts was determined using an assay that forms a complex with aluminum chloride [28]. The process began by mixing 100 μl of each diluted extract (100 mg/ml) with 100 μl of 5% sodium nitrate. This mixture was allowed to stand for 6 minutes. Subsequently, 150 μl of a 10% aluminum chloride solution was added and the solution was left to stand for an additional 5 minutes. Following this, a 200 μl solution of 1 M sodium hydroxide was added sequentially. The absorbance of the resulting reaction mixture was measured at 510 nm using a UV spectrophotometer. The total flavonoid content of the extracts was then calculated in terms of quercetin equivalents (expressed as mgQE/g).

All these procedures were performed in triplicate to ensure the accuracy and reliability of the results.

2.2.2. Fish Oil Extraction and Storage

After each collection campaign, the harvested *F. margarita* livers were meticulously sliced, amalgamated, and subsequently divided into five uniform batches for different extraction processes, some including stabilizers and some without. The first group was processed without stabilizers, acting as the control. The second group incorporated a common antioxidant, BHT, while the final three other groups were treated with various spices. The procedure was according to the protocol established by Womeni et al. [27]. Thus, the BHT was introduced into the liver samples at the legal limit of 200 ppm, and the spices, *A. sativum*, *P. nigrum*, and *M. myristic,* were each added at 2000 ppm. Then, the mixture was vigorously stirred for about 10 minutes to ensure the homogeneous diffusion of the antioxidants, and the oil extraction directly followed.

This extraction was performed using the exudation technique, following the methodology established by Takahashi and Mitsui as used by Bonilla and Hoyos-Concha [29]. Briefly, the samples were heated in a dry oven at 95°C for 10 minutes, after which the oil was collected. Anhydrous sodium sulfate (Na_2SO_4) was used to eliminate any traces of moisture from the oil. Immediately after extraction, each oil sample underwent physicochemical characterization (including quality index and Fourier transform infrared/FTIR profile) and the determination of antibacterial activities using standardized methods.

Each sample was then divided into two parts, stored in 50 mL opaque bottles, and kept for 180 days under different conditions—one portion in a refrigerator (4°C) and the other at room temperature (28 ± 2°C). Every 60 days of storage, the samples were analyzed for quality index and antibacterial activities. Additionally, the FTIR profile was analyzed only for the control sample stored at room temperature.

2.2.3. Chemical Characterization

The chemical characterization of oil involves assessing quality indicators and the Fourier transform infrared (FTIR) spectrum to ensure its freshness, purity, and suitability for use. The acid value measures free fatty acid levels to gauge freshness, while the iodine value indicates unsaturation and potential oxidation. Peroxide value points to initial oxidation stages, and both thiobarbituric acid reactive substances (TBARS) and anisidine values assess secondary oxidation products. The total oxidation value (TOTOX) offers a comprehensive view of oxidative stability. Meanwhile, the FTIR spectrum provides insights into the oil's chemical structure by identifying functional groups.

(1) Fish Oil Quality Indices. The critical quality parameters of oil including acid, iodine, peroxide, TBARS, anisidine, and TOTOX values were analyzed following the standard methods endorsed by the Official Methods and Recommended Practices of the AOCS [30], the International Union of Pure and Applied Chemistry as mentioned by Paquot [31], and the International Dairy Federation quoted by Zhang et al. [32].

For acid value assessment, a sample of 1 g of oil (m) was placed into a beaker containing 100 mL of ethanol at 95°C. To this mixture, two drops of 0.1 N phenolphthalein solution were added as an indicator. The solution was then titrated with 0.1 N (T) potassium hydroxide (KOH) until a color change was observed, indicating the endpoint of the titration. The volume (V1) of KOH required to reach this endpoint was recorded. A blank titration was also performed under the same conditions without the oil sample to account for any background reactions. The volume (V0) of KOH used in the blank titration was noted. The acid value (mgKOH/g oil) is calculated using the following formula:(1)Acid value=V1−V0m×56,1×T.

As for iodine value determination, a 0.2 g sample of oil (m) was accurately weighed and transferred into a flask. To this, 15 mL of 80% carbon tetrachloride (CCl_4) solution and 25 mL of Wijs reagent were added. The flask was then hermetically sealed, gently agitated, and stored in a dark location for 1 hour to allow the reaction to proceed. Following the incubation period, 20 mL of 10% aqueous potassium iodide (KI) solution, 15 mL of distilled water, and 5 drops of 1% starch solution were introduced to the mixture. The resulting solution was titrated with 0.1 N (T) sodium thiosulfate until a color change indicated the endpoint. The volume of sodium thiosulfate used in the titration (V1) was recorded. A blank titration was also conducted, following the same steps but without the oil sample, to measure the background volume (V0) of sodium thiosulfate. The iodine value (g I_2/100 g oil), which indicates the amount of iodine absorbed by the fats and oils in the sample, was calculated using the following formula:(2)Iodine value=V0−V1 m×12,69×T.

Concerning peroxide value assessment, in a clean glass tube, 50 mg of the oil sample was introduced. To this, 9.8 mL of a chloroform-methanol mixture in a 7 : 3 ratio was added, and the contents were thoroughly mixed. Next, 50 μL of a 30% ammonium thiocyanate solution was added, followed by another round of mixing using a vortex mixer for 2–4 seconds. This was immediately followed by the addition of 50 μL of an iron (II) chloride solution. The test tube was then vortexed again for 2–4 seconds to ensure complete mixing. The test tube was left to incubate at room temperature for 5 minutes. After incubation, the absorbance of the reaction mixture was measured at 500 nm, against a blank that contained all the reactants except the oil sample. The entire procedure was conducted in a dimly lit environment and completed within 8 minutes for each test to prevent light-induced changes. The peroxide value (mEqO_2/kg oil) was calculated from the ferric ion (Fe^{3+}) calibration curve using the following formula:(3)Peroxide value=As−Ab×y55,84×m,where As is the absorbance of the sample; Ab is the absorbance of the blank; y is the slope obtained from the calibration curve (with a value of 38.46); m is the mass of the oil sample in g; and 55.84 is the molar mass of iron.

Regarding the TBARS assay, a 1 g sample of oil was dissolved in 10 mL of carbon tetrachloride (CCl_4). To this solution, 10 mL of 0.67% thiobarbituric acid (TBA) solution and an equal volume of glacial acetic acid were added. The mixture was stirred intermittently over 2 hours and subsequently centrifuged at 1000 rpm for 5 minutes. The

aqueous phase was carefully separated and subjected to incubation in boiling water for 1 hour. After incubation, the absorbance of the solution was measured at 532 nm. The concentration of TBARS (μmol MDA/Kg oil) was calculated using the following formula:(4)Thiobarbituric acid value=Absd×m,where Abs is the absorbance reading at 532 nm; *d* is the path length of the curve (tank thickness); and *m* is the mass of the oil sample in g.

To determine the anisidine value, 0.5 g of oil were dissolved in a 25 mL volumetric flask. The volume was then made up to the mark with isooctane. Then, 5 mL of the above-prepared solution (oil and isooctane) was transferred into a test tube. To this test tube, 1 mL of p-anisidine (0.25 % in glacial acetic acid) was added. The mixture in the test tube was well stirred and then left to stand in the dark for 10 minutes to react. The absorbance of the solution was measured at 350 nm. A blank test was also performed using the same procedure but without the oil sample. The anisidine value was calculated using the following formula:(5)Anisidine value=25×1,2×As−Abm,where As is the absorbance of the lipid solution after reaction with anisidine; Ab is the absorbance of the lipid solution (blank); and *m* is the mass of the oil sample in g.

The TOTOX was determined using the equation: TOTOX = 2 × peroxide value + anisidine value. This formula quantifies the overall extent of oil oxidation by combining the peroxide value, which measures primary oxidation products, with the anisidine value, indicating secondary oxidation products.

(2) Fourier Transform Infrared Spectra Analysis. Infrared (IR) spectra ranging from 3800 to 500 cm^{-1} were captured using a tensor 27 (Bruker, Wissembourg, France). This device was paired with an ATR prism crystal accessory and an MCT (mercury cadmium telluride) detector. The resolution of the spectra was set at 4 cm^{-1}. The measurements were conducted at room temperature using approximately 2 μL of fish oils. These oils were placed on the surface of the ATR crystal and pressed with a flat-tip plunger until spectra with suitable peaks were obtained. The background was then subtracted using the OPUS version 6.3.2 spectrum software (PerkinElmer Inc.).

2.2.4. Antibacterial Activity

The antibacterial properties of the oil were assessed using the broth microdilution method in 96-well microplates. This method is in accordance with the standards set by the Clinical and Laboratory Standards Institute [33].

(1) Preparation of Stock Solutions of Oils and Antibiotic. The oils were prepared as stock solutions at a concentration of 1024 mg/mL in a 5% solution of Tween 80. Additionally, the antibiotic ciprofloxacin was prepared at a concentration of 256 μg/mL.

(2) Inoculum Preparation. Muller–Hinton agar culture was used to prepare the bacterial strain's inoculum. Bacterial suspensions were created with a concentration of approximately 1.5×10^8 CFU/mL, equivalent to McFarland turbidity standard no. 0.5. The inoculum was then obtained by diluting this suspension 100 times, resulting in a final concentration of 1.5×10^6 CFU/mL.

(3) Antibacterial Activity Evaluation of Fish Oils. Each well of the microplate was filled with 100 μL of culture broth (MHB). Then, 100 μL of oil was added to the top wells, and a series of twofold dilutions were performed to achieve a final concentration ranging from 2 to 256 mg/mL in a total volume of 100 μL/well. Each well was further diluted with 100 μL of inoculum. The plates were then incubated at 35°C for 18 hours. Growth was monitored using p-iodotetrazolium chloride (0.2 mg/mL) oil [34]. Viable bacteria changed the yellow dye of iodonitrotetrazolium into a pink color. The lowest concentration of oil, at which no visible color change was noted, was considered as the minimum inhibitory concentration (MIC). Ciprofloxacin was used as a positive control in this experiment.

2.3. Data Handling and Statistical Analyses

The area under the curve (AUC) from day 0 to day 180 was calculated for each parameter, for both quality indices and antibacterial activity, under each storage condition and treatment. The value at day 0 was used as the baseline. The AUC calculation was used to compare each parameter and the effects of storage temperature and different stabilizers.

The results were presented as the mean ± standard deviation. The statistical significance was determined using Student's *t*-test and one-way analysis of variance (ANOVA), followed by Turkey's post hoc tests for pairwise separation and comparison of means. The statistical analysis was performed using GraphPad Prism software version 5.9 (GraphPad Software; La Jolla, California, USA).

3. Results and Discussion

The phenolic content of the spices, determined through the Folin–Ciocalteu method relative to the dry extract, was found to be 25.67 ± 1.01 mgGAE/g for *A. sativum*, 21.37 ± 0.98 mgGAE/g for *P. nigrum*, and 22.5 ± 0.51 mgGAE/g for *M. myristica*. Regarding flavonoid levels, *A. sativum* exhibited 5.88 ± 0.24 mgQE/g, *P. nigrum* exhibited 6.71 ± 0.21 mgQE/g, and *M. myristica* exhibited 6.56 ± 0.51 mgGAE/g. The presence of these phenolic and flavonoid compounds underscores the potential of these spices as dietary antioxidants. Generally, the greater the concentration of these compounds, the stronger the antioxidant capacity is expected to be [25]. Of the three, *A. sativum* boasts the highest phenolic content, suggesting it may have the strongest antioxidant effects. Although *A. sativum* presents the lowest flavonoid concentration, both *P. nigrum* and *M. myristica* exhibit higher levels, potentially amplifying their antioxidant efficacy. This evidence further validates the selection in this study of these three spices as natural antioxidants, offering protection against the degradation of fish oil quality during extraction processes and storage. Additionally, the inclusion of these compounds in the extracted oil is likely to enhance its antibacterial properties [23, 25].

Tables 1 and 2 provide a comprehensive overview of the quality indices and antibacterial properties of *F. margarita* liver oil, respectively. These tables detail how these characteristics change over time, with different storage temperatures and stabilizers. Table 1 includes quality indices such as acid value, iodine value, thiobarbituric acid value, peroxide value, anisidine value, and TOTOX. These indices are crucial for assessing the quality and stability of the oil. However, Table 2 presents the antibacterial properties of the oil. This table shows how these properties vary with storage time, temperature, and the use of different stabilizers.

Table 1

Effect of spices and temperature storage on the quality indices of *Fontitrygon margarita* liver oil.

Parameters	Treatment	Standard	Day 0	Storage					
Room temperature (28 ± 2°C)			Refrigeration (4°C)			Day 60	Day 120	Day 180	Day 60
Day 120	Day 180	Acid value (mgKOH/g)	Control	**≤3**	**2.15 ± 0.84**	3.15 ± 0.04*	4.64 ± 0.65***	5.32 ± 0.20***	2.51 ± 0.03
2.87 ± 0.23	3.72 ± 0.10*	BHT	**1.68 ± 0.17**	2.52 ± 0.40*	2.80 ± 0.50**	3.62 ± 0.50***	2.22 ± 0.20*	2.45 ± 0.14*	2.86 ± 0.21**
A. sativum	**1.82 ± 0.21**	3.04 ± 0.02**	3.73 ± 0.32***	3.95 ± 0.41***	2.38 ± 0.39	2.78 ± 0.43*	3.20 ± 0.12**	*P. nigrum*	**1.95 ± 0.32**
3.12 ± 0.50**	3.51 ± 0.27**	3.75 ± 0.20**	2.30 ± 0.40	2.81 ± 0.05*	3.56 ± 0.14**	*M. myristica*	**2.05 ± 0.41**	3.40 ± 0.26*	3.63 ± 0.26*
4.01 ± 0.74**	2.39 ± 0.13	2.71 ± 0.05*	3.62 ± 0.63**	-					

Iodine value (gI_2/100 g)	Control	—	**106.65±5.00**	104.55±1.55	101.53±1.66	95.12±2.06*	104.52±2.69	100.81±2.39	98.12±3.10
BHT	**108.71±4.30**	104.27±2.80	100.44±3.90	98.21±3.10*	105.04±3.20	104.30±2.80	103.12±2.50	*A. sativum*	**107.50±6.10**
103.70±2.90	101.70±4.01	97.35±2.90*	104.20±3.30	102.20±4.02	100.54±3.20	*P. nigrum*	**108.35±5.20**	105.35±2.9	103.21±3.10
99.75±3.20	106.12±3.50	104.12±2.50	100.22±4.50	*M. myristica*	**106.86±3.70**	104.62±3.30	100.44±3.90	95.10±1.01*	104.61±4.70
101.30±2.80	99.33±1.40	-							
Thiobarbituric acid value (μmol MDA/Kg)	Control	**≤10**	**3.20±0.14**	4.77±0.27*	7.94±0.32**	10.54±0.30***	5.13±0.59*	6.64±0.28**	8.16±0.27***
BHT	**2.93±0.25**	4.60±0.88*	6.02±0.15**	7.32±0.15***	3.14±0.34	5.94±0.21*	6.81±0.60**	*A. sativum*	**2.95±0.21**
5.29±0.32**	6.58±0.50**	8.79±0.08***	4.59±0.04*	5.11±0.47**	6.40±0.01**	*P. nigrum*	**2.98±0.23**	5.84±0.32**	6.79±0.08**
7.62±1.00***	4.40±0.01*	5.14±0.21**	6.91±0.21**	*M. myristica*	**2.98±0.19**	5.12±0.16**	7.11±0.3***	8.43±0.20***	3.61±0.23*
5.47±0.11**	7.14±0.21**	-							
Peroxide value ($mEqO_2$/kg)	Control	**≤5**	**3.57±0.34**	4.60±0.04*	6.11±0.80*	7.50±0.20**	3.87±0.20	4.95±0.34*	5.83±0.60*
BHT	**2.99±0.37**	3.94±0.90*	5.68±0.01*	6.29±0.03**	3.69±0.10	4.16±0.22	5.22±0.30*	*A. sativum*	**3.27±0.22**
4.30±0.09*	5.28±0.26*	6.69±0.15**	4.12±0.30	4.82±0.20*	5.69±0.27*	*P. nigrum*	**3.46±0.16**	4.51±0.11*	5.20±0.32*
6.72±0.26**	3.77±0.44	4.52±0.12*	5.40±0.16*	*M. myristica*	**3.40±0.19**	4.69±0.02*	6.15±0.03**	6.94±0.80**	3.57±0.25

4.63±0.41*	5.11±0.12*	-							
Anisidine value	Control	**≤20**	**3.32±0.80**	6.81±0.59*	9.24±0.51**	13.08±1.11***	5.31±0.27*	7.16±0.89*	9.09±1.27**
BHT	**3.19±0.51**	5.33±0.97*	7.40±1.75*	10.88±2.11***	3.60±0.88	5.08±1.11*	7.05±1.20*	*A. sativum*	**3.26±0.70**
6.03±0.64*	8.44±2.26*	10.49±0.82***	4.27±1.80	6.18±0.97*	8.40±2.27*	*P. nigrum*	**3.26±1.13**	6.93±0.47*	9.39±0.41**
11.08±1.11***	4.06±0.67	6.40±2.27*	8.96±1.05**	*M. myristica*	**3.29±0.54**	6.57±0.39*	8.99±0.93**	11.65±2.06***	4.57±2.70
6.18±1.95*	8.77±2.01**	-							
TOTOX	Control	—	**10.46±0.57**	16.01±0.31**	21.46±0.65***	28.08±0.66***	13.05±0.23*	15.73±1.23**	20.75±0.94***
BHT	**9.17±0.44**	13.21±0.97*	18.76±0.88***	23.46±1.07***	10.74±0.57	14.34±0.76**	17.27±0.66***	*A. sativum*	**9.80±0.46**
14.63±0.37*	19.00±1.26***	23.87±0.49***	11.65±0.95	14.50±0.59**	18.84±1.29***	*P. nigrum*	**10.18±0.64**	15.95±0.29**	19.79±0.36***
24.52±0.69***	12.30±0.49	16.04±1.18**	20.34±0.66***	*M. myristica*	**10.09±0.37**	15.95±0.21**	19.29±0.48***	25.53±1.43***	12.11±1.57

BTH: butylhydroxytoluene; *significantly different at P<0.05 as compared to day 0; **significantly different at P<0.01 as compared to day 0; ***significantly different at P<0.001 as compared to day 0. In bold, we have values for oil freshly extracted.

Table 2

Effect of spices and temperature storage on the antibacterial activity of *Fontitrygon margarita* liver oil.

Bacteria		Treatment	CMI (mg/ml)					
ATB	FISH oil				Day 0	Storage		
Room temperature (28±2°C)		Refrigeration (4°C)			Day 60	Day 120	Day 180	

Day 60	Day 120	Day 180	*Escherichia coli*	ATCC 10536	Control	**16**	**32**	64	128	256
32	64	128	BHT	**32**	32	64	128	32	64	64
A. sativum	**16**	32	64	128	32	32	64	*P. nigrum*	**32**	64
128	256	32	64	64	*M. myristica*	**32**	64	64	128	32
64	128	EC 137	Control	**8**	**16**	32	64	128	16	32
64	BHT	**16**	32	128	128	32	64	64	*A. sativum*	**16**
32	64	64	32	64	64	*P. nigrum*	**16**	32	64	256
32	64	64	*M. myristica*	**32**	32	64	128	64	64	128
–										
Enterobacter cloacae	ENT 119	Control	**8**	**16**	32	64	128	16	32	64
BHT	**16**	32	64	64	32	64	64	*A. sativum*	**16**	32
64	128	32	32	64	*P. nigrum*	**16**	64	128	256	32
64	64	*M. myristica*	**32**	32	64	64	64	128	128	ENT 51
Control	**8**	**16**	32	64	128	16	32	64	BHT	**16**

64	128	128	32	64	128	*A. sativum*	**16**	32	64	128
32	32	64	*P. nigrum*	**16**	32	64	128	32	64	64
M. myristica	**16**	32	128	128	32	64	128			
Klebsiella pneumonia	KL 11	Control	**4**	**16**	64	128	256	32	64	64
BHT	**16**	32	64	128	32	64	64	*A. sativum*	**16**	32
64	128	32	64	64	*P. nigrum*	**32**	64	128	256	32
64	64	*M. myristica*	**16**	32	64	128	32	64	64	
Salmonella enterica serovar typhi	SAL 9	Control	**4**	**32**	64	128	256	64	64	128
BHT	**16**	64	128	128	32	64	64	*A. sativum*	**32**	64
128	128	32	32	64	*P. nigrum*	**16**	64	128	128	32
64	128	*M. myristica*	**32**	64	128	256	32	64	64	ATCC 28579
Control	**4**	**16**	64	128	256	32	64	128	BHT	**16**
64	128	128	32	128	128	*A. sativum*	**16**	32	64	64

64	64	64	*P. nigrum*	**16**	32	64	128	32	64	128
M. myristica	**16**	32	64	128	32	128	128			
Citrobacter freundii	CITB 81	Control	**16**	**32**	64	128	256	64	64	128
BHT	**32**	64	128	128	64	64	128	*A. sativum*	**32**	64
128	128	64	64	128	*P. nigrum*	**32**	64	128	256	64
64	128	*M. myristica*	**32**	64	128	128	64	64	128	
Yersinia enterocolitica	YERB 1	Control	**16**	**16**	32	64	128	32	64	64
BHT	**16**	64	128	256	32	64	128	*A. sativum*	**16**	32
64	128	32	32	64	*P. nigrum*	**16**	32	64	128	32
32	64	*M. myristica*	**16**	64	128	128	32	64	128	
Staphylococcus aureus	ST 120	Control	**8**	**32**	64	128	256	64	64	128
BHT	**32**	32	64	128	64	64	128	*A. sativum*	**16**	64
128	128	32	32	64	*P. nigrum*	**32**	64	128	256	32

BTH: butylhydroxytoluene. In bold, we have values for oil freshly extracted.

3.1. Effect of Stabilizers on Properties of *Fontitrygon margarita* Liver Oil during Extraction

3.1.1. Physicochemical Parameters

As indicated in Table 1, the acid, iodine, peroxide, anisidine values, and total oxidation of the extracted oil (day 0) did not significantly differ compared with the control, oil extracted with BHT, and those extracted with spices.

Similarly, there were no significant differences between the spices. However, these oil samples remain compliant with the 2021 Codex Alimentarius Commission [35]. This could be attributed to the limited time of exposure of the oil to heat (10 min), thus limiting the reaction of oxygen with double bonds. Furthermore, the infrared spectroscopy conducted between 3800 and 500 cm^{-1} to identify the functional groups present in *F. margarita* liver oil after extraction (Figure 1) revealed similar spectra of whether the oil was extracted with stabilizers or not. The five spectra had the same shape and intensity, as they originated from the same fish. This suggests that the nature and content of the chemical compounds present in the oil are relatively the same, which is consistent with the quality index results. Generally, the FTIR spectrum exhibited similar regions of functional group vibrations as reported previously for *Fontitrygon margarita* liver oils extracted without stabilizer oil [2].

[figure(s) omitted; refer to PDF]

3.1.2. Antibacterial Properties

As observed in Table 1, oil samples extracted with a stabilizer (spices or BHT) exhibited antibacterial activity immediately after extraction, compared with the control sample, regardless of the bacterial strain. Similarly, irrespective of the presence of stabilizers, the antibacterial activity of the different oil samples had a MIC between 16 mg/mL and 32 mg/mL for all the bacterial strains tested. This reflects the activities of these oils against bacteria responsible for food-borne diseases. For all these oil samples, the best antibacterial activity (MIC = 16 mg/mL) was obtained on the bacterial strain *Yersinia enterocolitica* (YERB 1), while the lowest antibacterial activity (MIC = 32 mg/mL) was obtained on the bacterial strain *Citrobacter freundii* (CITB 81). The antibacterial properties observed may be attributed to the presence of both saturated and PUFAs in the oil samples, notably the ω-3 family, in which EPA and DHA, along with the ω-6 family comprising linolenic acid and arachidonic acid, have been identified as key contributors. These fatty acids, as characterized in the fatty acid profiles from our previous research [2], are known for their potent antimicrobial activities. The harmful impact of these fatty acids on bacterial cells is due to their surfactant nature, which facilitates their interaction with the cell membrane, leading to the formation of temporary or permanent pores of varying sizes. This interaction increases the membrane's permeability and fluidity, causing the leakage of cellular contents, which may result in growth inhibition, cell lysis, or cellular death. Moreover, saturated fatty acids have been shown to trigger autolysis in bacterial cell walls for certain species, attributed to a decrease in membrane fluidity [4, 5].

3.2. Effect of Storage at Room Temperature on Properties of *Fontitrygon margarita* Liver Oil

3.2.1. Physicochemical Parameters

As indicated in Table 1, the acid, peroxide, thiobarbituric acid, and anisidine values, as well as the total oxidation of *F. margarita* liver oil extracted without a stabilizer (control) and stored at room temperature, significantly increased with storage time. This suggests a deterioration in the quality of fish oil during storage at room temperature. In fact, after 120 and 180 days of storage, respectively, the peroxide (6.11 ± 0.8 $mEqO_2$/kg) and thiobarbituric acid (10.54 ± 0.30 μmol MDA/Kg) values obtained from the oil were not compliant with the 2021 Codex Alimentarius Commission Standard guidelines [35]. However, the anisidine index (13.08 ± 1.11) remained within the standard limits even at the 180th day of storage. This deterioration in the quality of the oil is mainly due to its exposure at room temperature, as heat destabilizes the C=C double bond and initiates the oxidation reaction. Almeck et al. [36] showed that the acid and peroxide index of *Canarium schweinfurthii* fruit pulp oil packaged in transparent bottles increased significantly during storage at 25°C (room temperature). The acid index increased from 6.93 mgKOH/g on the extraction day to 8.77 mgKOH/g after 6 months of storage, while the peroxide index went from 3.4 $mEqO_2$/kg to 17.06 $mEqO_2$/kg in the same time interval. In addition, autoxidation could be the cause of the increased formation of peroxides during storage.

Figure 2 presents the FTIR spectra of *F. margarita* liver oil extracted without stabilizers (control) and stored at room temperature over time. Unlike the spectra in Figure 1, those in Figure 2 show the presence of peaks at 3400 cm^{-1}, indicating the presence of hydroxyl groups. This peak is more pronounced for oil samples stored for 180 days. This result shows that hydroperoxides are formed gradually during storage at room temperature. This could also suggest that long-term exposure of the oil at room temperature favors the hydrolysis of triglycerides, leading to the formation

of free fatty acids. Similar bands were found when analyzing the lipid oxidation of catfish after cooking and smoking by different methods oil [37], and in oils from Lutjanus dentatus oil extracted by drying at 45°C for 24 hours or by cooking in a pressure cooker at 95°C for 20 minutes [38]. The peak at 3014 cm^{-1} characteristic of the cis double bond (=CH) provides information on the degree of lipid unsaturation. This peak was high at extraction and low after 180 days of storage. The decrease reflects the degradation of the double bonds during storage. This observation correlated with the iodine value result. The peak at 1654 cm^{-1} attributed to the vibration strain of the elongation of C=C (cis) and those appearing at 718 cm^{-1} linked to the vibration in the molecules analyzed of the double cis bonds also reflect the double bond present in unsaturated fatty acid oil. These peaks are observable in the sample before storage and are less pronounced in the samples stored for 120 and 180 days at room temperature. The major structural modification of polyunsaturated lipids during oxidation reactions is the decrease in ethylenic double bonds caused by the attack of oxygen with the formation of oxidation compounds [39]. The band characteristic of the C=O ester group of triglycerides was found around 1745 cm^{-1}, whereas the values of 1746 cm^{-1}, 1743 cm^{-1}, and 1750 cm^{-1} were reported by Guillèn et al. [40], Giménez et al. [41], and Tenyang et al. [37, 42], respectively. The sample stored for 180 days presented the weakest band. This means that this sample had low carbonyl groups of ester linkage of triglycerides and stipulated that alterations in this sample are higher. This is in agreement with the results obtained by Tenyang et al. [37, 42].

[figure(s) omitted; refer to PDF]

3.2.2. Antibacterial Properties

As noted in the quality indices, the antibacterial activity of *F. margarita* liver oil gradually decreases during storage at room temperature, as shown in Table 2. In fact, the MICs of the oil on bacterial strains of *E. coli* (EC 137), *E. cloacae* (ENT 119 and ENT 51), and *Y. enterocolitica* (YERB 1) increased from 16 mg/mL on day 0 to 128 mg/mL after 180 days of storage at room temperature. A similar reduction in antibacterial activity was observed on the bacterial strains of *E. coli* (ATCC 0536), *S. typhi* (SAL 9), *C. freundii* (CITB 81), and *S. aureus* (ST 120), which increased from 32 mg/mL at day 0 to 256 mg/mL at the 180th day of storage at room temperature. This could be due to the reduction in fatty acids present in the oil, whose antibacterial properties have been demonstrated by several researchers. Generally, unsaturated FFAs have greater antibacterial potential than saturated FFAs with the same carbon chain length. Also, a direct correlation exists between the number of double bonds in an unsaturated FFA's carbon chain and its antibacterial efficacy [43, 44].

3.3. Stabilization of *F. margarita* Liver Oil Properties

3.3.1. Effect of Storage Temperature

(1) Physicochemical Parameters. Figure 3 illustrates the AUC of the quality indices of *F. margarita* liver oil according to the storage temperature. It is observed that, regardless of the quality index, the oil stored in the refrigerator had a significantly lower AUC than the oil stored at room temperature, for both oxidation markers and acid value. This indicates that refrigeration slows down the deterioration of fish oil, likely because low temperatures limit the destruction of C=C double bonds. Haffaf and Lardjane [45] demonstrated that oil stored in the refrigerator slowed down the evolution of the peroxide index compared with oil exposed to the sun. Similarly, Almeck et al. [36] showed that storage of *Canarium schweinfurthii* fruit pulp oil in a refrigerator slowed the formation of hydroperoxides compared with oil stored at 25°C (room temperature). As shown in Table 1, at the 180th day of storage, the acid and thiobarbituric acid values were 3.72 ± 0.10 mg KOH/g and 8.16 ± 0.27 μmol MDA/Kg when stored in a refrigerator, compared with 5.32 ± 0.20 mg KOH/g and 10.54 ± 0.30 μmol MDA/Kg for oil stored at room temperature, respectively. Also, when stored at room temperature, the peroxide value (6.11 ± 0.80 meqO_2/kg) exceeded the recommended limit after 120 days of storage, while it remained within the normal range (4.95 ± 0.34 meqO_2/kg) after the same period when stored in the refrigerator.

[figure(s) omitted; refer to PDF]

(2) Antibacterial Properties. Figure 4 illustrates the AUC of the antibacterial activity of *F. margarita* liver oil, with a focus on the impact of storage temperature. In particular, oil stored in the refrigerator demonstrated a significantly lower AUC across all tested bacterial strains than oil stored at room temperature. This suggests that the antibacterial

activity of *F. margarita* liver oil is better preserved in a refrigerator, potentially due to the refrigeration slowing down the degradation of the C=C double bonds in the oil's unsaturated fatty acids, which are believed to be responsible for its antibacterial properties. Herndon et al. [4] and Noutsa et al. [2] reported that the antibacterial activity of fish oil is positively correlated with unsaturation levels. As shown in Table 2, the MIC of oils ranged from 16 mg/mL to 128 mg/mL for the strains of *E. coli* (EC 137), *Y. enterocolitica* (YERB 1), *E. cloacae* (ENT 51), and *K. pneumoniae* (KL 11) at the 180th day when stored at room temperature. However, for oil stored in a refrigerator, the MIC only increased from 16 mg/mL to 64 mg/mL for these bacteria within the same period. Similarly, the MIC of oil increased from 32 mg/mL to 256 mg/mL for the strains of *E. coli* (ATCC 10536), *S. typhi* (SAL 9), *C. freundii* (CITB 81), and *S. aureus* (ST 120) at the 180th day of storage at room temperature, while the MICs only varied from 32 mg/mL to 128 mg/mL for these bacteria within the same period for oil stored in a refrigerator. In conclusion, storing *F. margarita* liver oil in a refrigerator appears to better maintain its antibacterial activity compared with storage at room temperature.

[figure(s) omitted; refer to PDF]

3.3.2. Effect of Stabilizers

(1) Physicochemical Parameters. Figure 5 demonstrates that *F. margarita* liver oils extracted with *A. sativum*, *P. nigrum*, *M. myristica*, and BHT had a significantly lower AUC than in the control oil (extracted without a stabilizer). This suggests that the inclusion of spices significantly mitigated the deterioration of fish oil stored at room temperature by decelerating the oxidation reaction. The antioxidant activity of these spices is often linked to the presence of phenolic compounds, which are known to capture free radicals and inhibit the release of superoxide radicals [46, 47]. This finding aligns with the work of Womeni et al. [27], who found that the rate of decrement in the iodine value of crude soybean oil was higher in the oil without spices than that containing the spices, *Z. officinale*, *X. parviflora*, *M. myristica*, *A. sativum,* and others. Similarly, Loungaing et al. [48] observed that the peroxide and anisidine index of palm oleic acid enriched with ginger extract had a significantly slower evolution during frying than in the oil sample not containing spices. In the comparative analysis of spices, *A. sativum* demonstrated the most significant efficacy, as evidenced by its lower AUC values relative to *P. nigrum* and *M. myristica* across various metrics, including acid, iodine, thiobarbituric acid, peroxide, and anisidine values. This potent activity correlates with the phenolic content found in these spices, with *A. sativum* extract showing higher levels than its counterparts. Consequently, *A. sativum* may offer superior effectiveness in maintaining fish oil quality throughout storage.

[figure(s) omitted; refer to PDF]

(2) Antibacterial Properties. As depicted in Figure 6, the AUC of the antibacterial activity of *F. margarita* liver oil stored at room temperature with the addition of spices shows interesting results. In particular, oil extracted with *A. sativum* and *M. myristica* had a significantly lower AUC than oils extracted without spices on 7 and 6 bacterial strains tested, respectively. In contrast, oil extracted with *P. nigrum* showed a significantly low AUC compared with the control oil extracted without spices on only 3 bacterial strains and was comparable on 4 other bacterial strains. This suggests that the addition of *A. sativum*, *M. myristica*, and *P. nigrum* helped to better preserve the antibacterial activity of *F. margarita* liver oil compared with when no spice was added. This could be due to the active principles contained in these spices as phenolic compounds, which, along with their antibacterial properties, may have acted as adjuvants by modulating the antibacterial activity of the oil. As shown in Table 2, after 180 days of storage at room temperature, the best antibacterial activity (CMI = 64 mg/mL) was obtained in oil extracted with *A. sativum* on *E. coli* (EC 137) and *S. typhi* (SAL 9; ATCC 28579). This was followed by oil extracted with *M. myristica,* which presented 7 CMIs of 128 mg/mL and 3 CMIs of 256 mg/mL. Cheradi and Sarni [49] demonstrated that bacterial strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *Escherichia coli* showed sensitivity to the aqueous extract of garlic. In addition to phenolic compounds, sulfur compounds such as allicin and diallyl disulfide present in garlic are believed to be responsible for this antibacterial activity. Oil extracted with *P. nigrum*, as well as the control sample without spices, each had 4 MICs of 128 mg/mL and 6 MICs of 256 mg/mL on the bacterial strains. Moreover, the addition of *A. sativum* and *M. myristica* overall better preserved the antibacterial activity of *F. margarita* liver oil during storage, especially on bacterial strains of *E. coli* (EC 137), *E. cloacae* (ENT 51 and ENT 119), *S. typhi* (SAL

9; ATCC 28579), and *Y. enterocolitica* (YERB 1) compared with the oil sample extracted with BHT.
[figure(s) omitted; refer to PDF]

3.3.3. Combined Effect of Storage Temperature and Stabilizers

Figure 7 provides an overview of the AUC of the oil quality indices, taking into account both storage temperature and the addition of spices. Notably, for the thiobarbituric acid index, anisidine values, and total oxidation, the oils extracted with *A. sativum*, *P. nigrum*, *M. myristica*, and BHT and stored in a refrigerator had significantly lower AUC than oils extracted with spices and stored at room temperature, and those extracted without spices and stored in a refrigerator. Furthermore, the iodine value of these oils had a significantly low AUC compared with oil extracted with spices stored at room temperature and was lower or equal to oils extracted without spices stored in a refrigerator. This suggests that the combination of adding spices to *F. margarita* liver oil and storing it in a refrigerator significantly reduced the deterioration process of the oil compared with just adding spices or only storing it in a refrigerator. As previously mentioned, both refrigeration temperature and the addition of spices are limiting factors for oil oxidation [27, 45]. Regarding acid and peroxide values, with the exception of *A. sativum* spice, oils extracted with stabilizers had a significantly lower AUC than oil extracted with spices stored at room temperature and were lower or equal to oil extracted without spices and stored in a refrigerator.
[figure(s) omitted; refer to PDF]

(1) Antibacterial Properties. Figure 8 provides a comparison of the AUC of the antibacterial activity of *F. margarita* liver oil, considering both storage temperature and the addition of spices. It is evident that oils extracted with various spices (*A. sativum*, *P. nigrum*, and *M. myristica*) and stored in a refrigerator had a significantly lower AUC than oils extracted with spices but stored at room temperature across all tested bacterial strains. The only exception was the oil extracted with *M. myristica* from the bacterial strains of *E. coli* (EC 137), *E. cloacae* (ENT 119), and *S. typhi* (ATCC 28579). This reinforces the observation that storage in a refrigerator helps preserve the antibacterial activity of the oil compared with storage at room temperature. Moreover, the oil extracted with these spices and stored in a refrigerator had an AUC less than or equal to oil samples extracted without spices but stored in a refrigerator, particularly on the bacterial strains of *E. coli* (ATCC 10536), *K. pneumoniae* (KL 11), *S. typhi* (ATCC 28579; SAL 9), *S. aureus* (ST 120), *Y. enterocolitica* (YERB 1), and *C. freundii* (CITB 81). The only exception was the oil extracted with *M. myristica* from the bacterial strains of *S. typhi* (ATCC 28579) and *Y. enterocolitica* (YERB 1). This indicates that the combination of spices and storage in a refrigerator incrementally sustained the antibacterial activity of the oil compared with just storing it in a refrigerator.
[figure(s) omitted; refer to PDF]

4. Conclusion

The study of *Fontitrygon margarita* liver oil has revealed that both storage duration and temperature significantly impact the oil's quality and antibacterial properties. Over time, the quality index and antibacterial activities of the oil tend to decrease. The degradation of oil quality is more pronounced when the oil is stored at room temperature. However, the addition of stabilizers to *F. margarita* liver oil prior to extraction, or storing the oil in a refrigerator, can mitigate this degradation by preserving the oil's chemical composition and antibacterial properties. The combination of adding stabilizers and storing *F. margarita* liver oil in a refrigerator proved to be the most effective method for preserving the oil's quality and antibacterial properties during storage. Given its antibacterial properties, *F. margarita* liver oil holds significant potential for the nutraceutical industry and could be used as a dietary supplement. This research underscores the importance of proper storage conditions and the use of stabilizers in maintaining the quality of such valuable natural resources.

Looking ahead, future research should focus on the oil's long-term stability, examining its shelf life over extended periods and the impact of various stabilizing agents. Studies should also delve into the influence of natural stabilizer concentrations and quantify the levels of phenolic compounds present in the oils. To establish the oil's suitability for health-related uses, clinical trials are essential to evaluate its effectiveness and safety profile as a dietary supplement or in other therapeutic contexts. Exploring alternative extraction techniques could potentially enhance the oil's quality or yield without compromising its intrinsic properties. Investigating the synergistic effects of

combining *F. margarita* liver oil extracted with other natural substances could further amplify its antibacterial capabilities. A thorough investigation into the molecular mechanisms underlying the oil's antibacterial action could pave the way for targeted medical applications. Additionally, conducting a market analysis would shed light on potential commercial prospects and consumer preferences for products derived from *F. margarita* liver oil.

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DETAIL

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Physicochemical and Rheological Characterization of a Novel Manna Exudate from Alhagi pseudalhagi (Iranian Tarangabin)

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ABSTRAK (ENGLISH)

Tarangabin manna (TM) is a resinous substance having a yellowish sticky character with a reasonably sweet taste. It is largely collected in Iran and Afghanistan. This study for the first time presents a comprehensive investigation of the techno-functional, rheological, and interfacial characteristics of water-soluble components for TM. The composition analysis revealed protein, moisture, fat, ash, and carbohydrate contents of 1.58, 2.98, 0.51, 2.04, and 92.90%, respectively. The effects of TM concentration on the physicochemical, structural, rheological, interfacial, emulsion, and foaming ability and stability were evaluated. X-ray diffraction analysis showed an amorphous structure for the purified sample and a crystalline structure for the raw sample. TM solutions exhibited Newtonian behavior,

with the apparent viscosity decreasing as temperature increased, fitting well with the Arrhenius model. The TM solutions exhibited weak viscoelastic properties, primarily demonstrating a dominant viscous character. The surface tension and interfacial tension of the TM solution prepared at a concentration of 50% were measured at 45.23 mN/m and 7.74 mN/m, respectively. The contact angle of the dry thin layer of TM was determined to be 31.74°. Remarkably, the TM solution at a concentration of 50% exhibited the highest foaming ability (76.80%), foaming stability (91.92%), and emulsifying activity index (24.53%). The findings, coupled with TM appropriate foaming ability and stability, sweetness, and characteristic flavor, suggest that TM holds potential as a special food ingredient.

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1. Introduction

Tarangabin manna (TM) is sweet, yellowish in color and semiliquid exudate, created on the aerial parts of some Alhagi genera, such as *Alhagi pseudalhagi* or *Alhagi maurorum* (Camelthorn). It is produced by an insect called Poophilus nebulosus Leth. (belongs to the genus Larinus, Cercopidae family, Homoptera phylum), which lives on the aerial parts of the plant. To be precise, TM is the exudation of this insect produced after nourishment on the plant, which crystallizes and dries on the plant. It should be pointed out that TM is not producible from all genera of Alhagi, while the climate condition also plays an important role in its formation. Special attention has been paid to TM in some major Materia Medica manuscripts of the Islamic era as one of the most commonly used medicinal matters in Islamic Traditional Medicine. In Greek medicine, “manna” is a collective term used to describe the extraction of sweet resin from the leaves and stems of the plant. *A. maurorum*, growing naturally in the Khorasan region (Iran’s North Eastern province), hosts an insect, which produces manna. Insects usually produce a gummy and sticky liquid, which is dried by the air and transformed into small, solid sticky particles. The manna sticks to the branches of *A. maurorum* [1–13].

When dry, it is fairly sweet yellowish resinous material resembling tear-shaped droplets measuring 1–3 mm. It has been widely used as a “herbal medicine.” However, its limited use as a food ingredient is likely due to low availability and lack of information. It has a significant carbohydrate content, specifically sucrose (up to 42%). It is also a good source of iron (782 mg/kg), zinc (18.6 mg/kg), and copper (23.3 mg/kg) [3, 4].

Extensive research has been conducted on the medicinal properties of TM, uncovering a multitude of benefits. These include its antiprotozoal, antibiotic, antimicrobial, antibacterial, antifungal, antinociceptive, anti-inflammatory, antiulcer, diuretic, antidiarrheal, antiurolithiasis, antiacetylcholinesterase, NADH oxidase activity, and antioxidant properties. Based on the Medica manuscripts of Islamic Traditional Medicine, TM is prohibited in acute fever, smallpox, typhoid, bloody diarrhea, and hemorrhage. Traditionally, TM has been used in combination with other substances to treat various ailments. For example, its combination with butter relieves dysuria, with fresh milk enhances libido, and with cumin alleviates flatulence. A study on the total aqueous fraction of Tarangabin manna has revealed its potential immunostimulatory effects in the human body [1, 5, 6].

There are several publications focusing on the insect (Poophilus nebulosus Leth.) and its host (A. maurorum) and sparse research on the medicinal properties of TM, but no published article is available covering a thorough research on the techno-functional, rheological, and interfacial characteristics of Tarangabin manna (TM). An article by Farahnaky et al. [7] published limited data on physicochemical and rheological properties of Gaz-angabin, an exudate from *Astragalus adscendens* by *Cyamophila dicora*.

Therefore, this study aims to provide comprehensive data on the characteristics of TM suitable for its possible application in the food and pharmaceutical industries. Hence, various aspects of TM collected from the northern region of Iran were thoroughly tested and analyzed. For this purpose, physicochemical characteristics such as protein, fat, ash, moisture, sugar type and content, zeta potential, molecular weight, contact angle, structural (XRD, FTIR, and SEM), rheological (apparent viscosity, temperature-dependent viscosity, amplitude, and frequency sweep), emulsifying (interfacial and surface tension, and emulsion activity), and foaming properties (foaming ability

and foaming stability) evaluated.

2. Material and Methods

2.1. Material

Tarangabin manna was collected from Kashmar, Khorasan Razavi, Iran. Canola oil was purchased from Narges Oil Company (Shiraz, Iran). Hexane, KBr, Toluene, and egg albumin were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. List of Abbreviations

Fourier-transform infrared spectroscopy (FTIR), double-distilled water (DDW), X-ray diffraction (XRD), molecular weight (MW), zeta potential (ZP), contact angle (CA), interfacial tension (IFT), scanning electron microscopy (SEM), water activity (aw), activation energy (Ea), linear viscoelastic region (LVE), emulsifying index (EAI).

2.3. Sample Purification

TM crude powder (400 g) was dissolved in 500 mL double-distilled water (DDW) for 12 hours and filtered by a cloth filter to remove soil, thorns, and rocks. The filtrate was centrifuged at 5000 g for 10 min to eliminate remaining solid residue. The supernatant was then lyophilized, and purified TM powder was kept in polyethylene bags at 4°C. Purified TM (water-soluble component) is used at 0, 5, 25, and 50% (W/W) concentration for further analysis.

2.4. Chemical Composition

The moisture, protein (expressed as % $N \times 5.75$), and ash content of the TM samples were determined according to the standard AOAC (2005) methods, specifically 925.1, 920.87, and 923.03, respectively. Each measurement was conducted in triplicate, and the reported values represent the average results [8].

2.5. Sugar Analysis by HPLC

Free sugar compositions were separately determined using the HPLC system (Knauer, Germany) equipped with Smartline pump 1000 and C18 column (Eurokat pb, particle size 10 μm, length 300 mm, internal diameter 10 mm, Knauer, Germany) and RI detector 2300 at 65°C. TM (10000 ppm) was dissolved in DDW. The mobile phase comprised a DDW at a flow rate of 0.8 mL/min. The findings were determined by internal normalization of the chromatographic peak area. Ingredients were reported by comparing the relative retention times (RT) of peaks with standard sugars. The standards were fructose, mannitol, sucrose, glucose, xylose, mannose, and arabinose at the concentration of 0–1000 μg/mL [9].

2.6. Fourier-Transform Infrared Spectroscopy (FTIR)

The lyophilized TM powder was converted to the pellets after mixing with KBr with a ratio of 1 : 99. The FTIR spectrum was recorded by Thermo Nicolet Avatar 370 (Madison, WI, USA) and was inspected in the wavenumber ranged from 4000 to 400 cm^{-1}.

2.7. X-Ray Diffraction (XRD)

XRD pattern was performed by an X-ray powder diffractometer (Bruker AFX D8, Germany) at 40 kV and 40 mA. Data were reported from 2θ of 5° to 70°. This test was carried out on both the physically cleaned crude manna and freeze-dried purified powder.

2.8. Molecular Weight (MW) Measurement

The MW of lyophilized TM powder was measured by a dynamic light scattering instrument (DLS, SZ100, Horiba, Japan) working under the static mode at scattering angle of 90° at room temperature [10]. Various concentrations (ranging from 0.625 to 5 mg/mL) of samples were prepared in DDW.

2.9. Zeta Potential (ZP)

ZP values of different TM solutions at concentrations of 1, 5, 25, and 50% were measured by the DLS technique (SZ100, Horiba, Japan) at room temperature using the Smoluchowski model. The samples were diluted with DDW in ratio of 1 : 100 before measurement [11].

2.10. Contact Angle (CA) and Interfacial Tension (IFT)

To measure the contact angle, thin layers of different TM solutions were applied onto glass slides and left to dry. A water droplet (2 μL) was then placed on the glass slide using a capillary tube. The contact angle of TM was determined using the static immobile drop method with a drop shape analyzer (DSA 100, KRÜSS GmbH, Hamburg,

Germany). A CCD high-definition camera with a soft-focus lens was used to capture an image of the droplet. The analysis was performed using the software provided with the drop-shape analyzer [12].

To assess the oil-water interfacial tension (IFT) of purified TM, the pendant drop method was employed using the DSA 100 drop-shape analyzer. A drop of the TM solution was formed from a capillary tube immersed in pure canola oil. A photograph of the solution drop was captured using a high-speed CCD camera equipped with a macro lens. The interfacial tension was measured at the point where the drop detached from the needle, and the calculation was based on processing the shadow of the digital photo and utilizing the Laplace–Young equation [13].

2.11. Scanning Electron Microscopy (SEM)

The surface morphology of the lyophilized TM powder was examined using a scanning electron microscope (SEM) (TESCAN Vega3, Czech Republic). The samples were affixed to an aluminum tape and coated with a thin layer of gold using a sputter coater (DSR1, Nanostructural Coating Co., Iran). Micrographs were captured at an accelerating voltage of 15 kV [14].

2.12. Rheological Properties

2.12.1. Apparent Viscosity

To assess the flow behavior of purified TM solutions (concentrations ranging from 1–50% w/v), measurements were conducted at 25°C using an Anton Paar MCR 302 rheometer (Graz, Austria). The rheometer was equipped with a cone-plate geometry (CP25-1) featuring a cone diameter of 25 mm, a gap size of 0.052 mm, and a cone angle of 1. To maintain a precise temperature control (±0.1°C), a Peltier system was employed [15].

2.12.2. Time Dependency

The time-dependent flow behavior characteristics of different TM solutions (1 to 50% w/v) were studied at shear rate (γ) of 100 s^{-1} over time (0 to 900 s).

2.12.3. Temperature Dependency

The apparent viscosity of purified TM solution at concentrations of 1, 5, 25, and 50% (w/v) was evaluated by heating the solutions from 20 to 60°C and cooling them from 60 to 20°C, at a constant shear rate of 100 s^{-1}. This study was conducted following the guidelines provided in Section 2.12.1. Furthermore, the observed viscosity behavior was fitted to an Arrhenius model using MATLAB R2018b (MATLAB®, ver.9.5.0, 2018).

2.12.4. Amplitude and Frequency Sweep Test

To assess the rheological properties of purified TM solutions, dynamic measurements were conducted at room temperature. A CP25-1 cone-plate geometry on an Anton Paar rheometer was utilized for these tests. The solutions were prepared at concentrations of 1, 5, 25, and 50% (w/v). Amplitude sweep tests were conducted, subjecting the solutions to shear strains ranging from 0.01% to 100% at a constant frequency of 1 Hz. Furthermore, frequency sweep tests were performed within the linear viscoelastic (LVE) region, varying the frequency from 0.01 Hz to 100 Hz while maintaining a steady shear strain of 1%.

For each test, a carefully measured volume of 2 mL TM solution was placed on the rheometer plate and allowed to settle for 15 minutes. RheoCompass™ software was employed to analyze the rheological parameters, providing insights into the flow behavior of the solution, whether it displayed elastic or viscous characteristics [16].

2.13. Surface Tension

To determine the surface tension of purified TM solution, samples of various concentrations (1, 5, 25, and 50%) were dissolved slowly in DDW using a magnetic stirrer for 15 minutes at 300 rpm. Subsequently, the samples were left at room temperature for one day to complete the dehydration process and allow the surface tension to reach equilibrium.

To accurately measure the surface tension at room temperature, a digital tensiometer (Kino Industry, A201, USA) was employed. This instrument provided precise readings of the surface tension for each sample, ensuring accurate analysis. During the experiment, the platinum plate was immersed in the liquid, and the sensor detected the balance value in this submerged state. Subsequently, this value was converted into the corresponding surface tension value, which was then displayed for further examination.

2.14. Emulsion Preparation and Characterization

To evaluate the emulsifying ability, purified TM solutions at concentrations 1, 5, 25, and 50% (w/v) were hydrated in 17.50 mL of DDW. Subsequently, 7.5 g of pure canola oil was slowly added to each solution. The mixtures were homogenized using an Ultra-Turrax (T18, IKA, Germany) at 15,000 rpm for 2 minutes, following the method described by Naji-Tabasi and Razavi [17]. The emulsifying ability of each concentration of TM solution was determined using the approach outlined by Naji-Tabasi and Razavi [17]. Specifically, emulsions of each concentration of TM (100 μL) were freshly added to 25 mL of DDW, and the absorbance of the resulting emulsions was measured at 500 nm. This allowed for the assessment of the emulsification effectiveness of TM at different concentrations.

2.15. Foam Preparation and Characterization

The capability of purified TM to prepare egg albumin foam was determined using the model discussed by Naji-Tabasi and Razavi [17]. Briefly, egg albumin (0.3% w/v) was dissolved with 20 mL of hydrated solution of TM at concentrations of 1, 5, 25, and 50% (w/v). Whipping was done intensely with speed of 15000 rpm by an Ultra-Turrax for 2 min. The amount of foam was calculated after production and then after 30 min. The ability of foaming and the stability of foaming were measured using the following equations:(1)Ability of Foaming %=Vf0V×100,(2)Stability of Foaming %=Vf30Vf0×100,where Vf0 is the volume of foam at the beginning, Vf30 is the volume of the foam after 30 minutes, and V is the whole volume of solution.

2.16. Statistical Analysis

All experiments were conducted using a completely randomized design with three replications. The analysis of variances was performed, and Duncan's test (SAS® software, ver. 9.1, SAS Institute Inc., NC, USA) was employed to compare the means. A significance level of $p<0.05$ was used to evaluate significant differences between the means in the statistical analysis of quantitative data [18].

3. Results and Discussions

3.1. Chemical Composition

Results demonstrated that TM contains 1.58% ± 0.23 protein, 2.98% ± 0.62 moisture, 0.51% ± 0.09 fat, 2.046% ± 0.06 ash, and 92.90% ± 0.53 carbohydrate. The TM is a rich source of carbohydrate. Aynehchi [19] reported that Tarangabin had 26.44% sucrose, 11.64% fructose, 12.4% mucilage, and 5.8% ash. Sherahi et al. [20] reported that moisture content, protein, ash, fat, and carbohydrate of *Descurainia sophia* seed gum were 5.14, 2.12, 3.01, 0.77, and 78.23%, respectively. Ramezany et al. [2] reported that the ash and moisture content of Persian Manna were 3.5% and 4.57%, respectively. The protein content in this manna has an important role in the emulsifying activity and reducing interfacial tension [21].

3.2. Sugar Analysis

HPLC is a highly accurate and reliable method for analyzing manna exudates. Since sugars are not typically detectable using ultraviolet light, RI detectors are employed for their detection. Figure 1(a) displays the HPLC chromatogram obtained from the analysis of manna exudates.

[figure(s) omitted; refer to PDF]

In this study, the important carbohydrates found in the TM exudates, namely, mannitol (538.85 ppm) and sucrose (418.27 ppm), were quantified by generating a calibration curve. Additionally, xylose (16.36 ppm) was detected in the sample with the retention time of 16.67, 8.98, and 8.08, respectively. However, fructose, glucose, and arabinose were not detected using HPLC.

Previous research by Fakhri et al. [22] highlighted mannitol as the primary sugar present in manna exudates from certain cotoneaster species. Similarly, Caligiani et al. [23] reported mannitol as the main sugar in manna exudates obtained from Sicilian Fraxinus excelsior L. Furthermore, Aynehchi [19] reported that Tarangabin contains 26.44% sucrose and 11.64% fructose. These findings provide valuable insights into the composition of TM and other manna exudates.

3.3. FTIR Spectroscopy

The purified TM powder underwent FTIR spectroscopy analysis to evaluate its functional groups and structural properties. Figure 1(b) displays the FTIR spectra of TM, and the corresponding peak assignments are provided in

Table 1.

Table 1

FTIR peak assignments of purified Tarangabin manna.

Wave number (cm^{-1})	Assignment
3276.25	-OH stretching vibrations
2924.21	-CH2- and >CH- stretching and bending vibrations
1627.21	C=C stretching vibration and C=O stretching vibration
1412.59	C=N stretching vibration
1335.46	HCOO-
1263.03	-OH bending vibration
1103.52	C-N (aliphatic amine)
1037.42	C-O-C stretching vibrations of glycosidic bonds
490.23	C-C-O and C-O-C

The broad absorption bands observed at 3276 cm^{-1} indicate the presence of various attributes, such as stretching bonds of free hydroxyl groups and O-H bands of carboxylic acid. Additionally, the absorption bands at 2924 cm^{-1} are assigned to stretching and bending vibrations of -CH2- and >CH- groups [24].

The absorption peaks at 1412 cm^{-1} and 1627 cm^{-1} indicate stretching vibrations of C=N and C=C bonds, respectively. Furthermore, the bands observed at 1037 cm^{-1} are attributed to stretching vibrations of C-O-C glycosidic bonds. The wide range of absorption between 3500 and 3000 cm^{-1} indicates the presence of functional groups like stretching bonds of free hydroxyl groups and O-H bands of carboxylic acid. The broad absorption bands at 3000 cm^{-1} and 2800 cm^{-1} are specified for stretching and bending vibrations of -CH2- and >CH- groups [25].

In the FTIR spectra, peaks observed in the range of 800–1200 cm^{-1} correspond to C-O bands in carbohydrates, while peaks in the range of 1300–1500 cm^{-1} are related to carboxyl groups of galacturonic acid, indicating the presence of sugars and carbohydrate structures in the manna. The peaks observed in the range of 3200–3400 cm^{-1} are associated with hydrogen bonding involving hydroxyl groups. Additionally, peaks in the range of 1600–1700 cm^{-1} correspond to the amide I group of proteins [25].

These findings obtained from the FTIR spectroscopy analysis provide valuable insights into the functional groups and structural properties of TM, shedding light on its composition and characteristics.

3.4. X-Ray Diffraction

XRD analysis is a valuable method for evaluating the crystalline and amorphous structure of carbohydrates. Each crystal structure exhibits a unique X-ray pattern. Figure 1(c) depicts the XRD curves of both the native and purified TM samples.

The results indicate that the structure of TM undergoes a transformation from crystalline to amorphous form after solution and drying. The purified TM sample exhibits a peak at 20.95°, which corresponds to the amorphous structure of carbohydrate samples. This amorphous structure is associated with higher solubility.

Previous research by Oetjen and Haseley [26] supports these findings, as they reported a significant increase in amorphous structure after freeze-drying a crystalline sample. Additionally, Rezaei et al. [27] reported that the solubility of almond gum is influenced by the crystalline structure of the gum. They found that the crystallinity index of the insoluble part, soluble part, and whole gum was 36.33%, 24.27%, and 26.46%, respectively. These observations from XRD analysis provide valuable insights into the structural changes of TM, indicating a shift from crystalline to amorphous form during the purification process. The resulting amorphous structure contributes to its higher solubility.

3.5. Molecular Weight (MW) and Zeta Potential

The average molecular weight (MW) of TM was determined based on DLS method. The MW of TM was 7.8 kD, which was pretty low. MW of 1130 kD for *Commiphora africana* exudate [28], 5.17 and 16.4 kD for peach gums [29], and 0.24 and 2.95 kD for Acacia gums [30] were reported. Low rheological properties may be attributed to the low molecular weight of TM in comparison with other known carbohydrates and hydrocolloids. High molecular weight hydrocolloids having long chain usually show higher water absorption and viscosity [27].

The zeta potential of TM is exhibited in Figure 2(a). The results showed that zeta potential of 1, 5, 25, and 50% (w/v) of TM was −8.93, −11.13, −21.56, and –39.46 mV, respectively. An almost 5-fold decrease in the zeta potential is observed as concentration increases by 50 times ($p<0.05$). The negative charge of TM is related to carboxyl and hydroxyl groups of TM, which detected in the FTIR spectra too. Zeta potential is an indication of the surface charge on the particle. Higher zeta potential is suitable for the production of stable colloidal systems, foaming, and emulsion system. By measuring the zeta potential of the solution, the amount of electrostatic repulsion forces between droplets of the same name (which prevents them from coming close to each other) can be determined [31].

[figure(s) omitted; refer to PDF]

3.6. Contact Angle

Contact angle was used for evaluating the material surface, hydrophobicity and hydrophilicity, and wettability of polymer surface. Contact angle measurement depends on various factors at macro- and microlevels. The contact angle of TM solution at various concentrations is demonstrated in Figure 2(b). The contact angle of 1, 5, 25, and 50% TM solutions after drying was 28.27, 28, 31.01, and 31.74°. There are not any significant differences between the samples ($p>0.05$). As shown in FTIR and zeta potential due to the presence of hydroxyl and carboxyl groups, the numerical value of contact angle shows that the TM had hydrophilic properties. Dupas et al. [32] evaluated the contact angle of sucrose at different particle sizes and maltodextrin at various molecular weights. They reported that the contact angle of sucrose is about to be 15.8 ± 0.3° and maltodextrin varies between 30 and 3° depending on molecular weight.

3.7. Scanning Electron Microscopy (SEM)

Figure 3 demonstrates SEM images of TM at 500 and 2000 magnifications. The results show that the sample had a unique and homogeneous surface without any porosity. Furthermore, the SEM photograms show that the size distribution of particles is mostly above 100 μm, which may, to a great extent, influence the solubility of samples. Evaluating the surface of TM is important for future application. The higher specific surface of TM in the image is related to hydration and solubility of TM. This parameter had an effect on the viscosity and molecular weight of the sample [33, 34].

[figure(s) omitted; refer to PDF]

3.8. Rheological Properties

3.8.1. Apparent Viscosity

The results depicted in Figure 4(a) demonstrate that all TM solutions exhibit Newtonian behavior. This characteristic may be advantageous in the food and pharmaceutical industries, particularly during pumping and filling processes, as Newtonian materials are less complicated to handle. Figure 4(a) illustrates the apparent viscosity of TM solutions at different concentrations. At a shear rate of 30 s^{-1} and a temperature of 25°C, the apparent viscosity of 1%, 5%, 25%, and 50% TM solutions was measured to be 1.1, 1.6, 2.4, and 12.4 MPa s, respectively.

[figure(s) omitted; refer to PDF]

These findings highlight the concentration-dependent viscosity of TM solutions, with higher concentrations exhibiting higher apparent viscosity values. This information is crucial for understanding the flow behavior of TM solutions and can aid in the design and optimization of processes in the food industry. Belay et al. [35] reported that Ethiopian monofloral honey had Newtonian behavior and their viscosity depends on moisture content, and they said that this could be because of the interaction of the ingredients with water, which reduce the honey liquid phase. So, the honey viscosity increased with decreasing the aw. Kamboj et al. [36] also said four different Indian honey varieties had Newtonian behavior because of their sugar structure.

3.8.2. Time Dependency

When some fluids are exposed to a constant shear rate, they become thinner, thicker, or might be time-independent over time, which is related to changes in the inner structure. Time dependency of different concentrations of TM is presented in Figure 4(c). Various TM solutions revealed the time-independent behavior over time. Time dependency of sugar solution was related to the type of sugar, moisture content, and composition of ingredient and sugar concentration [37].

3.8.3. Temperature Dependency

Assessing the impact of heating on the viscosity of solutions is a crucial aspect in the food industry, as different solutions exhibit varying behaviors when subjected to temperature changes. Figure 4(d) illustrates the influence of temperature on the viscosity of TM solutions at different concentrations (1%, 5%, 25%, and 50% w/v). Notably, the solution with a concentration of 50% exhibited significantly higher viscosity compared to the other samples. As the TM solutions were heated to 60°C at a fixed shear rate, a reduction in viscosity was observed across all concentrations. This decrease in viscosity can be attributed to the breakdown of hydrogen bonding and weakening of intermolecular bonds within the TM molecules.

Understanding the changes in viscosity induced by heating is vital for optimizing food processes, as it allows for better control of the flow behavior and consistency of the solutions. Sun et al. [38] reported that the viscosity of polysaccharide solution decreased following a temperature increase. Lowering the solution temperature to 50°C reduces the viscosity to lower values indicating that the strength of biopolymer bonds for absorbing the water molecules surrounding themselves with the help of hydrogen bonds at lower temperatures. Similar results on the effects of heating on the viscosity were reported by Farhoosh and Riazi [39] and Gahruie et al. [10]. The reduction of viscosity during heating is due to the mobility of molecules, the distance between molecules, and the weakening of interactions.

The temperature-viscosity relationship of TM solutions can be described by the Arrhenius model, as shown in Table 2. According to Eyring's theory, higher temperatures provide sufficient activation energy for the molecules to move more freely, resulting in increased fluid flow and filling of existing intermolecular spaces. The temperature dependency of hydrocolloid and sugar solutions can be well explained by the Arrhenius relation. Activation energy (Ea) represents the energy required for the occurrence of primary flow processes and is influenced by various factors, such as polymer concentration, ionic strength, physicochemical properties of the polymer, and applied shear stress. In the case of TM solutions, the Ea values for 1% and 50% concentrations were found to be the lowest (13493) and the highest (19514), respectively.

Table 2

Arrhenius model parameters of Tarangabin manna.

	Concentration (%)			
1	5	25	50	Ea⋆ (J/mol)
13493	15571	16384	19514	R2

99.44	99.31	99.17	98.48	RMSE

⁎Ea: activation energy.

The effect of temperature on viscosity is closely linked to the structural properties of the solution, as reported by Sun et al. [38]. Considering the various structural properties of the purified manna, such as sugar type, ash content, carbon content, protein content, and molecular weight, different temperature sensitivities have been observed [40]. Understanding the temperature-viscosity behavior and its relationship with the structural properties of TM solutions is crucial for determining their application in different food processes. These findings provide insights into the factors influencing the temperature sensitivity of TM solutions and contribute to the optimization of their utilization in the food industry.

3.8.4. Amplitude Sweep

Dynamic rheological methods are commonly used to investigate the rheological properties of a wide range of hydrocolloid solutions, which exhibit viscoelastic behavior. These methods involve various procedures at both macroscopic and microscopic scales, contributing to a comprehensive understanding of the material's response [41]. In the case of TM solution, the strain sweep test results (Figure 5) indicated a liquid-like behavior, with the storage modulus (G′) being lower than the loss modulus (G″) within the linear viscoelastic region (LVE). The LVE region is crucial for distinguishing between strong and weak gels. Notably, the elastic and viscous characteristics of TM increased with higher concentrations, suggesting a more pronounced viscoelastic response.

[figure(s) omitted; refer to PDF]

3.8.5. Frequency Sweep

The frequency sweep test is a commonly used oscillation test in rheology. In this test, the amplitude of the input stress or strain remains constant, while the frequency is increased. Figures 5(a) and 5(b) illustrate the results of the frequency sweep test conducted at a fixed shear strain of 0.1% and frequencies ranging from 0.06 to 62.8 rad/s. Within this frequency range, all solutions exhibited a fluid-like behavior, as indicated by the storage modulus (G′) being lower than the loss modulus (G″) throughout the entire experimental range. The ratio of G″ to G′, known as tan δ or the loss tangent, is shown in Figure 5(c). Typically, when tan δ is less than 1, the solution displays elastic characteristics, while values greater than 1 indicate a more viscous behavior. A tan δ higher than 0.1 suggests that the solution is not a true bulk gel [10].

Interestingly, the solution with a concentration of 50% exhibited a predominant elastic behavior or a weak gel-like composition across a wide range of applied frequencies, as depicted in Figure 5. In contrast, the other samples displayed liquid-like behavior.

Escriche et al. [42] reported that in eighteen honey samples, G″ was higher than G′, indicating a more viscous viscoelastic behavior. They also noted that the rheological properties of honey are influenced by polymeric compounds and the structure of sugars, as glucose and fructose solutions exhibit different rheological properties. These findings provide valuable insights into the frequency-dependent rheological behavior of the TM solutions, with the 50% concentration exhibiting a more gel-like composition, while the other samples displayed liquid-like behavior. The influence of polymeric compounds and the structure of sugars on the rheological properties are important factors to consider in understanding the behavior of these solutions.

3.9. Interfacial Tension (IFT) and Surface Tension

IFT among the water and oil was 14.3 ± 0.7 mN/m in the absence of TM. A meaningful reduction in the interfacial tension (IFT) was observed after addition of TM ($p<0.05$). Reduction in IFT was influenced by TM solution concentration (Figure 6(a)). The IFT samples at 1, 5, 25, and 50% concentrations showed a reducing trend and were 14.29, 13.09, 9.67, and 7.74 mN/m, respectively. The surface activity of hydrocolloids is attributed to the existence of proteinaceous moiety, the polysaccharide complex, and its hydrophobicity [43]. A similar trend in the variation of IFT was observed in the surface activity of the purified powder. In the concentration range of 1 and 50%, the surface activity varies between 65 and 45 mN/m, respectively (Figure 6(b)). A similar trend was reported by Osano et al. [43] on variation of surface activities by type of hydrocolloids and their concentrations. The surface tension of fenugreek,

pectin, guar, xanthan, Arabic gum, and methyl cellulose at 0.5% concentration was 50.3, 53.6, 55.2, 60.8, 46.9, and 52.9 mN/m [44]. Furthermore, Koocheki et al. [45] reported that low molecular polysaccharides reduce the surface tension more than bigger polysaccharides.

[figure(s) omitted; refer to PDF]

3.10. Emulsion and Foam Properties

The emulsifying activity index (EAI) is a measure of the ability of external active factors, such as molecules and their arrangement, to create and stabilize emulsions. Figure 6(c) presents the emulsifying activity index of TM solutions at different concentrations.

Remarkably, the EAI values showed a significant increase as the concentration of TM solutions increased from 1 to 50% ($p<0.05$). This indicates that higher concentrations of TM solutions have a greater capability to form and stabilize emulsions. Understanding the emulsifying properties of TM solutions is essential in various applications, particularly in the food industry where emulsions play a crucial role in product formulation and stability. These findings highlight the concentration-dependent emulsifying activity of TM solutions and provide valuable insights for optimizing emulsion-based processes and product development. Hydrocolloids have the capability to enhance emulsion stability by increasing the viscosity of the continuous phase [10].

TM exhibited lower emulsifying activity index (EAI) compared to other hydrocolloids, such as basil seed gum (0.3%, 32 m^2/g), asafoetida gum (1%, 38.6 m^2/g), and Arabic gum (1%, 88 m^2/g) [46].

The effects of TM concentration on foaming ability and foaming stability of albumin protein are shown in Figures 6(d) and 6(e). As the TM solution concentration increased from 1% to 50%, there was a notable increase in foaming ability, ranging from about 20% to 80%. No significant differences were observed between 1% and 5% samples, but the 50% sample exhibited significantly higher foam production ability compared to all other samples ($p<0.05$). Similarly, there were no significant differences in foaming stability between 1% and 5% samples, as well as between 25% and 50% samples.

Previous studies demonstrated that a 0.3% basil seed gum solution (containing 0.3% albumin) had a foaming ability of 28% [17]. The foaming stability and ability of different basil seed gums were associated with their surface activity and molecular weight. Hydrocolloids can enhance foam production capacity by reducing surface tension [17]. Foam ability refers to the capacity of a solution to sustain bubble size, foam mass, and liquid content over a period of time. Furthermore, the gas bubbles in foam ascend to the surface and eventually collapse [47]. Increasing the TM concentration resulted in increased foaming stability of albumin, which is influenced by the viscosity of the water phase.

4. Conclusion

The objective of this study was to comprehensively investigate the rheological, physicochemical, and interfacial properties of Tarangabin manna, an exudate obtained from A. maurorum. Analysis revealed that TM contains more than 90% carbohydrates and less than 2% proteins. The presence of proteins in TM contributes to its remarkable emulsifying activity and the reduction of interfacial tension. Additionally, the exceptional solubility of TM can be attributed to its amorphous structure. The relatively low molecular weight of TM likely contributes to its Newtonian behavior and low viscosity. Notably, the TM solution at a concentration of 50% exhibited the lowest interfacial tension for the range of concentration analyzed. This finding suggests that the TM solution possesses excellent emulsifying and foam-forming abilities. These results collectively indicate that TM solution holds significant potential as a foaming and emulsifying agent in the food industry. Understanding the rheological, physicochemical, and interfacial properties of TM provides valuable insights into its potential applications and advantages in various food formulations. The exceptional emulsifying and foaming properties of TM make it an appealing choice for enhancing the texture and stability of food products.

Authors' Contributions

All authors have significantly contributed to the development and the writing of this article.

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DETAIL

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CNFA: ConvNeXt Fusion Attention Module for Age Recognition of the Tangerine Peel

Deng, Fuqin; Li, Junwei; Fu, Lanhui; Qin, Chuanbo; Zhai, Yikui; dkk.

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ABSTRAK (ENGLISH)

Xinhui tangerine peel has valuable medicinal value. The longer it is stored in an appropriate environment, the higher its flavonoid content, resulting in increased medicinal value. In order to correctly identify the age of the tangerine peel, previous studies have mostly used manual identification or physical and chemical analysis, which is a tedious and costly process. This work investigates the automatic age recognition of the tangerine peel based on deep learning and attention mechanisms. We proposed an effective ConvNeXt fusion attention module (CNFA), which consists of three parts, a ConvNeXt block for extracting low-level features’ information and aggregating hierarchical features, a channel squeeze-and-excitation (cSE) block and a spatial squeeze-and-excitation (sSE) block for generating sufficient high-level feature information from both channel and spatial dimensions. To analyze the features of tangerine peel in different ages and evaluate the performance of CNFA module, we conducted comparative experiments using the CNFA-integrated network on the Xinhui tangerine peel dataset. The proposed algorithm is compared with related models of the proposed structure and other attention mechanisms. The experimental results showed that the proposed algorithm had an accuracy of 97.17%, precision of 96.18%, recall of 96.09%, and F1 score of 96.13% for age recognition of the tangerine peel, providing a visual solution for the intelligent development of the tangerine peel industry.

TEKS LENGKAP

1. Introduction

Tangerine peel, derived from Citrus reticulata Blanco, is an agricultural product made from citrus peels that have been dried or dried for storage [1]. Tangerine peel from Xinhui (Jiangmen City, Guangdong Province, China) holds valuable economic value because of its geographical advantage, climatic advantage, and the unique production techniques of tangerine peel [2]. The value of the tangerine peel industry chain was 19 billion CNY in 2022, accounting for 20% of the total GDP of Xinhui. Xinhui tangerine peel is considered to be the quality and is rich in the

flavonoid [3]. The flavonoid has great effects in anti-inflammatory, antiviral, and antiatherosclerosis [4]. As shown in Table 1, as the storage time of tangerine peel increases, the higher the flavonoid contained in the tangerine peel [3], the higher the medicinal value. No relevant literature is found for 20-year tangerine peel. The age of the tangerine peel is one of the important criteria for measuring the quality of tangerine peel.

Table 1

The total flavonoid content percentage and the price of the tangerine peel in different ages.

Age (years)	Total flavonoid content percentage (%)	Price (CNY/kilogram)
1	4.97	540
5	6.17	1,300
10	6.44	1,960
15	6.91	3,360
>20	—	11,960

As the years increase, market value tends to increase. The prices of tangerine peel are shown in Table 1, with its market price increasing exponentially as the age increases. During the recovery stage of Covid-19 pandemic, tangerine peel had a mitigating effect on symptoms [5]. However, many merchants have taken advantage of this gimmick by using young tangerine peel for craft processing to pass off as old tangerine peel as a way to gain more economic benefits, which not only harms the interests of consumers but also disrupts the market regulations to a certain extent [6]. Therefore, it is essential to develop a method that can flexibly, accurately identify the age of the tangerine peel.

Deep learning has been widely used in foods and agriculture in recent years [7, 8], and image recognition of plants has received a lot of attention from researchers. Following this trend, nondestructive age recognition of the tangerine peel contributes to the development of the intelligent tangerine peel industry. However, it still faces challenges as tangerine peels lack distinct shape differences and have similar colors. Therefore, the feature extraction of tangerine peels becomes more complex, leading to greater difficulty in recognition. Age recognition of the tangerine peel requires special attention to features such as oil bags, patterns, and color on the epidermis of the peel. Existing deep learning models struggle to capture these fine-grained details, and attention mechanisms are commonly used techniques to focus on such detailed features.

To effectively extract important features of tangerine peel, we designed a ConvNeXt fusion attention module (CNFA module) that uses a strategy to aggregate feature information extracted by ConvNeXt block and attention mechanisms. A high-level feature contains rich semantic information, which can be used for the localization of the tangerine peel. A low-level feature plays an important role in capturing crucial details of tangerine peel during feature extraction. In the CNFA module, the ConvNeXt block can effectively extract low-level feature information of images and aggregate hierarchical features. In addition, the cSE and sSE capture effective channel and spatial information adaptively in the image, including the local detailed feature of tangerine peel, and assign different attention weights to features of tangerine peel from different locations. The high-level feature generated by the attention module is utilized to guide the ConNeXt block in extracting the low-level feature. The CNFA module combines the obtained low-level feature information and high-level feature information, linking feature information to effectively extract features of tangerine peel images. We embedded the CNFA module into our network architecture, effectively extracting global contextual information and suppressing useless information. The main contributions of this work are as follows:

(1) We proposed a CNFA attention module aggregating low-level and high-level features in the network to improve the detection accuracy
(2) We validated the effectiveness of the CNFA module compared to other attention mechanisms through comparative experiments
The rest of this article is structured as follows. The second part reviews the related work. The third part introduces the network and implementation of age recognition of the tangerine peel. The fourth part introduces the experimental results and discussion. The fifth part includes the conclusion.

2. Related Work

The shapes and colors of tangerine peel are in different ages, which can be quite similar. It is difficult to recognize the age of tangerine peel for ordinary people. The main methods for identifying the age of the tangerine peel are the manual identification method and physical and chemical analysis method [9]. The former relies on experienced personnel to identify different ages of tangerine peel based on differences in color, shape, and odour. This is simple to operate, but it is susceptible to interference from subjective conditions and objective factors. The latter is mainly judged by detecting the content of components in the tangerine peel. Chen et al. used response surface methodology to optimize the process of microwave-assisted extraction of pectin polysaccharides from tangerine peel [10]. This method can analyze the age in terms of its material composition. Li et al. proposed a method to estimate the age of tangerine peel based on the trnL-trnF copy number [11]. The study explored the correlation between six DNA fragments and the age of tangerine peel. It was found that the trnL-trnF copy number showed a negative correlation with the age of the tangerine peel. Yue et al. extracted tangerine peel polysaccharides from five different-age tangerine peels and proposed the relationship between tangerine peel polysaccharides and their ages [12]. But these are tedious processes and destroy the sample, affecting secondary sales.
Age recognition of the tangerine peel is a novel research direction. Pan et al. used a handheld near-infrared spectrometer to scan the epidermis of tangerine peel and collected corresponding near-infrared diffuse reflection spectra [6]. After preprocessing, the data were used to identify the origin and age of tangerine peel using random forest, K-nearest neighbor, and linear discriminant analysis. Zhang et al. proposed a novel approach that combines near-infrared spectroscopy with machine learning to identify the age of the tangerine peel [13]. The method involves preprocessing the spectral data through Savitzky–Golay convolution smoothing, standard normal variate first-order derivatives, and principal component analysis (PCA) to yield characteristic spectral variables. The support vector machine (SVM) and K-nearest neighbor algorithms are employed for discrimination then. Pu et al. proposed a method for identifying the origins of tangerine peel using terahertz time-domain spectroscopy combined with CNN (convolutional neural network) [14]. Different spectral data were used to construct 1D CNN and 2D CNN models. Additionally, an Add-CNN model was developed by combining both spectral and image data. However, these works require specialized equipment that lacks flexibility.
Deep learning [15] is a machine learning technique in which machines simulate the human brain to analyze data, with the computer vision [16] being one of the more prominent applications. In the past few years, the image classification of agricultural products represented by tangerine peel is emerging. Chu et al. introduced a method to increase the data volume of tangerine peel by utilizing traditional data augmentation, deep convolution generative adversarial networks (DCGAN), and Mosaic [17]. The data volume of the original sample was increased by 23 times. They also used the CBAM module in conjunction with CSPNet to extract the endocarp features and classify, which can effectively extract feature information such as color, size, and shape on the endocarp of tangerine peel. However, they ignored the low-level feature information on the epidermis of tangerine peel, such as the connection between the oil bag and the surrounding textures on the epidermis, and the typhoon scars that are produced in old tangerine peel.
Networks based on attention mechanisms [18] have become mainstream research, with Swim Transformer [19] gaining significant success on a variety of vision tasks and effectively solving the problem of large computational costs. However, it is very difficult to deploy the Swim Transformer since the calculation of the sliding window is very complex. To solve this problem, Liu et al. proposed ConvNeXt [20] by modifying the structure of ResNet [21].

Through a series of experimental comparisons, ConvNeXt has faster inference speed and a higher accuracy rate than Swim Transformer at the same FLOPS. Various attention mechanisms have been proposed to address the problem of difficult feature extraction. Hu et al. presented a squeeze and excitation module (SE) [22], where he added an attention mechanism to the channel dimension to obtain the importance of each channel of the feature map and assign weights to each feature by the importance level, thus allowing the network to learn the important features. Because previous attention mechanisms focus more on the analysis of the channel dimension, CBAM [23] implemented a sequential attention structure from both channel and spatial scopes. Spatial attention allows the neural network to focus more on the pixel regions in the image that play a decisive role in classification and ignore irrelevant regions, while channel attention is used to deal with the assignment relationship of the feature map channels, thus enhancing the effect of the attention mechanism on model performance. But these methods ignore the linkage between global feature information and local feature information, which can affect the fusion of features and the generation of accurate attention maps. Deng et al. built a csRSE module for occupancy grid map recognition [24], which contains a residual block for generating hierarchical features, followed by a channel SE block and a spatial SE block for adequate information extraction along the channel and space. To achieve more flexible computation allocation and content awareness, Zhu et al. introduced the content-independent sparsity into the attention mechanism and proposed the BiFormer which selectively attended to relevant tokens in an adaptive manner, without dispersing attention to other unrelated tokens [25].

3. Materials and Methods

3.1. Workflow

Figure 1 shows the workflow of the age recognition of the tangerine peel model. In this study, we used the CNFA-integrated network to identify the age of tangerine peel. First, we used a digital camera to capture the images of the tangerine peel. Then, we labeled the tangerine peel samples according to their age and created dataset. Finally, the model is trained and evaluated using the dataset. We input tangerine peel samples into the model. After the model training is completed, the model outputs the corresponding year of the sample.

[figure(s) omitted; refer to PDF]

3.2. Image Acquisition

There is currently no publicly available tangerine peel dataset to use, so it is necessary to collect images of tangerine peel. The tangerine peel sample was collected from Huicheng (Xinhui, Jiangmen, Guangdong Province, China, longitude 113.034 and latitude 22.4583). We collected sample with two batches. The sample information is given in Table 2. The original species of the tangerine peel samples is Dazhongyoushen. We used a Canon 760D camera (Canon Inc., Tokyo, Japan) with a resolution of 6000×4000 pixels to capture the epidermis of Xinhui tangerine peel under the same lighting conditions. The tangerine peels used for image acquisition are stored in the same warehouse with a consistent temperature, humidity level, and lighting condition. The temperature was 21°C, and the humidity was 60% in the warehouse. According to the experts, it was indicated that the samples underwent traceability detection before being sold. Therefore, the accuracy of the age can be ensured.

Table 2

Sample information of 2 batches of different ages in the tangerine peel.

Location	Batch	Time	Category (year)	Sample size	Total sample size	The number of samples extracted	Total number of samples extracted
Huicheng, Xinhui	Batch 1	Mar 16, 2023	1	1,025	4,090	205	818
15	940	188	20	465	93	Batch 2	Mar 17, 2023

Since the tangerine peel sold online has a five-year interval between ages, the interval between each type of tangerine peel we collected is also five years. Under the premise of ensuring model performance and scientific sampling, we made efforts to achieve a concentrated distribution in our dataset. The 818 images of Xinhui tangerine peel were divided into five categories according to different ages. One sample corresponds to one image. The dataset was randomly divided into the training set, test set, and validation set in a ratio of 7 : 2 : 1. To decrease computational complexity and memory requirements, the training set images were uniformly converted to a resolution of 224×224 pixels. Figure 2 shows sample images of the dataset. The 1-year tangerine peel with bright orange skin and dense oil bags on the epidermis. The 5-year tangerine peel with reddish-brown skin and sparse oil bags on the epidermis. The 10-year tangerine peel with dark red skin and pig-bristle texture on the epidermis. The 15-year tangerine peel with black skin and more dense pig-bristle texture. The 20-year tangerine peel with typhoon scars on the epidermis.

[figure(s) omitted; refer to PDF]

Tangerine peel epidermis exhibits more age features. We used the tangerine peel dataset that distinguishes epidermis features in this experiment. The color is the most prominent low-level feature on the epidermis of tangerine peel, and the color of the epidermis will change with the increase of ages. However, the feature that oil bag and pig-bristle are the key to distinguishing the age of the tangerine peel. There are many sunken oil bags on the epidermis of tangerine peel, and the surrounding texture will combine with the oil bag, forming the pig-bristle texture of tangerine peel. With the increase of ages, the oil bags will dissipate, and the pig-bristle texture will become more obvious. In addition, old tangerine peel has increasingly obvious typhoon scars on the epidermis. Figure 3 shows the details of the oil bag, pig-bristle texture, and typhoon scar. The above features play a decisive role in the tangerine peel dataset.

[figure(s) omitted; refer to PDF]

3.3. CNFA-Integrated Network

Figure 4 shows the structure of the CNFA-integrated network, which consists of a stem convolution layer, a LN layer, CNFA stacked module with four stages, three downsampling layers, and a decision layer. The stem convolution layer has a kernel size of 4 and a stride of 4. The input image is processed by the stem convolution so that the continuous use of filters does not result in overlapping pixels. The use of the layer normalization (LN) layer [26] is to avoid the problems of gradient disappearance and gradient explosion. The proposed CNFA-integrated network is a multilevel design, with different feature map resolutions at each stage. The number of stacked modules of ResNet50 is (3, 4, 6, 3), with an approximate ratio of (1 : 1 : 2 : 1). In recent years, the number of stacked modules' ratio of most improved networks has been (1 : 1 : 3 : 1). To maintain the same network scale, the network is designed with the CNFA stacked module of four stages, with the number of modules stacked in each stage being (3, 3, 9, 3), and the input channel numbers being (96, 192, 384, 768), respectively. After passing through the CNFA module, the feature dimension of the feature map will change, leading to the loss of effective information. In order to ensure that effective information is retained, we added a separate downsampling layer between CNFA modules. The downsampling layer includes an LN layer and a convolution layer with a kernel size of 2 and a stride of 2. After passing through the different convolution layers, the final feature map is output and the feature map is processed by the decision layer (consists of a global average pooling, a layer normalization layer, and a linear layer) to calculate the probability of predicting the category. GAP sums up all the pixel values of a feature map and calculates their average, resulting in a numerical value corresponding to the feature map. This technique reduces the number of parameters and computational workload. LN normalizes the values generated by GAP to improve the stability and training effectiveness of the network. Finally, the feature vector is fed into a linear layer to map it to the probability distribution of output classes.

[figure(s) omitted; refer to PDF]

For the task of identifying the age of the tangerine peel, the CNFA module is the core of feature extraction in the network and can effectively emphasize or suppress mapped feature information. As shown in Figure 5, the components of the CNFA module are a ConvNeXt block, a cSE block, and an sSE block. The ConvNeXt block is

designed to capture the global low-level feature information and aggregate features at multiple levels. The sSE block determines the importance of specific positions in the input feature map and assigns corresponding weight parameters to highlight meaningful locations in spatial. Stacking the sSE block after cSE block can retain more intermediate layer spatial information. By placing the cSE block before the sSE block in sequential order, CNFA module models the correlations between channels and then further adjusts the spatial distribution of the feature map through the sSE. Through the calculations of the ConvNeXt block, the cSE block, and the sSE block, the ConvNeXt block extracts a low-level contextual feature and the attention module fusion generates a high-level contextual feature. The high-level features generated by the attention module are utilized to guide the ConNeXt block in extracting a low-level feature. These two features are aggregated into a global contextual feature. These features are weighted and averaged over all regions through an attention map.

[figure(s) omitted; refer to PDF]

The tangerine peel data input size is the same as the output size, and for the input feature map X∈RH×W×C, the network computes the output feature map Y∈RH×W×C. After the ConvNeXt block, the feature XCN is computed:(1) XCN=X⊗FCNX,where ⊗denotes element-wise multiplication.

The channel attention FC∈R1×1×C is then calculated from the cSE block to obtain the output feature map of channel attention XC:(2)XC=XCN⊗FCXCN.

The spatial attention FS∈RH×W×1 is then calculated from the sSE block to obtain the output feature map of spatial attention XS:(3)XS=XC⊗FSXC.

The ConvNeXt block output feature map XCN and the sSE block output feature map XS are aggregated and then output to obtain the final refined output feature map *Y*:(4)Y=XCN+XCN⊗XS.

The following summary will describe the details of the ConvNeXt block, the cSE block, and the sSE block.

3.4. ConvNeXt Block

Figure 6 shows the structure of the ConvNeXt block, which consists of a 7×7 deep convolution layer, two 1×1 general convolution layers, an LN layer, a nonlinear Gaussian error linear unit (GeLU) activation layer [27], and a layer scale [28].

[figure(s) omitted; refer to PDF]

The 7×7 deep convolution layer mainly mixes spatial information, and a larger convolution kernel provides a larger receptive field to capture large-scale feature information. The large-kernel convolution operation is performed with a smaller number of channels to reduce the number of model parameters. The 1×1 general convolution layer expands and compresses the feature maps in the channel dimension to deepen the channels. This structure has a deep convolution layer as the front layer, and the subsequent general convolution layers are similar to the feed-forward block of TransFormer [29]. The reverse bottleneck structure is used to make the calculation of the ConvNeXt block more efficient. Therefore, the ConvNeXt block effectively and economically extracts global and local features.

In the ConvNeXt block, the use of the LN layer after the deep convolution layer is to avoid differences between training and inference. Considering that the variation in the output of one layer will cause strongly correlated changes in the total output of the next layer, the LN layer solves the covariate shift problem by setting the mean (μ) and variance (σ) of the summed inputs within each layer. For all hidden cells in the same layer, the LN layer is calculated as follows:(5)μl=1H∑i=1Hailσl=1H∑i=1Hail−μl2,where *H* indicates the number of hidden cells in the layer in which it is located.

To improve the nonlinearity and generalization ability of the model, a GeLU is used after the first general convolution layer. This activation function incorporates the idea of random regularization in the activation, which can achieve the effect of adaptive dropout and ensure the robustness of the model training. For input *x*, GeLU can be expressed as follows:(6)GeLUx=xPX≤x=xφx=x×121+erfx/2.

The purpose of the layer scale is to scale the input feature data, which allows for a more refined and precise representation of the features.

Thus, we input the *x*, and the output of the ConvNeXt block is calculated as follows:(7)FCNX=X+X⊗f31×1GeLUf21×1μlf17×7,where *GeLU* is the GeLU function operation, μl is the LN layer operation, and f7×7 and f1×1 are the

convolution layer operations with convolution kernel sizes of 7×7 and 1×1, respectively.

3.5. Channel Squeeze-and-Excitation Block (cSE)

In the cSE block, we calibrate the correlation between image feature channels through spatial compression and channel excitation. As shown in Figure 7, the block first performs spatial compression. For the input feature vector X CN∈RH×W×C, we use global average pooling to compress global spatial information and generate a unique channel vector VC∈R1×1×C for each channel through the average value of global average pooling.

[figure(s) omitted; refer to PDF]

The block performs channel excitation to highlight channels with meaningful information. We take the dimensions obtained from the compression operation and run them through the multilayer perceptron (MLP) to count the weight values of the channels, which are then stimulated to the corresponding channels of the previous feature map for operation. The MLP consists of two fully connected layers, a ReLU linear activation function and a sigmoid nonlinear activation function.

Thus, we input the XCN, and the output of the cSE block is calculated as follows:(8)FCXCN=σW2δW1GAPXCN, where W1∈RC¯×C, W2∈RC×C¯ are the weights of the FC layers, δ is the ReLU activation function, and σ is the sigmoid activation function.

3.6. Spatial Squeeze-and-Excitation Block (sSE)

In the sSE block, it is able to transform various deformation data in spatial and automatically capture important regional features. The setting of this block is to determine the importance of specific positions in the input feature map and highlight meaningful positions in spatial. As shown in Figure 8, the input feature vector XC goes through several general convolution layers to generate an attention feature vector, which is then passed through a sigmoid function.

[figure(s) omitted; refer to PDF]

Thus, we input the XC, and the output of the sSE block is calculated as follows:(9)FSXC=σW∗XC=σf41×1f33×3f23×3f11×1XC,where σ is the sigmoid function, ∗is the convolution operation, and f1×1 and f3×3 are the convolution layers with convolution kernel sizes of 1×1 and 3×3, respectively.

3.7. Parameter Selection and Model Training

We conducted experiments on the tangerine peel dataset for age recognition of the tangerine peel using the PyTorch framework. The network training was running on the NVIDIA RTX 3090 GPU. In the preset values for training, the learning rate was 0.0002, batch size was 16, weight decay was 0.0001, and the number of epoch was 200. The Adam optimizer was used to optimize the parameters, and the input and output image resolutions of the network were both 224×224. In this experiment, we used the cross-entropy (CE) loss function [30] to train the network. CE is shown as follows:(10)L=−∑i=1nyilogpi,where yi is the true label and pi is the predicted probability of the ith item.

To observe the training situation in real time, we validated the trained model on the validation set after each epoch of training. As shown in Figure 9, the training loss and validation loss of the CNFA-integrated network were unstable and high at the beginning stage, but they tended to stabilize between 25 and 50 training epochs as the number of training epochs increased. The stable training loss and validation loss in the later stage of training indicate that the CNFA-integrated network did not have overfitting. The model converged under the input data, and both loss values were less than 1 after stabilization, proving that the CNFA-integrated network can be used for age recognition of the tangerine peel. As shown in Figure 10, the training accuracy and validation accuracy of the CNFA-integrated network on the tangerine peel dataset tend to stabilize between 25 and 50 training epochs as the loss and the learning rate decrease. The training accuracy reached 100, and the validation accuracy reached about 96.88, proving that the CNFA-integrated network learned the important features of tangerine peel.

[figure(s) omitted; refer to PDF]

3.8. Model Evaluation Metrics

For age recognition of the tangerine peel, we use accuracy to evaluate the model. In order to evaluate the detection of each category of tangerine peel by the model, precision, recall, and F1 will also be used in the evaluation metrics.

The formulas are as follows:(11)Accuracy=TP+TNTP+TN+FN+FP×100%,(12)Precision=TPTP+FP×100%,(13)Recall =TPTP+FN×100%,(14)F1=TPTP+1/2FP+FN×100%,where *TP* is a true positive, *FP* is a false positive, *TN* is a true negative, and *FN* is a false negative.

After the model training is completed, the network model is tested on the test set. The test sets are traversed, and we predict the category of each image, then the prediction is analyzed according to the ground truth to determine whether it is correct. For a certain category of tangerine peel, if it is predicted correctly, it is *TP* and if it is predicted incorrectly, it is *FN*. Other categories of tangerine peel are negative; if they are predicted correctly, they are *TN*, and if they are predicted incorrectly, they are *FP*.

4. Results and Discussion

4.1. Implementation Details

We evaluated the performance of each model on the tangerine peel dataset and conducted subsequent experiments. Each experiment was implemented on a computer equipped with 32 GB RAM, Intel i9 CPU, NVIDIA GeForce RTX 3090 GPU, and Ubuntu16.04 operating system. Each model was trained using the same training set. Also, each model used the same training parameters as the CNFA-integrated network.

4.2. Manual Identification Results

Before validating the model's performance, we conducted a manual identification experiment. This experiment evaluated the effectiveness of manual identification based on the accuracy of three experts. The identification was performed on the test set of the tangerine peel dataset, which consists of a total of 164 images.

As shown in Table 3, the accuracy rates of the three experts were 90.12%, 74.07%, and 83.95%, with an average of 82.71%. Each expert had a different accuracy rate, and there was a significant difference in accuracy among them. This is because manual identification can be influenced by subjectivity, making the accuracy of manual detection less stable. Compared to manual identification methods, deep learning methods are more stable and have higher accuracy.

Table 3

Results of manual identification experiments.

	Expert 1	Expert 2	Expert 3	Average
Accuracy (%)	90.12	74.07	83.95	82.71

4.3. Model Evaluation Results

We demonstrated the effectiveness of the CNFA-integrated network by comparing it with other mainstream network models on the tangerine peel dataset. While ensuring accuracy above 95%, we maintained the model scale in our comparative experiments. In the experiments, we compared with CNN, ResNet50 and ResNet50 variants, and ConvNeXt to evaluate their performance for age recognition of the tangerine peel.

As shown in Table 4, the CNN achieved the accuracy of 82.59%, precision of 82.60%, recall of 81.65%, and F1 score of 82.12%. It was the worst performing model among all models evaluated, indicating poor feature aggregation performance of CNN in task. Compared with CNN, the accuracy of the ResNet50 was increased by 13.39%, the precision was increased by 12.05%, the recall was increased by 13.27%, and the F1 score was increased by 12.57%. It indicates that the residual structure network performs well for age recognition of the tangerine peel. After adding the attention module to ResNet50, the metrics are improved. Adding SE module and CBAM module improved accuracy by 0.25% and 0.29%, improved precision by 0.75% and 0.88%, improved recall by 0.54% and 0.2%, and improved F1 by 0.73% and 0.63%, respectively. It indicates that there is not much difference in performance between ResNet-SE and ResNet-CBAM. Both CBAM module and csRSE module are dual attention modules. Compared with the csRSE module that focuses on global features, ResNet-csRSE had a higher accuracy, precision, recall, and F1 by 0.29%, 1.14%, 0.54%, and 0.84% than ResNet-CBAM. ConvNeXt achieved accuracy of 96.70%, precision of 96.18%, recall of 96.09%, and F1 score of 96.13%. ConNeXt performed better than previous

ResNet50 variant networks. BiFormer achieved accuracy of 96.38%, precision of 96.07%, recall of 95.91%, and F1 score of 95.99%. The performance of BiFormer performed slightly worse than ConvNeXt. The proposed CNFA-integrated network is a variant based on ConvNeXt. The accuracy was 97.17%, the precision was 96.71%, the recall was 96.86%, and the F1 score was 96.78%. CNFA-integrated had a higher accuracy, precision, recall, and F1 by 0.47%, 0.53%, 0.77%, and 0.65% than ConvNeXt. The metrics reached their maximum values, demonstrating the advantage of the CNFA-integrated network for age recognition of the tangerine peel. Through the comparative experiments results, the proposed CNFA-integrated network effectively captures global high-level and low-level information and aggregates information effectively through various modules. It validates the effectiveness of the CNFA module in detection accuracy.

Table 4

Results of model comparison experiments.

Network	Param (M)	FLOPS (G)	Accuracy (%)	Precision (%)	Recall (%)	F1 (%)	Time (s)
CNN	25.4	4.217	82.59	82.60	81.65	82.12	104.23
ResNet50	25.6	4.158	95.98	94.65	94.92	94.69	93.69
ResNet50-SE	28.1	4.162	96.23	95.40	95.46	95.42	92.02
ResNet50-CBAM	28.1	4.168	96.27	95.53	95.12	95.32	90.97
ReNet50-csRSE	28.1	4.175	96.67	96.67	95.66	96.16	**90.85**
BiFormer	25.5	4.5	96.38	96.07	95.91	95.99	98.52
ConvNeXt	28.6	4.546	96.70	96.18	96.09	96.13	91.67
CNFA-integrated (ours)	30.1	4.551	**97.17**	**96.71**	**96.86**	**96.78**	91.02

Bold indicates the best performance.

We also conducted experiments on the processing speed. The detection time of the CNN was the longest, at 104.23 seconds. The CNN performed poorly in both performance and speed. Compared with CNN, the detection time of ResNet50 reduced by 10.54 seconds. Compared with ResNet50, adding the SE module, CBAM module, and csRSE module reduced the detection time by 1.67 seconds, 2.72 seconds, and 2.84 seconds, respectively. Among them, csRSE had the shortest detection time. Compared with ResNet50-CBAM and ResNet50-csRSE, ConNeXt had an increased detection time. This is because ConNeXt has a larger number of parameters, resulting in increased computational complexity. BiFormer had a longer detection time compared to other attention-based networks. The attention mechanism in BiFormer has a certain degree of sequentiality. The model's computations depend on previous information, resulting in a large computational workload and increased detection time. Our proposed CNFA-integrated network reduced the detection time, which added attention mechanisms on ConNeXt. The CNFA module helps the model focus on important information in the input data, thereby reducing computational load and processing time. The CNFA-integrated network achieved a shorter detection time while ensuring the highest accuracy, with a small difference compared to csRSE. It validates the effectiveness of the CNFA module in detection efficiency.

4.4. Ablation Experiments

To validate the effectiveness of the CNFA module, we conducted a series of ablation experiments. The ablation experiments included four control groups. The control groups consisted of ConvNeXt, ConvNeXt and cSE, ConvNeXt and sSE, and CNFA. These four network structures were trained on the tangerine peel dataset. Table 5 shows the accuracy, precision, recall, and F1 of the models tested. CNFA performed the best, followed by ConvNeXt, in terms of performance. However, when adding the cSE block and sSE block individually to ConvNeXt, the performance decreased. CNFA had a higher accuracy, precision, recall, and F1 by 0.62%, 1.1%, 0.96%, and 1.03% than ConvNeXt and cSE. CNFA had a higher accuracy, precision, recall, and F1 by 1.91%, 2.02%, 1.95%, and 2.25% than ConvNeXt and sSE. This is because the exclusion of either channel or spatial information leads to the loss of important details and contextual in the tangerine peel dataset, resulting in incorrect data interpretation. Therefore, attention mechanisms should consider a balanced integration of both channel and spatial information.

Table 5

Ablation experiments result.

Model	Accuracy (%)	Precision (%)	Recall (%)	F1 (%)
ConvNeXt	96.70	96.18	96.09	96.13
ConvNeXt+cSE	96.55	95.61	95.9	95.75
ConvNeXt+sSE	95.26	94.15	94.91	94.53
CNFA (ours)	**97.17**	**96.71**	**96.86**	**96.78**

Bold indicates the best performance.

4.5. The Result of the Classification Metrics

As shown in Table 6, it records the prediction metrics of each category in the test set. The CNFA-integrated network trained on the tangerine peel dataset and obtained the prediction metrics for each category of tangerine peel on the test set: precision, recall, and F1 score. The classification metrics of each category of tangerine peel were relatively high after feature learning with the CNFA-integrated network, indicating good recognition performance. Because there were fewer test data for 20-year tangerine peel, the displayed metrics were lower than other categories.

Table 6

Various classification metrics for tangerine peel in different ages.

Category (year)	Precision (%)	Recall (%)	F1 (%)
1	97.56	97.56	97.56
5	97.14	97.14	97.14
10	96.77	100	98.35
15	97.37	94.87	96.10
20	94.73	94.73	94.73

We further tested the performance of the CNFA-integrated network by using a confusion matrix. The confusion matrix with prediction in the columns and real label in the row exhibited the performance of the CNFA-integrated network. Figure 11 shows a confusion matrix of the CNFA-integrated network. The CNFA-integrated network achieved 97.17% accuracy in the test set. From the confusion matrix, only one sample is misclassified from each category. It indicates that the CNFA-integrated network has good generalization performance.

[figure(s) omitted; refer to PDF]

5. Discussion

The CNFA module can rapidly and accurately identify the age of tangerine peel. Through our manual identification experiments, we found that the accuracy fluctuates up to 16%. The instability of the manual detection method can impact the assessment process of tangerine peel quality. Deep learning-based age recognition of the tangerine peel avoids subjectivity and provides more stable results. In our comparative experiments, all models demonstrated good performance. By utilizing deep learning models, the accuracy of identifying age had essentially reached 90% above. Our proposed CNFA-integrated network achieved the highest accuracy, precision, recall, and F1 scores in the comparative experiments. Additionally, the CNFA-integrated network exhibited fast processing speed. We also showed the various classification metrics for tangerine peel in different ages, and each metric achieved about 95%. This indicates that the CNFA module exhibits strong classification capability for each category of tangerine peel.

To visually demonstrate the effectiveness of the CNFA module, the heat map visualization method we use is called Grad-CAM [31]. It generates a heat map by analyzing the gradient information on the input image, visualizing the model's attention to different regions. As shown in Figure 10, the color scale of the heat map is in the bottom right corner. The values of the feature map are mapped to the range of [0, 1]. The heat map we generated shows red for high weight allocation positions and blue for low weight allocation positions. By overlaying the tangerine peel image with the result of Grad-CAM, we can effectively display the areas where the network has an impact on the task results.

As shown in Figure 12, the following are the input images of tangerine peel and their heat maps generated by different networks, including ResNet-SE, ResNet-CBAM, ResNet-csRSE, and CNFA-integrated network. The confidence score threshold was set to 0.5, and results with a confidence score less than 0.5 were considered to be wrong. These five images of tangerine peel were predicted incorrectly by ResNet-SE, ResNet-CBAM, and ResNet-csRSE. The samples were predicted successfully by the CNFA-integrated network, and the average confidence score is approximately 0.92. We will discuss the differences between the CNFA module and other attention mechanisms based on the features and heat maps of the output images.

[figure(s) omitted; refer to PDF]

Figure 12(a) shows a 1-year tangerine peel with a bright orange skin and dense oil bags on the epidermis. The sample was predicted to be five-year tangerine peel in ResNet variants because the colors of one-year tangerine peel and five-year tangerine peel are similar. In this sample, the dual-channel attention module performed well. However, the CBAM and csRSE modules still had issues with inaccurate localization of important features. The attention scope extended beyond the shape edges of the tangerine peel. The attention of the CNFA-integrated network was distributed in accurate regions, which can capture more areas of oil bags. Figure 12(b) shows a 1-year tangerine peel. Due to the high degree of curling in this sample, it is difficult to capture global features. The sample was predicted to be five-year tangerine peel in ResNet variants. The SE module failed to capture the shape of the sample, and the localization of the regions of interest was not comprehensive enough. Although the CBAM and csRSE modules extracted shape features more effectively, their ability to extract low-level features was insufficient, leading to incorrect prediction of the sample in Figure 12(b). The CNFA module located features of the age during decision-making, thereby improving the prediction ability of the network. Figure 12(c) shows a 10-year tangerine peel with a dark red skin and pig-bristle texture on the epidermis. The heat maps generated by the SE, CBAM, and csRSE modules were not ideal, as the red regions only covered a local position of the tangerine peel, ignoring the overall shape and inaccurate localization of the features of the age. Three ResNet variants predicted the sample to be 15-year tangerine peel. The attention in the CNFA-integrated network covered the overall shape of the tangerine

peel and accurately located the important feature areas. Figure 12(d) shows a 15-year tangerine peel with a black skin and a denser pig-bristle texture. In the heat maps generated by the three ResNet variants, red areas were mainly distributed in one section of the tangerine peel, ignoring the features of the age in other regions. Three ResNet variants predicted the sample to be 10-year tangerine peel or 20-year tangerine peel. The attention of the CNFA-integrated network was distributed in overall shape, which can capture more areas with a pig-bristle texture. Figure 12(e) shows a 20-year tangerine peel with typhoon scars on the epidermis. Three ResNet variants predicted the sample to be 15-year tangerine peel. The CNFA module accurately located the attention on the typhoon scars, while other attention modules ignore this important feature. This is mainly because the CNFA module can extract global feature information of tangerine peel. It helps the network accurately locate important feature positions, thus improving the accuracy of age recognition of the tangerine peel.

It can be seen that the CNFA proposed by us successfully recognizes the important features on the epidermis of tangerine peel in different ages. It aggregates low-level and high-level features on the epidermis of tangerine peel to provide more information for feature localization and can accurately locate the regions of interest to the important feature positions. By generating heat maps, we have effectively demonstrated that the CNFA module helps the network more accurately detect the appearance and details of different tangerine peels.

The method enables product quality control, traceability, and anticounterfeiting in the intelligent tangerine peel industry. By identifying the age of tangerine peel, producers can ensure that the products comply with standards and regulations. By utilizing age identification of the tangerine peel technology, it becomes possible to achieve anticounterfeiting and traceability for tangerine peel. Each tangerine peel can be associated with its unique age information, ensuring the authenticity and traceability of the age. By improving the database, this method can identify related varieties, counterfeit products, and artificially aged samples. Our algorithm has achieved a high level of accuracy. Additionally, the average detection time for detecting a single image is 0.55 seconds. Currently, the application is transmitting the data remotely to a server for processing and generating output results. The future work will focus on researching model lightweighting and efficiency improvement. This will facilitate the deployment of the model onto real-time terminals to achieve real-time detection.

6. Conclusions

This article proposes a method for age recognition of the tangerine peel based on the CNFA module to address the difficulty. Tangerine peel images are collected using a conventional digital camera and classified through the network model. This network can extract the global information of the tangerine peel and identify the important features that determine the age of the tangerine peel. The CNFA-integrated network had an accuracy of 97.17%, precision of 96.18%, recall of 96.09%, and F1 score of 96.13%, which did best in the comparison experiment. Furthermore, the CNFA module also exhibits fast processing speed. Finally, the validity of the model in the recognition task was verified through a visualization method based on heat maps, which concentrated the regions of interest on the important features of the tangerine peel and improved the detection accuracy of the age recognition. Therefore, this work has important application value for the identification of agricultural products represented by tangerine peel. Based on the excellent performance of this neural network, further exploration is needed. In future work, we will study multimodal network structures to improve detection accuracy and efficiency and achieve a lightweight structure to address the problem of extracting epidermis feature and endocarp feature.

Authors' Contributions

Fuqin Deng performed writing of the original draft; Junwei Li conducted experiment, data analysis, modelling, and writing of the original draft; Lanhui Fu performed optimisation of the language and writing of the original draft; Chuanbo Qin carried out sample resource collection and processing; Yikui Zhai collected literature and performed optimisation of the language; Hongmin Wang proofread the study; Ningbo Yi proofread the study; Nannan LI proofread the study; TinLun Lam proofread the study. All the authors have read and agreed to the published version of the manuscript. Fuqin Deng and Junwei Li contributed equally to this work and shared the first authorship.

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DETAIL

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Analysis of VOCs in Lueyang Black Chicken Breast Meat during the Steaming Process with GC-IMS and Stoichiometry

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ABSTRAK (ENGLISH)

Steamed chicken breast meat attracts people for its unique flavor and nutritional benefits. In this study, the sensory evaluation of Lueyang black chicken breast meat during steaming was first performed, and their volatile organic compounds (VOCs) were further analyzed by gas chromatography-ion mobility spectroscopy (GC-IMS) combined with stoichiometry. The sensory results demonstrated that the Lueyang black chicken breast meat steamed for 15–30 min was more acceptable in taste, flavor, and chewiness. A total of 60 volatile flavor signal peaks were obtained, and 46 VOCs were recognized from qualitative analysis, containing 24 aldehydes (51.19–72.57%), 8 ketones (10.15–16.97%), 9 alcohols (7.98-13.16%), 2 furans (2.24–10.85%), 2 esters (0.54–1.56%), and 1 ether (0.05–2.47%). A stable and reliable prediction model was set up by orthogonal partial least squares-discriminant analysis (OPLS-DA), and 18 characteristic VOCs (including 10 aldehydes, 3 alcohols, 3 ketones, 1 furan, and 1 ether) were picked out through variable importance in the projection (VIP>1.0 and $p<0.05$). Principal component analysis (PCA) results indicated that the cumulative contribution ratio was 92% with PC1 68.7% and PC2 23.3%, respectively, indicating that these characteristic VOCs could well discriminate the steaming time of Lueyang black chicken breast meat. Heatmap clustering analysis also demonstrated a similar distinguishing effect. These results could provide references for the research, development, and quality control of ready-to-eat steamed products for Lueyang black chicken breast meat in the future.

TEKS LENGKAP

1. Introduction

Chicken is the second favorite meat item after pork in the human diet. Among chicken varieties, black chickens are highly sought after due to their nutritional and medicinal value [1–3]. Lueyang black chicken is an ancient and excellent chicken breed in Lueyang County, Shaanxi Province of China, and has been certified as a geographical indication of agricultural products by the Ministry of Agriculture of China since 2017. Given its increasing quantity and scale, efforts have been made in the processing of Lueyang black chickens to increase their added value. To date, the genetics [4, 5], mitochondrial whole genome and molecular phylogeny [6], meat nutrition and transcriptome [7, 8], breeding [9–13], eggshell color [14, 15], and inosine acid content determination [16] of Lueyang black chicken have been studied. For a long time, slaughtered Lueyang black chicken has often been sold on the market, and there are fewer processed products. Meanwhile, there are several studies on the processed products of Lueyang black chicken, such as chicken sausage [17], chicken and *lentinus edodes* sauce [18], and chicken jerky [19]. Steaming, boiling, frying, and roasting are common cooking methods for chicken in daily life. Different cooking methods can lead to different texture and flavor profiles of the final products. Chicken breast meat is the tender part of chicken with high protein and low fat, and humans can easily cook, digest, and absorb it. Meanwhile, volatile flavor profiles of the processed chicken products are also very important quality parameters. There have been already several studies on the volatile organic compounds (VOCs) in raw chicken breast [20] and processed chicken breast (roasted, steamed, fried, etc.) based on gas chromatography-mass spectrometry (GC-MS) and gas chromatography-ion mobility spectroscopy (GC-IMS) [21–24].

Compared to GC-MS, GC-IMS is a novel emerging tool used for separating and detecting VOCs in food with low cost, rapid response, visualization, and high sensitivity [25, 26]. GC-IMS technology has been broadly utilized for the diagnosis and analysis of VOCs in restewed chicken breast [24], red-cooked chicken [27], braised chicken [28], and soft-boiled and boiled chicken [29, 30]. A large number of studies have proven that the GC-IMS is more effective than the common GC-MS, particularly for detecting trace volatile substances [21–24, 27–31], so the two complementary technologies can work together to provide the whole flavor profiles in foods.

Our previous studies analyzed the nutritional content and antioxidant effects of the soup made from Lueyang black chicken on aging mice induced by D-galactose [32] and optimized processing conditions of Lueyang black chicken sausage by single factor and orthogonal test [17]. However, there is still a lack of research on VOCs in the cooking process of Lueyang black chicken. This study aims to evaluate the sensory quality of Lueyang black chicken breast meat during the steaming process and further detect their VOCs by GC-IMS combined with stoichiometry, which would shed light on the research, development, and quality control of ready-to-eat steamed products of Lueyang black chicken in the near future.

2. Materials and Methods

2.1. Materials and Chemicals

Three 8-month-old fresh Lueyang black chicken cocks, with a body weight of 2.42 ± 0.43 kg, were purchased from Black Phoenix Black Chicken Breeding Co., Ltd. located in Lueyang County (Hanzhong, China). After killing the chickens, the breast meat was taken out and delivered to the laboratory on ice. Analytical grade n-ketones (2-pentanone, 2-butanone, 2-heptanone, 2-hexanone, 2-octanone, and 2-nonanone, purities ≥ 99%) were provided by Guoyao Reagent Co., Ltd. (Beijing, China).

2.2. Preparation of Steamed Breast Meat of Lueyang Black Chickens

The steamed chicken breast meat was referenced according to Fan et al. [21], with slight adjustments. The breast meat of Lueyang black chicken was first washed clean, cut into approximately 7 cm × 2.5 cm × 2 cm in size, and put in the steamer after steaming for 5, 15, and 30 min (labeled as Z5, Z15, and Z30, respectively). The raw breast meat (unsteamed, labeled as Z0) was used as control. It was removed and cooled, and then the steamed and unsteamed meat was crushed with a tissue masher homogenizer (JJ-2B, Changzhou, China) for analysis of VOCs.

2.3. Sensory Evaluation

Based on the Chinese national standard for sensory evaluation of meat products (20142773-T-601), 10 evaluators (including 5 men and 5 women) with experience in meat evaluation were invited to score three steamed chicken breast meat samples in four aspects, including taste, aroma, chewiness, and tissue state, with a total score of 100. The final sensory score = taste × 0.3 + aroma × 0.3 + chewiness × 0.3 + tissue status × 0.1, and the specific sensory assessment standards are listed in Table 1 [33].

Table 1

The sensory assessment standards of chicken breast meat.

Index	Scoring criteria	Score
Taste	Fresh and delicious	20~30
Not outstanding	10~19	No taste
0~9	-	
Aroma	Strong fragrance	20~30
Light fragrance	10~19	No fragrance
0~9	-	
Chewiness	Easy to chew	20~30
Easier to chew	10~19	Hard to chew
0~9	-	
Tissue status	Tight	8~10
Slightly fluffy	4~7	Fluffy

2.4. GC-IMS Assay of Breast Meat VOCs

GC-IMS was utilized to analyze the volatile flavor substances in breast meat samples of Lueyang black chicken at different steaming times. Each kind of meat sample was measured 3 times. Concisely, 2.0 g of breast meat at different steaming times was put in a 20.0 mL headspace vial and 500.0 μL headspace gas nitrogen (purity ≥99.99%) was injected into the injector after incubation at 60°C for 15 min and then detected by GC-IMS instrument (FlavourSpec®, Germany) [31]. The gas chromatographic separation was performed on the MXT-5 column (15 m × 0.53 mm) at 60°C with nitrogen (purity≥99.99%) as a carrier gas for 20 min. The start-up gas flow rate was 2.0 mL/min, maintained for 2 min, linearly enlarged to 10 mL/min within 10 min, and then linearly expanded to 100 mL/min within 20 min. The IMS was performed with 45°C IMS detector temperature and 150 mL/min nitrogen (purity ≥99.99%) flow rate and analyzed for 30 min. The n-ketones mentioned above were used as immigrant markers to obtain the relative ratio of VOCs in the breast meat according to the retention index (RI) and the drift time (DT) provided by the fragment library of the instrument.

2.5. Statistical Analysis

The VOCs were qualitatively identified using the built-in NIST 2014 and self-built IMS database software. The

Reporter plug-in allows for direct comparison of spectral differences between samples. The Gallery Plot plugin can be used to compare the fingerprint spectrum, allowing for intuitive and quantitative comparison of VOCs' differences between different samples. Excel 2010 was used to draw the bar graph of relative content changes of different components. PCA and the establishment and validation of OPLS-DA modeling were carried out by SIMCA-P 14.1 software. The cluster heatmap was drawn using the BioDeep tool assistant (https://www.biodeep.cn/home/tool).

3. Results and Discussion

3.1. Sensory Scores of Lueyang Black Chicken Breast Meat at Different Steaming Times

According to the sensory evaluation standards shown in Table 1, evaluators conducted a sensory evaluation on the steamed chicken breast meat samples of Z5, Z15, and Z30 (Figure 1(a)), and the sensory scores bar graph is shown in Figure 1(b). As can be seen from the results of the sensory evaluation (Figure 1), as the steaming time extended, the volume of breast meat continued to increase, the tissue was more fluffy, and the taste, aroma, and chewiness were better. The total sensory score of sample Z30 was the highest, followed by samples Z15 and Z5. Due to the short steaming time, the taste, aroma, and chewiness of sample Z5 were poor, and there were significant differences compared with samples Z15 and Z30, respectively ($p<0.01$). The taste and aroma of sample Z30 were better than those of sample Z15, while there was no significant difference between them, and sample Z30 is easier to chew and more fluffy than sample Z15. Therefore, based on different eating habits, consumers who prefer chewy meat can choose the breast meat steamed for 15 min, while consumers who prefer meat that is easy to chew can choose the breast meat steamed for 30 min. The Lueyang black chicken breast meat steamed for 15–30 min was more acceptable in taste, flavor, and chewiness.

[figure(s) omitted; refer to PDF]

3.2. GC-IMS 3D and 2D Spectrum of VOCs in Four Chicken Breast Meat Samples

The VOCs of the breast meat samples Z0, Z5, Z15, and Z30 were measured by GC-IMS. The GC-IMS 3D spectra of four breast meat samples were obtained (Figure 2(a)). The DT, ion relative retention time (RT), and signal intensity were the *X*, *Y*, and *Z* axes, respectively, on the 3D spectra. The signal intensity showed the amplitude of the peak [23, 31]. There were fewer VOCs on the spectrum of Z0 samples, while there were a higher variety and quantity of VOCs on the spectra of Z5, Z15, and Z30 samples steamed for 5, 15, and 30 min. When the meat was cooked, it could produce a greater variety and quantity of flavor substances. This is consistent with the results that the restewed chicken produced more variety and quantity of VOCs than the raw chicken [24] and the contents and types of VOCs of steamed sea bass were significantly higher than those of unsteamed sea bass [34]. However, it is difficult to tell the differences in VOCs on the 3D spectra.

[figure(s) omitted; refer to PDF]

To compare the variations of VOCs in the four breast meat samples more intuitively, 2D view spectra (Figure 2(b)) were converted by the 3D spectra in Figures 2(a), and 2D difference spectra (Figure 2(c)) were obtained by deducting Z0 spectrum as a base. Different VOCs have different horizontal migration times and vertical RT, and the same VOC content in different samples varied with an obvious trend of change (Figures 2(b) and 2(c)). The VOCs of Lueyang black chicken breast meat at different steaming times could be separated and distinguished effectively by GC-IMS, showing different characteristic GC-IMS spectra (Figures 2(b) and 2(c)).

3.3. Qualitative Analysis of VOCs in Four Chicken Breast Meat

The qualitative analysis of VOCs is conducted based on their DT and RT. Samples Z0 were taken as an example for qualitative analysis of VOCs (Figure 3), while those of other samples were not shown here. Each signal peak in Figure 3 represents a substance, marked with numbers. There were 60 signal peaks obtained from the breast meat of Lueyang black chicken at the different steaming time, from which 46 VOCs were identified, including 24 aldehydes (51.19–72.57%), 8 ketones (10.15–16.97%), 9 alcohols (7.98–13.16%), 2 furans (2.24–10.85%), 2 esters (0.54–1.56%) and 1 ether (0.05–2.47%) (Figure 3 and Table 2).

[figure(s) omitted; refer to PDF]

Table 2

Qualitative analysis and flavor description of VOCs in Lueyang black chicken breast meat during the steaming

process.

No.	Chemicals	RI	RT/s	DT/ms	Relative content (%)				Flavor description
Z0	Z5	Z15	Z30	1	2-Butanone-M	568.7	130.791	1.06064	12.994±1.449[a]
5.030±0.528[c]	8.153±0.189[b]	8.711±0.205[b]	Ether	2	2-Butanone-D	575.7	132.439	1.24613	0.104±0.011[c]
0.174±0.028[c]	0.460±0.012[b]	0.585±0.070[a]	Ether	3	Pentanal-M	687.6	162.092	1.19251	4.350±0.740[a]
3.648±0.211[a b]	3.278±0.011[b]	3.086±0.068[b]	Almond, malt, pungent	4	Pentanal-D	694	164.438	1.42362	0.523±0.124[d]
4.252±0.345[c]	5.803±0.095[a]	5.431±0.060[b]	Almond, malt, pungent	5	1-Pentanol-M	761.1	192.16	1.25622	1.948±0.346[b]
2.918±0.302[a]	1.875±0.011[b]	1.618±0.039[b]	Balsamic	6	1-Pentanol-D	761.3	192.23	1.51238	0.390±0.023[b]
3.686±0.297[a]	3.404±0.073[a]	3.537±0.018[a]	Balsamic	7	Hexanal-M	797.2	209.086	1.26376	11.318±0.713[a]

11.229 ± 0.521[a]	8.513 ± 0.118[b]	7.776 ± 0.144[b]	Grass, tallow, fat	8	Hexanal-D	792.6	206.824	1.56223	12.504 ± 2.674[b]
20.641 ± 0.487[a]	22.449 ± 0.099[a]	22.175 ± 0.160[a]	Grass, tallow, fat	9	2-Methylbutanal-M	658.1	153.69	1.16897	0.614 ± 0.036[a]
0.384 ± 0.014[c]	0.524 ± 0.008[b]	0.529 ± 0.016[b]	Cocoa, almond	10	2-Methylbutanal-D	651.9	151.976	1.40071	1.2855 ± 0.755[a]
0.376 ± 0.037[b]	0.255 ± 0.014[b]	0.230 ± 0.007[b]	Cocoa, almond	11	3-Methylbutanal-M	641.8	149.232	1.18336	1.550 ± 0.216[a]
1.208 ± 0.043[b]	1.171 ± 0.007[b]	1.164 ± 0.009[b]	Malt	12	3-Methylbutanal-D	642.4	149.404	1.40677	0.052 ± 0.006[c]
0.058 ± 0.009[c]	0.275 ± 0.018[b]	0.383 ± 0.030[a]	Malt	13	2-Pentanone	678.1	159.349	1.12126	3.018 ± 0.391[a]
0.393 ± 0.078[b]	0.199 ± 0.003[b]	0.185 ± 0.008[b]	Ether, fruit	14	1-Propene-3-methylthio	691.0	163.292	1.04401	2.471 ± 0.178[a]
0.230 ± 0.066[b]	0.046 ± 0.003[c]	0.060 ± 0.005[bc]	Garlic, onion	15	3-Hydroxy-2-butanone-M	710.0	170.665	1.06522	1.811 ± 0.373[a]

0.299 ± 0.066[b]	0.189 ± 0.003[b]	0.218 ± 0.005[b]	Butter, cream	16	3-Hydroxy-2-butanone-D	711.7	171.351	1.33104	0.121 ± 0.072[a]
0.073 ± 0.003[a]	0.092 ± 0.008[a]	0.092 ± 0.004[a]	Butter, cream	17	2-Methyl-propanal	543.5	124.967	1.28492	0.297 ± 0.005[c]
0.238 ± 0.043[c]	0.951 ± 0.046[b]	1.218 ± 0.094[a]	Pungent, malt, green	18	Methyl acetate	520.8	119.959	1.19416	0.433 ± 0.096[c]
0.287 ± 0.025[d]	0.741 ± 0.008[b]	0.910 ± 0.050[a]	Sweet, fruity	19	1-Penten-3-ol-M	669.7	156.949	0.9404	0.534 ± 0.173[b]
0.904 ± 0.050[a]	0.838 ± 0.009[a]	0.834 ± 0.033[a]	Butter, pungent	20	1-Penten-3-ol-D	691	163.32	1.35354	1.470 ± 0.113[b]
1.853 ± 0.201[a]	0.774 ± 0.047[c]	0.721 ± 0.037[c]	Butter, pungent	21	Octanal-M	1000.4	357.209	1.40732	2.301 ± 0.141[d]
4.911 ± 0.101[a]	3.905 ± 0.021[b]	3.489 ± 0.044[c]	Fat, soap, lemon, green	22	Octanal-D	1000.1	356.829	1.82369	0.476 ± 0.018[c]

3.004 ± 0.359[a]	2.505 ± 0.033[b]	2.286 ± 0.056[b]	Fat, soap, lemon, green	23	6-Methyl-5-hepten-2-one	983.5	339.799	1.17573	0.378 ± 0.080[a]
0.157 ± 0.013[b]	0.124 ± 0.009[b]	0.128 ± 0.011[b]	Pepper, mushroom, rubber	24	Benzaldehyde-M	953.7	312.134	1.14842	0.599 ± 0.169[b]
0.510 ± 0.037[b]	1.007 ± 0.025[a]	1.036 ± 0.012[a]	Almond, burnt sugar	25	Benzaldehyde-D	953.6	312.078	1.47255	0.132 ± 0.033[c]
0.084 ± 0.012[d]	0.320 ± 0.007[b]	0.400 ± 0.023[a]	Almond, burnt sugar	26	1-Octen-3-ol-M	974.3	331.012	1.15835	0.657 ± 0.065[c]
2.283 ± 0.118[a]	2.063 ± 0.013[b]	2.098 ± 0.050[b]	Mushroom	27	1-Octen-3-ol-D	973.5	330.27	1.59704	0.194 ± 0.009[d]
0.437 ± 0.113[c]	0.655 ± 0.034[b]	0.808 ± 0.015[a]	Mushroom	28	2-Pentyl furan	987.1	343.264	1.25468	0.204 ± 0.018[d]
0.554 ± 0.020[c]	0.651 ± 0.013[b]	0.716 ± 0.026[a]	Green bean, butter	29	Heptanal-M	895	264.184	1.33916	2.802 ± 1.004[a]

2.693 ± 0.279[a]	1.710 ± 0.036[b]	1.514 ± 0.022[b]	Fat, citrus, rancid	30	Heptanal-D	894.5	263.813	1.69931	0.352 ± 0.075[d]
3.054 ± 0.086[a]	2.540 ± 0.006[b]	2.390 ± 0.005[c]	Fat, citrus, rancid	31	2-Heptanone	883.0	256.388	1.63113	0.154 ± 0.016[a]
0.077 ± 0.003[c]	0.120 ± 0.002[b]	0.123 ± 0.008[b]	Soap	32	n-Hexanol-M	861.8	243.764	1.32879	2.399 ± 1.754[a]
0.785 ± 0.074[a][b]	0.516 ± 0.017[b]	0.536 ± 0.007[b]	Resin, flower, green	33	n-Hexanol-D	859.2	242.279	1.64002	0.340 ± 0.299[a]
0.186 ± 0.019[a]	0.216 ± 0.007[a]	0.275 ± 0.004[a]	Resin, flower, green	34	2-Hexenal-M	846.8	235.225	1.18058	0.213 ± 0.209[a]
0.129 ± 0.028[a]	0.118 ± 0.005[a]	0.142 ± 0.008[a]	Fat, rancid	35	2-Hexenal-D	840.8	231.906	1.51179	0.066 ± 0.003[b]
0.126 ± 0.042[a]	0.131 ± 0.006[a]	0.160 ± 0.009[a]	Fat, rancid	36	n-Nonanal-M	1104.1	503.508	1.47787	6.149 ± 0.571[b]

8.331 ± 0.259[a]	6.493 ± 0.135[b]	6.215 ± 0.175[b]	Fat, citrus, green	37	n-Nonanal-D	1104.5	504.205	1.94640	0.876 ± 0.096[c]
2.720 ± 0.323[a]	2.197 ± 0.130[b]	2.213 ± 0.127[b]	Fat, citrus, green	38	Butanal	590.3	135.976	1.29064	2.633 ± 0.085[a]
0.833 ± 0.019[b]	0.846 ± 0.049[b]	0.787 ± 0.057[b]	Pungent, green	39	Acetone	484.9	112.431	1.11614	8.387 ± 1.094[a]
3.952 ± 0.455[c]	5.636 ± 0.292[b]	6.328 ± 0.224[b]	Apple, pear	40	2-Penten-1-ol (Z)	758.6	191.039	1.45654	0.0513 ± 0.005[d]
0.110 ± 0.027[c]	0.161 ± 0.008[b]	0.198 ± 0.006[a]	Green, plastic, rubber	41	Butyl acetate	813.3	217.232	1.61174	0.106 ± 0.018[c]
0.448 ± 0.088[b]	0.664 ± 0.020[a]	0.654 ± 0.012[a]	Pear	42	2-Butyl furan	883.8	256.845	1.17934	10.648 ± 0.735[a]
2.602 ± 0.457[b]	1.671 ± 0.049[c]	1.522 ± 0.049[c]	Fruity, wine, sweet, spicy	43	2-Heptenal (E)	949.9	308.789	1.66653	0.536 ± 0.167[d]

2.850 ± 0.540[c]	4.509 ± 0.056[b]	5.208 ± 0.083[a]	Soap, fat, almond	44	2-Octenal (E)-M	1053.9	426.438	1.33121	0.623 ± 0.079[b]
0.752 ± 0.118[ab]	0.819 ± 0.016[a]	0.866 ± 0.043[a]	Green, nut, fat	45	2-Octenal (E)-D	1054.5	427.217	1.82421	0.209 ± 0.047[a]
0.073 ± 0.011[b]	0.068 ± 0.007[b]	0.078 ± 0.005[b]	Green, nut, fat	46	Decanal	1220.2	739.579	1.54424	0.727 ± 0.026[a]

The -M and -D following VOCs indicate monomer and dimer, respectively. Different lowercase letters in the same line indicate a significant difference ($p<0.05$). The flavor description comes from https://www.flavornet.org/flavornet.html and https://www.thegoodscentscompany.com/search2.html.

The RI, RT, DT, relative proportion, and flavor description of various VOCs are shown in Table 2. The relative proportion of hexanal was the highest among aldehydes, reaching 23.82%, 31.87%, 30.96%, and 29.95%, respectively, in the samples of Z0, Z5, Z15, and Z30 (Table 2), which can endow the breast meat of Lueyang black chicken with grass, tallow, and fat aroma, consistent with the results from Wang et al. [35]. Lueyang black chicken is a kind of free-range chicken in mountainous areas, and the hexanal content of free-range chickens is greater than that of cage-range chickens [36], and this may be one of the reasons for the high hexanal content of Lueyang black chicken breast meat. Research also showed that the *SLC27A1* gene and peroxisome proliferator-activated receptor (PPAR) pathway are closely related to hexal content in Chinese local chicken VOCs [37].

3.4. Fingerprint of VOCs in Four Chicken Breast Meat Samples

The fingerprint was constructed to display the VOCs' differences in four breast meat samples, including Z0, Z5, Z15, and Z30, by three parallel tests (Figure 4). The horizontal represents four breast meat samples (Z0, Z5, Z15, and Z30) from top to bottom, while the vertical represents the same volatile compound at different steaming times. The redder and larger the spot is, the higher the VOC content [38, 39]. There are significant differences in VOCs in four breast meat samples (Figure 4). The relative content of VOCs in samples of Z0, including butanal, 2-pentanone, 1-propene-3-methylthio, 6-methyl-5-hepten-2-one, 3-hydroxy-2-butanone monomer, 2-butanone, hexanal, and n-hexanol, were relatively higher (Figure 4). The variety and content of VOCs in samples Z5, Z15, and Z30 exhibited a clear increase compared to that of samples Z0, which is consistent with the observed trend of VOCs variation in chicken following thermal processing [22, 24]. The concentration of VOCs, including 1-penten-3-ol, octanal, n-nonanal, pentanal dimer, 1-pentanol, 2-methylbutanal monomer, 1-octen-3-ol, 2-pentyl furan, heptanal dimmer, 2-hexenal dimmer, 2-penten-1-ol (*Z*), and butyl acetate, exhibited relatively higher levels as the steaming time increased from 0 min to 5 min. After increasing the steaming time from 5 min to 15 min, there was a decrease in the levels of 1-penten-3-ol dimmer, octanal dimmer, 1-pentanol monomer, hexanal monomer, heptanal, n-nonanal, decanal, and octanal monomer, while there was an increase in the content of benzaldehyde monomer, 2-heptanone, acetone, butyl acetate, pentanal-D, 3-methylbutanal dimer, 2-methyl-propanal, methyl acetate, benzaldehyde dimer, 1-octen-3-ol dimer, 2-pentyl furan, 2-penten-1-ol (Z), and 2-heptenal (E). The content of VOCs, including octanal monomer, and heptanal dimer content decreased, and the contents of pentanal dimer, 3-methylbutanal dimer, 2-

methyl-propanal, methyl acetate, benzaldehyde dimer, 1-octen-3-ol dimer, 2-pentyl furan, 2-penten-1-ol (Z), and 2-heptenal (E) showed relatively higher levels as the steaming time increased from 15 min to 30 min.

[figure(s) omitted; refer to PDF]

During the hot processing of meat, various VOCs are generated, such as alcohols, aldehydes, ketones, esters, furans, ethers, and other substances [21–24]. To demonstrate the various VOC changes, the relative content of various VOCs in Lueyang black chicken breast meat at different steaming times was calculated based on the signal intensity of various substances on the fingerprint spectrum (Figure 5). The results showed that aldehydes, ketones, and alcohols were the main VOCs in steamed Lueyang black chicken breast meat, which was consistent with the research results of Fan et al. [21]. The relative content of aldehyde in breast meat of Lueyang black chicken was the highest, ranging from 69.14% to 72.57%, composed of hexanal, heptanal, decanal, butanal, octanal, pentanal, n-nonanal, benzaldehyde, 2-heptenal (E), 2-hexenal, 2-methyl-propanal, 2-octenal (E), 2-methylbutanal, and 3-methylbutanal. The relative content of ketones was 10.14%–16.37%, including 2-pentanone, 2-butanone, 2-heptanone, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one, and acetone. The relative content of alcohols was 10.50%–13.16%, which was composed of 1-octen-3-ol, 1-pentanol, 1-penten-3-ol, n-hexanol, and 2-penten-1-ol (*Z*). The relative content of furans, including 2-butyl furan and 2-pentyl furan, was 2.24%–3.16%. The relative content of esters, namely, butyl acetate and methyl acetate, was 0.74%–1.56%. The relative content of ether 1-propene-3-methylthio was 0.05%–0.23%.

[figure(s) omitted; refer to PDF]

The aldehydes, ketones, alcohols, furans, esters, and ethers mainly generated after the oxidation and degradation of chicken fat are important for chicken flavor [40]. From Figure 5, it can be seen that before steaming, the VOCs of Lueyang black chicken breast meat are mainly composed of aldehydes (51.19%), ketones (26.97%), furans (10.85%), and alcohols (7.98%), with a small amount of ethers (2.47%) and esters (0.54%). When the breast meat was steamed for 5 min, the content of aldehydes, esters, and alcohols increased, while the content of furans, ketones, and ethers decreased. With the steaming time extended from 5 min to 30 min, the content of aldehydes, alcohols, furans, and ethers in the breast meat of the steamed Lueyang black chicken gradually decreased, while the content of ketones and esters gradually increased. When steamed for 30 min, the VOCs in the breast meat of Lueyang black chicken are mainly aldehydes (69.14%), ketones (16.37%), and alcohol (10.625%).

Aldehydes have a low threshold, which is very important in the production of meat flavor [40]. They are the key VOCs in local Chinese chicken meat [41], which account for the highest proportion of VOCs in the unsteamed and steamed breast meats of Lueyang black chicken, and contribute grass, tallow, fat, almond, malt, pungent, citrus, green aromas to the breast meat. Aldehydes also contributed the largest to the overall breast meat flavor of Piao chicken and Yanjin silky fowl [20], Chai hen, and black chicken [35]. The thresholds of ketones and alcohols are higher than those of aldehydes, with pleasant flavors such as floral and fruity. Ketones are few in variety; however, they are important in the formation of chicken's characteristic flavor. The contents of ketones and alcohols in the breast meat of Lueyang black chicken steamed for 30 min were higher than that in the breast meat of many Chinese native chickens [21, 35].

3.5. OPLS-DA and Model Validation

OPLS-DA, different from PCA, is a supervised statistical method for discriminant analysis. The relationship model between the substance expression and sample category was established by partial least squares regression to predict the sample category. R2X and R2Y stand for the explanation rate of the constructed model for the *X* and *Y* matrices, respectively, and Q2 denotes the prediction ability of the model. A scoring plot in OPLS-DA was used to classify VOCs of 4 kinds of breast meat, and the results are shown in Figure 6. Parameters of Q2 greater than 0.5 and less than 1.0 are considered to be more accurate [38, 39, 42]. Most information on the VOCs of four breast meat was covered by this model with Q2 (cum) = 0.648, R2X(cum) = 0.96, and R2Y(cum) = 0.786 (Figure 6). Most breast meat samples at different steaming times could be classified by the OPLS-DA map (Figure 6(a)). To prevent overfitting, a permutation test was used to confirm the OPLS-DA model reliability, and the result is shown in Figure 6(b). The regression line of Q2 (−0.815) is less than 0 at the crossing point of the ordinate after 200 cross-

validations. R2 and Q2 are lower than the raw values in all tests, showing that the established OPLS-DA model is not overfitting and is stable and reliable [43].

[figure(s) omitted; refer to PDF]

3.6. Screening of Characteristic VOCs in Four Breast Meat

According to the VIP values in the OPLS-DA model, the importance of each variable for sample classification can be quantified. The characteristic VOCs were screened based on VIP values greater than 1.0 and significance levels less than 0.05. The screening method based on the OPLS-DA model and VIP values was used in many kinds of foods to select the characteristic flavor components [31, 34, 38, 39, 44]. The results showed that 18 characteristic VOCs were screened from 46 VOCs in four breast meat samples (Figure 7(a)), including 10 aldehydes (hexanal (monomer and dimer), pentanal (monomer and dimer), octanal (monomer and dimer), heptanal (monomer and dimer), 2-heptenal (E), and n-nonanal monomer), 3 ketones (acetone, 2-butanone monomer, and 2-pentanone), 3 alcohols (1-pentanol (monomer and dimer) and n-hexanol monomer), 1 furan (2-butyl furan), and 1 ether (1-propene-3-methylthio). Hexanal dimmer (with aromas of grass, tallow, and fat) had the highest VIP value, followed by 2-butanone monomer (with aromas of ether), 2-butyl furan (with aromas of fruity, wine, sweet, and spicy), hexanal monomer (with aromas of grass, tallow, and fat), 2-heptenal (*E*) (with aromas of soap, fat, and almond), acetone (with aromas of apple and pear), and pentanal dimmer (with aromas of almond, malt, and pungent). Hexanal is the main VOC in steamed, stewed, air-fried, boiled, fried, and roasted chicken breast meat [21–23, 35]. 2-Butanone is present in the volatile flavor compounds of fried and roasted chicken breast meat [22]. 2-Heptenal (*E*) is an olefinic aldehyde with a very low threshold that plays an important role in chicken breast flavor.

[figure(s) omitted; refer to PDF]

The 18 characteristic VOCs were then subjected to PCA and cluster analysis (Figures 7(b) and 7(c)). PCA results showed that the cumulative contribution ratio of 18 characteristic substances was 92% (PC1 and PC2 were 68.7% and 23.3%, respectively) (Figure 7(b)), which could explain the variation in the samples. In addition, 18 kinds of characteristic VOCs in the breast meat samples of Lueyang black chicken at the same steaming time were relatively concentrated, which could better distinguish the breast meat samples of Lueyang black chicken at different steaming times. Based on the signal intensities of these VOCs, a clustering heatmap was created (Figure 7(c)), and it was found that there were differences in 18 characteristic VOCs in the breast meat samples of Lueyang black chicken at different steaming times. The flavor characteristics of Lueyang black chicken breast meat could be divided into three categories: before steaming (Z0), the early stage of steaming (Z5), and the late stage of steaming (Z15, Z30). The results of PCA and heat map of 18 characteristic flavor substances could distinguish the breast meat of Lueyang black chicken with different steaming times. In this study, 18 potential characteristic VOCs were screened by GC-IMS and the OPLS-DA model with VIP > 1.0 and $p < 0.05$. However, quantitative analysis was lacking. In the future, it is necessary to combine GC-MS, GC-O, and relative odor activity values to further reveal the fine changes of VOCs during the steaming process of Lueyang black chicken breast meat.

4. Conclusions

In summary, a total of 46 VOCs were identified from the breast meat of Lueyang black chicken at different steaming times, mainly including aldehydes, ketones, alcohols, and furans. Compared with raw chicken breast meat, the relative contents of aldehydes and alcohols increased after steaming, whereas the relative contents of ketones and furans decreased. A stable and reliable OPLS-DA model was established, and 18 characteristic VOCs were screened including 10 aldehydes, 3 ketones, 3 alcohols, 1 furan, and 1 ether. Among them, the important substances affecting the flavor of chicken breast meat during the steaming process were hexanal dimmer, 2-butanone monomer, 2-butyl furan, hexanal monomer, 2-heptenal (*E*), acetone, and pentanal dimer (VIP > 1.50). Further, the PCA and cluster analysis of 18 characteristic VOCs showed that they could effectively distinguish the breast meat of Lueyang black chicken at different steaming times. Combined with sensory evaluation results, 15–30 min were recommended for steaming Lueyang black chicken breast meat. The GC-O aroma profiles, degradation of protein, and lipid of Lueyang black chicken breast during the steaming process will be reported elsewhere.

Authors' Contributions

Linlin He and Rui Chen conducted investigation, wrote the original draft, and performed plot analysis. Fei Lan and Hui Yang performed partial analysis, visualization, and language check. Ruichang Gao reviewed and edited the manuscript. Wengang Jin reviewed and edited the manuscript, supervised the work, and acquired the funding. Linlin He and Rui Chen contributed equally to this work.

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DETAIL

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Improvement of Quality Characteristics and Shelf Life Extension of Raw Chicken Meat by Using Black Mulberry Leaf (Morus nigra L.) Extracts

Yasemin Çelebi.

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ABSTRAK (ENGLISH)

The objective of this study was to examine the impact of different concentrations of black mulberry leaf extract (BMLE) on the microbial quality, lipid oxidation, biogenic amine content, color stability, and sensory attributes of raw chicken meat during a 12-day chilled storage period. The raw chicken meat was treated with 0.1% BHT (positive control), 0.1%, 0.3%, and 0.5% BMLE, and the outcomes were then compared to the results obtained from raw chicken meat with no additive (control). In comparison to the control group, the inclusion of BMLE resulted in a decrease ($P<0.05$) in pH and thiobarbituric acid reactive substances (TBARS), as well as an improvement in redness (a^*) ($P<0.05$). The addition of BMLE significantly extended the shelf life of raw chicken meats compared to the control, as it limited microbiological development and lipid oxidation during storage ($P<0.05$). Additionally, the BMLE exhibited the most potent inhibitory impact on the buildup of these four BAs (tyramine, cadaverine, histamine, and tyramine) in raw chicken samples at the 12-day storage period ($P<0.05$). Despite the 0.5% BMLE groups’ lowest

results for microbial counts, TBARS, and biogenic amines, the concentration of 0.3% BMLE proved to be the most advantageous in terms of sensory acceptability. These findings suggested that BMLE, rather than artificial chemicals, could be utilized in raw chicken products as a promising natural antioxidant and antibacterial agent.

TEKS LENGKAP

1. Introduction

Poultry meat is one of the most widely consumed foods in the world, and its production and consumption have increased significantly in the past several decades. The appeal of poultry meat stems from the fact that it is the most affordable and readily available meat source, and unlike beef or hog, in terms of culture or religion, there are no restrictions on it [1]. Its low-fat content has led to the designation of poultry meat as a low-calorie food. However, because of its high degree of unsaturation, the lipids in its muscles are pretty vulnerable to oxidation. Modern countries prefer poultry due to its accessibility, ease of use in further processed dishes, and healthier profile [2]. One of the main reasons meat quality deteriorates is lipid oxidation, which can lead to rancidity and the development of unwanted flavors and aromas. These effects reduce the functional, sensory, and nutritional value of meat products and their acceptance by consumers [3]. Internal factors such as iron content and antioxidant enzymes, along with external factors like feeding with oxidized foods, stress, slaughtering procedures, temperature, additional processing processes, and storage conditions, primarily influence the oxidation of poultry meat [2]. Poultry meat products can spoil due to either chemical deterioration or microbial growth. The main form of chemical deterioration is oxidative rancidity, which can cause significant changes in flavor, color, and protein structure and a loss of freshness that may discourage repeat purchases by consumers [4]. Synthetic antioxidants, including propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroxyquinone (TBHQ), can be added to poultry meat to delay, lessen, or avoid oxidative degradation [5, 6]. However, due to the potentially harmful effects of synthetic additives, consumers reacted negatively, prompting producers to turn to natural antioxidant sources [7]. In meat and meat products, edible extracts from plants and fruits high in phenolics have recently replaced synthetic substances to slow the oxidation of lipids and proteins, lessen discoloration, and inhibit the growth of microorganisms [8, 9].

Black mulberry leaves (*Morus nigra* L.) have been used as herbal medicines in China since ancient times and have recently become the most popular form of herbal medicine. The variations in nutritional components of mulberry leaves across different studies can be attributed to different factors such as varieties, genetics, environments, ecologies, and plant harvest conditions. Research findings indicated that dried mulberry leaf powder consists of moisture (ranging from 5.11% to 7.24%), crude protein (15.31% to 30.91%), total ash (14.59% to 17.24%), neutral detergent fiber (NDF) (27.50% to 36.66%), crude fat (2.09%), carbohydrates (9.70% to 29.74%), and energy content (113 to 224 kcal/100 g). Its bioactive compounds and phenolic substances contribute to its high antioxidant activity. The black mulberry leaves contain several primary phenolic acids, including caffeic acid, vanillic acid, chlorogenic acid, hydroxybenzoic acid, p-coumaric acid, sinapic acid, and ferulic acid. Furthermore, black mulberry leaves have demonstrated antimicrobial properties [10]. Despite these features, there is a significant lack of research on the use of black mulberry leaves in poultry meat. Hence, exploring the possible use of black mulberry leaves as a natural source of antioxidants in poultry products is crucial for enhancing their quality properties. So far as we are aware, no study is available to determine how black mulberry leaves affect the qualitative attributes of raw chicken meat. The objective of the investigation was to assess the impact of different concentrations of black mulberry leaf extract (BMLE) on the microbiological quality, lipid oxidation, biogenic amine content, color stability, and sensory attributes of raw chicken meat over a 12-day refrigerated storage period.

2. Material and Method

2.1. Materials and Chemicals

For this study, armless and skinless chicken thigh flesh was shipped in ice boxes to the laboratory from a nearby

poultry meat processing facility (Gedik Pilic Co).

2.2. Preparation of Black Mulberry Leaf Extract (BMLE)

Fresh black mulberry leaves (*Morus nigra*) were collected from diverse locations in Uşak, Turkey, using clean, dry, and sterilized plastic containers. Subsequently, these leaves were dried in the shade at room temperature in the clean, dry laboratory and were finely ground into a powder with a grinder for 2 minutes. The resulting powder was stored at −20°C to prevent enzymatic degradation. Black mulberry leaf extract was extracted following the procedure outlined by Martin-Garcia et al. [11]. Approximately 5 grams of black mulberry leaf powder were added to 25 mL of ethanol (96%) and water (50/50, V/V). The product blend was subjected to ultrasonication at 60°C for 45 minutes using an ultrasonic water bath (WB11, Daihan Scientific, Korea). Then, the mixture was centrifuged and filtered through the Whatman No: 1 filter paper. The liquid that passed through the filter was concentrated using a rotary evaporator (IKA, HB4 basic; RV 05 basic, Germany) in a vacuum at 40°C. After that, 20 mL of the condensed extract was put into 90 mm-diameter Petri dishes. These dishes were frozen at −40°C for 24 hours and subsequently subjected to lyophilization at 50°C.

2.3. Analyzing the Characteristics of BMLE

A pH measurement was conducted using a digital pH meter (Hanna Instruments, pH210, USA). A chromameter (Konica Minolta CR-410 from Osaka, Japan) was used to measure color values. Total phenolic content (TPC) was assessed using the Folin–Ciocalteu method, as detailed by Singleton et al. [12]. The DPPH radical scavenging activity, denoted as DPPH-RSA, was assessed through the methodology outlined by Blois [13] for measuring the capability to neutralize the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical.

2.4. Preparation of Raw Chicken Meat

To achieve this goal, around 1 kg of chicken thigh meat without skin or bones was soaked in 2 L of distilled water with varying levels of BMLE for 5 minutes, such as control (0% BMLE, without extract), 0.1% BMLE, 0.3% BMLE, and 0.5% BMLE. The other group dipped in a 0.1% BHT solution. Each piece of chicken meat from the extract solution was drained through a sieve for five minutes, then placed in polystyrene trays and covered with polyethylene film. Following packaging, the samples were kept at 4°C for 12 days, and analyses were conducted on days 0, 3, 6, 9, and 12. The complete research comprised two independent trials with three measurements for each analysis at distinct manufacturing times.

2.5. Analysis of Raw Chicken Meat

2.5.1. Approximate Composition

The analytical methods indicated by Gökalp et al. [14] were utilized to approximate the chicken flesh samples' ash, moisture, fat, and crude protein content.

2.5.2. pH

About 10 g of chicken meat were combined with 100 mL of distilled water and blended for 25–30 seconds utilizing an ultra-turrax. The pH levels were carried out at a temperature of about 20°C using a pH meter (Hanna Instruments, pH 210, USA).

2.5.3. Color Analysis

The color of the samples was evaluated using a chroma meter (CR-410, Konica Minolta, Osaka, Japan) equipped with an 8 mm-diameter aperture and the standard illuminant D50 at an observed angle of 10 degrees. In the CIE Lab color system, the L*, a*, and b* values correspond to black-white, red-green, and yellow-blue color characteristics, respectively. The L* value denotes the lightness ranging from black to white, the a* value indicates the presence of red or green hues, and the b* value represents the degree of yellowness or blueness in the color. The white standard plate was used to calibrate the instrument before color readings were taken.

2.5.4. Microbiological Analyses

Samples weighing ten grams of chicken meat were extracted from each pack and placed in an aseptic stomacher pouch. The samples were then homogenized using a stomacher for 90 seconds after adding 90 mL of pepton water. Following the decimal homogenate dilutions, a duplicate plate was constructed to count the microbes for each dilution using the surface spreading method. Petri plates were then incubated under aerobic conditions at 30°C for

2-3 days for total mesophilic aerobic bacteria (TMAB) and at 10°C for 5–7 days for total psychrotrophic aerobic bacteria (TPAB). After being cultured on MRS (de Man-Rogosa-Sharpe) and VRBD (Violet red bile dextrose) agars for three days at 30°C, respectively, lactic acid bacteria (LAB) and *Enterobacteriaceae* were enumerated. Colony-forming units, or log CFU, were used to express the results per gram of sample [15].

2.5.5. Determination of 2-Thiobarbituric Acid Reactive Compounds (TBARS)

The spectrophotometric technique calculated the samples' TBARS values [16, 17]. Samples were taken from both the surface and interior for TBARS analysis. Two grams of homogenized samples were double-extracted using 10 mL of 0.4 M perchloric acid each time. The volume of extracts was completed to 25 mL with 0.4 M perchloric acid, followed by centrifugation (1790×g for 5 min) (LAB 312 R, TD5, Turkey). Subsequently, 1 mL of the supernatant was transferred to a test tube with a glass stopper, and 5 mL of TBA reagent was added. The supernatant was then heated in a boiling water bath for 35 min. After cooling, the absorbance was measured at 538 nm (Spektrofotometre, Biochrom, Libra S70, England). The calibration curve was established using 1, 1, 3, 3- tetraethoxypropane (TEP).

2.5.6. Biogenic Amine Analyses

The determination of BA content was conducted using the HPLC chromatographic method, following the method outlined by Bulut et al. [18] and Çelebi et al. [17] with some adjustments. Two grams of chicken meat were treated with 25 mL of 0.4 M perchloric acid and centrifuged (LAB 312 R, TD5, Turkey) at 1500×g for 5 min. Then, 1 mL of the supernatant was alkalized using 200 μL of 2 N NaOH; 300 μL of saturated sodium bicarbonate was added as a buffer. 2 mL of dansyl chloride solution was then added, and the sample was incubated at 40°C for 75 min. 100 μL of 25% ammonia was added to suspend the residual dansyl chloride. After 30 min of incubation at room temperature, the sample was diluted to 5 mL with acetonitrile and then centrifuged at 1500×g for 5 min. The supernatant was filtered through a sterile micro-filter (0.45 μL). A gradient elution program was employed with mobile phases of acetonitrile (solvent A) and 0.4 M ammonium formate (solvent B), starting at 50% solvent A and 50% solvent B and concluding at 90% solvent A and 10% solvent B after 20 min. The temperature of the column was 40°C, and the flow rate was 1 mL/min^{-1}.

2.5.7. Sensory Analysis

All samples were prepared by cooking them in a heated oven at 175°C until the internal temperature of the meat samples reached roughly 70°C. The panelists were allowed to evaluate a range of attributes of the cooked chicken meats, including color, smell, flavor, texture, and overall acceptability, using a hedonic scale ranging from 9 to 1 (with 9 representing extreme liking, 5 representing moderate liking, and 1 representing dislike). The panelists were provided with water and a galette between samples to cleanse their palates and remove residual flavors [19]. Ten pre-informed and trained panelists carried out the sensory evaluation of chicken meats.

2.6. Statistical Analysis

The data of analyses were evaluated using the SPSS-20 (Armonk, NY, USA) package program. pH, color, TBARS, BAs, and microbiological counts data were subjected to multivariate analysis of variance (MANOVA) using the general linear model (GLM). Duncan's multiple comparison test was used to determine whether there were differences between the groups. Additionally, the results of BAs were treated with GraphPad Prism 10 Software, and the significance levels were indicated as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

3. Results and Discussion

3.1. BMLE's Physicochemical Characteristics and Antioxidant Capacity

Table 1 lists a few of the physicochemical (pH, L*, a*, b*) and antioxidant potential characteristics (TAC, TPC, TFC, DPPH-RSA) of BMLE. The phenolic compounds found in MLs can differ depending on factors such as variety, how they are grown, how long they are allowed to mature, and how they are processed [20]. Our findings are similar to the TPC (0.54–0.76 mg GAE/g) and TFC (105.33–143.94 mg QE/g) reported by Bülbül [21]. The physicochemical and antioxidant results of BMLE (Table 1) are consistent with information found in the literature about black mulberry leaves [22]. The primary naturally occurring active component of mulberry leaves (MLs) is polyphenol, an extremely potent antioxidant that may scavenge free radicals of oxygen, hydrogen peroxide, hydroxyl, etc [20]. These results suggest that BMLE may be a good source of antioxidants for fresh poultry items susceptible to oxidative processes

that cause rancidity and discoloration.

Table 1

Physicochemical properties and antioxidant activities of BMLE.

	TAC (mg/L)	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH-RSA (μg TE/mg)	pH	L*	a*	b*
BMLE	20.89 ± 0.15	0.83 ± 0.01	165.13 ± 1.90	52.13 μg ± 1.59	4.88 ± 0.03	49.52 ± 0.67	13.87 ± 2.64	17.11 ± 0.31

All values are expressed as mean ± SD of three replicates. TPC: total phenolic content; TAC: total anthocyanin content; TFC: total flavonoid content; DPPH-RSA: DPPH radical scavenging activity; GAE: gallic acid equivalent; QE: quercetin equivalent; TE: trolox equivalent.

3.2. Approximate Composition of Raw Chicken Meat

Table 2 displays the approximate composition (moisture, protein, ash, and fat) of chicken meat products containing varying amounts of BMLE (0.1%, 0.3%, and 0.5%) and BHT (0.1%). The moisture, fat, protein, and ash values of the raw chicken meat samples were not significantly affected by the BMLE application ($P>0.05$).

Table 2

Approximate composition of raw chicken meat samples.

Samples	Composition (%)			
Moisture	Protein	Ash	Fat	Control
52.75 ± 0.56	19.2 ± 0.08	1.55 ± 0.04	15.14 ± 0.09	BMLE 0.1%
52.45 ± 0.48	19.22 ± 0.04	1.6 ± 0.05	15.19 ± 0.33	BMLE 0.3%
52.89 ± 2.67	19.25 ± 0.02	1.52 ± 0.02	15 ± 0.70	BMLE 0.5%
52.98 ± 0.73	19.29 ± 0.05	1.55 ± 0.03	15.23 ± 0.60	BHT 0.1%
52.12 ± 1.58	19.28 ± 0.04	1.57 ± 0.03	15.28 ± 0.35	Significance

Values are expressed as mean ± SD. NS: nonsignificant, *$P<0.05$. BMLE: black mulberry leaf extract.

3.3. pH and Color Parameters of Poultry Meat

The pH levels of raw chicken meat indicate no statistical difference ($P>0.05$) (Table 3) in the A * S interaction. The pH levels of all raw chicken meats applied with BMLE were lower than those of the control samples, with a significance level of $P<0.05$ (Table 4). This drop could be explained by BMLE's average pH value of 4.88 (Table 1). The pH levels of the raw chicken meats rose during storage, with the most notable rises occurring in the control group (Table 4). Turan and Şimşek [23] found that throughout the storage period, the pH of beef patties with *Morus nigra* leaf extract progressively increased in all samples. After storage, the pH values of the 0.1%, 0.3%, and 0.5% BMLE groups were 0.31, 0.40, and 0.46 units lower than those of the control samples. The rise in pH levels in stored samples results from bacterial activity in meat, leading to the accumulation of microbial by products. When stored glucose is depleted, bacteria consume the amino acids generated during the breakdown of proteins, and ammonia from the breakdown of amino acids builds up and raises pH [24]. The presence of large quantities of *Enterobacteriaceae* microorganisms with proteolytic activity can also contribute to higher pH levels in meats [25].

Table 3

The effects of application (A), storage day (S), and correlation of A∗S on pH, TBARS, color, biogenic amine values, and microbial counts of raw chicken meats.

Effect	pH	Color values			Microbiological analyses				TBARS	Biogenic amines (Bas)					
L^*	a^*	b^*	TMAB	TPAB	LAB	*Enterobacteriaceae*	HIS	CAD	PUT	TYR	SPD	SPM	Application (A)	***	**
*	***	***	***	***	***	***	***	***	***	***	NS	NS	Storage days (S)	***	***
***	***	***	***	**	***	***	***	***	***	***	NS	NS	A∗S	NS	NS

NS: not significant, ∗P<0 .05, ∗∗P<0.01, ∗∗∗P<0.001; spermine: SPM, spermidine: SPD, tyramine: TYR; putrescine: PUT, cadaverine: CAD, histamine: HIS.

Table 4

pH and color values of raw chicken samples during chilled storage.

Samples	0 day	3 day	6 day	9 day	12day
pH					
Control	5.9b,B±0.1	6.12b,A,B±0.25	6.23a,A,B±0.24	6.46a,A±0.02	6.51a,A±0.02
BMLE 0.1%	5.75b,c,C±0.07	5.87b,c,B±0.01	5.93b,B±0.04	6.1b,A±0.02	6.2b,A±0.03
BMLE 0.3%	5.7c,D±0.02	5.78c,C,D±0.02	5.88b,B,C±0.02	6b,A,B±0.14	6.11b,c,A±0.01
BMLE 0.5%	5.78b,c,D±0.04	5.88b,c,C±0.01	5.94b,B,C±0.01	6b,A,B±0.07	6.05c,A±0.01

BHT 0.1%	6.35a,C±0.04	6.42a,B,C±0.02	6.46a,A,B,C±0.02	6.5a,A,B±0.02	6.55a,A±0.07
L*					
Control	55.83a,A±1.17	53.65a,b,A,B±0.49	51.52a,B,C±0.67	48.99a,C,D±2.81	47.04a,D±1.35
BMLE 0.1%	54.98a,A±1.21	52.98a,b,A,B±0.02	50.85a,B±1.21	48.32a,C±0.96	46.37a,C±0.88
BMLE 0.3%	54.00a,A±0.02	52.88a,b,A±0.03	50.75a,B±1.06	48.22a,C±1.10	46.27a,C±1.02
BMLE 0.5%	53.56a,A±0.79	52.50b,A,B±0.24	50.37a,B±1.94	47.84a,C±0.22	45.89a,C±0.15
BHT 0.1%	55.00a,A±1.41	54.74a,A±1.75	52.61a,A,B±1.95	50.08a,B,C±0.11	48.13a,C±1.22
a*					
Control	5.87a,C±0.09	5.40a,C±0.14	5.88a,B,C±0.02	6.25a,A,B±0.35	6.66c,A±0.22
BMLE 0.1%	5.87a,C±0.02	5.25a,C±0.70	6.20a,B,C±0.14	7.14a,A,B±0.19	7.89a,b,A±0.55
BMLE 0.3%	5.82a,C±0.02	5.18a,C±1.01	6.18a,B,C±0.25	7.30a,A,B±0.28	8.23a,A±0.17
BMLE 0.5%	5.80a,D±0.08	5.49a,C,D±0.72	6.55a,B,C±0.15	7.55a,A,B±0.07	8.33a,A±0.46
BHT 0.1%	5.85a,A±0.07	5.45a,A±0.63	6.00a,A±0.59	6.78a,A±1.10	7.00b,c,A±0.62
b*					
Control	12.38a,C±0.07	11.27a,b,D±0.22	12.85a,B,C±0.40	13.59a,b,B±0.62	14.5b,A±0.04
BMLE 0.1%	12.37a,A±0.14	10.95a,b,B±0.21	10.85c,B±0.21	12.13b,A±0.55	11.88b,A±0.04
BMLE 0.3%	12.30a,A±0.02	10.81b,B±0.20	11.00c,B±0.35	12.78a,b,A±0.58	11.15d,B±0.03

BMLE 0.5%	12.32a,A,B ± 0.17	11.46a,B ± 0.22	11.5b,c,B ± 0.70	12.88a,b,A ± 0.59	11.45c,B ± 0.04
BHT 0.1%	12.37a,C ± 0.07	11.37a,b,D ± 0.23	12.48a,b,C ± 0.39	13.88a,B ± 0.63	14.88a,A ± 0.05

A–E: in the same samples, the difference between the values expressed in different capital letters in the same rows on different storage days is statistically significant ($P<0.05$). a-b: The difference between values expressed with different lowercase letters in different samples in the same column on the same storage days is statistically significant ($P<0.05$).

The incorporation of BMLE had no effect ($P>0.05$) on the L^* levels of samples at storage days (Table 4). The study by Zhang et al. [22] yielded similar findings to our research, as they also observed no discernible trend in the changes of L^* and b^* values in the color of raw ground beef when treated with mulberry leaf extracts and stored in refrigeration. However, the L^* levels of raw chicken meats significantly decreased ($P<0.05$) during storage. Adding BMLE made the thighs slightly darker, leading to lower L^* values (Table 4). Lower L^* values during storage may be caused by the dipping solution containing BMLE, which has naturally occurring dark color pigments and a darker hue than the purified water and 0.1% BHT used in the control groups. Several studies have demonstrated that the inclusion of natural antioxidants led to a decrease in the L^* values of chicken meat samples [26, 27]. According to Turan and Şimşek [23]; using black mulberry water extract in packaging beef patties can decrease their lightness values. The extract's initial color values and high anthocyanin content are believed to cause this change. The a^* levels of the chicken meats were not significantly different on days 0, 3, 6, and 9. However, on day 12, a statistically significant difference was observed in redness between the samples (Table 4). Myoglobin oxidation caused a reduction in the a^* values of all treatments from day 0 to day 3. Iron atoms can oxidize or denature myoglobin molecules during oxidation, which results in a negative color change in the products and the conversion of myoglobin to methemoglobin [26]. Throughout storage, the TBARS levels of the groups in this investigation rose, correlated with decreasing a^* values, consistent with other studies in the literature [26, 28]. Following the storage time, the control samples showed the lowest a^* value, whereas the 0.5% BMLE samples showed the greatest a^* value. At the end of storage (day 12), the order of a^* values was as follows: 0.5% BMLE > 0.3% BMLE > 0.1% BMLE > 0.1% BHT > Control. These findings suggest that the extracts successfully maintained the red color of the meat. Turan and Şimşek [23] observed that beef patties with 0.2% black mulberry water extract had increased a^* compared to the control group. Additionally, the patties treated with 0.2% extract had higher a^* values than those treated with 0.4% after being stored aerobically for 15 days. Additionally, Zhang et al. [22] stated that the incorporation of mulberry leaf extracts caused a decrease in the a^* values of unprocessed ground beef. In the current investigation, yellowness (b^*) was found to be significantly influenced ($P<0.001$) by A * S interaction (Table 3). The yellowness values of chicken samples on days 0, 3, and 9 were not statistically ($P>0.05$) different. Nevertheless, there were notable variations between the treatments on the remaining days ($P<0.05$). b^* values of samples are influenced differentially by the extracts' color, which might vary from light green to dark yellow. The storage had a notable impact ($P<0.05$) on the b^* values of the treatments except for the % 0.5 BMLE group. Generally, the b^* values of all samples fluctuated throughout the storage. At the end of storage, the b^* values of the control and BHT groups increased compared to their initial values. However, there was a notable difference in the yellowness of the samples with BMLE addition ($P<0.05$). The use of BMLE in this study resulted in a decrease in the b^* values of chicken meat compared to control groups by the end of the storage period. These findings indicate that BMLE had a noticeable impact on the yellowness. In this study, the preservation of desired color parameters of chicken meat during chilled storage was achieved by BMLE. The reason for the enhancement in color values compared to the control can be attributed to its high amount of antioxidants and low pH level.

3.4. Microbial Counts of Raw Chicken Meats

The results presented in Table 3 demonstrate that the interaction of A * S had significant $P<0.05$, $P<0.001$, and $P<$

0.05 effects on the counts of TMAB, TPAB, and *Enterobacteriaceae* in raw chicken meat products, respectively. The variations in microbial counts that occur when chicken meats are stored, both with and without BMLE, are displayed in Figures 1(a)–1(d). No differences in TMAB numbers as of day 0 were observed among the control, 0.1%, 0.3%, 0.5% BMLE, and 0.1% BHT treatments ($P>0.05$) (Figure 1(a)). The TMAB and TPAB counts of chicken meats with BMLE added were considerably ($P<0.05$) lower than those of the control groups (Figures 1(a) and 1(b)). During the storage period, there was a continuous increase in the TMAB levels of chicken meats. It was found that the control groups (7.46 log CFU/g) and 0.1% BHT incorporated groups (7.25 log CFU/g) surpassed the limit value on day 9 in chicken meats (Figure 1(a)). Additionally, the TMAB counts of the BMLE and BHT-added chicken meats stayed within acceptable limits throughout the 9-day storage period (Figure 1(a)). The shelf life of raw chicken meats stored aerobically in refrigerated conditions is approximately 5 days, based on conditions for hygiene and preservation [29]. The current investigation showed a significant increase ($P<0.05$) in TPAB counts as the storage time increased. The chicken meats with 0.5% BMLE had the lowest TPAB counts, whereas the control samples had the highest values ($P<0.05$) (Figure 1(b)). Throughout the storage period, LAB numbers increased continuously in treatments ($P<0.05$). The LAB proliferation of chicken meats was promoted by the addition of BMLE, and this effect depended on the level of extract on day 12 of storage ($P<0.05$). The groups containing 0.3% and 0.5% BMLE, respectively, had the highest LAB number ($P<0.05$) at the end of storage (Figure 1(c)). The promotion of LAB development by BMLE was likely due to its ability to reduce pH and the potential stimulating impact of its components on LAB [23]. Despite initial counts ranging from 1.88 to 1.92 log CFU/g, there were no significant differences ($P>0.05$) in *Enterobacteriaceae* numbers on day 0 across all treatments studied. The counts of *Enterobacteriaceae* significantly increased ($P<0.05$) during storage. Chicken meat with 0.5% BMLE had the lowest counts ($P<0.05$) (Figure 1(d)). Turan and Şimşek [23] found that the initial levels of *Enterobacteriaceae* in aerobically packaged beef patties, whether or not lyophilized black mulberry water extract was applied, were between 2.43 and 2.52 log CFU/g. They also observed that the increasing number of *Enterobacteriaceae* in control groups (from 2.52 to 5.01) was higher than in groups with 0.4% BMWE extract (from 2.45 to 4.91) over a 15-day storage period. The inclusion of BMLE in samples resulted in a significant ($P<0.05$) decrease in microbial numbers (apart from LAB), indicating that BMLE positively affected the shelf life and microbial quality of chicken meat.

[figure(s) omitted; refer to PDF]

3.5. Lipid Oxidation

The quantity of secondary lipid oxidation products, mostly aldehydes (or carbonyls), that give oxidized meat and meat products an unpleasant flavor is represented by the TBARS values. The degree of lipid oxidation in meat samples during storage can be monitored using these measurements [30]. The results presented in Table 3 indicate that $A*S$ interactions had an important ($P<0.001$) impact on the TBARS levels of chicken meats. Including BMLE in chicken meat resulted in a beneficial impact on reducing TBARS levels (Figure 2). The chicken meat with BMLE exhibited a noticeably ($P<0.05$) reduced TBARS content compared to the control groups. The progressive rise in TBARS values over time is consistent with previous research findings that suggest an increasing formation of TBARS during storage [22, 23, 26]. The findings from chicken meat in Figure 2 demonstrate that TBARS values in the control group reached threshold values (<1 mg/kg) on the sixth day. There were values exceeding the limit on day 9 in the groups with 0.1% BMLE (1.12 mg/kg), 0.3% BMLE (1.00 mg/kg), and 0.1% BHT (1.24 mg/kg) ($P<0.05$). However, the TBARS value of the 0.5% BMLE group was 1.21 mg/kg and exceeded the threshold value on day 12 ($P<0.001$) (Figure 2). As a result, it was discovered that the application of 0.1–0.3% and 0.5% BMLE led to a delay in chicken meat lipid oxidation by 3 and 6 storage days, respectively, compared to the control groups. The TBARS values of chicken meat on day 12, which were incorporated with 0.1%, 0.3%, or 0.5% BMLE and 0.1% BHT, were 43.22%, 54.16%, 83.47%, and 18.08% lower ($P<0.001$), respectively, compared to the control sample (Figure 2). These findings suggest that BMLE may be a natural antioxidant to prevent lipid oxidation rather than synthetic antioxidants. Furthermore, it was discovered that 0.5% BMLE was superior to 0.1% and 0.3% BMLE in both packing techniques for postponing lipid oxidation. This extract's substantial phenolic and antioxidant content is responsible for BMLE's preventive activity against lipid oxidation. Phenolic compounds exhibit vigorous antioxidant activity using

mechanisms such as transition-free radical scavenging activity and single-oxygen quenching capacity [31]. According to Turan and Şimşek [23], the beef patties infused with black mulberry water extract exhibited lower and more consistent TBARS levels throughout the 15-day storage duration compared to the control group. In the same way, mulberry leaf extracts reduced the TBARS level of ground beef compared to the control sample [22].
[figure(s) omitted; refer to PDF]

3.6. Biogenic Amine Analysis

The high amount of protein in poultry meat leads to an increased breakdown of proteins and the release of amino acids. This, along with the presence of bacteria that can break down amino acids, speeds up the spoilage of meat and leads to higher levels of substances produced by microorganisms, such as biogenic amines [32]. Six biogenic amines (Bas)—cadaverine, putrescine, tyramine, histamine, spermine, and spermidine—were found and measured during storage. The results presented in Table 3 indicate that A∗S interactions had an important ($P<0.001$) impact on the histamine, tyramine, cadaverine, and putrescine levels of chicken meats. Additionally, Bas (without spermine and spermidine) in the samples with and without BMLE increased at the end of storage ($P<0.05$) (Figure 3). However, spermine and spermidine, crucial for cell division and growth, remained unaffected ($P>0.05$) by storage conditions, unlike the other biogenic amines analyzed in all samples. Notably, in addition to their role in cellular processes, spermine and spermidine also serve as a nitrogen source for bacteria [33]. The levels of spermine and spermidine vary between 12.80 and 13.30 mg/kg and 23.98–25.00 mg/kg, respectively, throughout the 12-day storage period ($P>0.05$) (Figures 3(a) and 3(b)). Decarboxylase-positive contaminating bacteria convert lysine into cadaverine, which can be utilized as a food hygiene indication. The cadaverine content in the control and sample groups with percentages of 0.1 BMLE, 0.3 BMLE, 0.5 BMLE, and 0.1 BHT increased to 6.11, 4.55, 3.50, 2.80, and 4.77 mg/kg at 12 days of storage ($P<0.05$). This could be attributed to the bacteria that produce cadaverine proliferating in raw chicken meats. Chicken meats treated with BMLE had a lower cadaverine content than the control group ($P<0.001$), suggesting that BMLE can effectively prevent cadaverine from building up (Figure 3(c)). According to Renes et al. [34], consuming too much putrescine might increase the toxicity of histamine and tyramine, in addition to causing poisoning. As seen in Figure 3(d), the putrescine in the control groups grew more quickly than in the other treatments, reaching 20.79 mg/kg at the end of storage ($P<0.05$; $P<0.01$; $P<0.001$). The addition of BMLE may inhibit putrescine accumulation, notably 0.5% BMLE, which at 12 days significantly decreased putrescine production by 32.65% ($P<0.001$) compared to the control. The most harmful BA found in food is histamine, which, when consumed in excess, might result in symptoms including headaches and diarrhea [35, 36]. Histamine formation was not observed in all samples at the beginning of storage, and their formation increased to 4.55, 3.11, 3.00, 2.79, and 3.90 mg/kg at 12 days for the control and samples with 0.1% BMLE, 0.3% BMLE, 0.5% BMLE, and 0.1% BHT, respectively ($P<0.01$, Figure 3(e)). BMLE exhibited effective inhibition of histamine. The histamine content in chicken meats with 0.5% BMLE was 34.06% lower than that of the control ($P<0.0001$) at 12 days (Figure 3(e)). The changes in the contents of tyramine exhibited a similar pattern, as indicated in Figure 3(f). The levels of these substances were significantly higher in the control groups compared to the samples treated with BMLE and BHT at the end of storage ($P<0.05$). Additionally, the group treated with BMLE demonstrated the greatest inhibition ($P<0.0001$). The BMLE exhibited the most potent inhibitory impact on the buildup of these four BAs in raw chicken samples at the end of storage. This could be due to its ability to suppress the growth of TMAB, TPAB, and *Enterobacteriaceae* (BA-positive bacteria) in chicken meats, as demonstrated in Figure 2 since BAs are predominantly produced by uncontrolled microbial enzymatic activity. On the other hand, it is believed that the BMLE group, having a higher lactic acid bacteria (LAB) count, can suppress the formation of biogenic amines (BAs).
[figure(s) omitted; refer to PDF]

3.7. Sensory Analyses

Figure 4 displays the sensory scores of the cooked chicken samples on day 5. No significant differences ($P>0.05$) existed between the control and BMLE-added chicken meats, even though flavor scores were highest in the 0.3% BMLE-added chicken meats. The color and texture of cooked chicken meats were enhanced ($P<0.05$) by adding 0.3% BMLE. The control groups and those with 0.1%, 0.3%, and 0.5% BMLE's smell scores were not statistically

significant (P>0.05). Similarly, Turan and Şimşek [23] showed that the addition of 0.1% and 0.2% black mulberry water extract did not impact the color, texture, smell, and flavor ratings of cooked beef patties. The samples with 0.3% BMLE added had the highest overall acceptance scores for cooked chicken meats (P<0.05). The addition of 0.3% BMLE can be regarded as the most appropriate quantity to avoid adversely affecting the sensory qualities of cooked chicken meats, taking into account all sensory features that have been studied. However, the results for lipid oxidation and microbial count indicated a more favorable effect with 0.5% BMLE. So, the suggested recommendation is to utilize 0.5% black mulberry leaf extract (BMLE) for raw meat preservation.
[figure(s) omitted; refer to PDF]

4. Conclusion

The findings suggest that incorporating BMLE significantly enhances the microbial quality, lipid oxidation, biogenic amine content, color stability, and sensory properties of chicken meat. The results demonstrate a substantial enhancement in microbial quality, lipid stability, and color retention, particularly at specific concentrations of BMLE. Additionally, the extract exhibits inhibitory effects on biogenic amine accumulation, contributing to raw chicken meat's overall freshness and safety. These outcomes underscore the potential of black mulberry leaf extract as a valuable natural ingredient for raw chicken products during refrigerated storage, preserving and enhancing their overall quality. Additionally, the application of BMLE is recommended not only for enhancing the quality of raw chicken meat but also for improving processed chicken products such as nuggets, schnitzel, and chicken patties.

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DETAIL

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ABSTRAK (ENGLISH)

Foliar calcium (Ca) treatment exhibits strong potential for enhancing yield and quality in some fruit crops. This study aimed to assess the impact of foliar application of Ca-organomineral (Ca-OM) suspension on total soluble solids (TSS) and Ca dynamics in leaves and berries across five red currant cultivars during the vegetation and storage. A randomized block design with two treatments: (1) Control (without Ca-OM treatment) and (2) foliar Ca-OM treatment, with three repetitions, was applied on five different red currant cultivars. Although foliar Ca-OM treatments did not impact Ca or TSS in leaves, they positively influenced Ca and TSS in fruits, displaying significant variability among cultivars. In addition, Ca-OM treatment increased berry density, reduced abscission, and inhibited the development of diseases, extending storage periods for “Lvovyanka,” “Vika,” and “Gazel” cultivars by three to seven days compared to the Ca-OM untreated control. Ca-OM treatment in the early stages of the ontogenesis of currants provided a high percentage of Ca intake in berries. At the stage of complete maturation, the Ca content in berries decreased and depended on the ripening period of the cultivars. Before harvesting, Ca-OM increased the strength of berries (Fc) and reduced the shedding of berries in the clusters (Fs). At the vegetation stage, Ca-OM increased TSS in berries, and the content of TSS depended on the genotype and weather conditions. The Ca-OM treatment and low temperatures contributed to preserving berry density, reducing the shedding of berries and PLW, and restraining the development of diseases during storage. In addition, the high content of TSS and Ca in berries against the background of a slow rate of decrease in berry density in the Ca-OM variants ensured an extension of the shelf life of “Lvovyanka,” “Vika,” and “Gazel” by three to seven days compared to the control untreated with Ca-OM. Clustering analyses identified these cultivars as similar in terms of TSS and calcium content in fruits, emphasizing their common traits. The study underscores the potential of foliar Ca treatment to enhance berry quality during growth and storage, significantly improve storage duration, and fortify resistance against adverse factors, presenting promising opportunities for elevating yield and quality in specific red currant cultivars.

TEKS LENGKAP

1. Introduction

The global demand for cultivating and consuming berry crops has increased in recent decades [1]. This trend is connected, firstly, with the beneficial qualities of berries for human health. Red currant berries are a source of biologically active compounds with a high content of ascorbic acid, phenolic compounds, anthocyanins, and high antioxidant activity [2, 3]; secondly, due to improved agrotechnological methods for growing berry crops. New elements of cultivation technology have made it possible to expand these crops’ availability and distribution area [4]. The production of currant berries ranks second in the world after strawberries. According to Faostat 2022, the world’s average production of currant berries is 45000 tons. The primary production of currants is located in Europe (Poland, France, Estonia, the Netherlands, Belgium, Russia, and Ukraine) [5]. Berry crops have a high value, so their production and sale can significantly contribute to the economy of the regions of several countries [6]. Most berry crops are intended primarily for use in processed products, but at the same time, the priority is the sale of fresh berry products [7].

Berry products are perishable raw materials [8]. After harvesting, the berries quickly lose their commercial qualities and organoleptic acceptance (berry weight loss, berry density reduction, berry rot) [9], and this affects the marketing of these products, which leads to significant economic losses [10].

To extend the shelf life of fruit and vegetable products and preserve the nutritional value and quality characteristics of fruits, chemical preparations (pesticides, preservatives), biological compounds (plant extracts) [11, 12], and physical methods (ultrasound, ultraviolet, electric field, pressure, temperature regimes) are used [12–15]. However, there is a scientific trend in switching to environmentally friendly and safe plant compounds to reduce crop losses during storage [16].

According to the International Federation of Organic Agriculture Movements (I.F.O.A.M.), such a production system

supports the health of soils, ecosystems, and people [17–19]. Many farmers often use freezing to increase the period of consumption of berries. Still, in the process of such storage (−12°C, −18°C, −24°C), several chemical processes change: sucrose is inverted, acidity increases, and the amount of tannins decreases [20]. There is evidence of a decrease in changes in the structure of red currant berries when stored in the refrigerator for up to 7–10 days [21, 22]. Rational nutrition considers the consumption of fresh fruits and berries as a vital factor. The berries' harvest quality is crucial and should be at the right point for improved storage. In this case, biological foliar fertilizing is becoming very important, significantly enhancing metabolic processes, yield, and fruit quality [23, 24]. The mineral composition of fruit crops affects fruits' quality and technological indicators, including their shelf-life capacity. One of the critical problems in ensuring the high quality of products and their safety is providing the optimal calcium content in fruits and berries [25–27].

The role of calcium is crucial to ensure the excellent storage ability of fruits and berry products; the higher the calcium content in berries, the greater and longer their preservation abilities, and, consequently, the possibility of more prolonged consumption of high-quality products rich in essential trace elements and vitamins [26]. Calcium is a critical component in maintaining the hardness of fruits during storage, as it is responsible for the integrity of the cell [25, 26]. Calcium ions create compounds between the peptic molecules in the middle of the plate, which are responsible for the integrity of the cell [25–27]. For instance, it was confirmed that apple fruits with a content of Ca >5 mg/100 g f.w., with a ratio (K + Mg)/Ca <25 mg/100 g f. w. and Ca/Mg >1 mg/100 g f. w. have high resistance to diseases during storage [27, 28]. Thus, the softening of the fruit may result from the loss of calcium in the middle of the plate and/or its absence in the bonds between the peptic molecules [28]. When treated during the preharvest period, the entrance of calcium into fruits delays the fruit's softening and ripening rate, thereby slowing down the decay of cell walls [28]. The preharvest use of calcium can slow down the aging of fruits without adversely affecting the consumer qualities of fruits [29, 30]. There are also studies of the positive effect of calcium-containing drugs on increasing the level of Brix in *Rubus Eubatus* Focke fruits [31]. The storage duration of berry crops, especially currants, is significantly influenced by calcium. It addresses a significant issue where red currants rapidly deteriorate within 3-4 days after the harvest, leading to logistical challenges in their distribution to major retail chains [32].

Thus, this study aimed to elucidate the effects of foliar Ca application in five red currant genotypes on (i) changes in total soluble solids (TSS) and calcium content in the biomass and fruits during the preharvest and postharvest period and (ii) the duration of the shelf life of berries under the influence of low temperatures.

2. Materials and Methods

2.1. Location, Facilities, Weather Conditions, and Agrochemical Measures

The study was conducted in 2021/2022 and 2022/2023 vegetation seasons at the site (0.2 ha) of the primary variety study of VNIISPK red currants. The experimental site was in the north-east of the central Chernozem region of Russia. The soil of the experimental site belonged to the Loamy Haplic Luvisol type (IUSS Working Group W.R.B., 2015), with a surface humus horizon of 0.55 m. During two growing seasons, the soil samples from three repetitions, in triplicates, were taken in spring (before the buds of red currant blossomed) from the rhizosphere at a depth of 0–0.2 m and 0.2–0.4 m and were subjected to chemical analyses. The exchangeable potassium content was determined using a flame photometric method with a flame spectrophotometer. The phosphorus content was determined by the spectrophotometric method using a Bio-RAD SmartSpec plus spectrophotometer (California, U.S.A.). Soil acidity (pH) was determined in a 20 g suspension with the addition of a 0.1 N KCl solution [33]. Measurements were carried out by the pH-150MI device (Moscow, Russia).

The experimental site was presented with five red currant cultivars of different ecological, geographical, and genetic origins: ("Jonkheer Van Tets" ("Faya Plodorodnaya" × "London Market"), Holland; "Vika" ("Chulkovskaya" × "Red Lake"), Russia; "Asya" ("Chulkovskaya" × "Maarses Prominent"), Russia; "Gazel" ("Chulkovskaya" × "Maarses Prominent"), Russia; Lvovyanka ("Weisse Hollandische" × "Jonkheer Van Tets"), Ukraine). The cultivars were of early, medium-early, and late ripening, planted in 2018 with a spacing of 2.8 × 0.5 m, and interrow plowing without irrigation.

The scientific institutions of Russia, Ukraine, and Europe provided the cultivars under the program «A unique

scientific set, a collection of living plants of the open field—bioresource collection of VNIISPK».

Ammonium nitrate (NH_4NO_3) was applied in an amount of 60 g. per plant, every vegetation season is in spring, the first decade of April. To protect against *Sphaerotheca mors-uvae*, the experimental plants were treated with a bio-phytoncides complex of botanical extracts on an organo-mineral basis. The preparation of systemic and contact action (pH = 7.5–8.0) has the form of a suspension of minerals of natural origin containing *Quassia amara, Cinnamomum zeylanicum,* and *Azadirachta indica* (the drug was obtained from AgroPlus, Russia). The treatments were performed before bud blossoming, during green berry formation, the initial ripening, and full ripeness, using a 5.0% solution.

The summary of weather conditions during the 2021-2022 and 2022-2023 vegetation seasons is presented in Table 1 and was obtained using a meteorological station iMetes 3.3. (Weiz, Austria) at the experimental site.

Table 1

Weather conditions at the experimental site during the vegetation periods.

Year	2022				2023			
T average (°C)	*T* min (°C)	*T* max (°C)	Precipitation amount (mm)	*T* average (°C)	*T* min (°C)	*T* max (°C)	Precipitation amount (mm)	April
								III decade
7.4	−0.5	17.1	28.7	9.8	−0.5	22.0	26.6	May
								I decade
9.8	1.7	20.8	8.3	7.8	−4.2	21.0	3.7	II decade
11.1	4.0	24.5	11.5	13.8	1.0	25.7	0.4	III decade
11.7	0	23.5	18.5	14.9	3.0	25.5	4.9	June
								I decade
17.9	6.5	28.5	0	14.6	0.5	27.5	1.3	II decade

19.0	8.5	30.5	17.6	16.7	8.0	28.8	1.5	III decade
20.4	10.0	32.0	25.0	16.5	8.0	29.0	34.0	July
								I decade
21.2	7.0	32.5	12.9	19.5	10.0	29.5	24.7	II decade

2.2. Experiment Design

2.2.1. Vegetation Period

To examine the impact of a foliar Ca application on red currant cultivars, a Ca-organomineral (Ca-OM) suspension derived from the oceanic bio flora containing Ca (1.31%), CaO (0.4%), SiO_2 (5.6%), Fe_2O_3 (0.4%), Al_2O_3 (0.16%), and MgO (0.4%) was applied. To determine the calcium content in the suspension, a solution of 10 ml was taken and subjected to burning in a muffle furnace at a temperature of +450°C. Burning was gradually carried out, raising the oven temperature by 50°C every 30 minutes. The total mineralization time was 8 hours. The resulting ash was dissolved, and a suspension was obtained. The complexometric method determined the calcium content in the test preparation suspension [34].

A randomized block design with two treatments: (1) Control (without Ca-OM treatment) and (2) foliar Ca-OM treatment, with three repetitions, was applied on five different red currant cultivars. There were five plants per treatment, with two plants between each treatment.

Foliar treatments were applied by the RT-16LI knapsack sprayer (Patriot, China), with a 1% Ca-OM solution and a consumption of 0.18 m^3/h. Treatments were carried out following the phases of ontogenesis of red currant plants (Table 2).

Table 2

Application of Ca-organomineral (Ca-OM) treatment.

Plant ontogenesis phase	Treatment date
Blossom	13.05.2022/4.05.2023
Formation of green berries (A)	16.06.2022/2.06.2023
At the beginning of ripening (20% of berries on the bushes acquired a characteristic color) (B)	24.06.2022/8.06.2023
At the beginning of ripening (50% of berries on the bushes acquired a characteristic color) (C)	30.06.2022/19.06.2023
Berry ripening (more than 90% of the berries acquired a characteristic color and taste) (D)	07.07.2022/26.06.2023

Note. A–D, stages of leaves and berries selection for analysis.

The TSS (Brix %) in leaves and fruits was determined using a refractometer (ATAGO, pocket PAL-1. Kyoto, Japan). The selection of plant material was carried out according to the experimental scheme (Table 2). The leaves and berries were selected five days after the treatments. To determine the soluble solids content in the leaves, a sample of 0.7 g was used. To determine the soluble solids content in the berries, a sample of 12 g was used. The sample was a mixture of leaves or berries from five plants of one repetition from the same cultivar.

Determination of the calcium content in the leaf tissue was performed by the complexometric method for organic substances at the beginning stages of the ripening of berries and the full ripening of berries [34, 35]. Dry ash was used for plant samples. Dry samples were burned in a muffle furnace at a temperature of +450°C, and the ash was obtained from a plant sample (Figure 1).

[figure(s) omitted; refer to PDF]

The resulting ash was dissolved and titrated with a 0.01 N. solution of complex III. The calcium content (X) in mmol/100 g of soluble solids was determined in the following formula:(1)X=a·n·p·100m,a is the volume of complexon III for titration, sm^3; *n* –0.01 N solution of complexon III, p is the ratio of the solution amount for dissolving ash to the amount of ash. In this experiment, p=20 (100 : 5 = 20), *m* is the weight of the sample, g.

2.2.2. Storage

The berries of tested red currant cultivars were harvested during biological ripening when >90% of the berries from the bush were mature. Berry ripening was accessed visually. To assess the extent of berry maturation, we determined the physico-mechanical parameters of the berries, specifically focusing on separation force and crushing force by the Dina-2 device (Siberian Institute of Physics and Technology of Agrarian Problems, Russia) [36]. The crushing force was determined using the Plodtest-1 device (Siberian Institute of Physics and Technology of Agrarian Problems, Russia). Mature berries were randomly selected from the experimental plants. The number of berries in the cluster varied from 8 to 16 pieces, depending on the genotype. Red currant berries can be stored at room temperature for no more than 20 hours before the appearance of the berries begins to deteriorate [37, 38]. Therefore, according to the experiment's design, the hand-picked berries of the varieties were placed in plastic disposable fruit containers with a volume of 0.8 liters. Within 1 hour, they were transported to the laboratory and stored in the refrigerator Polair CM105-Gm (Switzerland) at +2.8 to +4.0°C (the relative humidity of the air was 95%). The berries were stored for 27 days. The repetition of the experience was threefold. Before laying currant berries for storage, their weight was determined.

The measurements of TSS, the density of the berry skin, and Physiological Loss in Weight (PLW) were carried out at intervals of 3-4 days. Five currant clusters (60 berries) were selected from each container to determine TSS and the density of the berry skin. The TSS (Brix %) examination using a PAL-3 digital refractometer (ATAGO, Japan). The density of the berry skin was determined by the MEGEON 03004 penetrometer (Russia).

The Physiological Loss in Weight (PLW) was defined as the difference between the initial weight of the berries (*M*1) and the subsequent weight of the berries (*Mi*) in each container. It was determined by the following formula [39]:(2) PLW=M1−MiM1·100%.

The berries were weighed on the Scout Pro SP 202 laboratory scale (OHAUS, Parsippany, NJ, USA).

The values of TSS, the density of the berry skin, PLW, and the visual evaluation of spoiled berries determined the storage period. The maximum shelf life of the berries was the period during which the berries retained the optimal specified qualities, and the PLW did not exceed 10% [40].

The calcium content in berries during the postharvest period was determined by the previously specified method (Section 2.2.1) at three key stages: (a) initial, (b) midpoint, and (c) final phase of storage.

2.3. Statistical Analysis

The raw data were statistically summarized and graphically presented in Microsoft Excel. Then, an independent samples *t*-test (at a 5% significance level) was performed using SPSS version 22.0 to determine whether the control and applied treatments had a statistically different effect on the measured parameters. To compute and visualize the principal component analysis (PCA), the function of res.pca < -prcomp(df, scale = TRUE) from the factoextra R

package was used, and to compute and visualize cluster analysis, the function of res.hcpc<-HCPC (res) from the FactoMineR R package was used.

3. Results and Discussion

3.1. Vegetation Period

Foliar treatments in intensive technologies are essential elements in managing growth and production processes in the plant, as well as an important factor in the rapid impact on the processes that determine the yield and quality at the vegetation stage and the storage of fruits [41]. At the same time, Ca has a unique role in the nutrition of plants [28]. Calcium is an element that is not reutilized in the plant body, but young and growing organs and tissues constantly need this element [42]. For many fruit and berry crops, removing calcium per unit of yield is comparable to removing nitrogen [43–45]. The summary of agrochemical soil characteristics at the experimental site is presented in Table 3, confirming the acid soil conditions with a high content of available phosphorus and potassium [46].

Table 3

Agrochemical soil indicators of the experimental plot.

Indicator	0–0.2 m	0.2–0.4 m
pHKCl	4.7–4.8	4.7–4.9
Available potassium	41.7 $mg \cdot kg^{-1}$	21.0 $mg \cdot kg^{-1}$
Available phosphorus	110.0 $mg \cdot kg^{-1}$	128.0 $mg \cdot kg^{-1}$

The weather conditions guided foliar treatment with the Ca-OM during the growing seasons. In 2022, the beginning of red currant vegetation lagged the average annual values for the test crop by 10–15 days (Table 1). This difference affected the subsequent dates of Ca-OM treatments and physiological processes in the plant (Table 3). In 2023, the weather conditions at the experimental site conformed to the region's average, long-term climatic patterns, with no deviations observed in the progression of the ontogenetic stages of red currant development.

3.1.1. Effect of Foliar Calcium Treatments on Vegetative Mass

Monitoring the phases of ontogenetic development allows for specific adjustments in the implementation of the production process and the yield quality due to agrochemical measures [47]. The stages of fruit formation and quality management are of the most significant interest [48]. The Ca content in currant leaves depends on the cultivar and the vegetation stage. The studies of Hogue et al. [49] in apples, it was reported that the accumulation of Ca in the leaves is a complex process that depends on exogenous factors (weather conditions, abiotic and biotic stress) and endogenous factors (genotype, ontogenetic stages of development). The Ca content in the leaves of fruit crops [50–52] is lower than in berry crops [53]. The leaves of red currants are rich in calcium, potassium, and magnesium, and the content of these elements depends on the date of leaf collection [54–58]. A low seasonal variability of the Ca content in currant leaves is shown in Figure 2. Insignificant dynamics of the Ca content in the apple leaves were found during the growing season [59].

[figure(s) omitted; refer to PDF]

Foliar treatment with Ca-OM did not affect the Ca content in currant leaves. Similar results were obtained using different concentrations of calcium-containing preparations on strawberry, raspberry, BlackBerry, and blueberry cultivars [49, 59]. Lobos et al. [47] suggested that the elemental composition of the leaf is relatively stable and is little influenced by agrochemical techniques and weather conditions. The Ca content in the leaves increased with their ontogenetic development (Figure 2). The reports of Nour et al. [56] also showed that by the time the berries ripened, the calcium, magnesium, and iron content in currant leaves was the highest. An increase in Ca with leaf age was also revealed in apple cultivars and was explained by the immobility of Ca in leaf tissues and the absence of its redistribution to other plant organs [51]. At the stage of berry ripening, significant differences in the content of Ca in

the leaves were in the red current cultivars “Vika,” “Asya,” “Lvovyanka,” and “Jonkheer Van Tets” (Figure 2).
The TSS content in red currant leaves did not exceed 4% (Table 4). In the studies in apples [60] and in *Cydonia oblonga, Chaenomeles japonica, Ribes nigrum, Aronia melanocarpa, Vaccinium macrocarpon,* and *Vaccinium myrtillus* [61], the TSS content in the leaves also did not exceed 10%. Foliar treatments with Ca-OM at different stages of currant development did not significantly impact the TSS content in the leaves (Table 4). However, the reliability of the data is difficult to assess since no information has been found in the literature on the effect of foliar treatments with Ca on the TSS content in the leaves of fruit plants.

Table 4

The impact of foliar Ca-organomineral (Ca-OM) application on the content of total soluble solids (TSS; Brix %) in the leaves among tested red currant cultivars during the growing season.

Cultivar	Treatment	2022				2023			
16.06	23.06	27.06	5.07	2.06	8.06	21.06	26.06	Lvovyanka	Control
1.8*	2.0 ns	1.6*	2.3*	1.8*	2.1*	2.2*	2.3*	Ca-OM	2.8*
1.9 ns	2.0*	1.8*	2.9*	2.7*	2.7*	2.6*	·		
Asya	Control	1.8 ns	2.4 ns	2.2*	2.3 ns	2.0*	2.6 ns	2.8 ns	2.7 ns
Ca-OM	2.2 ns	2.3 ns	2.5*	2.2 ns	2.7*	2.7 ns	2.7 ns	2.6 ns	·
Vika	Control	1.4*	2.2*	1.3 ns	2.1 ns	2.4*	2.4 ns	3.3*	3.0 ns
Ca-OM	2.0*	2.6*	1.6 ns	2.5 ns	2.5*	2.4 ns	3.8*	2.9 ns	·
JVT	Control	2.5*	2.5 ns	2.4*	2.8 ns	2.8*	2.7*	2.7*	3.5 ns
Ca-OM	3.4*	2.2 ns	2.2*	2.6 ns	3.3*	3.3*	3.3*	3.7 ns	·
Gazel	Control	1.7 ns	2.3 ns	2.0 ns	1.9 ns	3.0*	2.9*	2.7 ns	2.5 ns

The small letters (ns) following the number of values (of the same cultivar) represent nonsignificance according to the t test, and the sign *represents the statistically significant difference.
A decrease in TSS in leaves during the adverse weather of 2022 is shown (Table 4). A positive correlation was found between TSS in currant leaves and temperature (r = +0.80–+0.92). The positive effect of temperature on TSS in leaves is shown in grapes [62].
At the berry ripening stage, the TSS content in the leaves decreased (Table 4). In “Jonkheer Van Tets” and “Lvovyanka” the decrease occurred when 20% of the berries on the bushes acquired a characteristic color; for other cultivars, this pattern occurred later, when a larger percentage of berries acquired a red color. Similar results were obtained in *Persica davidiana* Carr. [63]. The decrease in TSS content in the summer period may be explained by the intensification of hydrolytic processes in the leaves and the outflow of hydrolysis products from the leaves to the ripening fruits [63].

3.1.2. The Effect of Foliar Calcium Treatments on Berries

The mechanism of intake and distribution of Ca is complicated and is determined not only by the anatomy of the fruit but also by the genotype and stage of plant development [64].

The intake of Ca into the fruits occurs through the stomata on the surface of the fruits. Not only is the conductivity of stomata essential, but so is their number and distribution on the surface of the fruit [65]. A decrease in the number of stomata and a decrease in their conductivity reduce the intake and accumulation of Ca in fruits [47]. The Ca content in immature red currant berries is higher than in leaves (Figures 2 and 3). This is probably due to the functional activity of the stomata of the fruits.

[figure(s) omitted; refer to PDF]

The intake and distribution of Ca in fruits depends on the stage of plant ontogenesis [66, 67]. A high percentage of the Ca accumulation was at the initial stage of the development of currant berries. The Ca accumulation slowed and decreased when the berries were fully ripe (Figure 3). Thus, treating Ca-OM in the early stages of the ontogenesis of red currants provides a high percentage of the Ca intake in berries and ensures the high strength of berries. This again shows Ca's role in the development of fruits and determines their quality. In the early stages of the growth and development of fruits or berries, Ca is involved in cell division and metabolism. Ca is mainly involved in the intercellular junction in the later stages of fruit or berry development [42]. It is known that the movement of Ca through the plant depends on the xylem fluid. When the fruits are fully ripe, the movement of water switches from the xylem to the phloem, so the movement of Ca to other parts of the plant is limited [68]. Calcium accumulation in fruits decreases when the xylem losses function [67, 69]. The effect of dilution of the Ca content is observed as the fruit grows [70, 71].

The genetic characteristics of currants determined the date of the decrease in the content of Ca in fruits. In early-ripening cultivars "Lvovyanka," "Asya," "Vika," and "Jonkheer Van Tets," the decrease in Ca occurred 5-6 days earlier than in the late-ripening cultivar "Gazel." Similar results were obtained in blueberry [65], kiwi [67], and grape cultivars [69].

Varietal differences in the content of Ca in currant berries were revealed. "Asya" and "Gazel" had a significantly high content of it. By the time the berries ripened, Ca-OM minimized the decrease in Ca in the berries, and its content was 20% higher than in the control. Similar results on the content and distribution of Ca during the growing season were obtained in blueberry cultivars. Calcium accumulated rapidly at the initial stage of berry ripening; at the beginning of berry coloring, its accumulation slowed down and stopped when the berries were fully ripe [65].

The physical and mechanical parameters of currant berries (the crushing force of berries ((Fc) and the separation force of berries in the cluster (Fs)) were indicators of the period of biological maturity of the berries [36]. The data on the physical and mechanical qualities of the berries corresponded to the indicators of the Ca content in the fruits (Figure 3). Ca-OM increased the strength of berries (Fc) in currant cultivars and also reduced the shedding of berries in the cluster (Fs) compared to the control (Table 5). This is confirmed by the role of Ca in regulating fruit ripening and its quality. Foliar treatment with Ca stabilizes the cell wall of plants, maintains the elasticity of tissues, and preserves the hardness of fruits [42]. Pectin acid can combine with Ca and form calcium pectate, which is the structural basis of the cell wall, increasing its strength and preventing the gel layer's disintegration in the cell [72]. In the studies by Wójcik et al. [73] in cherries, Madani et al. [74] in papaya, Siddiqui et al. [75] in apples, Bonomelli et al. [76] and Martins et al. [77] in table grapes, Lobos et al. [47] in blueberry, and Souza et al. [78] in *Ficus carica* L. it is shown that the use of Ca before harvest increased the hardness of fruits.

Table 5

The physical-mechanical parameters of red currant berries before they are removed for storage.

Cultivar	Parameters	Treatment	
Control	Ca-OM	Lvovyanka	Fc

4.1 ns	4.3 ns	Fs	0.81 ns
0.76 ns	-		
Asya	Fc	4.44*	5.65*
Fs	0.86 ns	0.89 ns	·
Vika	Fc	5.01*	5.52*
Fs	0.65*	0.89*	·
JVT	Fc	5.65 ns	5.73 ns
Fs	0.72*	0.84*	·
Gazel	Fc	4.89*	5.43*

The small letters (ns) following the number of values (of the same cultivar) represent nonsignificance according to the t-test, and the sign *represents the statistically significant difference. Fc, force crushing of berry (*N*); Fs, force separation of berry from branches (*N*).

The TSS increased in immature berries during the growing season (Table 6). By the time the berries ripened, the TSS in all cultivars exceeded 10%. In “Vika” and “Lvovyanka,” a sharp increase in TSS (45–70% of the initial level) corresponded to the stage of TSS decline in the leaves (Table 4). A rise in TSS by the fruit ripening period was noted in grapes. It was explained by the high consumption of sugars at the early stages of berry growth and development. Subsequently, metabolic changes occurred during berry ripening that contributed to the accumulation of sugars in fruits [62, 79]. At the same time, Ca-OM essentially increased TSS in berries. Similar results were observed under foliar treatments of strawberries [80] and apples [81, 82] with Ca at different stages of ontogenesis. The variation coefficient (CV) of this trait in control and experimental variants exceeded 20% over the years of the studies. A sufficiently large spread and minimal alignment of values for the studied characteristics are once again confirmed by the dependence of the trait on climatic conditions and genetic origin. The significant influence of the genotype, stage of plant development, and climate conditions on the TSS content in berries $p \leq 0.05$ is shown in the blueberry studies of Yang et al. [65].

Table 6

The impact of foliar Ca-organomineral (Ca-OM) application on the content of total soluble solids (TSS; Brix %) in the berries among tested red currant cultivars during the growing season.

Cultivar	Treatment	2022				2023			
16.06	23.06	27.06	5.07	2.06	8.06	21.06	26.06	Lvovyanka	Control
3.1*	5.0 ns	6.7 ns	10.4*	4.7*	9.7*	10.8*	11.0*	Ca-OM	5.0*
4.6 ns	6.9 ns	11.2*	9.0*	10.6*	11.9*	12.4*	·		

Asya	Control	6.1*	4.5*	6.5*	10.7 ns	8.2 ns	10.5 ns	10.5*	11.5*
Ca-OM	5.2*	6.4*	6.2*	11.0 ns	9.4 ns	10.3 ns	11.8*	12.4*	·
Vika	Control	2.1*	2.2*	7.9*	7.8*	9.1*	9.8*	11.7*	11.8*
Ca-OM	4.8*	5.2*	8.4*	11.2*	10.3*	11.2*	12.1*	12.7*	·
JVT	Control	3.0*	5.1*	6.4 ns	9.7*	9.3 ns	9.7*	9.2*	10.7 ns
Ca-OM	4.8*	5.9*	6.2 ns	10.4*	9.4 ns	10.6*	10.9*	10.8 ns	·
Gazel	Control	4.4 ns	4.5*	6.5*	10.8 ns	9.6*	10.6 ns	10.0*	11.6*

The small letters (ns) following the number of values (of the same cultivar) represent nonsignificance according to the t test, and the sign *represents the statistically significant difference.

3.2. Storage Period

In storing berry products, there are several problems: a decrease in the hardness of berries, a color change (darkening), and the abscission of berries in the bunches, and the development of diseases [83].

The TSS of level and the density of berries are essential in assessing the quality of berries and extending their shelf life [83].

In red currant varieties, the TSS content increased slightly during storage until a certain period (Tables 7 and 8). This is due to the loss of moisture in the berries and the hydrolysis of carbohydrates to soluble sugars [84].

Table 7

The impact of foliar Ca-organomineral (Ca-OM) application on the content of total soluble solids (TSS; Brix %) in the berries among tested red currant cultivars during storage in 2022.

Cultivars	Treatments	Day 0	Day 4	Day 8	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
Lvovyanka	Control	10.0*	10.2*	10.9*	10.8*	11.0*	10.8*			
Ca-OM	11.2*	11.7*	11.5*	11.6*	12.1*	13.2*	12.4			·
Asya	Control	14.2 ns	14.3 ns	13.8*	15.0*	14.0 ns	12.1 ns			
Ca-OM	14.2 ns	14.0 ns	14.3*	13.9*	14.1 ns	11.9 ns				·
Vika	Control	12.0 ns	11.8*	11.7*	11.6*	12.3*	12.3*			
Ca-OM	12.3 ns	12.5*	12.1*	12.3*	13.5*	12.8*	13.4	12.5		·
JVT	Control	13.6*	12.4 ns	12.7 ns	13.2*	12.5 ns	11.9*			
Ca-OM	12.6*	12.4 ns	12.8 ns	12.1*	12.7 ns	12.7*				·

Gazel	Control	12.6*	12.1 ns	11.8 ns	12.0 ns	12.4*	12.4*	12.1*	11.8*	

The small letters (ns) following the number of values (of the same cultivar) represent nonsignificance according to the t test, and the sign *represents the statistically significant difference.

Table 8

The impact of foliar Ca-organomineral (Ca-OM) application on the content of total soluble solids (TSS; Brix %) in the berries among tested red currant cultivars during storage in 2023.

Cultivars	Treatments	Day 0	Day 4	Day 8	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
Lvovyanka	Control	13.8 ns	14.2 ns	13.6*	14.1*	14.2*	14.1*			
Ca-OM	13.8 ns	14.4 ns	14.3*	14.7*	15.4*	14.7*	12.5			
Asya	Control	10.7*	11.6 ns	11.6*	11.4*	11.9 ns	12.5 ns			
Ca-OM	11.6*	11.5 ns	11.4*	11.1*	12.0 ns	12.2 ns				
Vika	Control	13.8*	13.8*	14.6*	15.5*	14.8*	14.2*	12.9*		
Ca-OM	15.8*	14.6*	15.0*	16.2*	16.7*	16.8*	16.2*	13.7		
JVT	Control	10.9 ns	10.1*	10.2 ns	10.2*	11.3 ns	11.8*	11.9*		
Ca-OM	10.7 ns	10.8*	10.4 ns	10.9*	11.3 ns	11.6*	11.5*			
Gazel	Control	13.4*	12.6*	13.0*	13.6*	14.2*	14.2*	11.6*		

The small letters (ns) following to the number of values (of the same cultivar) represent nonsignificance according to the t test, and the sign *represents the statistically significant difference.

The introduction of Ca-OM increased the total TSS content in berries, and the decrease in TSS in the cultivars “Lvovyanka,” “Vika,” and “Gazel” was slower than in control $p \leq 0.05$. This is explained by the role of calcium cations in slowing down metabolic processes and cell respiration. Slowing down metabolic processes and respiration leads to a decrease in the rate of fruit ripening during storage [85]. Respiration slowdown reduces the synthesis and utilization of metabolites and decreases fruit TSS. Our results are consistent with those obtained in tomatoes [86] and strawberries [87]. Studies have reported that the amount of free sugars gradually increased during storage, and calcium cations noticeably slowed this increase.

The content of TSS is related to the strength of the fruit. A positive correlation between these indicators was noted in apples during storage [88, 89].

In this experiment, the density of berries gradually decreased, but the rate of decrease in berry density was slower in the variants with Ca-OM (Tables 9 and 10).

Table 9

The impact of foliar Ca-organomineral (Ca-OM) application on the density in the berries among tested red currant cultivars during storage in 2022 (*N*).

Cultivars	Treatments	Day 0	Day 4	Day 8	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
Lvovyanka	Control	3.1*	2.9*	2.5*	2.5*	1.7*	1.0*			
Ca-OM	3.8*	3.6*	3.9*	3.6*	2.5*	2.2*	1.0			
Asya	Control	2.5*	2.6*	1.4*	1.3*	0.7*	0.8 ns			
Ca-OM	3.2*	3.0*	2.5*	1.2*	0.9*	0.9 ns				
Vika	Control	2.3*	2.3*	2.7*	2.3*	1.6*	1.9*	1.0*		
Ca-OM	2.8*	3.3*	3.6*	3.0*	2.3*	2.4*	1.9*	0.9		
JVT	Control	2.2*	2.5*	1.9*	1.6*	1.3*	1.1 ns			
Ca-OM	2.9*	3.0*	2.4*	2.1*	1.8*	1.1 ns				
Gazel	Control	2.4*	2.6*	2.1*	2.0*	1.8*	1.7*	1.0*	0.9*	

The small letters (ns) following to the number of the values (of the same cultivar) represent nonsignificance according to the t test, and the sign *represents the statistically significant difference.

Table 10

The impact of foliar Ca-organomineral (Ca-OM) application on the density in the berries among tested red currant cultivars during storage in 2023 (*N*).

Cultivars	Treatments	Day 0	Day 4	Day 8	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
Lvovyanka	Control	2.2 ns	2.6 ns	2.8*	2.8*	1.7*	1.0*			
Ca-OM	2.2 ns	2.6 ns	3.2*	2.2*	2.0*	1.5*	0.9			
Asya	Control	3.5*	4.5 ns	4.2 ns	3.4*	2.0 ns	1.0 ns			
Ca-OM	4.4*	4.4 ns	4.2 ns	3.7*	2.2 ns	1.0 ns				
Vika	Control	4.6*	4.0*	3.8 ns	2.5*	2.4*	1.6*	1.0*		
Ca-OM	4.9*	4.9*	3.8 ns	3.7*	3.4*	2.2*	1.8*	1.0		
JVT	Control	4.5 ns	3.9 ns	3.3*	2.0*	1.5 ns	0.9 ns			
Ca-OM	4.4 ns	3.9 ns	3.5*	2.3*	1.5 ns	1.0 ns				
Gazel	Control	3.6*	3.9*	2.8*	2.9*	1.8*	1.7*	0.9*		

The small letters (ns) following to the number of the values (of the same cultivar) represent nonsignificance according to the t test, and the sign ⁎represents the statistically significant difference.

“Lvovyanka,” “Vika,” and “Gazel” in the Ca-OM variant maintained a high berry density compared to the control over a long storage period. The results of the study are consistent with data from Gupta et al. [90] and Rombaldi et al. [91] in peach, Changhoo et al. [92] in kiwi, and Ciccarese et al. [93] in grapes. Studies have reported that the addition increases the strength of fruits and prolongs the storage period of fruit products.

Gao et al. [30] and Vicente et al. [90] have shown that the use of Ca increases the density of the intercellular layer of the cell wall, prevents the penetration of hydrolase and the disintegration of the jelly-like layer, and also affects changes in the pectin component of the cell wall, thereby maintaining the stability of the cell wall and the hardness of fruits. Calcium, which is part of the structure of the cell wall of fruits, can reduce the availability of enzymes that destroy the cell wall and help preserve the postharvest qualities of fruits [95, 96]. Also, Ca, together with abscisic acid (A.B.A.), participates in the transmission of ethylene signals, which regulates the processes of softening, aging, and ripening of fruits [42, 90]. It has been proven that calcium is involved in transmitting the ethylene signal, where the SR1 gene encodes several calcium sensors (CaM, CML, and C.D.P.K.) responsible for fruit maturation. [97].

Studies of current cultivars have revealed genotypic differences in the calcium content of berries during storage. Foliar Ca-OM treatments increased the calcium content in berries (Figures 4 and 5).

[figure(s) omitted; refer to PDF]

There was a significant calcium increase compared with the control in “Lvovyanka,” “Vika,” and “Gazel.” Similar results were obtained by Tromp [70] in apples and by Fuentes et al. [25, 98] in grapes.

Changes in the calcium content in fruits are explained by the different viability of the cells of the genotypes of fruit crops to retain moisture for a specific time, thereby minimizing the percentage of calcium loss by fruits [42, 99].

In this experiment, an increase in the calcium content during storage was observed.

According to the research of White and Broadley [64], this result is explained, firstly, by the physiological loss of fruit weight and, secondly, by the peculiarity of calcium not being reutilized in the plant. In this experiment, physiological weight loss averaged 2–4% every three days.

Physiological Weight Loss (PLW) increased during the storage period (Table 11).

Table 11

The impact of foliar Ca-organomineral (Ca-OM) application on the physiological loss in weight (PLW) in the berries among tested red currant cultivars in a standard atmosphere at a storage temperature +2.8 to +4.0°C (%).

Cultivars	Year	Treatments	Day 0	Day 4	Day 8	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
Lvovyanka	2022	Control	0	1.0⁎	3.2⁎	3.8⁎	7.6⁎	15.1⁎			
Ca-OM	0	0	1.1⁎	1.2⁎	3.4⁎	6.6⁎	10.3			2023	Control
0	0.9⁎	2.9⁎	4.4⁎	8.5⁎	17.7⁎				Ca-OM	0	0
0.8⁎	1.0⁎	2.7⁎	8.9⁎	11.2			-				
Asya	2022	Control	0	0	2.0⁎	3.4⁎	6.7⁎	11.2 ns			

Ca-OM	0	0	0.7*	2.9*	5.4*	10.9 ns				2023	Control
0	0.4 ns	1.8 ns	4.1*	6.0 ns	12.0 ns				Ca-OM	0	0.1 ns
1.5 ns	3.0*	5.5 ns	11.8 ns				-				
Vika	2022	Control	0	0.5	1.0*	2.1*	4.4*	7.1*	10.1*		
Ca-OM	0	0	0.2*	1.0*	1.9*	3.2*	7.0*	10.9		2023	Control
0	0.2	0.9 ns	1.9*	3.4*	6.4*	12.1*			Ca-OM	0	0
0.5 ns	0.8*	1.2*	3.1*	6.4*	11.8		-				
JVT	2022	Control	0	0.9*	3.9*	8.8*	10.9 ns				
Ca-OM	0	0.1*	2.8*	4.1*	10.1 ns					2023	Control
0	1.0 ns	4.6*	8.1*	11.2 ns					Ca-OM	0	0.8 ns
2.1*	6.4*	10.6 ns					-				
Gazel	2022	Control	0	0.9	2.4*	4.8*	6.9*	7.7*	8.4*	11.8*	
Ca-OM	0	0	0.6*	2.1*	4.0*	5.2*	7.1*	9.1*	12.2	2023	Control
0	0.2	1.8*	3.1*	7.7*	9.6*	11.2*			Ca-OM	0	0

The small letters (ns) following to the number of the values (of the same cultivar) represent nonsignificance according to the *t* test, and the sign * represents the statistically significant difference.

These results are similar to those of Dhillon et al. in mango [100], Gupta et al. in peach [90], Gangwar et al. in aonla [101], and Mahajan et al. in guava [102].

It is reported that calcium is adequate for maintaining the integrity of cell membranes, and it reduces the loss of phospholipids, proteins, and ions, which may be the reasons for reducing weight loss during storage [103].

The influence of the cultivar and the use of Ca-OM on the duration of storage and preservation of the quality of currant berries have been revealed. Ca-OM extended the shelf life of “Lvovyanka,” “Vika,” and “Gazel” berries by an average of 3–7 days compared to the control (Tables 7–11). These data are consistent with the results of Blažek et al. [104], Pissard et al. [105], and Tokala et al. [106] for apples. The shelf life is reported depending on the cultivar

and cultivation technology and storage technology.

This is due to the varietal reactions to foliar application of Ca and the peculiarity of Ca cations that slow down the processes of spoilage of berries. At low temperatures, the additional content of Ca-pectate can contribute to the thickening of cell walls and slowing down metabolic processes in cells [107]. Under visual observation, the shedding of berries, the development of spoilage, and the darkening of berries decreased in “Lvovyanka,” “Vika,” and “Gazel” treated with Ca-OM (Figure 6). Symptoms of a decrease in the quality of berries were observed in the treated cultivars “Lvovyanka,” “Vika,” and “Gazel” after 18 days of storage.

[figure(s) omitted; refer to PDF]

3.3. Cluster Analysis and Variety Similarity

The results of PCA biplot according to Ca and TSS content in leaves and fruits of five different berry cultivars during growth periods are given in Figure 7 [108]. According to the results, it can be concluded that the cultivars closest to each other (with similar characteristics) are the “Asya” and “Gazel” cultivars [109]. These two cultivars are primarily similar in terms of TSS content in the control treatment and Ca content in the fruits of the Ca-OM treatment.

[figure(s) omitted; refer to PDF]

The Ca content was notably higher in the fruits of the control of the “Lvovyanka” cultivar than the “Gazel” and “Asya” cultivars, highlighting a distinctive characteristic of this cultivar. Similarly, TSS fruit content increased with Ca-OM treatment, but it was not determined as a defining characteristic for any cultivar. On the other hand, the “Vika” cultivar was determined to have a defining characteristic, especially regarding calcium content in leaves. The “Jonkheer Van Tets” cultivar was determined to be superior in terms of TSS content in leaves compared to other cultivars. It can be observed from Figure 7 that the OMP application did not have a significant effect on the TSS content in the leaves.

Consistent with the results analysis, the cluster analysis (CA) results (Figure 8) revealed similar groupings among the tested cultivars. Specifically, “Gazel” and “Asya” cultivars were grouped, demonstrating comparable calcium and TSS, and this grouping was also observed with the “Vika” cultivar. The remaining cultivars exhibited similarity at the 4th level of significance, indicating a considerable distance from each other in terms of these characteristics.

[figure(s) omitted; refer to PDF]

4. Conclusion

The application of foliar Ca-OM treatment should be strategically timed with different periods of plant ontogenesis, considering the specific characteristics of the cultivar and the prevailing climatic conditions during cultivation. Ca-OM treatment in the early stages of the ontogenesis of red currants provides a high percentage of Ca intake in berries. It improves berries’ physical and mechanical parameters by the period of full ripening. Notably, the stability of calcium content in leaves was less influenced by agrotechnical and natural factors than by fruits, underscoring its potential as a consistent indicator. The significant reduction in calcium content during the full ripening of red currant berries was attributed to their inherent biological characteristics. Additionally, the content of TSS in both vegetative mass and berries is contingent on genotype, ontogenetic stage, and weather factors.

Furthermore, exposing red currant berries to low temperatures during storage and applying Ca-OM in the berry vegetation stage demonstrated favorable outcomes, including increased TSS and berry density, reduced berry abscission within the bunch, and an extension of the storage period for “Lvovyanka,” “Vika,” and “Gazel.”

Notably, CA identified these cultivars as similar to TSS and calcium content in fruits, emphasizing their common traits. Moreover, the elevated calcium content in berries emerged as a positive influence, enhancing consumer characteristics. Contrary to this trend, the results from PCA biplot indicated that for “Asya” and “Jonkheer Van Tets,” TSS content does not determine berry quality during the storage period.

In summary, the findings of this experiment pave the way for the development of agrochemical methods aimed at managing the quality of berry products, offering valuable insights for interventions during both the vegetation and storage stages, taking into account the cultivar characteristics of berry crops. The obtained data are relevant both for agricultural producers and researchers studying the role of calcium in the growth and development of horticulture crops [94].

Authors' Contributions

All authors contributed to writing the conception, designing the study, analyzing the data, and discussing the findings. All authors have read and approved the final manuscript submission for publication.

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DETAIL

Subjek:	Fruits; Vegetation; Low temperature; Weather; Storage life; Weather conditions; Crops; Calcium content; Leaves; Food quality; Calcium; Precipitation; Genotypes; Abscission; Cultivars; Temperature; Consumption; Fruit crops; Berries; Genetic diversity; Genotype &phenotype; Cluster analysis
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Pengarang:	Panfilova, Olga1 ; Ibrahim Kahramanoğlu2 ; Ondrasek, Gabrijel3 ; Ryago, Nelli1 ; Leonteva, Larisa1 ; Chunpeng (Craig) Wan4 ; Tsoy, Mikhail1 ; Okatan, Volkan5 ; Golyaeva, Olga1 ; Loretts, Olga6 ; Kukhar, Viktor6 ; Karpukhin, Mikhail6 1 Russian Research Institute of Fruit Crop Breeding (VNIISPK), Zhilina, Orel District, Orel Region 302530, Russia2 Department of Horticulture, Faculty of Agricultural Sciences and Technologies, European University of Lefke, Gemikonagi, via Mersin 10, Ankara 99780, Northern Cyprus, Türkiye3 Faculty of Agriculture, University of Zagreb, Zagreb 10000, Croatia4 Jiangxi Key Laboratory for Postharvest Technology and Nondestructive Testing of Fruits &Vegetables, College of Agronomy, Jiangxi Agricultural University, Nanchang 330045, China5 Department of Horticulture, Faculty of Agriculture, Eskisehir Osmangazi University, Eskişehir 26160, Türkiye6 Ural State Agrarian University, ul. Karl Liebknecht, 42, Yekaterinburg 620075, Russia

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Formulation, Process Optimization, and Biochemical Characterization of Cereal-Based Sweet Potato and Mulberry Instant Beverage

Singh, Vishakha; Das, Mamoni; Sukh Veer Singh; Narashans, Alok Sagar.

ABSTRAK (ENGLISH)

Most of the beverages have a high glycemic index, which is attributed to a sudden rise in blood sugar. The beneficial role of functional foods combination provided the tool to perform and design our study to develop an instant beverage mix (IBM) that might be revealed as the favorable therapeutic potential for the treatment of hyperglycemia and act as a functional beverage. Therefore, resistant fibre-rich ingredients/raw materials were used to formulate the cereal-based instant beverage (CIB). CIB was formulated using black rice flour (40–70%), germinated lentil flour (10–20%), sweet potato flour (10–20%), and mulberry powder (10–20%). The product formulation was optimized with respect to the following responses such as color and appearance, texture, flavor, taste, and overall acceptability using a D-optimal mixture design. The results revealed that the variation in raw ingredients significantly affected the organoleptic properties of trials. The ratio 40 : 20 : 20 : 20 of black rice: germinated lentil: sweet potato: mulberry was found to be optimum for the development of CIB. Optimized CIB had 9.71 ± 0.10 g/100 g of crude protein, 4.73 ± 0.09 g/100 g of fat, and 4.48 ± 0.06 g/100 g of crude fibre. Moreover, the total mineral content and carbohydrate content were found to be 1.08 ± 0.07 g/100 g and 72.45 ± 0.44 g/100 g, respectively, whereas, the energy value was 371.21 ± 4.23 kcal. *In vivo* glycemic index was also performed for the optimized CIB. The findings showed a lower glycemic response (37.70) than the diabetic control group, and blood glucose was found to be lowered (279.67 ± 20.06 to 227.17 ± 13.44 mg/dL) via the hypoglycemic mechanism. Thus, the optimized CIB exhibited a therapeutic effect against diabetic conditions and might be a healthy instant beverage for human consumption.

TEKS LENGKAP

1. Introduction

Instant beverage mix is referred to as a dry mix which can be blended with water or other liquid foods (i.e., milk) to prepare the instant beverage [1]. The hectic lifestyle of consumers promoted the consumption of instant beverage mixes due to their easy preparation and health benefits. Recently, due to the availability and increasing demand for “on-the-go” food and beverages, instant beverage mixes have received much demand across the developed countries [2]. Many research studies showed that plant-based diets can prevent the type 2 diabetes [3]. The constituents (fibre, minerals, phytonutrients, and antioxidants) present in plant-based diets play a key role in preventing and treating type 2 diabetes as well as reducing the risk of other complications, such as overweight, abdominal obesity, high blood pressure, hyperlipidemia, and inflammation. [4]. According to the World Health Organization (WHO) and Food and Agriculture Organization (FAO) [5, 6], diets rich in plant foods are not only effective in reducing health-related complications but also more environmentally sustainable than diets rich in animal products.

Instant foods such as ready-to-eat (RTE), ready-to-drink beverages (RTD), and semicooked or ready-to-cook breakfast and snack items are commonly prepared by using malted or unmalted cereals alone or by mixing with

legumes, nuts, fruits and/or vegetables. Among cereals, rice is the second most important cereal in the world, consumed as a staple food by the Asian population, providing energy and other nutrients compared to other staple foods [7]. Black rice is one of the healthiest foods and contains a variety of constituents including antioxidants, essential amino acids, minerals, and dietary fibre. Regular consumption of black rice can help in weight management and detoxification of the body as it gives a feeling of fullness which reduces food intake [8]. Among all colored rice varieties, anthocyanin content is the highest (327.60 mg $100\,g^{-1}$) in black rice, which is able to prevent the accumulation of bad cholesterol over the heart valves and also reduces the level of cholesterol in the blood. Thus, black rice results in controlling and preventing chronic diseases, such as coronary infarction, cancer, type 2 diabetes mellitus, allergies, and Alzheimer's disease [9, 10]. Many ready-to-serve (RTS) and RTE foods use white rice as the main ingredient; however, it can be replaced with black rice to develop a new food product with increased nutritional status [11]. Among legumes, lentils have the highest content of functional components, such as total phenolic content, than other common legumes [12], which have potential health benefits as complementary medicinal foods, that exert antioxidant, hypolipidemic, cardioprotective, anti-inflammatory, and anticancerous effects [13]. Sweet potato flour contains 2-3% of fibre [14]. Potential benefits of soluble dietary fibre include lowering blood serum cholesterol levels, influencing glucose metabolism, and thus regulating postprandial blood sugar levels and secretion of insulin in diabetic patients [15]. Lund et al. [16] compared 28 varieties of fibre-rich test samples prepared from tropical fruits and vegetables for their capacity to bind cholesterol. On the other hand, the sweet potato-based test sample had the highest cholesterol-binding ability. Therefore, sweet potato genotypes with a high polyphenol content and used as a vegetable, tea, beverage breakfast food, food ingredient, and as a nutritional supplement can be developed to be used for the promotion of health [17]. A natural antioxidant in black mulberry (*Morus nigra*) is the anthocyanin pigment which has excellent free radicals scavenging activity and leads to limit and cure the diseases caused by oxidative damage in human beings [18].

The literature reported that black rice (*Oryza sativa* L.), sweet potato (*Ipomoea batatas*), lentil (*Lens culinaris*), and mulberry fruit (*Morus nigra*) have been used for the prevention and treatment of adult-onset diabetes via a specific mechanism of insulin interaction [19]. The beneficial role of these functional food combinations provided us with the tool to think and design our study to develop an instant beverage mix (IBM) from functional ingredients of plant origin and to determine its efficacy on blood glucose levels of alloxan-induced diabetics [20]. IBM thus might reveal the favorable therapeutic potential for the treatment of hyperglycemia and act as a functional beverage.

There is no research work available on the development of cereal-based instant beverages using the abovementioned functional ingredients. If properly processed, such a product would be novel and could fetch substantial revenue to the beverage processing industry. Apart from this, there is a need to enhance the postharvest processing of these underutilized grains by formulating value-added convenience and healthy products such as instant beverage mixes. This functional beverage meets the consumers' demand by supplying functional components. Thus, the present study was carried out to optimize the ratio of raw ingredients of a CIB, and the effects of the ingredient variations were observed on the organoleptic properties of it as well as the biochemical parameters of the final optimized CIB was also investigated for its therapeutic suitability.

2. Materials and Methods

2.1. Raw Materials

Black rice (*Oryza sativa* L.) and black mulberry (*Morus nigra*) were procured from the counter of the Agricultural Technology Information Centre (ATIC) and the sericulture farm of Assam Agricultural University (AAU), Jorhat, respectively. Both lentils (*Lens culinaris*) and pale yellow-fleshed sweet potato (*Ipomoea batatas*) were purchased from the weekly market of Jorhat town, Assam, India. Soy milk (Life Health Foods, Jorhat, Assam, India) and sucralose with 100 purity (Brand: ProFoods Nutrition, Jaldhara and Co. Jorhat, Assam) were taken as an alternative to sugar in CIB preparation.

2.2. Pretreatment of Raw Materials and Its Processing

Black rice was first cleaned and washed, followed by soaking in water for half an hour. The rice sample was steamed for 45–50 min. and dried using a hot air oven at 60°C for 8 h (Castro Engineering Pvt. Ltd., Howrah, India).

Finally, rice was ground into flour using paddy dehuller (Paddy Dehusker, Osaw Industrial Product Pvt. Ltd., Assam, India) and sieved with 150 mess size. For germinated lentil flour, lentil seeds were first washed, followed by soaking in water by portioning into a cup and adding water (lentil: water = 1 : 2, w/w) for 24 h, and covering with a muslin cloth. After that, legume seeds were washed and allowed to germinate in a muslin cloth at 20°C for 5 days. Germinated samples were steamed for 20 min (a ratio of 1 : 1.5, w/w of lentil and water) and finally, dried at 40°C for 72 h. The germs or sprouts were separated by rubbing. After dehulling, legumes were ground into flour using a paddy dehuller machine and sieved with 150 mesh sieves [21]. Sweet potato was processed according to the method given by Hernandez-Aguilar et al. [22]. In brief, yellow flashed sweet potato was peeled off, washed, and dabbed in a muslin cloth to remove surface water. Thereafter, the washed sweet potato was uniformly sliced in 10 × 10 × 5 mm size. Sweet potato slices were oven-dried at 60°C for 10 h and ground into flour using an electric grinder. The flour was screened with a 100-mesh sieve [22]. Fresh ripe black mulberries were sorted and dried at 55°C for 30 h using a cabinet dryer (XDL315 Dry Cabinet, Hibex India Pvt. Ltd., Assam, India). The dried fruit sample was ground into powder using an electric grinder and sieved with a 100-mesh sieve size. All the flour and powders were separately packed in high-density polyethylene (HDPE) virgin airtight containers and stored at room temperature.

2.3. Experimental Design of Optimization

The selection of the different ingredients for the development of CIB was based on preliminary experiments. The range of flours (independent variables) comprising of black rice (C1: 40–70%), germinated lentil (C2: 10–20%), sweet potato (C3: 10–20%), and mulberry (C4: 10–20%) was taken and experimental formulations were designed using a D-optimal mixture design. The effect of formulations was investigated on responses such as appearance, color, taste, texture, flavor, consistency, and overall acceptability, as shown in Table 1. A total of 20 experiments were performed for the optimization of raw material ratio in CIB, including 4 replicates to obtain an estimate of pure error, as shown in Table 1. The accuracy of fit and the significance of linear and quadratic models were performed by Design-Expert® Version 13 (Stat-Ease, Inc., Minneapolis, MN, USA).

Table 1

Organoleptic responses of different CIB formulations.

Samples	Cereal (C1)	Pulse (C2)	Fruit (C3)	Vegetable (C4)	Responses						
Appearance	Color	Flavor	Taste	Texture	Consistency	Overall acceptability	TS1	53.83	20	10.40	15.77
7	7.20	7.10	7.30	7.30	7.3	7.30	TS2	40	20	20	20
7.90	7.90	7.60	8	8	7.80	7.60	TS3	66.21	10.00	13.78	10
6.60	6.50	6.70	6.60	6.90	6.70	6.70	TS4	62.86	17.14	10	10
6.70	6.70	6.80	6.50	6.80	6.80	6.90	TS5	54.79	10.00	15.34	19.87
7.10	7.20	7	6.90	7.50	7.30	7.20	TS6	50.02	19.99	19.99	10

7.70	7.60	7.30	7.20	7.60	7.40	7.20	TS7	45.44	19.92	15.23	19.41
7.40	7.50	7.50	7.70	8.20	7.70	7.50	TS8	53.29	15.65	16.35	14.71
6.70	6.80	7.20	7.70	7.00	7.20	7.10	TS9	66.46	10	10.01	13.53
6.80	6.70	6.60	6.70	6.70	6.60	6.70	TS10	57.39	17.98	13.20	11.42
6.70	7	7	7	7.20	7.30	7	TS11	57.79	11.36	20	10.84
7.10	7.10	6.90	7.20	6.90	7.20	6	TS12	46.22	18.55	20	15.23
6.90	7.60	7.70	7.90	6.50	7.60	7.50	TS13	53.83	20	10.40	15.77
7	7.20	7.10	7.30	7.30	7.30	7.30	TS14	59.05	12.98	10.52	17.44
7.20	7.30	7	7	7.30	7.30	7.10	TS15	62.86	17.14	10	10
6.70	6.70	6.80	6.50	6.80	6.80	6.90	TS16	57.79	11.36	20	10.84
7.30	7.10	7	6.80	7.20	6.80	7.10	TS17	51.09	16.18	12.72	20
7.40	6.90	7.10	7.20	6.80	7.10	7.30	TS18	49	11.01	19.99	20
7.80	7.60	7.50	7.40	7.50	7.40	7.30	TS19	40	20	20	20
7.90	7.90	7.60	8	8	7.80	7.60	TS20	50.02	19.99	19.99	10

TS1 to TS20, test formulations 1 to 20 based on the D-optimal mixture design.

2.4. Preparation of CIB

The raw ingredients (30 g) were taken from each formulation (20 experiments) and added to 100 ml of warm soy milk containing 2 g of sucralose. Thereafter, it was stirred well for proper mixing. The organoleptic evaluation was conducted on the prepared instant beverage (CIB) of each formulation. It was carried out with respect to the

following responses such as appearance, color, taste, texture, flavor, consistency, and overall acceptability as shown in Table 1.

2.5. Organoleptic Evaluation

After the preparation of the CIB of each formulation, organoleptic evaluation was conducted in the Department of Food Science and Nutrition, Assam Agricultural University, Jorhat, (India). Organoleptic evaluation of the fresh instant was conducted by a total number of 15 semitrained sensory panelists comprising five males and ten females. The coded CIB samples assigned with three-digit numbers were randomly presented to the trained panelists. All the panelists were asked to rinse their oral cavity with lukewarm water for palate cleansing in between each sample at 20°C and RH of 62%. The organoleptic evaluation was performed using a 9-point hedonic scale ranging from 1 to 9, where 1 depicted “dislike extremely” and 9 depicted “like extremely” [23].

2.6. Optimization Using D-Optimal Design

The ratio of raw ingredients/materials was optimized using organoleptic response values on the basis of better-set goals which were maximum for each response (appearance, color, taste, texture, flavor, consistency, and overall acceptability). The criterion of the set goal for optimization was determined according to Pérez-Báez et al. [24]. The lower and upper limits for black rice flour (C1: 40–70%), germinated lentil flour (C2: 10–20%), sweet potato flour (C3: 10–20%), and mulberry powder (C4: 10–20%) were fixed on the basis of our preliminary trials. For carrying out the optimum solution of multiple responses, the individual goals were combined into an overall composite function called the desirability function [25]. The desirability of optimized CIB was found to be closer to one. Contour plots for all responses showed better results with respect to the used ingredient levels. Moreover, optimized CIBs were further subjected to proximate, free radical scavenging activity, and biochemical analysis (*in vivo*).

2.7. Proximate Analysis of Optimized CIB

Standard AOAC analytical methods [26] were used to analyse the proximate composition i.e., moisture, protein, fat, fibre, and ash of optimized CIB. The carbohydrate content was determined by the differential method and the energy value was determined by multiplying the proportion of protein, fat, and carbohydrate with its respective physiological energy value.

2.8. Determination of Free Radical Scavenging Activity of Optimized CIB

Free radical scavenging activity was estimated using the DPPH method given by Vani et al. [27]. Analysis was carried out in the analytical laboratory of the Department of Food Science and Nutrition and Department of Food Science and Technology, Assam Agricultural University, Jorhat, Assam.

2.9. Biochemical Analysis of Optimized CIB

2.9.1. *In Vivo* Glycemic Index (GI)

Twelve adult healthy male Wistar rats (150–250 g) from Chakraborty Enterprises, Kolkata were used to determine the glycemic index. All the animals were reared in the Department of Pharmacology and Toxicology, College of Veterinary Sciences, Assam Agricultural University, Khanapara, Assam. The rats were housed in polypropylene cages (five rats per cage). The rats were incubated in a controlled condition with a 12 h light and dark cycle for acclimatization. Deionized distilled water was offered *ad libitum*. The animals were divided into two groups, namely, group A (control group) and group B (optimized CIB as a feed). Each group consists of 10 animals. The control group (group A) was fed with rat ration along with standard glucose and group B was fed with optimized CIB. The guidelines of the Institutional Animal Ethics Committee had been followed during animal experimentation (Approval no. 770/GO/Re/S/03/CPCSEAFVSc/AAU/IAEC/18-19/709 dated 28.12.2018).

(1) Glucose Tolerance Test (GTT). GTT was performed using blood samples taken from overnight fasting animals. Fasting blood sugar was taken from Group A before administration of 0.15 g of standard glucose solution (dissolved in distilled water). Blood glucose was determined after feeding at the interval of 15, 30, 45, 60, 90, and 120 mins. The same method was performed with Group B (feed supplied as optimized CIB) after determining their fasting blood sugar level according to the method proposed by Thannoun [28].

A blood sample was collected from the experimental animals by the tail-tipping method. Blood glucose was determined by using the Accu-Check Roche blood glucose meter (Active blood glucometer kit, Pillbox Pharmacy,

Assam, India).

The glycemic index (GI) for each diet was determined by calculation of the incremental area under two hours of blood glucose response or curve (iAUC) for each diet and compared with iAUC for glucose solution standard according to the method of Jenkins [29] by using the following equation:(1)GI=IncrementalAreaUnder2hbloodglucoseCurveforfoodIncrementalAreaUnder2hbloodglucoseCurveforglucose×100.

2.9.2. Supplementation Study

Sixty male Wistar rats (150–250 g) were used to study the effect of optimized CIB supplementation on blood glucose levels. The animals were divided into five groups: alloxan free, control group fed with rat ration, group A (diabetic control) fed with rat ration, group B treated with metformin and fed with rat ration, and group C (C1, C2, and C3) fed diet loaded with 50%, 60%, and 70% of optimized CIB, respectively (Table 2). Diabetes was induced by a single intraperitoneal administration of alloxan monohydrate (160 mg/kg) with 4% saline (average 0.90 ml per specimen) after overnight fasting. Animals were left undisturbed for 48 h. After 48 h, blood samples were collected from the surviving rats by retro-orbital puncture and blood glucose level was checked. The rats that had a value above 200 mg/dl were considered as diabetic.

Table 2

Proportion of diets for assessing the hypoglycemic effect of optimized CIB.

Experimental group	Proportion of diet	Number of animals
Control	Rat ration (100%)	10
A (diabetic control)	Alloxan + rat ration (100%)	10
B (standard metformin)	Alloxan + rat ration (100%)	10
Group C (CIB)		
C1	Alloxan + rat ration (50%) + CIB (50%)	10
C2	Alloxan + rat ration (40%) + CIB (60%)	10
C3	Alloxan + rat ration (30%) + CIB (70%)	10

CIB, cereal-based instant beverage; C1 to C3, group C subgroups.

(1) Determination of Blood Glucose Level. Blood samples were collected from the experimental animals on days 0, 2, 7, 14, and 21 by retro-orbital puncture and centrifuged at 3000 rpm for 20 min. Serum was collected in a microcentrifuge and glucose level was determined using a commercially available assay kit with an auto analyser. The standard laboratory method was used for blood glucose estimation by using a spectrophotometer (iCE 3000 series, Systronics, India).

2.10. Statistical Analysis

In the present study, a D-optimal mixture design was applied to design the experiments and to analyse the data of organoleptic observations. All experiments were performed independently with at least three determinations. Mean values ± standard deviations for all quantitative parameters were calculated using Microsoft Excel® 2016 (Microsoft Co., Ltd., Washington, USA). Paired "*t*" test, analysis of variance, and *F*-test were used to compare the effect of a specific treatment, the ratio of between-group variability to within-group variability, and the equality of the different treatments on the time of the interval using IBM® SPSS® Statistics version 22.0 (New York, USA). A probability value ≤ 0.05 was considered to be significant.

3. Results and Discussion

3.1. Effect of Ingredients Ratio on Organoleptic Properties of CIB

The role of the different raw ingredients on organoleptic properties was determined by the second-order polynomial model which examined the effect of the significant difference between the independent variable (linear effect) and combined variable (interactive effect) on the organoleptic responses of the developed products. The linear and interactive effects of all the ingredients on the individual organoleptic parameters of the formulated CIB are shown in Table 3.

Table 3

Linear and interactive effect of each variable on organoleptic responses of CIB formulations.

Variables	Appearance	Color	Flavor	Taste	Texture	Consistency	Overall acceptability
Independent variables	Linear effect (individual effect)						
β1 (cereal)	5.99	6.20	6.59*	7.23*	7.18*	6.84	6.67*
β2 (pulse)	7.10*	7.40*	7.91*	7.82*	6.43*	7.72*	7.40*
β3 (fruit)	7.80*	8.31*	8.62*	8.54*	7.29*	6.66	7.87*
β4 (vegetable)	7.23*	7.52*	8.10*	8.23*	6.16	7.77*	7.50*
–							
Combined variables	Interactive effect (combined effect)						
β1β2 (cereal and pulse)	5.82	6.87*	5.72	6.22*	5.74	5.40	5.82*
β1β3 (cereal and fruit)	6.29	6.00	7.20*	7.10*	6.43*	6.89*	6.80*
β1β4 (cereal and vegetable)	5.94	6.32	5.89	6.12	6.12	6.34	6.12*
β2β3 (pulse and fruit)	7.23*	7.43*	8.10**	8.42**	7.83*	7.21*	7.70*
β2β4 (pulse and vegetable)	6.23	6.40	6.82*	6.55*	6.92	7.22*	6.69*

β3β4 (fruit and vegetable)	6.89*	7.12**	7.72*	7.92**	6.43	7.22*	7.22*
–							
ANOVA value							
P value	0.01	0.01	0.01	0.001	0.05	0.01	0.001
R2	0.80	0.81	0.81	0.86	0.83	0.80	0.89

*Significance level at $p<0.05$; **Significance level at $p<0.01$; R2 = coefficient of regression.

The independent variables showed a significant ($p<0.05$) effect on the organoleptic properties of the product. Organoleptic evaluation of the 20 test samples obtained through a D-optimal mixture design revealed that the appearance of CIB formulation ranged from 6.6 to 7.9 in different test samples. Appearance and color of the product were linearly and significantly ($p<0.05$) improved by pulse, fruit, and vegetable. The texture was linearly and significantly ($p<0.05$) improved by cereal, pulse, and fruit, while consistency was linearly and significantly ($p<0.05$) improved by pulse and vegetable. Moreover, the texture ranged from 6.7 to 8.2. The flavor, taste, and overall acceptability of the product were linearly and significantly ($p<0.05$) improved by all the independent variables.

The interactive effect between pulse and fruit and fruit and vegetable significantly ($p<0.01$) improved the appearance. Similarly, the interactive effect between cereal and pulse, pulse and fruit, as well as fruit and vegetable significantly improved the color. It ranged from 6.5 to 7.9. The effect between cereal and pulse, pulse and fruit, as well as fruit and vegetable significantly improved the flavor (6.6 to 7.7) and consistency (6.6 to 7.8) of the product. The interactive effect between cereal and pulse, cereal and fruit, pulse and fruit, pulse and vegetable, as well as fruit and vegetable significantly ($p<0.01$) improved the taste of the product. Similarly, the interactive effect between cereal and fruit as well as pulse and fruit significantly ($p<0.01$) improved the texture of the CIB. All the independent combined variables (interactive effect) significantly ($p<0.05$) raised the overall acceptability of the product. The organoleptic responses including appearance, color, flavor, taste, texture, and consistency of CIB together contributed to 89% overall acceptability of the product. Sensory evaluation indicated that the overall acceptability of the different test samples ranged from 6.7 to 7.6 as shown in Table 1. The 2D representation of the D-optimal mixture design in relation to the independent variables involved in the organoleptic properties of CIB is shown in Figures 1(a)–1(n).

[figure(s) omitted; refer to PDF]

3.2. Optimization of Formulation and Verification

Optimization of the independent variables was performed for the organoleptic responses based on better-set goals for each response, which were “in range” for all ingredients and “maximum” for all responses. The results of the optimization process showed that a maximum desirability could be obtained at a level of 40% incorporation of cereals and 20% incorporation of pulses, fruits, and vegetables. At this optimal level, the values of the predicted responses were found to be appearance (7.90), color (7.90), flavor (7.60), taste (8.00), texture (8.00), consistency (7.80), and overall acceptability (7.60). For verification of the optimized level of variables (40% of cereal, 20% of pulse, 20% of fruit, and 20% of vegetable), organoleptic evaluation was again performed at this level and observed values for each response were found to be closer to predicted values. The variation was found to be 1% in all the organoleptic parameters. Thus, the ratio of raw ingredients with 40 : 20 : 20 : 20 (cereal: pulse: fruit: vegetable) was termed as “optimized CIB” formulation and later it was subjected to proximate, free radical scavenging activity and *in vivo* studies.

3.3. Proximate of Optimized CIB

The proximate composition of optimized CIB is shown in Table 4. The moisture content of the optimized CIB was

7.55±0.16g/100g, which was within the recommended limit given by the Food Safety and Standards Regulation of India, 2011 [30]. The protein content of the optimized CIB formulation was 9.71±0.10g/100g. The optimized CIB contained a range of crude protein levels, varying from 13.92g/100g to 22.12g/100g, attributable to the inclusion of germinated lentils [31]. This could be related to the germination and malting process, in which lentil seeds absorb water by imbibition process to initiate sprouting [32]. Sprouting increases the crude protein content, reduces the phytate level of legumes, and promotes the activity of protease and phytase enzymes, which makes solubilization of phytates easier and releases soluble protein and minerals that increase protein content [33]. The fat content of optimized CIB was 4.73±0.09g/100g, which was higher than the fat value (1g/100g) of the healthy drink mix available in the market. This could be due to the use of fat-rich lentil flour in optimized CIB formulation. A similar range of fat content from 3.75g/100g to 7.26g/100g was reported by Qiu et al. [34]. The total mineral content of optimized CIB was 1.08g/100g. This was in agreement with Santos et al. [35], who reported a total mineral content of 1.9g in black rice flour. Chen et al. [36] reported that black rice bran had a higher mineral content than other cereal brans. The crude fibre content of CIB was 4.48g/100g. Cereal's outer bran layer is rich in crude fibre content. According to the World Health Organization (WHO), dietary fibre promotes metabolism and prevents noncommunicable diseases [37]. The total carbohydrate content of the optimized CIB was 72.45g/100g and the energy value was 371.21kcal/100g as shown in Table 4. Similar energy content from 309kcal/100g to 350kcal/100g in different samples of sprouted legume-based composite diet was reported by Okorie et al. [38]. Thus, an increased metabolic process and degradation of starch into simple sugar provide energy during the germination process [39].

Table 4

Proximate composition of optimized CIB.

Parameters	Nutrients (per 100g of dry weight basis)
Moisture (g)	7.55±0.16
Crude protein (g)	9.71±0.10
Crude fat (g)	4.73±0.09
Crude fibre (g)	4.48±0.06
Total mineral content (g)	1.08±0.07
Total carbohydrate content (g)	72.45±0.44
Energy (kcal)	371.21±4.23

Data are expressed as the mean±standard deviation (SD).

3.4. Free Radical Scavenging Activity of Optimized CIB

The free radical scavenging activity of optimized CIB was 75.65%. The free radical scavenging activity was observed to be significantly ($p<0.05$) higher in CIB than in PIB. This could be due to the presence of black rice flour as the main constituent which has a potent source of phytochemicals, comprising of anthocyanins, flavones, flavonoids, glycosides, carotenoids, and tocopherols. The study also reported that the most abundant anthocyanin present in black rice extract is cyanidin 3-glucoside which prevents diseases associated with hyperlipidemia and hyperglycemia by regulating the hepatic lipogenic enzyme activities, inhibiting a-glucosidase activity, and inducing pancreatic beta cells regeneration, thereby causing an increase in blood insulin level and reducing blood glucose level [40, 41].

3.5. Glycemic Index of Optimized CIB

Group A animals fed with glucose standard had 75 mg/dl of initial fasting blood glucose level, followed by 92 mg/dl at 60 min and again decreased gradually to 77 mg/dl after 120 min. Group B animals fed with optimized CIB had 85 mg/dl of initial fasting blood glucose level, followed by 98 mg/dl at 60 min and again gradually decreased to 79 mg/dl after 120 min. In experimental groups, the peak blood glucose level was observed at 45 min and 60 min after consumption of optimized CIB, which significantly ($p<0.05$) decreased within 120 min, indicating slow digestion and absorption of the optimized CIB (Figure 2). Comparatively and significantly ($p<0.05$) minimum rise in blood glucose level was observed in group B fed with optimized CIB, and this may be due to the higher dietary fibre presence in optimized CIB whereas the glucose standard contains no dietary fibre. Several studies have reported that the soluble dietary fibre promotes the satiety feeling which decreases the quantity and frequency of food intake and exerts hypoglycemic effects as well as reduces the regulatory systems stress related to glucose homeostasis through the activation of endocrine L cells in the colon by their physiological ligands and short chain fatty acids. They promote proglucagon expression and GLP-1 secretion, thereby controlling insulin secretion and maintaining glucose homeostasis. Thus, the optimized product (CIB) is able to combat and maintain the higher blood glucose level. Hence, this could be suggested for the treatment of diabetes mellitus [42].

[figure(s) omitted; refer to PDF]

3.5.1. Incremental Area under Blood Glucose Response Curve (iAUC) and Glycemic Index of Optimized CIB

The data of the blood glucose response were used to calculate the glucose response incremental area. The iAUC value of the reference and sample are shown in Table 5. The mean iAUC of glucose standard and optimized CIB was 1132 and 427, respectively. The iAUC values expressed that CIB had a lower level of glucose than orally given glucose. The glycemic index is an important parameter when considering diet as a treatment for metabolism-related disorders, such as adult-onset diabetes mellitus, an improvement in postprandial blood glucose concentration after having a meal [43]. The glycemic index of optimized CIB was calculated from their iAUC and iAUC of glucose standard. The glycemic index of CIB was 37.70. The studies reported that the low glycemic index of foods, such as black rice and germinated lentil seed, are rich in dietary fibres, proteins, and phytonutrients. Fibers and protein play crucial roles in producing short chain fatty acids (SCFAs) and amino acids (AAs). These SCFAs and AAs, once produced, stimulate insulin signaling and activate the insulin secretion channels. Consequently, a diet rich in dietary fiber and amino acids helps in maintaining stable blood glucose levels [44]. Several studies have reported that a water-soluble dietary fibre is low or resistant to digestion and absorption in the small intestine. It may be fermented by gut microflora in the large bowel or as such excreted through feces, thus maintaining the blood glucose levels [45].

Table 5

Mean incremental area under the curve (iAUC) and glycemic index for formulated CIB[1].

Samples	iAUCmg.min/100 ml	GI
Glucose standard	1132.50	—
CIB	427.00	37.70

GI, glycemic index.

3.6. Effect of Supplementation of Optimized CIB on Blood Glucose Level

At the end of the supplementation period, the diabetic control experimental group A showed a significant ($p\leq0.05$) increase in the blood glucose level from the initial value of 284.17 ± 16.36 mg/dl to 293.83 ± 20.80 mg/dl, after induction of alloxan and fed with rat ration, whereas, the experimental group B showed a significant decrease ($p< 0.05$) in the blood glucose level (279.17 ± 36.60 mg/dl to 167.23 ± 10.35 mg/dl) which was injected with standard metformin and fed with rat ration. At the end of the supplementation period, the average reduction in blood glucose

level was observed to be 111.94 mg/dl. Under group C, the subgroup C3 fed with 70% of optimized CIB showed the highest significant decrease ($p<0.05$) in blood glucose level from 279.67 ± 20.06 mg/dl to 227.17 ± 13.44 mg/dl than subgroups C1 and C2 after the supplementation. At the end of the supplementation period, the highest average reduction in blood glucose level was observed in subgroup C3 (52.50 mg/dl), followed by subgroup C2 (50.34 mg/dl) and subgroup C1 (42.33 mg/dl) as shown in Table 6. This might be due to the presence of crude fibre, which binds with water to form a viscous gel and passes through the small intestine. This is relatively unchanged until it reaches the colon, where it is fermented by the gut microflora and produces short-chain fatty acids, resulting in the decrease of serum-free fatty acids. This reduces blood glucose levels through competition in insulin-sensitive tissues, which is most beneficial for diabetic patients [46]. Furthermore, the presence of phytonutrients in black rice and mulberry powder reduces the blood sugar level as it decreases the intracellular production of H_2O_2, resulting in the reduction of apoptosis and *β*-cells' destruction thus maintaining the insulin secretion in the blood [47, 48]. Franco San et al. [49] reported that anthocyanin compounds in functional foods help in re-growth and maintenance the function of *β*-cells. These cells are responsible for the synthesizing and secreting of insulin into the bloodstream, helping to control the blood glucose level during diabetic complications. Rathna et al. [50] reported that the whole grain black rice is digested slowly and is thereby important to slow releasing of glucose in the blood as well as also promoting obesity reduction which is a main cause of type 2 diabetes mellitus. Several studies documented that zinc from sweet potato has the ability to raise adiponectin hormone levels in the blood that appears to play a crucial role in protecting against insulin resistance and minimizing the chance of developing adult-onset diabetes mellitus [51, 52].

Table 6

Effect of optimized CIB supplementation on blood glucose levels (mg/dL) of alloxan-induced diabetic rats and mean decrease in blood glucose level (mg/dL) after supplementation[1].

Experimental groups	Day 0	2-day diabetic rat	Supplementation period (days)			Mean
7	14	21	Group A	87 ± 12.14	284.17 ± 16.36	284.67 ± 20.63
288.50 ± 20.43	293.83 ± 20.80	287.79 ± 83.53^{A}	Group B	76 ± 14.42	279.17 ± 36.60	248.99 ± 30.51
215.50 ± 14.47	167.23 ± 10.35	227.72 ± 76.59^{CDE}	*Group C*			
C1 (50%)	89.83 ± 13.42	270 ± 24.53	261.33 ± 20.05	247.17 ± 19.18	227.67 ± 19.17	251.54 ± 71.30^{BCD}
C2 (60%)	79.67 ± 13.72	258.17 ± 31.67	245.33 ± 36.27	231.33 ± 35.49	207.83 ± 32.02	235.66 ± 71.71^{EF}
C3 (70%)	85.67 ± 10.71	279.67 ± 20.06	259.83 ± 16.76	249.50 ± 17.20	227.17 ± 13.44	254.04 ± 73.14^{BCD}
Mean time	83.13 ± 14.34^{a}	271.14 ± 30.82^{b}	256.10 ± 30.26^{c}	242.58 ± 30.27^{d}	218.56 ± 33.32^{e}	

Data are expressed as the mean ± standard deviation (SD). The mean values on the same column with different lower-case superscripts and row with different upper-case superscripts represent significant differences ($p<0.05$) based on analysis of variance (ANOVA) and Duncan's multiple range tests. CIB, cereal-based instant beverage; C1 to C3, group C subgroups, group A (diabetic control) fed with rat ration, group B treated with metformin and fed with rat ration, group C (C1, C2, and C3) fed diet loaded with 50%, 60%, and 70% of optimized CIB, respectively.

4. Conclusion

The present study explored the utilization of different functional food matrix formulations to prepare cereal-based instant beverage that has a lower GI value of 37.70. Supplementation study found that the incorporation of optimized CIB in animal diets at 50%, 60%, and 70% significantly decreased the level of glucose in blood up to 42.33 mg/dl, 50.34 mg/dl, and 52.50 mg/dl, respectively. The findings of this study provide evidence for the selection of suitable ingredients for the formulation of CIB that can manage and control the blood glucose and lipid profile, thereby exerting additional health benefits in the control and treatment of several noncommunicable diseases. The technology for the production of instant beverage mixes with enhanced nutritional and functional properties can be transferred for commercialization to local food, health and wellness industries, and entrepreneurship. Thus, it can be accessible to people to have safe nutritious and convenient ready-to-serve food. The obtained findings will hopefully provide crucial supporting data for further study on other noncommunicable diseases. The effect of a specific amount of optimized CIB can also be investigated for liver diseases, CVDs, and obesity.

Ethical Approval

Ethical approval for the involvement of animal subjects in this study was granted by Institutional Animal Ethics Committee, Approval no: 770/GO/Re/S/03/CPCSEAFVSc/AAU/IAEC/18-19/709, dated 28.12.2018.

Consent

Verbal informed consent was obtained for experiments involving human voluntary participation.

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DETAIL

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Combined Addition of Citric Acid and Ascorbic Acid Significantly Inhibits Browning in Chinese Yam (Dioscorea polystachya Turczaninow) Processing

Yang, Wentao; Song, Xiaoning; Wang, Qingsong; Wang, Wenting; Zhao, Zhifeng.

ABSTRAK (ENGLISH)

Chinese yam (*Dioscorea polystachya* Turczaninow) is widely cultivated in East Asia, whose edible stem is a common vegetable and herb in traditional Chinese medicine. In fruit and vegetable processing, browning is estimated to be a major reason of waste. Browning lowers the nutrition value and brings undesired characteristics in food processing. To develop a secure and low-cost browning inhibiting protocol in yam processing, different thermal treatment conditions and color protectants were tested for their color-protecting ability. Color difference ΔE was calculated to evaluate the browning with a colorimeter. To ensure that the color-protecting treatment does not influence the quality of yam, texture properties and nutrition compositions were quantified. The optimal treatment is as follows: deactivate yam in water bath of 60°C for 10 min and then incubate in 2 g/L citric acid and 1 g/L ascorbic acid for 1 hour. The treatment led to significant decrease of the color difference, with no obvious changes in the texture properties and nutrition value. To summarize, this research provides an ideal color-protecting solution in yam processing.

TEKS LENGKAP

1. Introduction

Chinese yam is the edible stem of *Dioscorea polystachya* Turczaninow, which is native to China and cultivated in China, Japan, and Korea [1]. Yam is also used in traditional Chinese medicine. Modern food processing and packaging technology, especially the rapid development of precooked food industry in China, bring up various yam products, including salted yam, yam chips (a resemblance of potato chips), yam oatmeal, and yam biscuit. Inhibition or minimizing uncontrolled enzymatic and nonenzymatic browning should be one of top priorities in fruit and vegetable processing industry because browning leads to unfavourable changes in the sensory properties and lowers nutritional value [2–4]. In yam processing, browning's negative effect is especially significant: the peeled yam represents a pleasant and appetizing snow-white appearance and browning largely darkens the surface and softens the texture. Therefore, browned yam intermediate in the processing should be discarded or catalogued into the secondary product. A low-cost, stable, repeatable, and secure method to inhibit browning in yam processing should be developed.

In food industry, browning refers to all biological and chemical reactions which darken products and/or intermediates. Polyphenol oxidase (PPO) and peroxidase (POD) contribute to most enzymatic activities in food browning [2]. PPO (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) contains copper ions in the active site. PPO can catalyze two reactions: (1) adding a hydroxy group to the *o*-position of the benzene ring of a phenol molecule, forming a diphenol; (2) oxidizing a diphenol, producing an *o*-benzoquinone. The two reactions require oxygen. *O*-Benzoquinone can undergo a series of nonenzymatic reactions, forming melanin. Melanin is an insoluble and black pigment, which renders food the undesired and unfavourable appearance. POD (peroxidase; EC 1.11.1.7) can oxidize phenol compounds with hydrogen peroxide as the cosubstrate. Some studies reported that PPO from strawberry and mango harboured more enzymatic activities than POD after thermal treatment [5, 6].

Nonenzymatic browning normally is the result of Maillard reaction and/or caramelization [7]. Maillard reaction is an extreme complex reaction network, whose substrates are a reducing sugar, like glucose, and a compound with a free amino group. The amino group can be the R-group of an amino acid, e.g., lysine or the N-terminal end of a peptide [8, 9]. Maillard reaction is a mixed blessing: it can give food characteristic aroma, flavor, color/appearance, and texture properties. But it also has negative effects. As Maillard reaction starts from the condensation of a reducing sugar and a free amino group, it lowers the nutrition value of products. Moreover, acrylamide is an early product of Maillard reaction, whose correlation with cancer is still in debate [10–13]. It must be noticed that, in most cases, expected Maillard reaction takes place in the backing process, e.g., cake, bread, coco, coffee, and tea, while unexpected Maillard reactions normally take place inside the package or in the processing progress, e.g., on the shelf and in the transportation process, and lead to disgusting flavors and textures. Caramelization is widely used in food preparation since ancient times. It requires high temperature (>120°C) and starts from hydrolysis of sugar. Products of caramelization provide food special aroma and flavor. But inappropriate caramelization renders food undesired “burnt like” aroma and flavor, which in many cases result from overcooking or excessive thermal treatment [14, 15].

Various methods have been developed and applied in food industry to inhibit and minimize unexpected browning. Low temperature can inhibit activities of PPO and POD and lower rates of Maillard reactions. Fridge and cold chain logistics are quite common in food supply chain, supermarkets, and retailers. Dadali et al. found that microwave treatment is effective in reducing browning of lily bulbs. They suggest that the reduction in the browning should be results of decreased enzymatic activities of PPO and POD [16, 17]. Modified and controlled atmosphere package technology has been widely used in fruit and vegetable products. This technology controls the composition of the atmosphere around the product. At low O_2 or high idle gas (N_2, CO_2) condition, the respiration of cells slow down, leading to decreased PPO and POD activities and less browning. Moreover, lower O_2 content could inhibit degradation of oxygen-sensitive compounds [18]. Color protectants have important roles in inhibiting browning in research literature [6, 19]. To find out a low-cost color protectant suitable for yam processing, this research studies the browning inhibiting effect of sodium chloride, L-cysteine, citric acid, sodium erythorbate, and ascorbic acid on yam. Also, the influence of thermal treatment on yam browning was investigated.

2. Materials and Methods

2.1. Materials

Yam (length 0.5–1.0 m, diameter 8–12 cm) was purchased from local supermarkets in Yibin City, Sichuan Province, China, and stored at 4°C overnight for further research. Deionized water was provided by the water purification system (Milli-Q EQ 700, Merk, US). Unless otherwise stated, all chemicals are of analytical purity and supplied by Kelong Chemical Co. Ltd., Chengdu, China.

2.2. Methods

2.2.1. Pretreatment

Yam with similar size was screened, trimmed, and peeled with porcelain knife (to avoid oxidation caused by metal ions). Peeled yam was cleaned with deionized water, cut into 2-3 mm thick slices, incubated in water bath (time: 4–12 min, temperature: 40–80°C, and volume/weight = 20 : 1) to deactivate enzymes.

2.2.2. Color Protectant Treatment

Deactivated yam slice was cooled to room temperature in the air and then soaked in color protectant solution (sodium chloride: 4 g/L, 6 g/L, 8 g/L, 10 g/L, and 12 g/L; L-cysteine: 0.5 g/L, 1.0 g/L, 1.5 g/L, 2.0 g/L, and 2.5 g/L; citric acid: 0.5 g/L, 1.0 g/L, 1.5 g/L, 2.0 g/L, and 2.5 g/L; sodium erythorbate: 0.2 g/L, 0.4 g/L, 0.6 g/L, 0.8 g/L, and 1.0 g/L; ascorbic acid of 0.2 g/L, 0.4 g/L, 0.6 g/L, 0.8 g/L, and 1.0 g/L; combination: citric acid of 2 g/L and ascorbic acid of 1 g/L) for 1 h. Afterwards, yam slice was washed with deionized water, drained with tissue, and stored in the beaker at room temperature on the lab bench. To observe long-term effects of color protectant treatment, yam slice after color protectant treatment was washed with deionized water, drained with tissue, packaged via a vacuum pump, and stored on the lab bench for 1 month. The color difference ΔE was measured regularly.

2.2.3. Color Difference Measurement

Color difference was measured with the method described elsewhere [17]. Briefly, lightness (L), redness (a), and yellowness (b) of yam slice were measured with a colorimeter (UltraScan VIS, Hunterlab, US). The freshly sliced yam without any treatment was used as the blank. The color difference ΔE was calculated using the following equation:(1)ΔE=Ls−L02+as−a02+bs−b02.

In the formula, Ls, as, and bs represent the lightness, redness, and yellowness of the treated yam. L0, a0, and b0 represent the lightness, redness, and yellowness of the blank.

2.2.4. Texture Property Measurement

Texture properties of yam slices were measured with a texture analyzer (TA-XT Plus, Ronghua Instruments Co. Ltd., Changzhou, China). P/36 type flat bottom probe was used. The sample was condensed two times with the interval being 1s. Parameters: pretest speed, 1.0 mm/s; test speed, 3.0 mm/s; posttest speed, 3.0 mm/s; pressure level, 50%; force, 5 g; pressure height, 30 mm. The hardness was defined as the maximum force in the force-deformation curve. Number of peaks (Np) and the slope of the first peak (Sp) were used to quantify the brittleness.

2.2.5. Sensory Analysis

Sensory analysis of yam slices was performed according to China National Standard GB/T 29605-2013 (Sensory analysis-Guide for food sensory quality control). The taste panel was composed of 5 male and 5 female undergraduate students in Sichuan University of Science and Engineering. The yam slice after water bath (60°C, 10 min, and volume/weight = 20 : 1) without color protectant treatment was used as the reference. The panel was asked to grade the odor and taste differences between test samples and the reference. A five-point grading format was used. 5 meant no difference or slight difference; 4 meant slight to intermediate difference; 3 meant intermediate difference; 2 meant intermediate to relatively huge difference; 1 meant relatively huge to huge difference.

2.2.6. Nutrient Quantification

Starch and protein were quantified according to methods described by Smith and Zeeman [20] and Jung et al. [21], respectively. Yam slices to be analyzed were freeze-dried and homogenized, and dry weight was recorded. For starch content measurement, soluble interferences were washed away with 80% ethanol three times (3000×g, 10 min, Eppendorf 5804 R). Starch granules were gelatinized at 100°C for 10 min. Starch was converted to glucose in digestion buffer (200 mM sodium acetate, 6 units of amyloglucosidase (Roche Life Science, Germany), and 0.5 units of α-amylase (Sigma-Aldrich, Germany)) at 37°C for 4 hours. Glucose was enzymatically quantified, in which hexokinase and glucose 6-phosphate dehydrogenase were used to convert glucose to 6-phosphogluconate with concomitant reduction of NAD to NADH [22]. NADH was quantified with a spectrophotometer (UV-1800, Shimadzu, Japan), and starch content was calculated. Protein was quantified with the Kjeldahl method. Briefly, samples were digested in concentrated sulfuric acid with cupric selenite and potassium sulfate as the catalysts. 40% sodium hydroxide was used to release ammonia, which was then captured by 4% boric acid. The titration was performed with standardized 0.1 N hydrochloric acid, with 0.12% methyl red and 0.08% methylene blue as the indicator.

Allantoin and dioscin contents were measured according to Wu's work [23]. Allantoin was extracted with 80% ethanol two times and then separated on Agilent 1260 HPLC system. HPLC colum: Waters PAH C18 column, particle size 5 μm, diameter 4.6 mm, length 250 mm. Flow rate: 0.5 ml/min. Mobile phase: 10% of methanol and 90% of water. Detection wavelength: 224 nm. Allantoin was identified by comparing the retention time of the sample to the standard (National Institutes for Food and Drug Control, China) and then quantified *via* a standard curve.

Dioscin was extracted with 95% methanol two times, dried at 60°C, and then dissolved in methanol for further analysis on Agilent 1260 HPLC system. HPLC colum: Waters PAH C18 column, particle size 5 μm, diameter 4.6 mm, length 250 mm. Flow rate: 1.0 ml/min. Mobile phase: 88% of methanol and 12% of water. Detection wavelength: 210 nm. Dioscin was identified by comparing the retention time of the sample to the standard (National Institutes for Food and Drug Control, China) and then quantified via a standard curve.

2.2.7. Data Processing

Values in bar charts are means of three biological replicates. Error bars are standard deviations. $*p<0.05$; $**p<0.01$. Raw data were recorded with Microsoft Office Excel. Curves, bar charts, and significance analysis were produced and performed with Microsoft Office Excel.

3. Results

As shown in Figure 1(a), temperature has significant influence on the color difference ΔE. When temperature increases from 40°C to 50°C, color difference increases almost two times (from 5.33 ± 0.46 to 9.17 ± 0.38). When temperature reaches 60°C, the color difference dramatically drops to 2.74 ± 0.44, which is the lowest in this experiment. At temperatures higher than 60°C, the color difference and temperature are basically in lineal correlation, with the color difference at 80°C being 12.26 ± 0.56. This demonstrates that the optimal thermal treatment temperature of yam slices is 60°C.

[figure(s) omitted; refer to PDF]

Figure 1(b) shows that water bath time also has significant influence on the color difference. Insufficient water bath time (shorter than 10 min) leads to significant increase of color difference. In other words, when water bath time increases from 4 min to 10 min, the color difference drops from 4.21 ± 0.2 to 3.06 ± 0.17. However, longer water bath time (12 min) leads to an increase of color difference, compared with 10 min treatment. This might be attributed to nonenzymatic browning. Therefore, 10 min is the optimal treatment time.

Sodium chloride has the poorest color protecting effect in this study (Figure 2(a)). Even worse, sodium chloride of 10 g/L and 12 g/L treatment increases the color difference compared with the control; this indicates that inappropriate sodium chloride treatment can intensify browning. Sodium chloride of 4, 6, and 18 g/L can lower the color difference to about 60% of the control.

[figure(s) omitted; refer to PDF]

The color protection ability of L-cysteine varies with concentration (Figure 2(b)). L-cysteine of 1.5 g/L can lower the color difference to ~50% of the control. Higher or lower concentrations than 1.5 g/L intensify browning, but the color difference is still lower than the control. Similar phenomenon could be also observed in citric acid (Figure 2(c)) and sodium erythorbate (Figure 2(d)). Citric acid has very nice color protection ability, which also varies with concentration (Figure 2(c)). The optimal concentration of citric acid is 2.0 g/L, which can decrease the color difference to ~25%–50% of the control. It is noteworthy that after 25 hours, the color difference of 2.0 g/L treatment is about one quarter of the control; this indicates that citric acid is especially effective in long time. Sodium erythorbate and ascorbic acid are edible and extensively used antioxidants in food industry. Browning inhibiting effect of ascorbic acid increases with concentration (0.2 g/L–1.0 g/L). However, at low concentrations, the color difference of ascorbic acid is higher than 60% of the control at 5 h and 10 h (Figure 2(e)). This implies that the browning inhibiting effect of L-ascorbic acid is relatively lower than other antioxidants in the early stage (Figure 2(d)).

According to the above results, citric acid and ascorbic acid were combined and used as the color protectant in yam processing. The browning inhibiting effect of combination solution was compared with single antioxidant (citric acid and ascorbic acid). In line with Figures 2(c) and 2(e), citric acid and ascorbic acid both have very strong browning inhibiting effect (Figure 3(a)). Moreover, combination of ascorbic and citric acid even provides better color protecting effect. The color difference in the combination is significantly lower than ascorbic acid and citric acid, reaching only about 10% of the control. This demonstrates that combination of ascorbic acid and citric acid provides the best browning inhibiting effect in this study. Furthermore, the sensory properties of yam slices were analyzed instrumentally and subjectively separately to ensure that citric acid plus ascorbic acid treatment does not lead to undesired flavor and texture changes. The brittleness and hardness of yam, measured by texture analyzer, did not change in the next 25 hours after treatment (Figures 3(b)–3(d)). This phenomenon can also be observed in the sensory analysis performed with panelists (Table 1). Furthermore, nutrition components (starch, protein, allantoin, and dioscin) were measured. As shown in Figures 3(e) and 3(f), nutrients were stable after color protectant treatment. These results suggest that citric acid plus ascorbic acid treatment could effectively protect color, with no influence on the texture, flavor, and nutrient contents.

[figure(s) omitted; refer to PDF]

Table 1

Sensory analysis.

	Odor	Taste
24 h	5[a]	4.8±0.4
30 d	5[a]	4.7±0.5

[a]All 10 panelists gave a grade of 5; therefore, SD = 0 and not labelled.

Browning happens in processing, also inside the package. To explore whether citric acid plus ascorbic treatment could inhibit browning inside the package, yam slices after color protectant treatment were sealed with airtight plastic membrane, and then the color difference Δ*E*, texture properties, and nutrition composition were recorded in 30 days. Also, the texture and flavor properties were measured. As shown in Table 2, above parameters have few changes in the first 30 days, which is a typical shelf life for many yam products. Therefore, citric acid plus ascorbic treatment not only inhibits browning in yam processing but also helps to minimize browning in the storage and retail stage.

Table 2

Color difference, texture properties, and nutrition composition of vacuum-packaged yam after color protectant treatment.

	0 d	5 d	10 d	15 d	20 d	25 d	30 d
Δ*E*	0.32±0.02	0.32±0.01	0.32±0.02	0.31±0.05	0.29±0.02	0.32±0.01	0.32±0.01
Np	14.3±0.58	11.3±0.58	11.0±0	12.67±0.58	11.0±0	11.0±0	11.3±0.58
Sp	1.1±0.2	1.2±0.1	1.1±0.3	1.2±0.1	1.3±0.1	1.1±0.1	1.1±0.2
Hardness	3.42±0.2	3.42±0.3	3.42±0.4	3.42±0.5	3.42±0.6	3.42±0.7	3.42±0.8
Starch	80.0±3.1	80.4±4.2	82. ±3.9	79.4±3.4	81.9±3.9	78.9±3.7	82.4±3.8
Protein	10.4±0.2	10.4±0.3	11.4±0.8	10.1±0.5	10.9±0.6	11.4±0.3	11.1±0.3
Allantoin	1.4±0.2	1.42±0.3	1.2±0.1	1.0±0.1	0.9±0.1	0.8±0.1	1.2±0.3
Dioscin	0.12±0.02	0.12±0.03	0.13±0.01	0.08±0.01	0.09±0.01	0.13±0.01	0.12±0.01

4. Discussion

Browning is an important negative effect in food and vegetable processing industry; it leads to lower sensory quality, less nutrients, and disgusting off-flavors [2, 3]. In fact, browning is estimated to be the second major reason of food waste, with the biggest reason being spoilage caused by microorganism. The causes of food browning include enzymatic reactions, which require polyphenol oxidase (PPO) and peroxidase (POD), and nonenzymatic reactions, which normally refer to Maillard reaction and caramelization. Solutions to control food browning include thermal treatment, low temperature storage, and color protectant. The aim of thermal treatment is to deactivate PPO and POD to inhibit enzymatic browning. However, it must be noticed that high temperature could destroy thermolabile nutrients, such as vitamin C and E. Also, thermal treatment could lead to undesired characteristics in texture. Low temperature storage is normally applied in the transportation and storage stage. The aim of this study is to find a solution to inhibit and/or minimize browning in the processing stage. So, the optimal thermal treatment condition was

explored. As shown in Figure 1(a), yam slices after 60°C water bath have the lowest color difference, indicating 60°C is the optimal temperature to minimize browning. 40 and 50°C water bath treatments have higher color difference. This can be explained by the fact that PPO and POD are relatively thermal-stable enzymes [2, 6, 19, 24], while the high color difference in 70 and 80°C should be the result of nonenzymatic browning. There are studies reporting that the reaction rate of nonenzymatic browning increases 2–8 times as the temperature increases by 10°C [25].

Figure 1(b) shows that the optimal thermal treatment temperature for yam is 10 min. Insufficient deactivating time shorter than 10 min leads to higher color difference, while longer time (12 min) will accumulate products of nonenzymatic browning, causing higher color difference. The poorer color-protecting effect in shorter time must be due to residual enzymatic activities of PPO and POD. Therefore, in yam processing, the optimal thermal treatment condition is 60°C for 10 min.

Sodium chloride, L-cysteine, citric acid, sodium erythorbate, and ascorbic acid were tested for their color protecting effect in yam processing in this study. Sodium chloride is different from other color protectants in principle. High concentrations of sodium chloride can (1) denature proteins to deactivate PPO and POD and (2) decrease the solubility of oxygen in the solution to reduce the contact between substrate phenol and oxygen [26]. As shown in Figure 2(a), sodium chloride of 4 g/L, 6 g/L, and 8 g/L can inhibit browning. But the color difference of the treated yam is above 50% of the control, which is poorer than other color protectants and could be seen in the next figures. Even worse, 10 g/L and 12 g/L of sodium chloride lead to higher color difference compared with the control. This indicates that sodium chloride with concentration higher than the threshold can promote browning.

L-cysteine, citric acid, sodium erythorbate, and ascorbic acid are antioxidants [27–30]. Antioxidants can protect phenols and nutrients from oxygen's attack. It can be observed that the color difference of L-cysteine, citric acid, and sodium erythorbate does not always decrease with the concentration increase (Figures 2(b)–2(d)). When the concentration is higher than a threshold, the color difference starts to bounce. In L-cysteine-treated yam, the threshold is 1.5 g/L. Lower or higher concentration leads to the increase of the color difference. In citric acid, the threshold is 2 g/L, and in sodium erythorbate, the threshold is 0.6 g/L. It could be easily explained that the color difference in the low concentration is higher because of insufficient contact between the substrate and the antioxidant. The higher color difference in the high concentration might be due the pro-oxidation effect of antioxidant [31].

The browning inhibiting effect of citric acid is rather strong, as shown in Figure 2(c). It must be noticed that citric acid of 2 g/L and 2.5 g/L can keep color difference an almost constant, while in other concentrations, the color difference increases with time dramatically. Moreover, citric acid of 2 g/L has lower color difference than 2.5 g/L. 2 g/L citric acid's inhibiting effect can maintain the color difference down to ~30% of the control, which is the lowest among all antioxidants. It is noteworthy that after 25 hours, the color difference of 2.0 g/L treatment is about one quarter of the control; this indicates that citric acid is especially effective in long time. Therefore, citric acid has the best browning effect in some concentrations; this indicates that usage of citric acid should be careful to maintain the optimal concentration to avoid the pro-oxidation effect. So, citric acid was chosen to be one component in the further combination experiment.

Sodium erythorbate and ascorbic acid are a pair of isomers which share similar structures, with the only difference being the configuration of one carbon atom. In sodium erythorbate, the threshold concentration is 0.6 g/L. Lower and higher concentrations have much poorer inhibiting effects (Figure 2(d)). The reason of this concave curve should be similar with the phenomenon observed in L-cysteine, while in ascorbic acid, no pro-oxidation effects could be observed. Therefore, sodium erythorbate and ascorbic acid both work in minimizing the browning in yam processing. Considering (1) ascorbic is a beneficial vitamin, while sodium erythorbate has no physiological functions *in vivo*; (2) ascorbic acid's inhibiting effect does not fluctuate dramatically with concentration; and (3) sodium erythorbate and ascorbic acid of food grade have similar prices, ascorbic acid was chosen to be another component in the experiments.

According to the above results, citric acid, a strong but fluctuating color protectant, and ascorbic acid, a relatively

weaker but stable color protectant, were combined to check whether their collaboration could provide better browning inhibiting effects. As shown in Figure 3(a), combination of citric acid and ascorbic acid significantly lowers the color difference, compared with citric acid and ascorbic acid. Moreover, after 25 hours at room temperature, the color difference of the combination is only 15% of the control, which is obviously better than any other treatment in this study. Although in combination treatment, there is a significant difference of color difference between 0 h (right after the color protectant treatment) and 25 h ($p<0.05$, Figure 3(a)), the surfaces of yam slices are very similar, i.e., the change of color cannot be observed with naked eyes. This might be explained by the fact that the significant but small increase of color difference cannot lead to macroscopic changes in the appearance. The contents of starch and protein do not have significant changes after 25 hours; this indicates that the color difference should be products of reactions between amino acid and reducing sugar or phenol compounds and oxygen, while starch and protein do not contribute to the color difference.

It is necessary to prove that the color protecting methods described in this research bring very few or no negative influence on nutrients, flavor, and texture. Starch and protein are two main essential nutrients in yam, which in sum occupy ~90% of the dry weight. Allantoin and dioscin are secondary metabolites which are present in yam. Allantoin can decrease plasma glucose in streptozotocin-induced diabetic rats and inhibit the increase of total inflammatory cells in rats [32, 33]. Dioscin is a natural compound with therapeutic potential in metabolic diseases, cancer, inflammation, and infections [34]. To ensure that citric acid and ascorbic acid treatment is applicable for yam processing, hardness, brittleness, and contents of starch, protein, allantoin, and dioscin of treated yam slices were measured every 5 hours in 25 hours. As shown in Figures 3(b)–3(f), the hardness, brittleness, and nutrient contents of yam after color protectant treatment have very few changes over 25 hours. Furthermore, sensory evaluation was performed with panelists. It can be seen in Table 1 that there was no obvious sensory difference between the treated sample and the control. Therefore, citric acid plus ascorbic acid treatment does not influence sensory and nutrient quality of yam.

To explore whether citric acid plus ascorbic treatment could inhibit browning inside the package, yam slices after color protectant treatment were sealed with airtight plastic membrane, and then the color difference ΔE, texture properties, and nutrition composition were recorded in 30 days. It is noteworthy that texture properties were measured subjectively and instrumentally, respectively. As shown in Tables 1 and 2, above parameters have few changes in the first 30 days, which is a typical shelf life for many yam products. Therefore, citric acid plus ascorbic treatment not only inhibits browning in yam processing but also helps to minimize browning in the storage and retail stage.

5. Conclusion

To summarize, 2 g/L citric acid plus 1 g/L ascorbic acid treatment gives very strong browning inhibiting effects and brings no negative influence on the sensory and nutrient quality, which is a very effective color protectant solution to minimize browning in yam processing.

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All persons who have made contributions to this work are listed as authors.

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DETAIL

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Biopriming of Momordica charantia Seeds with Enterobacter to Improve Nutritional and Biochemical Attributes

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ABSTRAK (ENGLISH)

The increasing world population needs a standard balanced diet to address malnutrition problems. For this purpose, seed priming is one of the best techniques, which helps to increase the production of functional and nutritional food crops. Different techniques have been used for seed priming, but biological priming is the most frequently used because biocontrol agents offer a friendly environment for the growth of food crops. In this study, *Momordica charantia* L. seeds were subjected to a strain of *Enterobacter* sp. FD17 as a biocontrol agent at different time exposures (i.e., 24 h, 48 h, and 72 h). Leaf growth, flavonoids, chlorophyll content, amino acids, soluble sugars, protein, and total soluble phenolics were studied in the vegetative stage. The yield of nutritive components was evaluated from fruit, peel, and pulp of *M. charantia.* Biopriming was revealed to improve the final emergence rate, mean emergence time, seedling vigor, emergence index, and vigor indices I and II. Among the growth parameters, the root (0.45 ± 0.045 g) and shoot fresh weight (1.23 ± 0.05 g), leaf area (15.52 ± 1.5 cm), shoot length (30.33 ± 0.58 cm), number of flowers (6 ± 1.0), fruit weight (96.33 ± 1.15 g), and germination percentage (56.67 ± 11.55%) were also improved. Among biochemical analyses, biopriming improved chlorophyll a (6.33 ± 0.58 mg/g) and b (8.58 ± 2.5 mg/g), total soluble sugar (33.13 ± 2.24%), and total chlorophyll content (9.0 ± 1.5 mg/g). The nutritional analysis showed that free amino acids (1.43 ± 0.02 mg/g), total soluble sugar (42.53 ± 1.65%), ash (20.53 ± 2.57%), and catalase (347.47 ± 34.76 U/g) were increased in fruit, while crude fiber (3.62 ± 0.1%) and peroxidase (5.61 ± 0.34 U/g) in peel and protein and metabolizable energy in peel and fruit were increased. Among the water, acetone, and methanol extracts, the maximum antibacterial activity was shown by methanol extracts of leaves against Gram-negative and Gram-positive bacterial species (i.e., *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively) with inhibitory diameters of 3 mm. Biopriming also improved the phenolic contents in the leaves and fruits of *M. charantia*. Biopriming treatment was also revealed to be directly correlated with antiglycation activity. Therefore, biopriming treatment on seeds could be used to manipulate plant cell metabolism with a substantial improvement in phenolic content, antibacterial activity, and growth of *M. charantia*.

TEKS LENGKAP

1. Introduction

Our agriculture faces issues related to climate change and reductions in production, biodiversity, and resources. Nutraceuticals and functional foods are a cheap solution to this problem. Specific defense mechanisms in plants are strongly dependent on the state of seed priming. Priming has been divided into three different states, including a priming phase, a postchallenge primed state, and a transgenerational primed state [1]. In developing countries like Pakistan, herbal medicines have become more important in solving the problems of the health-seeking populations of these countries. Seed priming is a technique that is applied to seeds before sowing. In this technique, the seeds are moderately hydrated to a point where metabolic processes related to pregermination begin but without actual germination. The seeds are dried almost completely to handle normally. Among the different priming techniques,

biopriming gives an extra advantage to manage biotic stress, and therefore, this priming method has gained more attention [2]. In seed biopriming, beneficial microbes are utilized to improve the physiological performance of seeds and enhance their ability to withstand stress, and therefore it is an environmentally friendly and advantageous technique of seed priming [3].

A wide range of bacteria (e.g., *Azotobacter*, *Bacillus*, *Burkholderia*, *Arthrobacter*, *Klebsiella*, *Azospirillum*, *Agrobacterium*, *Rhizobium*, *Streptomyces*, phosphate-solubilizing bacteria, *Pseudomonas fluorescens*, and *Enterobacter*) and/or fungi (e.g., vesicular-arbuscular mycorrhiza, *Trichoderma viride*, and *Trichoderma harzianum*) have been found to be promising biopriming agents, whether these agents are used as biopesticides or biofertilizers [4].

Momordica charantia is usually used as a vegetable and is also an important medicinal plant economically. The common name of *M. charantia* is bitter gourd [5]. *M. charantia* is beneficial in the physiological and metabolic processes of the human body. *M. charantia* juice increases body stamina and prevents chronic fatigue. Various parts of *M. charantia* are used for the treatment of different diseases, including anemia, blood disorders, diabetes, cancer, diarrhea, cholera, and bronchitis [6]. Bitter gourd has biologically active plant chemicals such as proteins, steroids, alkaloids, triterpenes, flavonoids, saponins, and other acids that exhibit antibacterial, antifungal, antiviral, antifertility, anticarcinogenic, antidiabetic, and hypoglycemic properties [7].

Bitter gourd can reduce blood glucose in diabetic patients. Bitter gourd decreases the amount of glucose in the blood by inhibiting the enzyme *α*-glucosidase, which causes the breakdown of oligosaccharides to monosaccharides [8]. Bitter gourd affects glucose transport channels, which decrease glucose transport into the blood. This impact is important for the treatment of both types of diabetes (i.e., type I and type II). Bitter gourd contains a chemical known as charantin that is used to reduce blood glucose. A regular high sugar concentration has been observed in type I and type II diabetes, which could increase the risks of inflammation, blindness, oxidation in the whole body, kidney diseases, and heart attack [9]. The aim of this research work was to compare the growth parameters (i.e., mean germination time and percent germination, determination of vigor indices, shoot and root lengths, and total fresh and dry weights), biochemical changes (i.e., photosynthetic pigment, total soluble sugar and free amino acids, and antibacterial activity), nonenzymatic antioxidant (i.e., total phenolic contents and total flavonoid contents), and antiglycation activity induced in *M. charantia* by a biological priming method (i.e., treated with *Enterobacter* sp. strain FD17 for different time exposures of 24 h, 48 h, and 72 h).

2. Materials and Methods

2.1. Sample Collection

The seeds of *M. charantia* of a local variety were provided by AARI (Ayub Agriculture Research Institute), Faisalabad, Pakistan. Taxonomical identification of *M. charantia* seeds was carried out at the Department of Botany of Government College University, Faisalabad. The pot experiment was conducted with three replicates in a completely randomized manner in June under natural conditions of the environment to study growth and biochemical and antibacterial activity. A field experiment with a complete randomized block design was conducted in March with five replicates under natural environmental conditions to study the nutritional and nutraceutical potential.

2.2. Selection and Level Optimization

2.2.1. Biological Treatment

Before biopriming, *M. charantia* seeds were thoroughly cleaned and then surface sterilized using mercuric chloride solution (1%) for three minutes. A set of samples with three replications and each sample comprising 40 seeds were used. The seeds were thoroughly washed with distilled water before subjecting to biopriming. *Enterobacter* (FD17) was applied to bitter gourd seeds. An inoculum of the selected strain (FD17) was prepared in a 200 ml Erlenmeyer flask containing 10% TSA broth (Trypton soya agar). The flask was inoculated with the selected bacterial strain and incubated in a shaking incubator (Firstek Scientific, Tokyo, Japan) at 100 rpm at optimized temperature levels, i.e., 24 h, 48 h, and 72 h at 28 ± 1°C. An absorbance of 0.5 was achieved, measured with an optical density meter (Biolog1 Model-21907; Biology Inc.) at wavelength 535 nm, by dilution to maintain a uniform cell density (10^7–10^8 CFU/ml) prior to seed inoculation. Finally, the suspension of bacterial strain was injected into sterilized peat (100

ml/kg, seed to peat ratio: 1 : 1 w/w). For inoculation, seed dressing was carried out on inoculated peat. In case of control, the seeds were coated with the same slurry but autoclaved without inoculum. Seeds inoculated/uninoculated were seeded in pots having soil (5 kg) [10].

2.3. Growth Parameters

2.3.1. Mean Germination Time and Percent Germination

The incubation proportion was measured at the end of the seventh day of incubation by the process designated by Soad et al. [11].(1)Gp=NgNp×100,where “Ng” is the last number of seeds emerged and “Np” is the total number of seeds seeded.

Similarly, the mean growth time (MGT) in days was calculated as follows:(2)MGT=∑Dn∑n,where “*n*” is the number of seeds germinated on day “*D*” and “*D*” is the number of days counted from the beginning of the germination test.

2.3.2. Determination of Vigor Index and Number of Leaves

Following equations were used to determine seedling vigor [12].(3)Vigor index I=germination percentage×seedling length,Vigor index II=germination percentage×seedling dry weight.

The leaves of all the plants in the field were counted manually in every row and the mean was taken. Roots and shoots were used to determine length and dry weight of seedlings.

2.3.3. The Length of the Shoot and Primary Root (cm)

The length of the shoot and the primary root from the ground to the ligule of the upper leaf of the plant in each pot was measured using a tape and the average length of each plant/shoot was determined. For the length of the primary root, the plants were taken from the land at the time of harvest. A scale was used to record the root length and the average was used.

2.3.4. Total Fresh and Dry Weight (g)

The total fresh weight was measured by adding weight of the root and shoots of every plant, while the total dry weight of the plant was measured based on the dry weights of the root and shoot.

2.4. Biochemical Parameters

2.4.1. Photosynthetic Pigments

The method described by Arnon [13] was used to determine photosynthetic pigments. Total 0.5 g of fresh *M. charantia* leaves were ground using pestle and mortar in acetone solution (80%). After grounding, the solution was filtered and distilled water was used to make the final volume up to 10 ml of the filtrate. A spectrophotometer was used to note the absorbance at 480, 645, and 663 nm.

2.4.2. Total Soluble Sugar and Free Amino Acids

Total soluble sugars were measured using the method defined by Van Handel [14] and the method of Hamilton et al. [15] was used to determine total free amino acids.

2.4.3. Antibacterial Testing

Three extractions (i.e., water, acetone, and methanol) of fresh leaves of *M. charantia* were prepared. One hundred milligrams of leaf sample were used for each extraction. Broth Micro-Dilution Method [16] was employed to measure the antibacterial activity. Briefly, to prepare microdilution trays, a 2–6 fold dilution of the sample extract was used volumetrically. Each subsequent dilution step was performed using a new pipette and the extract was dispensed into the microdilution tray. Cultures of selected bacterial species (i.e., *P. aeruginosa* and *S. aureus*) were grown in their proper growth medium for inoculum preparation, and suspension (0.01 ml) was carefully transferred to broth. The growth of each bacterial species was maintained at 5 × 105 CFU/ml. Standardization of the inoculum was performed using the growth method and used for inoculation of each well of the microdilution tray. A volume not exceeding 10% was delivered in the well. Incubation was carried out in an ambient air incubator after a colony count of inoculum suspension for 16–20 hours at 35 ± 2°C. Finally, the lowest concentration (which completely inhibited the microbial growth) of leaf extract was determined in the microdilution wells and expressed as minimal inhibitory concentration (MIC).

2.5. Nonenzymatic Antioxidant

2.5.1. Total Phenolic Content (TPC)

The Folin–Ciocalteu reagent method [17] was used to measure total phenolic contents. To confirm oxidation of the Folin–Ciocalteu reagent (1 ml), dilutions were prepared and 7.5% sodium carbonate (2 mL; w/v) was used for neutralization. A final volume of 7 ml was maintained by adding distilled water. A spectrophotometer with a 1 cm cell was used to measure the absorbance at 765 nm of the resulting blue color after two hours of incubation at room temperature in the dark. To calibrate the curve, a standard (i.e., gallic acid) was used.

2.5.2. Total Flavonoid Content

The colorimetric assay [18] was employed to determine total flavonoid content with minor modifications. Briefly, one milliliter of dilute sample was taken in a volumetric flask containing distilled water (4 ml) followed by the immediate addition of 5% $NaNO_2$ (0.6 ml), 10% $AlCl_3$ (0.5 ml) after 5 min, and 1 m NaOH (2 ml) after 1 min. The reaction flask was subsequently diluted by adding distilled water (2.4 ml) immediately and mixed. The absorbance at 510 nm was observed for the pink solution. To calibrate the curve, quercetin (μg/g) was taken as a standard. The total flavonoid content of the samples was measured with the help of the following linear equation ($y = 0.0019x + 0.6157$) based on the calibration curve.

2.6. Enzymatic Antioxidant

2.6.1. Catalase Activity

The method of Aebi [19] was employed to check catalase activity with minor modifications. Briefly, in 1.5 ml of 1 m phosphate buffer (pH 7.0), half grams of leaves, peel, and fruit of the plant were ground in a prechilled mortar to homogenize. After centrifugation (15,000 rpm, 15 min at 40°C), catalase activity of the supernatant was checked. In a cuvette, phosphate buffer and H_2O_2 (3.0 ml) were taken and 40 μl of enzyme extract was added rapidly and thoroughly mixed. The time taken to decrease the absorbance by 0.05 units was measured at 240 nm on a spectrophotometer (Genesys 10-S, USA). The enzyme quantity required to decrease the absorbance at 240 nm by 0.05 units was taken as one enzyme unit.

2.6.2. Peroxidase Activity

The method of Sadasivam and Manickam [20] was employed to determine peroxidase (POD) activity using 20 mm guaiacol and hydrogen peroxide as a substrate. Briefly, total 0.5 g of plant material was ground and extracted in a prechilled mortar by adding 3 ml of 0.1 m phosphate buffer (pH 7.0). After centrifugation (18,000 rpm, 15 min at 5°C), the supernatant was used as an enzyme source within 2–4 hours and stored on ice until POD analysis. Then the buffer solution (3 ml), guaiacol solution (0.05 ml), enzyme extract (0.1 ml), and hydrogen peroxide (0.03 ml) were pipetted into a cuvette. The mixture was shaken well, and the absorbance was recorded in a spectrophotometer. The time required for the mixture to increase absorbance by 0.1 (Δt) at 430 nm was recorded and used in the following calculations:(4)the enzyme specific activity units g−1f. wt.=500Δt×11000×TVVU×1f. wt.,where Δt = change in time (min), TV = total volume of extract (ml), UV = volume used (ml), and f. wt. = weight of fresh leaf tissues (g).

2.7. Antiglycation Activity

2.7.1. Sample Extraction and AGE Assay

Methanol (50%) was added to one gram of the sample. The solution was centrifuged at 1500 rpm for ten minutes. To check the antiglycation activity, the supernatant was employed. A characteristic absorbance was used to check advanced glycation end products (AGEs) as given by Matsuda et al. [21]. Briefly, bovine serum albumin (BSA) (150 μl in 1 ml of Na_3PO_4 buffer at pH 7.2), d-glucose (150 μl), and sample (150 μl) were added and incubated for seven days at room temperature. Absorbance at 440 nm was calculated with a spectrophotometer. D-glucose was not added in the control and was used as a blank. The readings were taken in duplicates.

2.8. Percentage Inhibition (IC_{50}%)

The IC_{50} value is used to show the amount of a substance that is required to inhibit (i.e., *in vitro*) a specific biological component or process by 50%. The following equation was used to calculate the % inhibition:(5)% inhibition=absorbance of control−absorbance of sampleabsorbance of control×100.

2.9. Statistical Analysis

Complete randomized experiments were used in all analyses. Analysis of variance was applied to measure the significant differences in various factors. The least significant difference test was used to compare means to the final

significant difference. The computer software CoStat (CoHort 2003 v.6.2) was used to check the overall interaction of all factors for significance.

3. Results and Discussion

3.1. Effect of FD17 Bacterial Strain Treatment on Germination

3.1.1. Final Emergence Rate

The *Momordica charantia* seeds were treated with the bacterial agent *Enterobacter* sp. The FD 17 strain was used for this purpose. The seeds were dipped in bacterial culture at time exposures of 24 h, 48 h, and 72 h (Figure 1).

[figure(s) omitted; refer to PDF]

In Figure 1, it was found that *M. charantia* seeds treated at 72 hours with the FD17 bacterial strain have a high emergence rate of 70% compared to 24 and 48 hours. It was revealed that the control group treated with water had a high final emergence rate of 22% compared to the control group without water treatment, which had a final emergence rate of 18%. Therefore, compared to untreated samples, the percentage of the final emergence rate in seeds treated with FD17 bacterial strain was found to be significant ($p<0.05$).

3.1.2. Mean Emergence Time

The *M. charantia* seeds treated at 72 hours with FD17 bacterial strain have the highest mean emergence time compared to 24 and 48 hours (Figure 2). Furthermore, it was also revealed that the control group treated with water had a higher mean emergence rate compared to the control group without water treatment. Therefore, the mean emergence time was significant ($p<0.05$) in *M. charantia* seeds treated with bacterial strain FD17 compared to untreated samples.

[figure(s) omitted; refer to PDF]

3.1.3. Seedling Vigor

It was found that *M. charantia* seeds treated at 72 hours with the FD17 bacterial strain were found to have the highest seedling vigor compared to 24 and 48 hours (Figure 3). Furthermore, it was explored that the control group (treated with water) had higher seedling vigor compared to the control group without water treatment. Therefore, compared to untreated samples, the seedling vigor of *M. charantia* seeds treated with the bacterial strain FD17 was found to be significant ($p<0.05$).

[figure(s) omitted; refer to PDF]

3.1.4. Emergence Index

Figure 4 shows that *M. charantia* seeds treated with bacterial strain for 72 h had the highest emergence index compared to treatments of 24 h and 48 h and the control groups with and without water treatment. Therefore, compared to untreated samples, the emergence index in the seeds of *M. charantia* treated with the bacterial strain FD17 was found to be significant ($p<0.05$).

[figure(s) omitted; refer to PDF]

3.1.5. Vigor Indices I and II

M. charantia seeds treated with *Enterobacter* sp. for 72 hours showed the highest values for vigor indices I and II compared to *M. charantia* seeds treated for 24 and 48 hours and control groups with and without water treatment (Figures 5(a) and 5(b)). Therefore, compared to untreated samples, the vigor indices I and II of *M. charantia* seeds treated with the bacterial strain FD17 were found to be significant ($p<0.05$).

[figure(s) omitted; refer to PDF]

3.2. Growth Attribute

The plant phenotype after biopriming with its improved germination and growth is shown in Figure S1. The growth parameters of the *M. charantia* seeds treated with the bacterial strain FD17 are given in Table 1. The fresh weight of the root was found to be 0.45 ± 0.045 g, while the weight of the root of the control group was 0.37 ± 0.02 g. Similarly, the fresh weights of the shoots from the treated and controlled seeds were found to be 1.23 ± 0.05 g and 0.84 ± 0.08 g, respectively. The leaf area of the treated seeds was found to be 15.52 ± 1.50 cm and that of the control group was 11.37 ± 0.55 cm. The length of the shoot and the length of the root of the treated seeds were found to be 30.33 ± 0.58 cm and 2.97 ± 0.06 cm, respectively, while in the case of the untreated seeds, the length of the root and the length of

the shoot were 4.33 ± 0.29 cm and 21.83 ± 2.75 cm, respectively. The weight of the fruits of the treated seeds was 96.33 ± 1.15 g and that of the control group was 94.67 ± 5.03 g. The percentage of germination of treated seeds was found to be 56.67 ± 11.55 and that of the control group was 26.67 ± 5.77. From these growth attributes, it was explored that there is a significant difference between all growth parameters compared to the control group. The length of the root and shoot, the weight of the root and shoot, the leaf area, and the germination parameters were found to be higher in the treated samples compared to the seeds of the untreated sample.

Table 1

Growth parameters of *M. charantia* seeds treated with bacterial strain FD17.

Growth parameters	Control *M. charantia*	Treated *M. charantia*
Root fresh weight (g)	$0.37^{a} \pm 0.02$	$0.45^{a} \pm 0.045$
Shoot fresh weight (g)	$0.84^{b} \pm 0.08$	$1.23^{a} \pm 0.05$
Leaf fresh weight (g)	$0.20^{a} \pm 0.045$	$0.16^{a} \pm 0.02$
Leaf area (cm)	$11.37^{b} \pm 0.55$	$15.52^{a} \pm 1.50$
Shoot length (cm)	$21.83^{b} \pm 2.75$	$30.33^{a} \pm 0.58$
Root length (cm)	$4.33^{a} \pm 0.29$	$2.97^{b} \pm 0.06$
No. of flowers	$1.33^{b} \pm 0.58$	$6.0^{a} \pm 1.0$
Fruit weight (g)	$94.67^{a} \pm 5.03$	$96.33^{a} \pm 1.15$
Germination (%)	$26.67^{b} \pm 5.77$	$56.67^{a} \pm 11.55$

The results are presented as means of 3 replicates. Values are given as mean (±SD). “a” and “b” show treatments with a mean significant difference ($p<0.05$).

3.3. Protein and Total Soluble Sugar

The statistical analysis of total soluble sugar revealed that there was a nonsignificant ($p<0.05$) effect ($p<0.05$) of bacterial treatment, while according to the analysis of variance data ($p<0.05$), the protein in the leaves showed a significant difference. Compared to control plants, the protein level was found to be lower in plants that were treated with the FD17 microbial strain (Table 2).

Table 2

Biochemical parameters of the leaves of *M. charantia* seeds bioprimed with the bacterial strain FD17.

Biochemical analysis	Control	Bioprimed with FD17
Phenolic content (mg/g)	$10.211^{a} \pm 0.01$	$5.56^{b} \pm 0.03$
Total soluble sugar (%)	$28.431^{a} \pm 3.11$	$33.13^{a} \pm 2.24$

Protein (mg/g)	27.40[a] ± 1.162	5.68[b] ± 0.07
Free amino acid (mg/g)	4.071[a] ± 0.06	2.0[b] ± 0.1
Total chlorophyll content (mg/g)	7.16[a] ± 1.03	9.0[a] ± 1.5
Chlorophyll-a (mg/g)	2.850[b] ± 0.0	6.33[a] ± 0.58
Chlorophyll-b (mg/g)	3.780[a] ± 0.66	8.58[a] ± 2.50
Flavonoid (mg/g)	6.86[b] ± 0.01	1.03[a] ± 0.05
Catalase (U/g)	542.4[a] ± 17.86	459.8[a] ± 70.78
Peroxidase (U/g)	5.681[a] ± 0.01	2.68[b] ± 0.64
Anthocyanins (mg/g)	0.66[a] ± 0.002	0.67[a] ± 0.01
Carotenoids (*μ*g/g)	5.031[a] ± 0.06	3.17[b] ± 0.29

The results are shown as means of 3 replicates. Values are given as mean (±SD). “a” and “b” show the treatments with a significant difference ($p<0.05$).

3.4. Phenolic Content and Free Amino Acids

Phenolic content and free amino acids decreased significantly in plants treated with the bacterial strain FD17 according to statistical analyses ($p<0.05$). The phenolic content and free amino acids were found to be higher in the control group (Table 2).

3.5. Chlorophyll Content and Flavonoids

According to analysis of variance data ($p<0.05$), both chlorophyll a and chlorophyll b revealed a significant increase after bacterial treatment. Compared to the control group, the plants treated with FD17 bacterial strain showed a positive effect on chlorophyll a and chlorophyll b levels, while the total chlorophyll result was nonsignificant ($p<0.05$) for the treated plants (Table 2). Bacterial treatment was explored to reduce flavonoids in *M. charantia* plants after treatment with *Enterobacter* strain FD17 (Table 2).

3.6. The Nutritive Analysis of Peels and Fruits

3.6.1. Free Amino Acids

Statistical analysis of free amino acids in fruit and peel revealed nonsignificant results ($p<0.05$) in *M. charantia* plants after seeding with *Enterobacter* strain FD17 (Table 3). The free amino acids decreased slightly in the peel of the treated plants, while these increased slightly in the fruit of the treated plants compared to the control group.

Table 3

Proximate and nutritive analyses of the peel and fruit of *M. charantia* after seed priming with *Enterobacter* strain FD17.

Proximate analysis	Control *M. charantia*		Treated *M. charantia*	
Peel	Fruit	Peel	Fruit	Free amino acids (mg/g)

11.03[b] ± 1.05	0.44[a] ± 0.05	9.57[a] ± 0.31	1.43[a] ± 0.02	Crude fiber (%)
3.4[a] ± 0.44	3.08[a] ± 0.04	3.62[b] ± 0.10	2.81[a] ± 0.02	Protein (mg/g)
26.78[b] ± 0.68	26.62[b] ± 1.57	28.63[a] ± 0.15	76.20[a] ± 3.56	Total soluble sugar (%)
31.16[a] ± 0.07	29.0[b] ± 3.46	30.82[b] ± 0.12	42.53[a] ± 1.65	Ash (%)
15.82[a] ± 0.75	14.93[a] ± 0.46	15.38[a] ± 0.75	20.53[a] ± 2.57	Peroxidase (U/g)
2.43[a] ± 0.57	2.24[b] ± 0.41	5.61[a] ± 0.34	3.52[a] ± 0.53	Catalase (U/g)
374.3[a] ± 17	322.9[a] ± 48.52	290.13[a] ± 4.69	347.47[b] ± 34.76	Metabolizable energy (kcal/100 g)
17.04[b] ± 0.76	17.1[b] ± 0.79	28.94[a] ± 0.86	28.87[a] ± 0.81	Oil (%)

The results are shown as means of 3 replicates. Values are given as mean (±SD). “a” and “b” show the treatments with a significant difference ($p<0.05$).

3.6.2. Crude Fiber

The crude fiber in the peel indicated a significant effect of microbial treatment according to the variance of the data ($p<0.05$). The treated plants with the bacterial strain FD17 exhibited lower crude fiber in the fruit compared to control plants and therefore expressed nonsignificant ($p<0.05$) results in the treated plants (Table 3).

3.6.3. Protein

Statistical analysis of protein in peel and fruit had shown significant results ($p<0.05$) in treated plants, and protein content was found to increase in both peel and *M. charantia fruit* after seed priming with *Enterobacter* stain FD17 (Table 3).

3.6.4. Total Soluble Sugar

The results of statistical analysis according to the variance of data ($p<0.05$) of total soluble sugar in *the* fruit and peel of *M. charantia* demonstrated significant effects of biopriming. For fruit, treated plants showed better results compared to control plants as the percentage of total soluble sugar increased significantly, while it decreased in the peel of treated plants (Table 3).

Similarly, the percentage of oil and ash content showed nonsignificant results in both the peel and the fruit of *M. charantia* after treatment with the *Enterobacter* strain FD17. Bacterial treatment showed a significant increase in the concentration of peroxidase and catalase enzymes in the fruit of treated plants. Metabolizable showed a significant increase in both the peel and the fruit of the treated plants (Table 3).

3.7. Antiglycation

Antiglycation activity was found to increase significantly in *M. charantia* fruit after seed preparation with *Enterobacter* strain FD17 (Table 4).

Table 4

Antiglycation activity of *M. charantia* fruit after seed priming with *Enterobacter* strain FD17.

Activity	Control *M. charantia*	Treated *M. charantia*

Antiglycation	$0.45^{a} \pm 1.49$	$0.90^{b} \pm 0.052$

The results are shown as means of 3 replicates. Values are given as mean (±SD). 'a' and 'b' show the treatments with a significant difference ($p<0.05$).

3.8. Antibacterial Activity of *M. charantia* Leaf Extracts

The antibacterial activity of various leaf extracts (i.e., water, acetone, and methanol) of *M. charantia* was explored against *S. aureus* and *P. aeruginosa* bacteria (Figure 6). In the case of Gram-negative bacteria, the maximum activity was shown by methanol extract followed by acetone and water (Figure 6(a)). The microbial treatment enhanced the antibacterial activity in all extractions. However, in the case of Gram-positive *Staphylococcus aureus* bacteria, the highest activity was displayed by methanol followed by water and acetone. Treatment with the FD17 bacteria strain had reduced antibacterial activity in the extraction of methanol and acetone compared to the control (Figure 6(b)).

[figure(s) omitted; refer to PDF]

3.9. Phenolic Profile

The freeze-dried samples of *M. charantia* (i.e., leaves and fruits) indicated the presence of several phenolic acids. According to the analysis of variance, the quantitative variation was found to be significant in microbial FD17 treated and untreated plants. In *M. charantia* leaves, quercetin, benzoic acid, and sinapic acid levels improved significantly after treatment with FD17, while chlorogenic acid, syringic acid, *M*-coumaric acid, and cinnamic acid decreased in leaves of treated plants (Table 5, Figure S2). In the case of the *M. charantia* fruit, there was a significant increase in chlorogenic acid and *M*-coumaric acid, while a decrease was observed for quercetin and sinapic acid (Table 5, Figure S3).

Table 5

Phenolic acid profile of *M. charantia* leaves and fruit after treatment with *Enterobacter* strain FD17 by HPLC.

Sr. no.	Phenolic acids	Retention time (min)	Concentration (ppm)			
Control leaf	FD17 treated leaf	Control fruit	FD17 treated fruit	1	Quercetin	2.82
3.77	6.46	2.96	0.86	2	Benzoic acid	14.65
1.64	40.81	ND	ND	3	Chlorogenic acid	15.87
5.19	ND	ND	6.08	4	Syringic acid	17.01
3.31	ND	ND	ND	5	M-coumaric acid	19.73
5.81	ND	ND	2.22	6	Cinnamic acid	24.71

16.25	ND	11.58	ND	7	Sinapic acid	26.19

ND: not detected.

3.10. Correlation of Antibacterial Activity *vs* Primary and Secondary Metabolites

The antibacterial activity of *M. charantia* leaf extracts against a Gram-negative *P. aeruginosa* after treatment with *Enterobacter* strain FD17 showed a direct correlation with catalase enzyme and secondary metabolites such as vanillic acid and coumaric acid, while showing an indirect correlation with ferulic acid and a nonsignificant correlation with peroxidase, total chlorophyll contents, chlorophyll a, chlorophyll b, soluble sugar, protein, amino acids, anthocyanin, flavonoids, carotenoids, quercetin, sinapic acid, syringic acid, cinnamic acid, chlorogenic acid, and benzoic acid (Table 6).

Table 6

Correlation matrix of antimicrobial activity with enzymes, primary, and secondary metabolites of *M. charantia* leaves.

Bacteria	Enzymes		Primary metabolites						Secondary metabolites	
Peroxidase	Catalase	Chl-tot	Chl-a	Chl-b	Proteins	Soluble sugar	Amino acids	Carotenoid	Anthocyanin	*P. aeruginosa*
ns	0.7***	ns	ns	ns	ns	ns	ns	ns	ns	*S. aureus*
ns	ns	−0.9**	−0.9**	ns	0.8***	−0.7***	0.7***	ns	ns	
Bacteria	Secondary metabolites									
Flavonoid	Quercetin	Vanillic acid	Sinapic acid	*M*-coumaric acid	Cinnamic acid	Syringic acid	Benzoic acid	Chlorogenic acid	Ferulic acid	
P. aeruginosa	ns	ns	1***	ns	1***	ns	ns	ns	ns	−1***
S. aureus	ns	−1***	1***	−1***	1***	1***	1***	−1***	1***	−1***

*, **, and ***indicate significant correlation; ns: nonsignificance at $p<0.05$.

Similarly, Gram-positive *S. aureus* presented a positive correlation with biochemical parameters such as proteins and free amino acids and showed a negative correlation with chlorophyll a, total chlorophyll contents, and soluble

sugar. *S. aureus* expressed a direct correlation with cinnamic acid, *M*-coumaric acid, vanillic acid, chlorogenic acid, and syringic acid. On the other hand, it was indirectly correlated with quercetin, sinapic acid, benzoic acid, and ferulic acid (Table 6).

3.11. Correlation of the Phenolic Profile

The phenolic profile of the *M. charantia* fruit after seed priming with *Enterobacter* strain FD17 showed a direct correlation with ferulic acid and coumaric acid to metabolizable, peroxidase, and protein, while it revealed an indirect correlation with catalase and soluble sugar. Benzoic acid in fruit had a positive correlation with metabolizing energy, peroxidase, and protein, whereas there was a negative correlation with catalase and soluble sugar. Sinapic acid, cinnamic acid, and quercetin showed an indirect correlation to metabolizable, peroxidase, and protein, while a direct correlation to catalase and soluble sugar was obtained, and a nonsignificant correlation with ash, crude fiber, oil, and free amino acids was observed. The phenolic components also had a positive correlation with catalase and soluble sugar and negatively correlated with the metabolism of energy, peroxidase, and protein (Table 7). The metabolizable energy is physiologically useful that is obtained when carbohydrates, fats, and proteins are catabolized.

Table 7

Correlations between fruit phenolics and nutritional characteristics of *M. charantia* after seed bioprimed with FD17 strain of *Enterobacter*.

Acids	Metabolizable energy	Peroxidase	Catalase	Soluble sugar	Protein	Ash	Oil	Crude fiber	Amino acid
Ferulic acid	0.9***	0.97**	−0.97**	−0.9*	0.9**	Ns	Ns	Ns	Ns
Coumaric acid	0.9***	0.97**	−0.97**	−0.9*	0.9**	Ns	Ns	Ns	Ns
Benzoic acid	0.9***	0.97**	−0.97**	−0.9*	0.9**	Ns	Ns	Ns	Ns
Sinapic acid	−0.9***	−0.97**	0.97**	0.9*	−0.9**	Ns	Ns	Ns	Ns
Cinnamic acid	−0.9***	−0.97**	0.97**	0.9*	−0.9**	Ns	Ns	Ns	Ns
Quercetin	−0.9***	−0.97**	0.97**	0.9*	−0.9**	Ns	Ns	Ns	Ns
Phenolic acid	−0.9***	−0.97**	0.97**	0.9*	−0.9**	Ns	Ns	Ns	Ns

*, **, and ***indicate significant correlation; Ns indicates nonsignificance at $p<0.05$.

3.12. Antiglycation Level Correlation

The level of antiglycation increased after microbial treatment of *M. charantia* seeds. A positive correlation with catalase (−0.98*), protein (−0.97**), and the phenolic profile, i.e., coumaric acid (0.94**), sinapic acid (−0.94**), quercetin (−0.94**), and chlorogenic acid (0.94**), was observed in fruit. Antiglycation exhibited a negative correlation with peroxidase, ash, soluble sugar, crude fiber, and free amino acids in the *M. charantia* fruit.

4. Discussion

To improve the speed and uniformity of seed germination, seed priming has been used as a strategic presowing technique [22, 23]. Upon seed priming, various biochemical processes of seeds are stimulated which play a vital role

in the breakdown of dormancy of the reserved seed food and its mobilization. During germination, seed priming also improves enzymatic activity, resulting in an early emergence of the embryonic part [24] with better synchrony [25]. In response to presown treatments, altered germination characteristics are supposed to be correlated with improved metabolic activities, which eventually lead towards enhanced plant growth [26]. For example, in a study, the magnetic field was applied for the manipulation of plant cell metabolism, resulting in an improvement in antimicrobial activity, plant growth, and phenolic contents of *M. charantia* [5]. Similarly, in another study, for the development of food crops with improved pharmaceutical and nutritional values, $ZnSO_4$ was used for the seed priming of *M. charantia* [9]. Previously, different types of chemicals, plant growth regulators, and vitamins have been investigated to get better agronomic yield [27]. Despite their successful findings, these chemicals were not found to be very effective in their regular application in developing countries (e.g., Pakistan) [9].

Enterobacter sp. strain FD17 had positive reports for growth and improvement in yields of some crops (e.g., maize) and the current work also proved similarity to the findings of Naveed et al. [10], where the fresh weight of the root (21%), the fresh weight of the leaf (112%), the fresh weight of the shoot (48%), and the length of the shoot (38%) increased after seed priming with this species. The response of *M. charantia* to FD17 differed from the previous work of our own research group [28]. Another variety of *M. charantia* was compared for its phytochemicals in two growth stages. In that study, in the seedling stage, there was no effect of treatment on total chlorophyll content, but later in the flowering stage, control plants remained with similar content, whereas the leaves of treated plants showed a significant increase. The present findings can be partially agreed on since the data presented here are for leaves in the vegetative stage. Furthermore, the difference in outcomes could be due to the varietal difference or due to the difference in the FD17 inoculum protocol followed in this study. In addition to bacterial strains, researchers have also employed fungal strains as biopriming agents. For example, in a study, Afrouz et al. [29] used *Trichoderma harzianum* as a biopriming agent and investigated the tolerance of seedlings from two genotypes of maize to cold stress. The emergence of the seedlings and the physiological parameters were revealed to be enhanced as a result of pretreatments with *T. harzianum*.

Enterobacter spp. has been confirmed to be nonhemolytic by blood hemolysis test and therefore has been revealed safe for animals and humans [30]. There are many bacterial species (e.g., *Enterobacter cloacae*, *Enterobacter amnigenus*, *Bacillus anthracis*, *Bacillus cereus*, and *Klebsiella pneumoniae*) that have been well studied for their association with plant growth-promoting abilities, and all these bacterial species have been considered as opportunistic pathogens for humans [31]. However, based on genetic differences in their virulence-associated genes possessed by some of their strains, pathogenicity and virulence factors of a bacterial species have been found to vary between different strains of similar species [32].

Primary metabolites act as feedback to metabolic pathways as metabolic precursors, which include photosynthetic pigments [33], enzymes [34], and soluble sugars [35]. In the event of better accumulation of primary metabolites, there would be activation of enzymes and consequently improved secondary metabolites. The leaves of primed *M. charantia* plants showed an accumulation of soluble sugars better than those of nonprimed plants. Formerly, Shahzad et al. [28] had observed an enhancement in catalase activity in response to FD17 priming in a local race of *M. charantia*. In contrast to their findings, in the current study, the Black King variety of *M. charantia* showed a decrease in catalase activity in plants treated with FD17, pointing out that the allosteric response of catalase to exogenous applications differs from variety to variety for the same species. However, a minor difference in seed treatment method and timing could also be the reason behind this difference in the FD17 priming effect on catalase activity.

Kumar et al. [36] pointed out the importance of plant-derived secondary metabolites as a potential antimicrobial remedy. Different phenolics have been studied for their antimicrobial activities [37, 38] against pathogenic bacterial species [39]. A similar trend was shown in the current study. With very few exceptions, sinapic acid, vanillic acid, p-coumaric acid, and ferulic acid showed highly significant correlation with the antibacterial activity of the leaf extract of *M. charantia* against *S. aureus* and *P. aeruginosa*. The different extractions used for the antibacterial analysis differed in their MIC against both pathogens, where the best was exhibited by methanol followed by acetone and

water. The antibacterial activity against both species differed for their correlation with secondary metabolites. The differences in the constituents of the cell membrane of Gram-positive and Gram-negative bacterial species could be the reason for the increased sensitivity of Gram-positive bacterial species towards different extractions [40]. However, for the primary metabolites, there was no significant correlation. At later stages, the response of the blackberry variety might be linked with some better outcomes that could be explored in future studies. Although the possibility of this attribute being varietal specific could not be completely ignored, as in previous studies, a different metabolic response of *Trachyspermum ammi* was observed after similar seed treatment [41].

Glycation can cause damage to proteins and induction of oxidative stress. In the body, the excessive accumulation of advanced glycation end products (AGEs) has been found to be associated with various adverse health conditions, including metabolic disorders such as diabetes mellitus [42] and even sometimes tumor [43]. Antiglycation has been proposed as a tactic that slows human aging and lowers inflammasome activity [44]. Phenols are involved in the human antiglycation process through antioxidant ability and protein interactions. Furthermore, they can trap or block receptors for advanced glycation end products [45, 46]. In the current study, it was revealed that antiglycation activity could be enhanced by FD17 seed treatment. The plants primed with FD17 for their antiglycation capacity were directly correlated with the amounts of coumaric acid and chlorogenic acid. The results related to these treatments confirmed the previous findings of Aljohi et al. [47]. In the future, the secondary metabolites reported in this study should be tested *in vivo* in different animal models to explore and confirm their medicinal properties.

5. Conclusions

The biological seed priming technique has been preferred because the bioagents used in this technique provide a friendly environment for the growth of nutritional food crops and vegetables. In the current study, after treatment of *Momordica charantia* seeds with *Enterobacter* strain FD17, the percentage of germination, growth, total amino acids in the peel, chlorophyll content, and phenolic content improved. FD17 treatment could be used for the manipulation of plant cell metabolism with increased growth and antibacterial activity. Treatment also showed a direct correlation with antiglycation activity, and therefore it can be concluded that the treatment of *Enterobacter* strain FD17 could be followed by pharmacists to overproduce metabolites of interest in *M. charantia* with better antiglycation activity.

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DETAIL

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Physicochemical, Antioxidant, and Sensory Characteristics of Sponge Cake Fortified with Quinoa Flour, Oolong, and White Tea Powder

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ABSTRAK (ENGLISH)

Nowadays, there is a growing demand for healthy foods enriched with various functional bioactive ingredients. Cakes are ready-to-eat baked products consumed worldwide and are suitable for the development of functional food products. In this study, a Box–Behnken design was used to investigate the effect of three independent variables, including oolong tea powder (OT, 0–20%), white tea powder (WT, 0–15%), and quinoa flour (QF, 0–40%), on the quality characteristics of sponge cake during 21 days of storage. Following the evaluation of the model, the optimum levels of ingredients for the preparation of sponge cake were 15% WT, 17.17% OT, and 24.97% QF. Total phenolic content of 52.09 mg gallic acid/100 g, antioxidant activity of 0.068 mg/mL, overall acceptability of 4.89, lightness of 47.94, and peroxide value of 0.68 mEq/kg were obtained under optimized conditions. The growth of molds and yeasts was prevented during storage time. The hardness, gumminess, and chewiness of the optimized sponge cake improved with increasing WT, OT, and QF levels in comparison to the control. Scanning electron micrographs showed a more porous structure in optimized cake samples. In conclusion, the utilization of OT, WT, and QF as phenolic compounds in sponge cake led to an increase in its nutritional value and improved shelf life.

TEKS LENGKAP

1. Introduction

Bakery products especially cakes are the most popular and widely consumed snacks in many parts of the world due to their ease of use, nutritional value, suitable organoleptic properties, and shelf life of about four weeks [1, 2]. The global cake market was valued at 46.06 USD billion in 2022 and is expected to expand to 58.76 USD billion in 2030, growing at an annual rate of 3.09% (Research and Markets. Global Cake Market-Growth, Trends, and Forecast, 2023–2030).

Sponge cake is a type of air-leavened cake made from flour, sugar, eggs, fat, and other ingredients.

Using fats in the formulation create a softer texture in the final product, but they are susceptible to lipid oxidation resulting in rancid odors, unpleasant flavors, and discoloration of products which consequently decrease safety and nutritional quality [3]. Furthermore, sponge cakes are intermediate moisture foods (with aw of 0.75–0.90) prone to microbial spoilage caused by molds that reduces their shelf-life [4]. In this regard, using antimicrobial and antioxidant compounds for maintaining the product quality are inevitable. However, consumers' willingness for healthy foods has directed the researchers and the food industry to seek for natural additives such as plant-based substances [5]. The researchers concluded that some herbal ingredients, such as oolong tea powder (OT) and white tea powder (WT) could be considered good alternatives to synthetic antioxidants [6].

Unfermented tea, made from young tea leaves or unopened buds, is called white tea [7]. White tea contains several polyphenolic compounds belonging to the catechin family. These compounds are known for their wide range of biological activities as antioxidants, antivirals, anticancer, antibacterial, and antifungal [8]. Oolong tea is a traditional type of tea obtained by the incomplete fermentation of fresh tea leaves. The main constituents of oolong tea are glutamic acid, epicatechin, alkaloids, polyphenols, flavonoids, tannins, vitamins, amino acids, minerals, proteins,

polysaccharides, and organic acids with desirable antioxidant properties [9]. Due to the lack of fermentation processes in the production of white tea, the extract of this plant has more polyphenolic compounds than black tea and oolong tea and also has stronger antimicrobial properties compared to other types of tea [10]. It has been highlighted that chiffon cake containing 20% tea powders (green tea, oolong tea, and black tea powders) showed good antioxidant activity and acceptable sensory characteristics [11]. In another study, it has been shown that the addition of 10% green tea powder to sponge cake formulation improved antioxidant properties, sensory attributes, and glycemic potential [12].

Cakes have high energy and calorie with high levels of fat and sugar that can lead to health problems, but this can be tackled through fortification of cake formulation with health-promoting compounds and creating functional cake. One of the approaches used for improving nutritional profile of cake is partial replacement of wheat flour with other flour sources such as nonwheat grains, legumes, tubers, and pseudo-cereals [13]. In this context, adding quinoa flour (QF) can increase the nutritional value of wheat flour. Quinoa is a highly digestible and rich source of bioactive compounds. It contains proteins, phytosterols, omega 3, and 6 fatty acids, as well as carbohydrates with a low glycemic index, which have high benefits in reducing cardiovascular risks in humans [14]. QF contains 70% unsaturated fatty acids, including linoleic acid (38.9%) and oleic acid (27.7%). Also, quinoa proteins contain high amounts of essential amino acids, including methionine, lysine, and cystine [15]. It has been stated that due to high content of dietary fibers of quinoa, its inclusion in bread formulations improved gastrointestinal transit and decreased the level of cholesterol [16]. Quinoa flour has been used for production of gluten-free breads, biscuits, muffins, and cookies. Gluten-free cake formulated with 20 and 30% quinoa flour showed improved nutritional profile, high specific volume, low hardness, good color, and acceptable sensory properties [17]. In another study, the addition of 50% quinoa flour to cake formulation resulted in an improved rheological of cake batters and consequently the physical, chemical, nutritional, and sensory properties of cupcakes [18]. In this study, the effect of QF, OT, and WT on the physicochemical, textural, and overall acceptability of sponge cake samples was investigated, and an optimized formulation with desirable quality characteristics is presented.

2. Materials and Methods

2.1. Materials

OT and WT were purchased from local market (Refah Company, Lahijan, Iran). Dried OT and WT dried leaves were ground to a fine powder with a grinder (Moulinex-Grinder; MC300, France). QF was obtained by grinding quinoa seeds and then screening them to a particle size in the range of 300–500 μm. Other materials, including flour (Golha factory, Iran), sunflower oil (Bahar Company, Iran), baking powder (Cisaron Shimi Company, Iran), granulated sugar (Varamin sugar factory, Iran), vanilla powder (Jivadan company, Switzerland), and milk powder (Sigma, USA), were used for sponge cake preparation.

2.2. Chemical Composition of Quinoa Flour, White, and Oolong Tea Powder

Proximate composition of quinoa flour, white, and oolong tea powder was determined according to the methods of the Association of Official Analytical Chemists (AOAC) [19] for moisture (934.01), protein (984.13), fat (920.39), and ash (923.03). The carbohydrate content was determined by difference.

2.3. Preparation of Sponge Cake

The sponge cake was prepared according to Mau et al. [11] method with some modifications (Table 1). Wheat flour was replaced with different levels of OT, WT, and QF. For cake preparation, all the powdered ingredients, including wheat flour, emulsifier, sugar, salt, milk powder, baking powder, OT, WT, and QF, were poured into a container. Then, sunflower oil was added and mixed at medium speed for 5 min with a KitchenAid Professional mixer (Model 5KSM7990, Whirlpool, MI, USA). Eggs were added and mixed at high speed for 3 min. The sponge cake batter was poured into cake pans and baked at 180°C for 40 min in an electric oven (Europa, Malo, VI, Italy). The sponge cakes were allowed to cool for 1 h at room temperature (25°C) and then removed from the pans and packed in polypropylene bags. Sponge cake samples were stored in a dry, cool place away from sunlight until further evaluation.

Table 1

Formulation of sponge cake.

Ingredients	Amount (g)
Flour	100
Baking powder	3.75
Sucrose	72
Sodium chloride	1.25
Sunflower oil	50
Egg	72
Distilled water	50
Milk powder	10

2.4. Formulation Optimization of Sponge Cake

To optimize the factors affecting sponge cake quality, Design-Expert software (v.7.0.0, State-Ease, Inc., Minneapolis, USA) with Box–Behnken design (BBD) and response surface methodology (RSM) were used. The BBD/RSM is a technique applicable for optimizing the responses which are influenced by a range of process factors. In this method, the interactions of factors with the responses are evaluated and the optimal conditions for the process with the least number of experiments are determined. In BBD, three levels for each factor are considered, and the required experiments are set based on the combination of the factors [20].

Three independent variables were chosen as the WT level (X1), OT level (X2), and QF level (X3). The coded levels, actual values of the independent variables, and a set of 17 experiments was employed with five replicates (used to estimate experimental error) of the center point are presented in Table 2.

Table 2

Independent variables and their levels used in Box–Behnken design for the formulation of sponge cake samples containing different levels of OT, WT, and QF.

Independent variables	Symbol	Coded levels	Coded levels	Coded levels
−1	−1	−1	WT level	X1
0	7.5	15	OT level	X2
0	10	20	QF level	X3
0	20	40	-	
Samples	Wheat flour (%)	OT[A] (%)	WT[B] (%)	QF[C] (%)

–				
1	62.5	10	7.5	20
2	45	20	15	20
3	62.5	10	7.5	20
4	92.5	0	7.5	0
5	80	0	0	20
6	62.5	10	7.5	20
7	62.5	10	7.5	20
8	65	0	15	20
9	72.5	20	7.5	0
10	65	10	15	0
11	90	10	0	0
12	50	10	0	40
13	32.5	20	7.5	40
14	35	10	15	40
15	60	20	0	20
16	62.5	10	7.5	20
17	72.5	0	7.5	20

[A]OT: oolong tea powder; [B]WT: white tea powder; [C]QF: quinoa flour.

2.5. Determination of Viscosity and Specific Volume of Cake Batter

The viscosity of the sponge cake batter was determined using a rotational viscometer (Brookfield, DV2T, RV, USA). The specific volume of batter was measured by dividing the weight of 100 mL of batter by the weight of 100 mL of water [21].

2.6. Physicochemical Characteristics of Sponge Cake

2.6.1. pH and Moisture Content

The moisture of cake samples was determined by AACC method 44-15.02 (AACC, 2010), and the pH of the cake was measured using a pH meter (SP-701, Suntex, Taiwan).

2.6.2. Color Characteristics

The upper surface color of the cakes was measured by HunterLab (Hunter Lab, Color Flex, USA) and expressed as L* (lightness), a* (redness), and b* (yellowness). Three measurements were taken from the surface of the cakes at room temperature (25°C), and the mean value was recorded [22].

2.6.3. Peroxide Value

The lipid fraction was extracted from cake samples according to the method of Iranian National Standard No. 37 [23] using 200 mL n-hexane mixed with 100 g cake samples in a laboratory shaker at 25°C. After filtration and separation of the lipid fraction, the solvent was removed by a rotary evaporator at 40°C.

Peroxide value (PV) was determined by titration of lipid fractions of samples (5 g) dissolved in 30 mL of chloroform: glacial acetic acid mixture (2 : 3; v/v) in the presence of saturated potassium iodide solution and starch as an indicator with 0.02 M sodium thiosulphate solution from a purple to a slightly yellow or colorless endpoint. The results of PV were shown in milliequivalents of active oxygen per kg of fat sample (meq O_2/kg) and were calculated according to the following equation:(1)PV=V−V09cm,where V and V0 are the volume (mL) of sodium thiosulphate exhausted by the test sample and blank, respectively, *m* is the mass of the lipid fraction sample (g), and *c* is the concentration of sodium thiosulphate (mM).

2.6.4. Total Phenolic Content and Antioxidant Activity

The cake sample was mixed with ethanol (50% v/v) at a ratio of 1 : 10 and shaken for 24 h at room temperature (25°C). The mixture was filtered using Whatman filter paper, and then the solvent residue was removed at 40°C, and the obtained extract was kept in the refrigerator for further analysis. The method of Singleton and Rossi [24] was used for the determination of total polyphenol content in cake samples, with some modifications. 0.5 mL of extract was added to 2.5 mL of Folin–Ciocalteu reagent (10%, v/v) and 2 mL of $NaHCO_3$ solution (7.5%, w/v), vortexed for 15 seconds, kept in a dark place for 30 min at 25°C, and then the absorbance was measured at 760 nm by spectrophotometer. Total phenolic content was calculated according to the standard curve of gallic acid and expressed as mg gallic acid equivalents (GAE) per g dry weight.

The antioxidant activity of the cake extracts was measured according to the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by Sangsrichan and Wanson [25]. A 0.1 mL extract sample was added to a 3.9 mL DPPH solution (0.1 g/L in ethanol) and mixed. The mixture was kept for 30 min at 25°C in a dark place until the reaction took place. Afterwards, the absorbance was measured at 517 nm by spectrophotometer, and antioxidant activity was expressed as a percentage of DPPH radical scavenging activity and calculated using the following formula:(2)DPPH radical scavenging activity%=ADPPH−AExtractADPPH×100,where ADPPH is the absorbance value of the DPPH blank sample and AExt is the absorbance value of the test solution.

2.7. Texture Analysis

The effect of optimum levels of OT, WT, and QF on the texture properties of sponge cake samples was evaluated using a texture analyzer (TA-XT2 Texture Analyzer, Stable Microsystems, Surrey, UK), according to Conte et al. [26]. Texture profile analysis involved compressing the sample twice and quantifying the mechanical properties, such as hardness, springiness, cohesiveness, chewiness, and resilience. Texture profile analysis was carried out under the following conditions: pretest speed = 0.8 mm/s; test speed = 1 mm/s; post-test speed = 5 mm/s; strain = 30%. 25 mm thick cake samples were cut using a cylindrical probe with a diameter of 25 mm to obtain samples with a 25 mm height and a 25 mm diameter. The mean values of ten measurements were reported. The cake crust structure was observed using a scanning electron microscope (JEOL JSM-5600LV, Tokyo, Japan). The cake samples were fixed with 3.0% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, dehydrated with ethanol, silver-coated by a sputter, and observed with a microscope.

2.8. Molds and Yeasts Count

Molds and yeasts count were determined according to the method of Iranian National Standard No. 10899-2 [27] using the pour plate method at 0, 7, 14, and 21 days of storage. Plates containing samples were incubated at 30°C for 72 h in an incubator, and then the number of colonies was counted.

2.9. Sensory Evaluation

The five-point hedonic test was used to determine the overall acceptance of sponge cakes (1 = strongly dislike, 5 =

strongly like). Three-digit random codes were assigned to the samples, and 30 untrained assessors (15 male and 15 female, age between 25 and 40 years old) evaluated the overall acceptance of the samples [28].

2.10. Statistical Analysis

The parameter used for cake optimization was analyzed by standard response surface methodology (RSM). In this study, BBD with three variables at three levels was used to evaluate the production of sponge cake using Design-Expert software version 9.0 (Stat-Ease Inc., Minneapolis, MN, USA) [29]. All the experiments were performed in triplicate, and all data were expressed as mean ± standard deviation using one-way analysis of variance (ANOVA). Also, Duncan's multiple range test was used to identify significant differences between means at the significance level of $P<0.05$ in the SPSS 12.0 software (SPSS Inc., Chicago, USA).

3. Results and Discussion

3.1. Proximate Composition

Table 3 demonstrates chemical composition of QF, OT, and WT. The proximate composition of quinoa flour was consistent with previous studies: moisture content of 8.9–12.41%, protein content of 13.46–18.5%, fat content of 5.01–6.60%, ash content of 2.5–2.82%, and carbohydrate content of 13.4–15.36% [17, 30, 31]. Regarding WT and OT, the results were similar to [6, 32].

Table 3

Proximate composition of QF, WT, and OT.

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrate (%)
QF	10.38 ± 0.15	17.45 ± 0.24	5.64 ± 0.03	2.39 ± 0.02	64.13 ± 0.82
WT	7.76 ± 0.01	29.81 ± 0.11	3.82 ± 0.02	7.39 ± 0.03	50.12 ± 0.92
OT	8.11 ± 0.01	28.22 ± 0.09	4.13 ± 0.02	7.45 ± 0.02	51.67 ± 0.95

Values represent mean ± standard deviation for triplicate determinations. OT: oolong tea powder; WT: white tea powder; QF: quinoa flour.

3.2. Cake Batter Viscosity and Specific Volume

According to Figure 1, it was observed that the viscosity of the cake batter increased with increasing levels of OT, WT, and QF. Regression coefficients showed that QF had a greater effect on the viscosity and volume of cake samples compared to WT and OT.

[figure(s) omitted; refer to PDF]

Mau et al. [11] reported that by replacing green tea, oolong, and black tea powder in the chiffon cake formulation, the viscosity of the batter increased. This could be attributed to the high water binding capacity of the fibers in OT, WT, and QF. The low lignin content and high content of cellulose and hemicellulose results in higher water binding capacity [33]. Similar results have been observed by incorporation of date powder [34], mushroom powder [35], and drumstick leaves powder [36].

With increasing the level of each of the variables, the specific volume of cake decreased (Figure 2). A suitable batter for the cake should have the ability to create an appropriate viscosity to prevent joining bubbles and leaving the surface in the initial stage of heating [33]. The reduction of specific volume can be attributed to the replacement of wheat flour with flours containing high fiber content, as these compounds weaken the three-dimensional network of gluten (gluten network structure consisting gliadins and glutenins which are connected through covalent and noncovalent bonds), which is responsible for storing carbon dioxide and water vapor during the baking process [37]. Similarly, Aydogdu et al. [38] reported a decrease in the specific volume of cake batter by increasing the level of pea, oat, apple, and lemon fibers. It was highlighted that fibers disturbed the starch-gluten matrix and caused a decrease in the gas retention capacity and a low specific volume. However, the obtained results are in contradiction

with some studies reporting an increase in specific volume by an increment of fiber level, which can be attributed to the nature and level of dietary fiber used in the formulation [33].

[figure(s) omitted; refer to PDF]

3.3. Physicochemical Characteristics

The results revealed that adding QF, OT, and WT had a significant effect on the moisture content of cake samples. It was found that increasing the amount of QF led to an increase in the moisture content in different samples, which is consistent with the results of Levent [39]. QF absorbed moisture due to the presence of carbohydrates, starch, and protein in its structure [40]. Starch increased the water absorption capacity and moisture in cake samples by absorbing water in the batter formation stage. Also, the presence of protein in QF assisted to the absorption of water and increased the moisture level in cake samples [41]. As the levels of OT and WT increased, the moisture content decreased. Also, Lu et al. [42] and Mashkour et al. [12] reported a decrease in moisture content from 36 to 35% and 25 to 22% by supplementation of green tea powder in cake formulation. Furthermore, with increasing storage time, the amount of moisture showed an increasing trend (Figure 3), which could be due to the absorption of moisture from the surface of the cake samples during storage.

[figure(s) omitted; refer to PDF]

With increasing OT, WT, and QF in the formulation, pH showed a decreasing trend (Figure S1). This is due to the presence of high amounts of polyphenols and organic acids, such as ascorbic acid, that decrease pH due to their acidic nature [41]. pH decreased in all sponge cake samples during the storage period. This can be due to the destructive reactions of ascorbic acid in cake samples, the formation of melanoidin, furfural, and aldehyde compounds such as malondialdehyde and ketones, the oxidation of reducing sugars and triglycerides, and the degradation of amino acids. Also, the baking process at high temperatures, which was accompanied by the Maillard reaction and the presence of amino compounds and reducing sugars, resulted in a reduction of pH in cake samples [43]. It was found that QF level had the least effect on pH, while WT showed the greatest effect. The active ingredients in tea, phenolic acids, gallic acid and its derivatives, and catechins caused an acidic and antioxidant nature in sponge cake samples fortified with white and oolong tea powders. In QF-supplemented samples, phenolic compounds such as polyphenols with a hydroxyl structure reduced the pH of cake samples.

All three variables had a significant effect ($P<0.05$) on acidity. WT and OT increased the acidity of samples (Figure 4) due to their high antioxidant compounds and various organic acids [7]. The effect of WT on acidity was greater than that of other variables. This can be attributed to the presence of high amounts of polyphenols and organic acids, such as ascorbic acid. During the storage period, acidity increased in the sponge cake samples, which can be attributed to the breakdown of simple sugars into lactic acid, lowering the pH, denaturation of simple and complex proteins, breakdown of triglycerides, and production of hydroperoxides [44].

[figure(s) omitted; refer to PDF]

It was observed that with increasing the level of variables, the amount of total phenolic content increased in cake samples (Figure 5). White tea contains much higher levels of phenolic compounds than oolong tea and QF due to the lack of fermentation. The phenolic compounds in cake samples decreased during shelf life. Mau et al. [11] replaced green tea, oolong, and black tea powder as a part of flour in chiffon cake and reported that with the increase in powder levels, the total phenolic content and antioxidant activity increased. In another study by Pourzafar et al. [45], increasing the level of green tea, white tea, and ginger extract increased total phenolic content of sponge cake.

[figure(s) omitted; refer to PDF]

According to Figure 6, it was shown that the inhibition of DPPH increased with the increase of WT, OT, and QF levels, which could be explained by the presence of high levels of phenolic compounds and the probability of hydrogen donation to free radicals [40]. Also, regression coefficients showed that WT had the greatest free radical scavenging activity, followed by OT and QF. The antioxidant properties of cake samples decreased during their shelf life. Similarly, Ahmad et al. [46] reported an increase in the inhibition of DPPH in cookies with an increase in the amount of green tea powder that was attributed to the polyphenols and natural antioxidant compounds like myristic

acid and palmitic acid. Also, the incorporation of 1 g green tea powder into 100 g whole-wheat flour enhanced the antioxidant activity by 18.5%. It was stated that catechins, vitamin C, carotenoids, and selenium in green tea exhibited antioxidant activity [47]. In a study by Xu et al. [48], the addition of quinoa flour (5–15%) to wheat bread increased total phenolic content and DPPH radical scavenging activity. According to Gil et al. [49], the predominant compounds in the extractable fraction of quinoa bread were p-hydroxybenzoic acid, quercetin, and rutin, while ferulic and sinapic acids were the most abundant compounds in the hydrolyzable fraction.

[figure(s) omitted; refer to PDF]

With increasing the concentrations of WT, OT, and QF, the peroxide value decreased in cake samples (Figure S2). WT had a greater effect on peroxide value than OT and QF due to the presence of antioxidant compounds. As white tea has the highest amount of catechins compared to OT and QF, the samples containing the highest concentration of WT had the lowest peroxide value after 21 days of storage. With increasing storage time, the peroxide value increased, which can be attributed to the breakdown of triglycerides and the production of free fatty acids, aldehydes, and ketones during storage [50].

According to Iranian National Standard No. 2553 [51], the peroxide value for cake should not exceed 2 meq O_2/kg. The results indicated that the peroxide value of lipid fractions extracted from sponge cake samples was lower than this limit during 21 days, except in three samples containing the lowest levels of OT, WT, and QF. According to Taghvaei and Jafari [52], tea polyphenols are a suitable mixture of antioxidants with the capability of scavenging oxygen radicals and chelation of metal ions. Similarly, in a study by Kozlowska et al. [53], the addition of green tea extract to sponge cake resulted in a lower peroxide value in the lipid fraction after baking compared to the control samples. Addition of grapefruit peel powder to cake decreased peroxide value during 14 days of storage due to the high amounts of phenolic compounds in the powder [54].

Color is one of the most essential characteristics of foods, being supposed as a quality key that determine their acceptance. By increasing OT, WT, and QF levels, the L* value of cake samples decreased (Figure S3) and QF addition showed less impact on L* compared to the other two variables. The presence of pigments such as chlorophyll, carotenoids, and polyphenols, as well as fiber compounds with moisture retention ability caused a darker color in cake samples [9]. The samples with the highest concentration of WT showed the highest decrease in L*. Also, during the shelf life, the L* of all samples decreased, which could be attributed to the oxidation of fats and ascorbic acid and the decomposition reactions of amino and organic acids [55]. Singh et al. [56] reported a decrease in the L* value in the crust of gluten-free and egg-free muffins using modified rice flour and jambolan fruit pulp in the presence or absence of xanthan gum. Similar results have been obtained by incorporation of *Clitoria ternatea* extract [3], olive stone powder [33], dried button mushroom powder [35], drumstick leaves powder [36], *Rubus coreanus* powder [57], and jujube powder [58] to sponge cake formulation.

The results showed that a* value increased in different cake samples over time (Figure S4). This can be explained by the production of by-products from browning reactions, such as melanoidin and furfural, which increased a* in sponge cake samples [11]. Increasing WT and OT level led to a decrease of a* value and a greener cake, while increasing QF level resulted in an increase of a* value and redness in the sample. According to Bozdogan et al. [18], the increasing redness of cake crumb fortified with quinoa flour could be associated with its natural color. Moreover, redness in the crust of cakes can be ascribed to the caramelization and Maillard reactions. An increased protein content of cake as a result of adding quinoa flour reacting with reducing sugars during baking increased the speed and intensity of browning reactions and formation of dark-brown compounds [56].

Stikic et al. [59] added 20% QF to the bread formulation and obtained a product with a reddish-yellow color and a crispy texture. This is in agreement with previous work of El-Sohaimy et al. [15] and El-Sohaimy et al. [60], who reported that increasing the level of quinoa flour in bread formulation increased redness of bread due to high content of protein. a* value increased during the shelf life as a consequence of a decrease in humidity and increase in the levels of pigments, such as furfural and methyl furfural.

The trend of changes in b* value was similar to that of a* value, and the variables (QF, WT, and OT) reduced the b* value of cake samples. Tea powders decreased the L*, a*, and b* values, while QF reduced the L*, but caused the

cake samples to turn yellow and red. In accordance with this result, addition of green tea powder to whole-wheat flour pan bread decreased b* value. It was stated that green tea powder influenced bread crumb as a result of oxidation of catechins and formation of compounds with deep color such as theaflavins, thearubigins, and theabrownines, during high temperatures in the baking process. Also, break-down of chlorophyll and generation of magnesium chlorophyll with brown color affected the color of bread [47].

3.4. Molds and Yeasts Counts

Molds and yeast growth were not detected in most of the sponge cake samples during the storage period, which can be due to the presence of antimicrobial compounds in the samples, packaging in impermeable plastics to oxygen and moisture, as well as storage temperature. According to Iranian National Standard No. 10889-2 [27], the number of mold and yeast in cake samples should be below 100, and the amount of mold and yeast in the samples was within the allowable range after 21 days of storage. Polyphenols in oolong tea and white tea produce hydrogen peroxide under certain conditions and have an inhibitory effect on microorganisms [44]. It should be noted that the synergistic effect of the phenolic compounds plays an important role in reducing the microbial load. In a study by Wu et al. [55], adding instant green tea powder to the sponge cake resulted in a decrease in mold and yeast counts compared to the control sample.

3.5. Sensory Evaluation

The results showed that with increasing OT, WT, and QF, the overall acceptability of cake samples decreased (Figure S5). Low levels of OT, WT, and QF increased overall acceptability, while at higher levels, a decreasing trend was detected. It was also found that samples containing low levels of QF received higher scores, and samples with high levels of WT received lower scores. Similarly, Wu et al. [55] reported that adding 12.5% instant tea powder to cake formulation resulted in the highest overall acceptability and an excessive amount of tea powder lowered the acceptability score of the product. Mashkour et al. [12] reported that cake samples with 10% green tea powder received the highest score in overall acceptability. Rothschild et al. [61] studied the effect of QF on the consumers' overall acceptability of a gluten-free cake formulation. They reported that no significant differences were observed between commercial chocolate cake, and cakes containing QF, which had the highest sensory scores. Also, Demir and Kilinç [14] stated that the addition of QF improved all sensory properties of cookie samples. In a study by Mohammad et al. [41], substitution of wheat flour with 25% quinoa flour increased the sensory acceptability of sponge cake.

3.6. Optimization of Sponge Cake Preparation

Optimum operating conditions were performed to determine the optimized sponge cake sample using the numerical optimization technique with Design-Expert software (Figure 7). For this purpose, the optimum conditions were first selected by the software. The response surface method was able to estimate the optimized formulation of sponge cake samples with high desirability (0.95). Optimum conditions for sponge cake were determined as 15% WT, 17.17% OT, and 24.97% QF which was equivalent to the desirability of 0.95, and total phenolic content of 52.09 mg gallic acid/100 g sample, antioxidant activity of 0.068 mg/mL, overall acceptability of 4.89, lightness of 47.94, and peroxide value of 0.68 mEq/kg. Therefore, using this optimized formulation, the optimum sponge cake was produced, and its texture properties were compared with the control sample.

[figure(s) omitted; refer to PDF]

3.7. Microstructure and Texture Analysis

According to the scanning electron microscopy (SEM) graphs for control and optimized samples (Figure 8), there was a low number of pores in the microstructure of the control sample, but enriched cake samples had a more porous structure. In control samples, a lump was observed on the crust, which could be due to the presence of sodium bicarbonate and the baking process at a temperature above 180°C, which was accompanied by moisture loss. In the optimized samples, in addition to this lump, pores were observed in all areas of the product, which was due to the effect of WT, OT, and QF on the crust and texture of the cake sample.

[figure(s) omitted; refer to PDF]

Texture analysis of control and optimized cake samples (Table 4) showed that the hardness of optimized cake

samples increased and the texture strength improved, which can be related to the reduction of moisture in the samples. According to Premi and Sharma [36], the interactions between fibers and other components such as gluten, starch, and fat in batter affected the hardness of sponge cake. The fibers decreased air entrapment and prevented sponge cake expansion during baking. According to Mashkour et al. [12], increasing the green tea powder replacement increases the phenolic compounds, which leads to more hydrogen bond formation between protein molecules and phenolic groups and consequently a harder texture. The hardness of cake is directly related to its density and inversely correlated with specific volume [33]. The results showed that by increasing QF, the texture hardness increased, which could be explained by the weakening of the gluten network [62]. Increased hardness of the cake texture due to the increase in the level of QF is directly related to the high viscosity of the batter [56]. Cohesiveness indicates the degree of internal resistance of food tissue. The results showed that cohesiveness was lower in optimized cake samples compared to the control. Similarly, Pasukamonset et al. [3] reported a significant decrease in the cohesiveness of sponge cake fortified with *Clitoria ternatea* compared to control sample.

Table 4

Texture analysis of control and optimized sponge cake samples.

Samples	Hardness (g)	Cohesiveness	Gumminess (g)	Springiness	Chewiness (mJ)
Control[A]	215	0.67	144	12.69	17.91
Optimum[B]	317	0.61	233	12.97	29.70

[A]Control: sponge cake sample contains 100% wheat flour. [B]Optimum: sponge cake sample contains 42.86% wheat flour, 17.17% OT (oolong tea powder), 15% WT (white tea powder) and 24.97% QF (quinoa flour).

Gumminess is calculated by multiplying hardness and cohesiveness. The results showed that gumminess increased in optimized cake sample compared to control. This result is in agreement with previous studies illustrating that sponge cake containing tea powder and chickpea flour caused an increase in gumminess [11, 63]. The extent of recovery between the first and second compression represents the elasticity of sample cakes and is described as the springiness value. Springiness was higher in optimized cake sample compared to control. On the contrary, adding *Clitoria ternatea* powder and green tea powder to sponge cake decreased springiness that was attributed to the weaker and less elastic gluten structure of the cakes caused by the polyphenols [3]. Chewiness demonstrates the amount of energy required to disintegrate a food for swallowing, indicating the rate of cake breakdown. The chewiness improved in the optimized sample compared to the control sample. According to Qasem et al. [64], the chewiness and hardness of samples are strongly interrelated and they follow similar trend.

4. Conclusions

A novel formulation of sponge cake with quinoa flour, white and oolong tea powders was developed. Incorporation of these ingredients improved quality characteristics of sponge cake. The presence of phenolic compounds in the powders contributed to the reduction of peroxide value and microbial growth during storage. Also, the cake samples produced by the optimized formulation (15% WT, 17.17% OT, and 24.97% QF) showed improved hardness, gumminess, and chewiness compared to control samples. Therefore, the use of quinoa flour, white, and oolong tea powders represents a new direction for the development of functional sponge cake with improved nutritional profile, antioxidant activity, and potential health benefits. Further research could investigate in vitro digestibility and potential health effects of fortified sponge cake and the feasibility for commercial production. In addition, quinoa flour, white, and oolong tea powders can be used in other bakery products such as bread, cookie, and biscuit.

Additional Points

Highlights. (i) A novel functional sponge cake using oolong tea powder, white tea powder, and quinoa flour was developed. (ii) Increasing the levels of WT, OT, and QF improved texture properties of the optimized sponge cake compared to control. (iii). Incorporation of OT, WT, and QF in combination could improve the quality, safety, and

sensory characteristics of sponge cake.

Ethical Approval

This research has received ethical approval from Shahid Beheshti University of Medical Sciences.

Authors' Contributions

F.F. and M.M. conceptualized the study. A.A. performed investigation. M.M. and N.K. contributed to formal analysis. F.F. performed data curation. A.A. wrote the original draft. F.F. and M.M. reviewed and edited the manuscript. All authors have read and agreed to the finalized version of the manuscript.

Glossary

Abbreviations

BBD:Box–Behnken design
RSM:Response surface methodology
PV:Peroxide value
OT:Oolong tea powder
WT:White tea powder
QF:Quinoa flour
GAE:Gallic acid equivalents
DPPH:2,2-Diphenyl-1-picrylhydrazyl
SEM:Scanning electron microscopy.

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DETAIL

Subjek:	Foods; Phenols; Quinoa; Yeasts; Viscosity; Nutritive value; Functional foods &nutraceuticals; Antioxidants; Wheat; Fatty acids; Storage life; Amino acids; Eggs; Vegetable oils; Water hardness; Acceptability; Flour; Proteins; Cholesterol; Lipids; Health risks; Dietary fiber; Polyphenols; Oolong tea
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Inhibitory Activities of Thai Culinary Vegetables against Key Enzymes Relevant to Diabetes Mellitus and the Kinetics of Enzyme Inhibitions

Ratananikom, Khakhanang; Nitisuk, Panorjit; Wongpreedee, Panida; Kubola, Jittawan.

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ABSTRAK (ENGLISH)

Diabetes mellitus (DM) is one of the most challenging noncommunicable diseases, as it causes significant costs for medical treatment as well as high morbidity and mortality rates. Dietary plants with antidiabetic properties have been explored as an alternative to synthetic medicines to treat DM because of their safety and nutrition. Hence, the objective of the present study was to determine the inhibitory activities of twenty commonly consumed Thai culinary vegetables against *α*-glucosidase and *α*-amylase. All vegetables were extracted using deionized water, ethanol, and hexane at 150 rpm and 30°C for 24 hours. The enzyme inhibitory activities were performed using a colorimetric assay. Diverse results for *α*-glucosidase and *α*-amylase inhibitory activities were found for all vegetable extracts. The most potent anti-*α*-glucosidase activity was obtained from the ethanolic extract of *Leucaena leucocephala* (Lamk.) de Wit with the half maximal inhibitory concentration (IC_{50}) of 13.39 ± 0.14 *μ*g/mL, followed by the aqueous and ethanolic extracts of *Polygonum odoratum* Lour with IC_{50} of 25.60 ± 0.42 and 49.03 ± 0.72 *μ*g/mL, respectively. All the samples exhibited mixed, noncompetitive, and uncompetitive inhibition. It can be concluded that the *α*-glucosidase and *α*-amylose inhibitory effects of the investigated extracts may be an indicator of antidiabetic potency,

and these extracts might potentially be beneficial as functional components for postprandial hyperglycemia treatment.

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1. Introduction

Diabetes mellitus (DM), a modern lifestyle-related disease, has been classified as one of the most challenging global public health problems. According to the World Health Organization (WHO), an estimated 463 million adults were living with diabetes as of 2021, and the number is projected to rise to 578 million by 2030 [1]. In addition, diabetes is a leading cause of disability and mortality, accounting for an estimated 4.2 million deaths annually [2]. According to the International Diabetes Federation (IDF), an estimated 6.0 million adults in Thailand were living with diabetes as of 2021, representing a prevalence of 10.6% [2]. Furthermore, an estimated 2.4 million adults in Thailand were estimated to have undiagnosed diabetes. The prevalence of diabetes in Thailand is projected to increase in the coming years, with an estimated 6.7 million adults living with diabetes by 2030. Diabetes is one of the leading causes of death in Thailand, and it is responsible for an estimated 62,000 deaths annually [2]. Undoubtably, the rising number of DM patients every year generates tremendous expenses in medical care as well as high morbidity and death rates.

DM is a chronic health condition that occurs when the body does not produce enough insulin or does not use the insulin effectively. Insulin is a hormone produced by the pancreas that plays a key role in the regulation of glucose metabolism. It helps the body use glucose for energy and store excess glucose in the liver and muscles for future use. Insulin helps cells take up glucose from the blood, preventing it from reaching excessively high levels. It also stimulates the liver to take up and store glucose, helping to maintain stable blood glucose levels [3]. Without insulin, too much glucose can build up in the bloodstream, resulting in several health complications. To control blood glucose levels, most DM patients take synthetic medicines, which have an inhibitory effect against the activity of the enzymes, especially *α*-amylase and *α*-glucosidase which break down carbohydrates in the gut, leading to a slowdown of glucose absorption into the bloodstream. The digestion process is initiated by *α*-amylase by breaking down starches into smaller oligosaccharides and disaccharides, which are then further broken down by *α*-glucosidase to release glucose and other simple sugars. These monosaccharides are then absorbed into the bloodstream. By acting on the final step of carbohydrate digestion, *α*-glucosidase directly influences the rate at which glucose enters the bloodstream [4]. As a result, glucose levels can be maintained at a more consistent level, preventing spikes or drops in blood sugar. Nevertheless, these drugs often have unpleasant side effects such as flatulence, inflation, diarrhea, nausea, and loss of appetite. Therefore, a long-term use of these medications would constitute a burden to DM patients and reduce their quality of life [5]. To reduce the side effects caused by the synthetic antidiabetic medications and to lower the cost of medical treatments, many bioactive compounds from natural sources, especially plants, have been investigated.

Plants are rich in many secondary metabolites possessing potent biological properties to maintain blood glucose levels [6]. Thus, many plants have recently been investigated for their inhibitory effects against *α*-amylase and *α*-glucosidase, including bitter gourd (*Momordica charantia*) [7], Java plum (*Syzygium cumini*) [8], turmeric (*Curcuma longa*) [9], and king of bitters (*Andrographis paniculata*) [10]. Some have been studied and provided valid scientific evidence, while others have not been scientifically demonstrated. To pursue the finding, in this study, twenty commonly consumed Thai culinary vegetables in the northeastern region of Thailand with herbal remedy backgrounds in diabetes management were screened and examined for their effect on *α*-amylase and *α*-glucosidase inhibition *in vitro*. The knowledge gained from this study would be useful to identify potential culinary vegetables with *α*-amylase and *α*-glucosidase inhibitory activity as functional foods for postprandial hyperglycemia management as well as help establish the scientific validity of folk medicine.

2. Materials and Methods

2.1. Chemicals and Reagents

The 3, 5-dinitrosalicylic acid, *α*-amylase from *Aspergillus oryzae*, *α*-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl-*α*-D-glucopyranoside, and starch were purchased from Sigma-Aldrich (USA). Other chemicals were purchased from Fisher Scientific (USA).

2.2. Sample Preparation

Twenty Thai culinary vegetables were studied, namely ma klam (*Adenanthera pavonina* L), king daeng (*Alpinia purpurata* (Vielle.) Schum), phak khom (*Amaranthus lividus* L), sadao (*Azadirachta indica* A. Juss. var. siamensis Valeton), phak pang (*Basella alba* L), phak kard hin (*Brassica juncea* (L.) Czern), tam-leung (*Coccinia grandis* (L.) Voigt), fucktong (*Cucurbita moschata* Decne), phak naam (*Lasia spinosa* (L.) Thwaites), kra thin (*Leucaena leucocephala* (Lamk.) de Wit), ka-yang (*Limnophila aromatica*), kan jong (*Limnocharis flava* (L.) Buchenau), buap (*Luffa acutangula* (Linn.) Roxb), phak tob Thai (*Monochoria hastata* (L.) Solms), ma rum (*Moringa oleifera* Lam), cha phlu (*Piper sarmentosum* Roxb), phak peaw (*Polygonum odoratum* Lour), khae (*Sesbania grandiflora* (L.) Desv), mek (*Syzygium gratum* (Wight) S.N. Mitra var. gratum), and buap ngo (*Trichosanthes anguina* Linn). Fresh vegetables were obtained from three representative markets in Kalasin Province from January to May 2022. At each market, 1-2 kg of samples were collected from three representative outlets. A single composite sample for each representative market was prepared by combining about 500 g of a homogenized single sample of the same vegetable variety from three representative outlets and then homogenizing again to obtain a uniform single composite sample. The characteristics of vegetables are presented in Table 1.

Table 1

The characteristics of the selected vegetables.

Scientific name	Common name	Thai name	Part of use
Adenanthera pavonina L	Red sandalwood tree	Ma klam	Leaf
Alpinia purpurata (Vielle.) schum	Red ginger	King daeng	Rhizome
Amaranthus lividus L	Purple amaranth	Phak khom	Leaf
Azadirachta indica A. Juss. var. siamensis Valeton	Siamese neem	Sadao	Leaf
Basella alba L	Malabar spinach	Phak pang	Leaf
Brassica juncea (L.) Czern	Chinese mustard	Phak kard hin	Leaf
Coccinia grandis (L.) Voigt	Ivy gourd	Tam-leung	Leaf
Cucurbita moschata Decne	Pumpkin	Fucktong	Flower
Lasia spinosa (L.) thwaites	—	Phak naam	Leaf
Leucaena leucocephala (Lamk.) de Wit	Pearl wattle	Kra thin	Young shoot
Limnophila aromatica	Rice Paddy herb	Ka-yang	Leaf

Limnocharis flava (L.) Buchenau	Sawah lettuce	Kan jong	Leaf
Luffa acutangula (Linn.) Roxb	Angled loofah	Buap	Fruit
Monochoria hastata (L.) solms	Monochria	Phak tob Thai	Flower
Moringa oleifera Lam	Drumstick tree	Ma rum	Leaf
Piper sarmentosum Roxb	—	Cha phlu	Leaf
Polygonum odoratum Lour	Vietnamese coriander	Phak peaw	Leaf
Sesbania grandiflora L Desv	Vegetable hummingbird	Khae	Flower
Syzygium gratum (Wight) S.N. Mitra var. gratum	—	Mek	Leaf
Trichosanthes anguina Linn	Snake gourd	Buap ngo	Fruit

2.3. Plant Extraction

Ten grams of freeze-dried vegetables were macerated using extraction solvents with different polarity including deionized water, ethanol, and hexane, in a ratio of 1 : 10 in a 30°C water bath shaker for 24 hours at 150 rpm. Vegetable debris was removed by centrifugation, and the vegetable extract was obtained after the removal of the extraction solvent by rotary evaporation. The vegetable extract was kept in darkness at 4°C for further analysis.

2.4. α-Glucosidase Inhibitory Assay

The α-glucosidase inhibitory properties were analyzed using the method, explained by Kim et al. with minor modifications [11]. Briefly, 50 μL of 10 mg/mL vegetable extract was preincubated with 0.1 M phosphate buffer, pH 6.8 containing α-glucosidase for 10 min. After preincubation, 1 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer, pH 6.8 was added and further incubated at 37°C for 10 min. The reaction was stopped by adding 1 mL of 0.1 M sodium carbonate. The α-glucosidase inhibitory activity was followed by the measurement of absorbance at 405 nm. The α-glucosidase inhibitory property was expressed as the percentage of α-glucosidase inhibition and calculated according to the following equation: $(1)\text{Percentage of inhibition}\,\% = \frac{A-B}{A} \times 100$, where A and B were the absorbance values for the control and sample, respectively. A control was prepared using the same procedure to replace the vegetable extract with distilled water. The experiment was conducted in five replicates.

2.5. α-Amylase Inhibitory Assay

The α-amylase inhibitory property was analyzed using the method, explained by Kidane et al. with minor modifications [12]. Briefly, 50 μL of 10 mg/mL vegetable extract was preincubated with 0.02 M phosphate buffer, pH 6.9, containing α-amylase for 10 min. After preincubation, 1% starch in phosphate buffer, pH 6.9 was added and further incubated for 10 min. The reaction was stopped by adding 1 mL of the 3, 5-dinitrosalicylic acid reagent, then incubated in boiling water for 5 min and cooled to room temperature. The α-amylase inhibitory activity was followed by the measurement of absorbance at 540 nm. The α-amylase inhibitory property was expressed as the percentage of α-amylase inhibition and calculated according to the equation: $(2)\text{Percentage of inhibition}\,\% = \frac{A-B}{A} \times 100$, where A and B were the absorbance values for the control and sample, respectively. A control was prepared using the same procedure, replacing the vegetable extract with distilled water. The experiment was conducted in five replicates.

2.6. Determination of the IC_{50}

The vegetable extracts with more than 50% of α-glucosidase inhibitory or 50% of α-amylase inhibitory were selected for the evaluation of IC_{50} value. The IC_{50} is defined as the concentration of vegetable extract that could reduce the α-glucosidase activity by 50% which was only determined for the vegetable extract with inhibition ≥50%. The IC_{50} was

obtained graphically for the plot of percentage of inhibition versus concentration [11]. The experiment was conducted in triplicate.

2.7. Kinetics of Enzyme Inhibition

In the enzyme-kinetic measurement, an inhibition assay was performed according to the protocol described by Kim et al. Inhibition modes of selected vegetable extracts against *α*-glucosidase were determined by increasing concentration of *p*-nitrophenyl-*α*-D-glucopyranoside solution in the absence or presence of selected vegetable extracts. The experiment was conducted in triplicate. The type of inhibition of the vegetable extracts was determined by a Lineweaver-Burk plot [11].

3. Results

3.1. Screening of *In Vitro* *α*-Glucosidase and *α*-Amylase Inhibitory Activity

The *α*-glucosidase inhibitory activities of the vegetable extracts, intentionally chosen for their Thai remedial background in diabetes management are presented in Table 2. Using different extraction solvents to extract anti-*α*-glucosidase agents from each vegetable resulted in different anti-*α*-glucosidase activity levels. Nine out of twenty vegetables showed an inhibitory effect against *α*-glucosidase, whereas eleven showed no inhibition. Hexane did not appear to be a suitable solvent to extract anti-*α*-glucosidase substances. Most of the *α*-glucosidase inhibitory activities were discovered from the extracts using water and ethanol as extraction solvents. *L. leucocephala* (Lamk.) de Wit and *P. odoratum* Lour generated the most promising vegetable extracts with very high anti-*α*-glucosidase activity. The *α*-glucosidase inhibitory activity of the ethanolic extract from *L. leucocephala* (Lamk.) de Wit was 96.10 ± 1.64% and the aqueous and the ethanolic extract of *P. odoratum* Lour produced the anti-*α*-glucosidase activity with values of 93.47 ± 1.66% and 96.19 ± 1.15%, respectively. Moderate *α*-glucosidase inhibitory effects were also obtained from *S. gratum* (Wight) S.N. Mitra var. gratum, which gave lower anti-*α*-glucosidase activity when compared to the formers. Its inhibition rates against *α*-glucosidase were 55.12 ± 1.71% from the aqueous extract and 53.11 ± 1.44% from the ethanolic extract, respectively. The ethanolic extract from *C. grandis* (L.) Voigt showed 67.30 ± 1.39% of inhibition, but no inhibition was found from the aqueous or hexane extract. Besides, other vegetable extracts produced either no or insufficient anti-*α*-glucosidase activity; their inhibitory activities were less than 25% of inhibition against *α*-glucosidase activity.

Table 2

The *α*-glucosidase and *α*-amylase inhibitory activities of vegetable extracts.

Scientific name	Percentage of inhibition against *α*-glucosidase (%)			Percentage of inhibition against *α*-amylase (%)		
Water	Ethanol	Hexane	Water	Ethanol	Hexane	*Adenanthera pavonina* L

nd	nd	nd	nd	nd	nd	*Alpinia purpurata* (*Vielle.*) schum
nd	nd	nd	nd	nd	nd	*Amaranthus lividus* L
nd	nd	nd	nd	nd	nd	*Azadirachta indica* A. Juss. var. siamensis Valeton

nd	nd	nd	nd	nd	nd	*Basella alba* L
nd	nd	nd	nd	nd	nd	*Brassica juncea* (L.) Czern
nd	nd	nd	nd	nd	nd	*Cocciniagrandis* (L.) Voigt
nd	67.30 ± 1.39	nd	nd	35.12 ± 3.06	nd	*Cucurbitamoschata* Decne

nd	nd	nd	nd	nd	nd	*Lasiaspinosa* (L.) thwaites
nd	nd	nd	nd	nd	nd	*Leucaenaleucocephala* (Lamk.) deWit
22.40 ± 1.54	96.10 ± 1.64	nd	nd	31.81 ± 3.19	nd	*Limnophilaaromatica*

nd	nd	3.69±1.62	nd	nd	nd	*Limnocharis flava* (L.) Buchenau
nd	nd	nd	nd	nd	nd	*Luffa acutangula* (Linn.) Roxb
nd	15.56±0.79	nd	nd	nd	nd	*Monochoria hastata* (L.) solms

nd	24.69±2.76	nd	nd	nd	nd	*Moringa oleifera* Lam
nd	nd	nd	nd	nd	nd	*Piper sarmentosum* Roxb
12.69±1.40	6.66±1.91	nd	nd	nd	nd	*Polygonum odoratum* Lour
93.47±1.66	96.19±1.15	nd	nd	7.67±1.05	nd	*Sesbania grandiflora* (*L.*) Desv

nd	nd	nd	nd	nd	nd	*Syzygium gratum* (Wight) S. N. Mitra var. gratum
55.12 ± 1.71	53.11 ± 1.44	6.17 ± 2.92	nd	22.42 ± 0.16	nd	*Trichosanthes anguina* Linn

Remarks: All data was expressed as mean ± standard deviation (S.D.). Nd indicates “not detected.”

Table 2 also shows the *α*-amylase inhibitory activities of the vegetable extracts. It was interesting that most of the vegetable extracts were unable to inhibit *α*-amylase. Few of them, including the ethanolic extracts from *C. grandis* (L.) Voigt, *L. leucocephala* (Lamk.) de Wit, *P. odoratum* Lour, and *S. gratum* (Wight) S.N. Mitra var. gratum occupied inadequate anti-*α*-amylase activity. Their *α*-amylase inhibitory activities were measured at 35.12 ± 3.06%, 31.81 ± 3.19%, 7.67 ± 1.05%, and 22.42 ± 0.16%, respectively.

These screening results indicated that *L. leucocephala* (Lamk.) de Wit, *P. odoratum* Lour, *S. gratum* (Wight) S.N. Mitra var. gratum, and *C. grandis* (L.) Voigt appeared to be good potential sources of anti-*α*-glucosidase agents. Therefore, these vegetable extracts were selected for further analysis on IC_{50} determination and study for the kinetics of enzyme inhibition.

3.2. The IC_{50} and Kinetics of Enzyme Inhibitions

In the *α*-glucosidase inhibition assay, the ethanolic extract of *L. leucocephala* (Lamk.) de Wit was the most influential on *α*-glucosidase inhibitory activity with an IC_{50} value of 13.39 ± 0.14 μg/mL, while the extracts from *P. odoratum* Lour and *C. grandis* (L.) Voigt could inhibit *α*-glucosidase with IC_{50} values ranging from 25.60 ± 0.42 to 82.74 ± 1.39 μg/mL, respectively. The IC_{50} values of the aqueous and ethanolic extracts of *S. gratum* (Wight) S.N. Mitra var. gratum were

516.92 ± 5.08 and 542.50 ± 0.90 μg/mL, respectively (Table 3).

Table 3

The IC_{50} and kinetics for enzyme inhibition of the vegetable extracts.

Scientific name	Extraction solvent	IC_{50} (μg/mL)
Syzygium gratum (Wight) S.N. Mitra var. gratum	Ethanol	542.50 ± 0.90
Water	516.92 ± 5.08	·
Coccinia grandis (L.) Voigt	Ethanol	82.74 ± 1.39
–		
Polygonum odoratum Lour	Ethanol	49.03 ± 0.72
Water	25.60 ± 0.42	·
Leucaena leucocephala (Lamk.) de Wit	Ethanol	13.39 ± 0.14

Remarks: All data was expressed as mean ± standard deviation (S.D.).

To determine the inhibition mechanism of selected vegetable extracts with high inhibitory activity against α-glucosidase, the inhibitory kinetics of the vegetable extracts were measured at various concentrations of substrate, and the data were exported using the method of Lineweaver-Burk plot. Table 4 shows the Km and Vmax values of the vegetable extracts towards α-glucosidase. Compared to the uninhibited reaction (reaction containing α-glucosidase without inhibitor), a decrease in Vmax was found for all vegetable extracts, but the effects of the vegetable extracts on Km values were different. The Km values were reduced in the presence of an aqueous extract of *S. gratum* (Wight) S.N. Mitra var. gratum and the ethanolic extract of *C. grandis* (L.) Voigt. The Km values were increased in the presence of the ethanolic extract of *S. gratum* (Wight) S.N. Mitra var. gratum and *P. odoratum* Lour. However, the aqueous extract of *P. odoratum* Lour and the ethanolic extract of *L. leucocephala* (Lamk.) de Wit did not change the Km values of the reactions. These results demonstrated the mode of inhibition for the vegetable extracts on α-glucosidase. The aqueous extract of *S. gratum* (Wight) S.N. Mitra var. gratum and the ethanolic extract of *C. grandis* (L.) Voigt exhibited an uncompetitive inhibition mode. The ethanolic extract of *S. gratum* (Wight) S.N. Mitra var. gratum and *P. odoratum* Lour were in a mixed inhibition mode, while the aqueous extract of *P. odoratum* Lour and the ethanolic extract of *L. leucocephala* (Lamk.) de Wit demonstrated a noncompetitive inhibitor mode.

Table 4

Kinetic parameters for α-glucosidase inhibition of the vegetable extracts.

Scientific name	Extraction solvent	Km (mM)	Vmax (μM/min)
α-glucosidase without inhibitor		0.39 ± 0.001	16.0 ± 0.002
–			
Syzygium gratum (Wight) S.N. Mitra var. gratum	Ethanol	0.41 ± 0.001	4.99 ± 0.001

Water	0.15 ± 0.001	5.98 ± 0.001	·
Coccinia grandis (L.) Voigt	Ethanol	0.06 ± 0.002	2.32 ± 0.001
–			
Polygonum odoratum Lour	Ethanol	0.53 ± 0.001	6.41 ± 0.002
Water	0.39 ± 0.001	5.71 ± 0.001	·
Leucaena leucocephala (Lamk.) de Wit	Ethanol	0.39 ± 0.001	7.13 ± 0.002

Remarks: All data was expressed as mean ± standard deviation (S.D.).

4. Discussion

There is growing interest in developing novel and potential antidiabetic properties with minimal adverse effects that can be derived from plants that have known, scientifically validated antidiabetic characteristics. Numerous phytochemicals, such as polyphenols, phenolic acids, stilbenes, lignin, glucosinolates, and carotenoids are abundant in plants. These phytoconstituents play a vital part in plant metabolism and offer considerable health advantages to slow the progression of diseases as well as prevent them from occurring. A decrease in developing metabolic syndromes, including DM, can eventually result from an increase in plant consumption, whether through direct ingestion or dietary supplements. Plants are thought to contain a variety of substances that have hypoglycemic effects driven by several different mechanisms, including insulin sensitization, insulin release, and carbohydrate-hydrolyzing enzyme inhibition [6, 13, 14].

In the present study, the inhibition of carbohydrate-hydrolyzing enzymes of regularly consumed vegetables in Thailand was the subject of investigation. The enzyme inhibitory property may be a practical method for regulating the control of blood glucose levels. The target enzymes in this investigation were the hydrolyzing enzymes: *α*-amylase and *α*-glucosidase. These enzymes are primarily responsible for the decomposition of starch into glucose. The inhibition of these enzymes results in a delay in glucose absorption into the blood vessels.

The inhibition of carbohydrate-hydrolyzing enzymes of sixty different vegetable extracts from different twenty vegetables was tested. It was discovered that anti-*α*-glucosidase and anti-*α*-amylase activities were affected by the type of vegetables and solvent utilized, which was in agreement with several previous studies [15–18]. Plants have a wide range of phytochemicals that are structurally different, resulting in distinct polarity [16, 17]. In this study, solvents with a wide range of polarity from nonpolar to polar were used to ensure that plant materials that differed in their polarity were sequentially extracted based on their polarity, and all components were presented in the screening study. It was found that only four out of twenty vegetables evaluated in this study had high anti-*α*-glucosidase activity (>50% of inhibition). This circumstance could be explained by the phytochemical components present in plants varying contingent on plant origin, plant genotype, geography, climate, soil fertility, and stress level. These elements affect how plants create bioactive compounds, which might vary in quantity and form [19–21]. In addition, the results of the current study suggested that the anti-*α*-glucosidase agents from these vegetable extracts were also likely composed of functional groups that appear to be hydrophilic with polarity indices between 5.2 (absolute ethanol) and 10.2 (water) because the anti-*α*-glucosidase activities were found in the ethanolic and aqueous extracts. Five out of the twenty vegetables evaluated, however, had minimal inhibitory effects on *α*-glucosidase. Additionally, a few of the vegetable extracts exhibited minor inhibitory effects on *α*-amylase. Therefore, it is probable that these vegetables may not contain effective or sufficient anti-*α*-glucosidase and anti-*α*-amylase agents. As mentioned previously, the type and concentration of bioactive chemicals found in various plants may vary.

Measuring enzyme inhibition at a fixed concentration provided rather limited information [22]. The IC_{50} value was

therefore used to compare the efficiency of the vegetable extract to inhibit *α*-glucosidase in this study. The IC_{50} value means the concentration of vegetable extract that generates 50% inhibition under a particular assay condition, resulting in a difference in the IC_{50} value found among the conditions used [23].

The IC_{50} results revealed that the aqueous and ethanolic extracts of *S. gratum* (*Wight*) *S.N. Mitra* var. *gratum* were approximately the same with values of 516.92 ± 5.08 and 542.50 ± 0.90 *μ*g/mL, respectively. This result implied that the active compounds found in these two extracts were possibly hydrophilic-like compounds, which corresponded to the study from Syabana et al. [24]. In their study, the leaf extract of *S. gratum* (*Wight*) *S.N. Mitra* var. *gratum* was found to be a potential source of *α*-glucosidase inhibitors, especially the fractions extracted by acetone-water 4 : 1 and 3 : 2. The IC_{50} of these two fractions were 24.8 and 31.8 *μ*g/mL, respectively [24]. According to the study from Syabana et al. [24], the *α*-glucosidase inhibitory activity of *S. gratum* (*Wight*) *S.N. Mitra* var. *gratum* could be a result of myricetin-3-O-rhamnoside (myricitrin) and epigallocatechin-3-gallate (EGCG) [24]. Additionally, both aqueous extract and ethanolic extract from the leaves of *S. gratum* (*Wight*) *S.N. Mitra* var. *gratum* showed strong antioxidant and intercellular oxygen scavenging activity, according to the study from Senggunpri et al. [25]. The aqueous extract also showed a cytoprotective effect *in vivo*. The activity of heme oxygenase (HO-1), a potent cytoprotective enzyme in the antioxidant defense system, was significantly increased in the high-dose-treated C57BL/6J mice, and the expression of HO-1 gene had a tendency to increase when treated with the aqueous extract. The data extrapolated the benefit of *S. gratum* (*Wight*) *S.N. Mitra* var. *gratum* as a source of natural antidiabetic agents and antioxidants, and it could induce cytoprotective enzymes without toxicity being observed.

The IC_{50} values of the *P. odoratum* Lour extracts were 49.03 ± 0.72 *μ*g/mL for the ethanolic extract and 25.60 ± 0.42 *μ*g/mL for the aqueous extract, respectively. These results were supported by the study of Thongra-ar et al. [26], which specified that the ethanolic extract of *P. odoratum* Lour strongly inhibited *α*-glucosidase with an IC_{50} value of 9.82 ± 1.64 *μ*g/mL [26]. Moreover, Dedvisitsakul and Watla-Iad [27] further reported the inhibitory effect of the ethanolic extract from *P. odoratum* Lour towards *α*-glucosidase with the IC_{50} of 0.66 ± 0.08 mg/mL. Their study also discovered that the ethanolic *P. odoratum* Lour extract demonstrated significant inhibitory activity towards the formation of advanced glycation end product (AGEs) which derived from glucose using a BSA-glucose system with the IC_{50} of 0.03 ± 0.01 mg/mL [27]. The phenolic compound (gallic acid and chlorogenic acid) and flavonoid (isorhamnetin) were believed to respond to the inhibitory effect of *α*-glucosidase in accordance with their phytochemical study [26]. The *in vivo* study from Deng et al. [28] also indicated saponins found in this vegetable presented antidiabetic activity, whereas flavonoids influenced antioxidant activity.

The ethanolic extract of *C. grandis* (L.) Voigt proved its IC_{50} against *α*-glucosidase at 82.74 ± 1.39 *μ*g/mL. Likewise, the study by Pulbutr et al. indicated the IC_{50} value of the ethanolic extract against *α*-glucosidase at 77.66 ± 9.16 *μ*g/mL [29]. The antidiabetic properties of the ethanolic extract were supported by the study from Astiti et al. [30], which identified the compounds responsible for the antidiabetic effect from the extract of *C. grandis* leaves, including rutin, kaempferol 3-*O*-rutinoside or nicotiflorin, kaempferol 3-*O*-robinobioside, quercetin 3-*O*-robinobioside, quercetin 3-*O*-*β*-D-apiofuranosyl-(12)-[*α*-L-rhamnopyranosyl-(16)]-*β*-D-glucopyranoside or CTN-986, kaempferol 3-*O*-*β*-D-apiofuranosyl-(12)-[*α*-L-rhamnopyranosyl-(16)]-*β*-D-glucopyranoside, and kaempferol 3-*O*-*β*-D-apiofuranosyl-(12)-[*α*-L-rhamnopyranosyl-(16)]-*β*-D-galactopyranoside.

The most efficient anti-*α*-glucosidase activity in this study was obtained from the ethanolic extract from *L. leucocephala* (Lamk.) de Wit with the lowest IC_{50} of 13.39 ± 0.14 *μ*g/mL. In addition, the study by Renganathan et al. [31] revealed that *L. leucocephala* (Lam.) De Wit leaf extract inhibited enzyme activity in a dose-dependent manner. The study from Wan-Nadilah et al. [32] also found the *in vitro α*-glucosidase inhibitory activity from the seed of *L. leucocephala* (Lam.) De Wit with the IC_{50} of 30.80 ± 2.50 *μ*g/mL. Parts of use in the study by Renganathan et al. [31] and the present study were not the same; their extracts were obtained from leaves, while the extracts in this study were from young shoots. This might have contributed to the difference in IC_{50} values. However, these data indicated that leaves, seeds, and young shoots of *L. leucocephala* (Lam.) De Wit were a good source of anti-*α*-glucosidase constituents. Moreover, *in silico* virtual screening was used to identify the phytochemicals involved in *α*-amylase enzyme inhibition, while hexadecenoic acid and oleic acid ((Z)-octadec-9-enoic acid) were identified as *α*-amylase

inhibitors.

Line-weaver-Burk plots revealed that the inhibition modes of all samples might be mixed–uncompetitive and noncompetitive inhibition. A mixed inhibitor can either bind to the free enzyme or the enzyme-substrate complex which results in an alteration of Km and Vmax values (an increase in Km and a decrease in Vmax). For the uncompetitive inhibition and noncompetitive inhibition, the binding of these types of inhibitor can influence the binding of the substrate by changing the conformation of the enzyme. The uncompetitive inhibitor binds the enzyme-substrate complex, resulting in a decrease of Km and Vmax, while the noncompetitive inhibitor either binds to a free enzyme or the enzyme-substrate complex, which results in a decrease of Vmax value and no change in Km [11, 18, 23]. It is likely that the capacity of these extracts as mixed, uncompetitive, and noncompetitive inhibitors to bind to extensive areas of the enzyme other than the active site allows them to show a broader specificity of inhibition when compared to acarbose as a competitive inhibitor [11, 18, 23]. The explanation for various inhibition modes from the extracts could be as a result of the different bioactive constituents available in the extracts [18, 22, 23]. The various bioactive compounds presented in the extracts probably had different binding modes to *α*-glucosidase. Contrary to acarbose, these extracts may not be affected by increased quantities of the substrate, which is one advantage they have over acarbose. With increased carbohydrate meal consumption, higher dosages of acarbose as a competitive inhibitor would be necessary to have the same impact, but with the mixed, uncompetitive, and noncompetitive inhibition, the inhibitor would be effective at lower concentrations [18, 23, 33]. Moreover, the stronger inhibition activity of the *α*-glucosidase than the *α*-amylase activity of these extracts revealed their medicinal potential to prevent some negative effects of utilizing synthetic *α*-glucosidase and *α*-amylase inhibitors. The side effects of using synthetic enzyme-inhibitor drugs can include abnormal bacteria fermentation of undigested carbohydrates in the colon because these drugs strongly inhibit *α*-amylase over *α*-glucosidase. Therefore, more potent inhibitors for enzymes should have a strong inhibitory effect on *α*-glucosidase and a moderate inhibitory effect on *α*-amylase, which can improve the management of postprandial hyperglycemia with the fewest side effects [34, 35].

The results of the current study showed the potential of vegetable extracts toward enzyme-hydrolyzing carbohydrates. As a result, individuals should be encouraged to consume more of these vegetables as an alternative course for diabetic prevention and treatment. However, more information on *in vivo* bioactivity and the absorption of these bioactive components needs to be established before deciding on their applications.

5. Conclusion

Vegetables that are frequently consumed in the northeastern region of Thailand showed a varying range of *α*-glucosidase and *α*-amylase inhibitory effects. Promising *α*-glucosidase inhibitory activities were reported from *L. leucocephala* (Lamk.) de Wit, *P. odoratum Lour*, *C. grandis* (L.) Voigt, and *S. gratum* (Wight) S.N. Mitra var. gratum. The results of this investigation suggest that these vegetables may be good dietary sources of extractable anti-*α*-glucosidase agents for preventing or managing postprandial hyperglycemia-induced complications. Nevertheless, this was an *in vitro* study with potential relevance concerning phytochemicals.

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DETAIL

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Chemical Profiling and Biological Activities of Ziziphus Mauritiana var. spontanea (Edgew.) R.R. Stewart ex Qaiser &Nazim. and Oenothera Biennis L.

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ABSTRAK (ENGLISH)

Bioactive compounds of medicinal plants, including polyphenols, flavonoids, terpenoids, and alkaloids, are essential sources for developing analgesic, anti-inflammatory, and antidiarrheal drugs. In the current study, secondary metabolites were assessed through phytochemical screening and GC-MS analysis whereas analgesic activity was carried out through hot plate (HP) and acetic acid-induced method (AAI), anti-inflammatory through paw edema model (PEM), and antispasmodic activity via charcoal meal test (CMT) using ethyl acetate and ethanolic extract of *Ziziphus mauritiana* var. *spontanea* and *Oenothera biennis*. The phytochemical screening revealed that the ethyl acetate and ethanolic extracts of *Z. mauritiana* and *O. biennis* were rich in alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins. GC-MS analysis of *Z. mauritiana* and *O. biennis* of ethyl acetate and ethanolic extract showed the existence of many bioactive substances at various retention durations (min). These included pharmacologically active compounds such as heptadecane, 2-methoxy-4-vinylphenol, hexadecanoic acid, and tetradecanoic acid. The results of the HP method revealed that ethanolic and ethyl acetate extracts of *Z. mauritiana* and *O. biennis* at 300 mg/kg increased basal reaction time significantly ($p<0.001$) after 90 min. The results of the AAI method revealed that the ethanolic and ethyl acetate extract of *Z. mauritiana* and *O. biennis* showed significant ($p< 0.001$) peripheral analgesic activity at the dose of 200 and 300 mg/kg body weight. The dosage of 100, 200, and 300 mg/kg body weight of the ethyl acetate and ethanolic extracts of *Z. mauritiana* and *O. biennis* showed significant (p<

0.001) anti-inflammatory activity. According to PEM, the ethanolic extract of *O. biennis* showed the highest reduction in paw volume (73.3%) at 300 mg/kg. The results of CMT revealed that ethanolic and ethyl acetate extract of *Z. mauritiana* and *O. biennis* significantly ($p<0.001$) inhibited charcoal movement at 300 mg/kg. The maximum percent inhibition (67.2%) was shown by ethyl acetate of *O. biennis* at 300 mg/kg. From the present study, it can be concluded that ethanolic and ethyl acetate extracts of *Z. mauritiana* and *O. biennis* have the potential to manage inflammation, pain, and diarrhea-related problems mainly at a higher dose, i.e., 300 mg/kg. The presence of alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins might be among the responsible bioactive constituents. These plants showed significant medicinal and therapeutic efficacy which are novel. However, further studies are required to investigate the mechanism responsible for the activity.

TEKS LENGKAP

1. Introduction

Medicinal plants are naturally God-gifted with a lot of chemical compounds that are formed in different plant parts and used in the treatment of various ailments. These chemical compounds are also known as secondary metabolites. These secondary metabolites can be utilized in the formulations of novel medications and have been reported as very effective in curing serious health problems. The World Health Organization (WHO) reported that about 80% of the population in the world still depends on medicinal plants for their basic healthcare due to their effectiveness and easy availability [1]. Advancements in biotechnology have made it possible to manufacture therapeutic protein drugs in significant quantities [2]. Historically, fruits have served as a basis for various medicinal and liquor products [3]. Additionally, plant hydrocolloids have shown potential in mitigating cardiovascular disease risk, decreasing blood cholesterol levels, and enhancing immune function [4].

Aromatic medicinal plants are widely used by people for various purposes including the food industry, perfumery, textile industry, and pharmaceutical industry. The medicinal properties of plants are due to the presence of bioactive compounds such as saponins, glycosides, quinones, alkaloids, and flavonoids [5].

Pain is associated with potential or actual tissue damage. Pain is not only a symptom used to diagnose several diseases and conditions but also has a protective function [6]. Analgesic drugs have been used for the elimination of pain without significantly altering consciousness. The use of synthetic analgesic drugs has several side effects including gastrointestinal disorders, bleeding, and ulcers [7]. A physiological response that protects a body from tissue injury is called inflammation. There are two types of inflammation, i.e., acute inflammation and chronic inflammation [8]. The nonsteroidal anti-inflammatory drugs are used to treat inflammation but it has a lot of side effects, including gastrointestinal and cardiovascular complications. Therefore, the development of new drugs is necessary for the management of pain and inflammation. Plant-based beverages typically contain advantageous bioactive compounds, including flavonoids, phenolic acids, lignans, and phytosterols, known for their exceptional antioxidant, analgesic, and anti-inflammatory properties, contributing significantly to health benefits [9, 10]. In the past twenty years, numerous research studies have underscored the pivotal role of the gut microbiome in influencing health and disease. Alterations in the composition of the intestinal microbiome have been linked to diverse intestinal and metabolic disorders, including inflammatory bowel disease, diabetes, and obesity [11].

An imbalance in the gut microbiota plays a crucial role in the pathogenesis of functional Dyspepsia (FD). This imbalance disrupts the intestinal environment, ultimately causing a decrease in beneficial probiotics and triggering a range of acute and chronic diseases [12]. The condition of increased stool frequency, liquidity, or volume is called diarrhea. It mainly affects neonates and babies and is a significant cause of illness and mortality in developing countries [13]. Medicinal plants are reservoirs of essential antidiarrheal bioactive constituents without any side effects and can be used to treat gastrointestinal disorders, for example, constipation and diarrhea [14].

Ziziphus mauritiana belonging to the family Rhamnaceae Juss., locally called Ber, is a fruit tree that grows worldwide in tropical and subtropical regions [15]. *Z. mauritiana* is a medicinal plant used to cure several disorders such as

ulcers, asthma, allergies, depression, digestive problems, weakness, obesity [16, 17], diabetes, urinary issues, and skin infections [18].

Oenothera biennis L. also known as evening primrose belongs to Onagraceae Juss. It is distributed in Peshawar Pakistan and eastern and central North America. The seed oil of *O. biennis* is used for the treatment of several ailments such as asthma, eczema, breast problems, rheumatoid arthritis, menopausal syndrome [19] fistulas, and lung disease [20]. The present study will analyze the plant in terms of drug standardization and evaluating its pharmacological effect which can be used as a potential candidate for future drug developments.

2. Materials and Methods

2.1. Plant Collection

Ziziphus mauritiana was collected from the area of Palosai Peshawar, and *Oenothera biennis* was collected from the Department of Botany, University of Peshawar KPK Pakistan which is located at 34.0011°N and 71.4874°E in August 2022. The identification of the plant was carried out with the help of Flora of Pakistan and Ghulam Jelani (plant taxonomist) and kept in the herbarium for future reference with Voucher Number Ambrin Bot. 33 (PUP) and Ambrin Bot. 34 (PUP).

2.2. Extraction of Plant Material and Sample Preparation

Whole plants were washed with distilled water, shade dried in the air dryer, and ground into a fine powder, and 50 g was soaked in 250 mL each in ethanol and ethyl acetate, respectively, which were supplied by U.M. enterprises. After 48 hours, the extract was passed through muslin cloth followed by filtration through filter paper. The extract was concentrated using a rotary evaporator (RE-100D Phoenix) supplied by MED Lab Services. The derived ethyl extracts (10.1 g) and ethanol extract (9.5 g) of *Z. mauritiana* and ethyl extracts (10.4 g) and ethanol extract (10.12 g) each were kept at 4°C in capped bottles before use [21].

2.3. Phytochemical Screening

Phytochemical analysis of the extracts was carried out to detect flavonoids, alkaloids, tannins, steroids, triterpenoids, and tannins following protocols [22].

2.4. Gas Chromatography-Mass Spectrometry (GC/MS)

A gas chromatography-mass spectrometry of the crude extract of *Z. mauritiana* and *O. biennis* was carried out by coupling Thermo GC-Trace Ultra version 5.0 with Thermo MS DSQ II which was supplied by MED Lab Services. The sample was prepared by adding 2 mg crude extract in 5 mL of respective solvents. To purify the samples, the mixtures were divided on a ZB 5-MS capillary regular nonpolar column (30 m 0.25 mm ID 0.25 lm FILM). The temperature of the column was kept at 70°C with an increasing rate of 2°C/minute. Finally, the increase in temperature was raised to 260°C at 6°C/minute with a holding time of about ten minutes. The splitless mode was used to introduce the particle-free, diluted sample (10 mL/min split flow and 1 min spitless period). Helium was used as the carrier gas at a constant flow rate of 1 mL/min, and 1 L of sample was injected. Peak area normalization was used to quantify the relative percentages of crude extract elements. In full scan mode, the mass spectral scan range was adjusted to 50 to 650 (m/z). By comparing the retention indices of the compounds with those of real samples stored on the Wiley and Main Lab computer library search software, the compounds were identified [23].

2.5. Analgesic Activity

2.5.1. Acetic Acid-Induced Writhing

Ziziphus mauritiana and *Oenothera biennis* ethanolic and ethyl acetate extracts (sample was prepared by adding 10 mg crude extract in 25 mL of respective solvents) were tested for their analgesic activity following the method of [24] with few modifications. Mice were divided into five groups containing five mice in each group. Group 1 served as control and administered only with normal saline (10 ml/kg i.p.) Group 2 was administered with standard diclofenac sodium (25 mg/kg), and groups 3–5 were administered with three doses *of Z. mauritiana* and *O. biennis* ethanolic and ethyl acetate extracts (100 mg/kg, 200 mg/kg, and 300 mg/kg), respectively, and these extracts were administered orally one hour before intraperitoneal injection of 0.6% v/v acetic acid and after five mins of postinjection; the number of writhing was counted for the next 20 min. The percent analgesic effect was calculated by the following formula:(1)% analgesic effect=100−no of writhing in tested animals no of writhing in control animals X

100.

2.5.2. Eddy's Hot-Plate Method

Its requirements were similar to the previous method. To perform this activity, the method of [25] was followed, the albino mice (male) were divided randomly into 5 groups, and each group consisted of 5 mice. Group 1 served as control and administered only with normal saline (10 ml/kg ip), group 2 was treated with the standard drug diclofenac sodium (25 mg/kg), and groups 3–5 were orally administered with different concentrations (100, 200, and 300 mg body weight) of ethanolic and ethyl acetate extracts, respectively. The initial reaction time of control and test group animals was recorded by placing them on the hot plate (55 ± 0.5°c), and the licking of the paw or jumping was taken as the index of reaction of heat: the post-treatment reaction time of each animal after the administration of plant extracts recorded at 30 min, 60 min, and 90 min.(2)elongation % latency test − latency control × 100 latency test statistical analysis

2.6. Anti-Inflammatory Activity

The anti-inflammatory activity of the *Z. mauritiana* and *O. biennis* ethanolic and ethyl acetate extracts was investigated on carrageenan-induced inflammation in mice paws following the procedure [26]. The sample was prepared by adding 10 mg crude extract in 25 mL of respective solvents. Animals were divided into 5 groups comprising five animals per group. In all groups, acute inflammation was produced by subplantar injection of 0.1 ml freshly prepared 1% suspension of carrageenan. The paw volume was measured plethysmometrically from 0 to 180 min after carrageenan injection. All the animals were orally premedicated with diclofenac sodium (10 mg/kg b.wt), two hours before the infection. The mean increase in paw volume was measured, and the percentage was calculated for all the extracts. Percentage inhibition of paw volume was calculated by the following formula:(3)Vc − Vt % inhibition of paw edema = x 100 Vc, where Vt = increase in paw volume in mice treated with test compounds and Vc = increase in paw volume in the control group of mice.

2.7. Antispasmodic Activity

The antispasmodic activity of *Z. mauritiana* and *O. biennis* ethanolic and ethyl acetate extracts was carried out by adopting the method of [27]. The sample was prepared by adding 10 mg crude extract in 25 mL of respective solvents. The selected mice were divided into four groups of five mice each. At first, 1 ml of castor oil was given orally to every mouse in each group to produce diarrhea. After 1 hr, group I (control group) orally received saline (10 ml/kg). Group II received the standard drug (atropine sulfate 10 mg/kg b. wt in), and group it-V (the rest of the three groups) received ethanolic and ethyl acetate extracts of plants (100, 200, and 300 mg/kg b. wt ip, respectively). After 1 h, all animals orally revived of the charcoal meal (10 charcoal is a pension in 5% gum acacia). After one hour following the charcoal meal administration, all animals were sacrificed, and the distance covered by the charcoal meal in the intestine, from the pylorus to the caecum, was measured and expressed as a percentage of the distance moved intestinal transit.(4)% = D/L × 100, where *D* = distance covered by charcoal (in meters) and *L* = intestinal length (in meters).

2.8. Ethical Approval

The animal study was reviewed and approved by the Ethical Committee Pharmacy Lab, Qurtuba University of Science and Information Technology Peshawar, Pakistan, under permit no (148/VIEC/VRIP).

2.9. Statistical Analysis

The data were analyzed by Dunnett's *t*-test statistical methods using SPSS Software 22.0. For the statistical tests, $p < 0.001$, 0.01, and 0.05 was considered as significant.

3. Results

3.1. Phytochemical Screening

The phytochemical screening of ethanolic and ethyl acetate extracts of *Z. mauritiana* and *O. biennis* revealed the existence of different bioactive compounds. The ethanolic extract of *Z. mauritiana* showed the presence of alkaloids, flavonoids, tannins, steroids, and triterpenoids whereas saponins were found absent. Similarly, the ethyl acetate extracts of *Z. mauritiana* detect alkaloids, flavonoids, saponins, tannins, and triterpenoids while steroids were absent. Likewise, the ethanolic extract of *O. biennis* revealed the presence of alkaloids, flavonoids, saponins, and

steroids and the absence of tannins and triterpenoids. The ethyl acetate extract of *O. biennis* unveiled the presence of alkaloids, flavonoids, tannins, steroids, and triterpenoids and the absence of tannins (Table 1).

Table 1

Phytochemical screening of *Ziziphus mauritiana* var. *spontanea* and *Oenothera biennis*.

Chemical constituents	*Ziziphus mauritiana* var. *spontanea.*		*Oenothera biennis*	
Ethyl acetate extract	Ethanol extract	Ethyl acetate extract	Ethanol extract	Alkaloids
+	+	+	+	Flavonoids
+	+	+	+	Saponins
−	+	−	+	Tannins
+	+	+	−	Steroids
+	−	+	+	Triterpenoids

3.2. Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry profiling identified the probable phytochemicals in the ethanolic and ethyl acetate extract of *Z. mauritiana* and *O. biennis*. In ethanolic and ethyl acetate extract of *Z. mauritiana*, fourteen phytoconstituents were detected by GC–MS (Tables 2 and 3; Figures 1 and 2). Similarly, eight phytoconstituents were detected in the ethanolic extract of *O. biennis*, and eight phytoconstituents were detected in its ethyl acetate extract (Tables 4 and 5; Figures 3 and 4). The results showed that phytoconstituents found in maximum concentration in ethanolic extract of *Z. mauritiana* was heptadecane (9.06%) followed by 2-methoxy-4-vinylphenol (6.54%) and dodecanoic acid (4.50%) (Table 2; Figure 1). In the ethyl acetate extract of *Z. mauritiana*, tetracosane (11.52%) was detected in the highest concentration followed by dodecane, 1,1-dimethoxy- (4.06%), and 2-methoxy-4-vinylphenol (6.36%) (Table 3; Figure 2). In an ethanolic extract of *O. biennis*, bioactive constituents detected in the maximum amount were phytol (7.45%), followed by furfural (6.53%) and 4-vinyl-2-methoxy-phenol (2.36%) (Table 4; Figure 3). Likewise, the phytoconstituents detected with maximum concentration in ethyl acetate extract of *O. biennis* include 9,12-octadecadienoic acid (Z, Z)- (7.33%), followed by hexathiane (1.06%) and ethyl hexadecane (4.37%) (Table 5; Figure 4).

Table 2

GCMS analysis of ethanol extract of *Ziziphus mauritiana* var. *spontanea*.

S. No	Name of the compound	Compound formula	Retention time (min)	Peak area (%)	Compound structure	Molecular weight

1	Heptadecane	$C_{17}H_{36}$	7.2	9.06		240.475 $g \cdot mol^{-1}$
–						
2	2-Methoxy-4-vinyl phenol	$C_9H_{10}O_2$	10.5	6.54		150.17 g/mol
–						
3	Dodecanoic acid	$C_{12}H_{24}O_2$	17.4	4.50		201.31 g/mol
–						
4	2,3,6-Trimethyl decane	$C_{11}H_{24}$	25.3	8.43		156.31 g/mol
–						
5	Octadecanoic acid	$C_{18}H_{34}O_2$	30.2	5.36		282.5 g/mol
–						
6	Methyl 14-methyl pentadecanoate	$C_{17}H_{34}O_2$	39.5	3.40		270.5 g/mol
–						
7	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	42.6	2.53		330.5 g/mol

Table 3

GCMS analysis of ethyl acetate extract of *Ziziphus mauritiana* var. *spontanea*.

S. no	Name of the compound	Compound formula	Retention time (min)	Peak area (%)	Compound structure	Molecular weight
1	Dodecane, 1,1-dimethoxy-	$C_{12}H_{26}O_2$	6.3	4.06		202.33 g/mol
–						
2	2-Methoxy-4-vinyl phenol	$C_9H_{10}O_2$	10.0	6.36		150.17 g/mol
–						
3	9-Octadecene	$C_{18}H_{36}$	14.5	3.45		252.5 g/mol
–						

4	Methyl 11-octadecenoate	$C_{19}H_{36}O_2$	19.4	10.65		296.5 g/mol
–						
5	Hexadecanoic acid	$C_{16}H_{32}O_2$	24.4	7.06		287.61 g/mol
–						
6	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	$C_{18}H_{32}O_2$	27.6	5.43		280.4 g/mol
–						
7	Tetracosane	$C_{24}H_{50}$	30.6	11.52		389.0 g/mol

[figure(s) omitted; refer to PDF]

Table 4

GCMS analysis of ethanol extract of *Oenothera biennis.*

S. No	Name of the compound	Compound formula	Retention time (min)	Peak area (%)	Compound structure	Molecular weight
1	Furfural	$C_4H_5O_2$	9.3	6.53		96.08 g/mol
–						
2	4-Vinyl-2-methoxy-phenol	$C_9H_{10}O_2$	14.2	2.36		150.17 g/mol
–						
3	Hexadecanoic acid and methyl ester	$C_{17}H_{32}O_2$	16.5	1.42		268.4 g/mol
–						
4	7-Methyl hexadecane	$C_{17}H_{36}$	20.2	4.06		240.5 g/mol
–						
5	2,3,6-Trimethyl decane	$C_{11}H_{24}$	22.4	3.40		156.31 g/mol
–						
6	Squalene	$C_{30}H_{50}$	26.5	2.53		410.7 g/mol
–						
7	Phytol	$C_{20}H_{40}O$	30.3	7.45		296.5 g/mol

Table 5

GCMS analysis of ethyl acetate extract of *Oenothera biennis.*

S. no	Name of the compound	Compound formula	Retention time (min)	Peak area (%)	Compound structure	Molecular weight
1	Hexathiane	C_6H_8	15.3	1.06		80.13 g/mol
–						
2	Ethyl hexadecanoic	$C_{98}H_{184}O_{10}$	18.6	4.37		1522.5 g/mol
–						
3	2,3,6-Trimethyl decane	$C_{11}H_{24}$	22.4	3.16		156.31 g/mol
–						
4	n-Eicosane	$C_{41}H_{86}$	25.3	6.05		579.1 g/mol
–						
5	1,19-Eicosadiene	C_8H_{14}	28.5	5.32		110.20 g/mol
–						
6	9,12-Octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$	33.4	7.33		280.4 g/mol
–						
7	Tetradecanoic acid	$C_{14}H_{28}O_2$	45.5	1.56		228.37

[figure(s) omitted; refer to PDF]

3.3. Analgesic Activity

3.3.1. Writhing Method

The ethanolic and ethyl acetate extract of *Z. mauritiana* and *O. biennis* exhibited significant ($p<0.001$) analgesic activity when measured by acetic acid-induced writhing inhibition method at a dose of 300 mg/kg body (Table 6). The ethyl acetate and ethanolic extract of *Z. mauritiana* showed percent inhibition of 22.0%, 57.5%, 25.0%, and 72.6% at a dose of 100 mg/kg and 200 mg/kg body weight whereas the ethyl acetate and ethanolic extract of *O. biennis* showed percent inhibition of 24.0% and 64.1% and 29.1% and 59.4%, respectively, at a dose of 100 mg/kg and 200 mg/kg body weight which was comparable with the positive control diclofenac sodium (68.5%). The ethanolic extract of *Z. mauritiana* was more effective than *O. biennis* extracts.

Table 6

Analgesic activity of *Ziziphus mauritiana.* var *spontanea* and *Oenothera biennis* L. by writhing method.

Treatment	Dose (mg/kg)	*Ziziphus mauritiana*	*Oenothera biennis*

Average no. of writhing	% Inhibition	Average no. of writhing	Inhibition %	Normal saline	5 ml/kg
70.6±5.03	—	70.6±5.03	—	·	
Diclofenac sodium	10 mg/kg	22.3±2.51***	68.5	22.3±2.51***	68.5
–					
Ethyl acetate	100 mg/kg	60.66±9.45	14.1	58.66±0.57	17.0
200 mg/kg	55.00±4.00**	22.0	53.66±3.21**	24.0	300 mg/kg
30.00±5.00***	57.5	25.33±5.50***	64.1	·	
Ethanol	100 mg/kg	57.66±6.65*	18.4	61.66±6.65	12.7
200 mg/kg	53.00±4.35**	25.0	50.00±1.73***	29.1	300 mg/kg

Values are expressed as mean±standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

3.3.2. Hot-Plate Method

The ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* showed a significant ($p<0.001$) increase in latency time after 90 min at a dose of 200 mg/kg and 300 mg/kg body weight (Tables 7 and 8). Both the plants showed dose-dependent effects, and a significant result was obtained after 90 min at 300 mg/kg comparable with diclofenac sodium. The inhibition percentage at 200 and 300 mg/kg of ethyl acetate and ethanolic extract of *Z. mauritiana* was 46.7%, 56.0% and 38.1%, 52.3% while that of *O. biennis* 40.3%, 53.7% and 48.5%, 59.5% after 90 min, respectively. The highest central analgesic effect was shown by the ethanolic extract of *O. biennis* which was higher than diclofenac sodium.

Table 7

Analgesic activity of *Ziziphus mauritiana* by hot-plate method.

Treatment	Dose (mg/kg)	After 0 min	After 30 min	After 60 min	After 90 min	Percent decrease in latency time
Normal saline	10 ml/kg	3.30±0.62	4.20±0.36	3.73±0.41	4.53±0.80	…
–						
Diclofenac sodium	10 mg/kg	3.70±0.17	8.10±0.45***	9.23±0.37***	10.60±0.10***	57.2
–						

Ethyl acetate	100 mg/kg	3.50 ± 0.30	4.56 ± 0.15	4.13 ± 0.55	6.40 ± 0.52**	29.2
200 mg/kg	3.53 ± 0.87	4.63 ± 0.45	5.23 ± 0.95*	8.50 ± 0.17***	46.7	300 mg/kg
3.63 ± 0.20	4.73 ± 0.45	6.13 ± 0.50***	10.30 ± 0.45***	56.0	-	
Ethanol	100 mg/kg	3.40 ± 0.79	4.26 ± 0.40	3.80 ± 0.36	5.56 ± 0.15	18.5
200 mg/kg	3.30 ± 0.45	4.56 ± 0.11	4.70 ± 0.62	7.33 ± 1.05***	38.1	300 mg/kg

Values are expressed as mean ± standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

Table 8

Analgesic activity of *Oenothera biennis* L. by hot-plate method.

Treatment	Dose (mg/kg)	After 0 min	After 30 min	After 60 min	After 90 min	Percent decrease in latency time
Normal saline		3.30 ± 0.62	4.20 ± 0.36	3.73 ± 0.41	4.53 ± 0.80	…
–						
Diclofenac sodium	10	3.70 ± 0.17	8.10 ± 0.45	9.23 ± 0.37	10.60 ± 0.10***	57.2
–						
Ethyl acetate	100	3.40 ± 0.62	4.63 ± 0.41	3.96 ± 0.47	5.83 ± 0.41	22.2
200	3.50 ± 0.60	4.73 ± 0.90	4.40 ± 0.20	7.60 ± 0.60***	40.3	300
3.66 ± 0.90	4.86 ± 0.28	6.80 ± 0.62***	9.80 ± 0.55***	53.7	-	
Ethanol	100	3.46 ± 0.90	4.23 ± 0.45	4.33 ± 0.05	7.23 ± 0.95***	37.3
200	3.63 ± 0.45	4.30 ± 0.80	5.50 ± 0.26**	8.80 ± 0.45***	48.5	300

Values are expressed as mean ± standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

3.4. Anti-Inflammatory Activity

3.4.1. Carrageenan-Induced Paw Edema

A significant reduction ($p<0.001$) in the paw volume was revealed by ethanolic and ethyl acetate extract (100, 200, and 300 mg/kg) of *Z. mauritiana* and *O. biennis* at the 5th hour dose-dependently. The diclofenac sodium caused a significant ($p<0.001$) reduction at the 4th and 5th hours in the volume of the paw (Tables 8 and 9). The inhibitory effect of the ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* at 100 mg/kg, 200 mg/kg, and 300 mg/kg was 64.6%, 68.6%, 70.0%, 57.3%, 60.6%, and 66.0% at 5th hour while the inhibitory effect of the ethyl acetate and ethanolic extract of *O. biennis* at 100 mg/kg, 200 mg/kg, and 300 mg/kg was 53.3%, 68.0%,71.3%, 66.6%, 69.3%, and 73.3%, respectively, at 5th hour. The percent inhibition of ethanolic extract of *O. biennis* was more than the *Z. mauritiana* but less than diclofenac sodium (10 mg/kg) which showed the highest 75.4% inhibition in paw volume at the 5th hour.

Table 9

Anti-inflammatory activity of *Ziziphus mauritiana.* var. *spontanea*.

Treatment	Dose (mg/kg)	Paw volume after drug administration (mean + SEM)				
1 hour	2 hour	3 hour	4 hour	5 hour	Normal saline	10 ml/kg
0.90 ± 0.02	0.77 ± 0.08	0.86 ± 0.10	0.95 ± 0.02	1.50 ± 0.27	.	
Diclofenac sodium	10 mg/kg	0.80 ± 0.04 (11.11%)	0.54 ± 0.04* (29.8%)	0.45 ± 0.03* (47.67%)	0.52 ± 0.04*** (45.2%)	0.38 ± 0.08*** (75.4%)
–						
Ethyl acetate extract	100 mg/kg	0.86 ± 0.06 (4.44%)	0.68 ± 0.08 (15.58%)	0.75 ± 0.08 (12.7%)	0.65 ± 0.04** (31.57%)	0.53 ± 0.06*** (64.6%)
200 mg/kg	0.88 ± 0.07 (2.22%)	0.57 ± 0.08 (26.0%)	0.66 ± 0.06 (23.2%)	0.55 ± 0.08*** (42.1%)	0.47 ± 0.05*** (68.6%)	300 mg/kg
0.76 ± 0.09 (15.5%)	0.59 ± 0.06 (23.3%)	0.64 ± 0.07 (34.3%)	0.53 ± 0.09*** (44.2%)	0.45 ± 0.04*** (70.0%)	.	
Ethanolic extract	100 mg/kg	0.87 ± 0.03 (3.33%)	0.71 ± 0.04 (7.79%)	0.75 ± 0.18 (12.7%)	0.70 ± 0.14* (26.3%)	0.64 ± 0.19*** (57.3%)

200 mg/kg	0.86 ± 0.12 (4.44%)	0.66 ± 0.10 (23.2%)	0.70 ± 0.24 (26.3%)	0.64 ± 0.12** (57.3%)	0.59 ± 0.04*** (60.6%)	300 mg/kg

Values are expressed as mean ± standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

3.5. Antispasmodic Activity

3.5.1. Charcoal Meal Test

The ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* significantly ($p \leq 0.001$) decreased the distance travelled by a charcoal meal at a dose of 300 mg/kg body weight which was comparable to atropine sulfate (10 mg/kg) (Tables 10 and 11). The 300 mg/kg dose of ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* presented 64.4%, 56.8%, 67.2%, and 50.7% percent inhibition in the charcoal meal movement whereas the ethyl acetate extract of *Z. mauritiana* and *O. biennis* showed maximum percent inhibition of 64.4% and 67.2% in charcoal meal movement which was comparatively higher inhibition than the standard drug atropine sulfate. However, the ethyl acetate extract of *O. biennis* showed the highest percent inhibition (67.2%) than the ethyl acetate extract (64.4%) of *Z. mauritiana*.

Table 10

Anti-inflammatory activity *Oenothera biennis* L.

Treatment	Dose (mg/kg)	Paw volume after drug administration (Mean + SEM)				
After 1 hour	After 2 hour	After 3 hour	After 4 hour	After 5 hour	Normal saline	
0.90 ± 0.02	0.77 ± 0.08	0.86 ± 0.10	0.95 ± 0.02	1.50 ± 0.27	.	
Diclofenac sodium	10 mg/kg	0.80 ± 0.04 (11.11%)	0.54 ± 0.04* (29.8%)	0.45 ± 0.03* (47.67%)	0.52 ± 0.04*** (45.2%)	0.38 ± 0.08*** (75.4%)
–						
Ethyl acetate extract	100 mg/kg	0.86 ± 0.01 (4.44%)	0.67 ± 0.07 (12.98%)	0.72 ± 0.15 (16.27%)	0.64 ± 0.10** (57.3%)	0.70 ± 0.11*** (53.3%)
200 mg/kg	0.85 ± 0.02 (5.55%)	0.58 ± 0.08 (24.6%)	0.66 ± 0.05 (23.2%)	0.55 ± 0.11*** (42.1%)	0.48 ± 0.05*** (68.0%)	300 mg/kg
0.83 ± 0.03 (7.77%)	0.61 ± 0.09 (20.7%)	0.68 ± 0.11 (20.9%)	0.49 ± 0.09*** (48.4%)	0.43 ± 0.02*** (71.3%)	.	
Ethanolic extract	100 mg/kg	0.87 ± 0.07 (3.33%)	0.64 ± 0.11 (16.8%)	0.73 ± 0.15 (15.1%)	0.61 ± 0.07** (35.7%)	0.50 ± 0.06*** (66.6%)

200 mg/kg	0.87 ± 0.06 (3.33%)	0.59 ± 0.11 (23.3%)	0.67 ± 0.100 (22.0%)	0.5733 ± 0.13051*** (26.0%)	0.4633 ± 0.06028*** (69.3%)	300 mg/kg

Values are expressed as mean ± standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

Table 11

Antispasmodic activity of *Ziziphus mauritiana* var. *spontanea* and *Oenothera*.

Treatment	*Ziziphus mauritiana* var. *spontanea*				*Oenothera biennis* L.		
Dose	The mean length of the intestine	Mean distance travelled by charcoal	Percent inhibition (%)	The mean length of the intestine	Mean distance travelled by charcoal	Percent inhibition (%)	Normal saline + castor oil
10 ml/kg	55.2	46.06 ± 2.51	16.6	55.2	46.06 ± 2.51	16.6	
Atropine sulfate	10 mg/kg	54.3	20.60 ± 0.70***	62.0	54.3	20.60 ± 0.70***	62.0
–							
Ethyl acetate extract	100 mg/kg	50.6	34.63 ± 3.61	31.6	49.5	36.63 ± 10.66	26.0
200 mg/kg	51.3	29.13 ± 3.82*	43.2	54.3	30.80 ± 4.96*	43.2	300 mg/kg
52.3	18.65 ± 0.21***	64.4	54.6	17.90 ± 2.26***	67.2		

Ethanolic extract	100 mg/kg	52.5	37.3 ± 3.70	28.9	53.2	34.80 ± 15.39	34.5
200 mg/kg	49.4	29.3 ± 2.72*	40.6	54.4	32.30 ± 9.83	40.6	300 mg/kg

Values are expressed as mean ± standard deviation. Significance is shown as *significant, **more significant, and *** highly significant.

4. Discussion

For peripherally acting drugs, the acetic acid-induced abdominal constriction test is used. The pain induction occurs by releasing endogenous substances and other pain mediators such as prostaglandins [28]. The dosage of 300 mg/kg of ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* significantly ($p<0.001$) inhibited the writhing's paralleled to diclofenac sodium ($p<0.001$). The ethanolic extract of *Z. mauritiana* was more effective in inhibiting the writhing response which showed comparatively higher percent inhibition 72.6% in writhing than the standard drug diclofenac sodium (68.5%) (Table 6). The results indicated that the reduction in pain was dose-dependent; hence, the 300 mg/kg dose proved to be most effective. These results are in line with [29] who reported effective analgesic results from methanolic extracts of *Phyllanthus seeds*. Pain inhibitory activity of plant extracts may be interrelated to the inhibition of prostaglandin synthesis [30]. The analgesic activity of *Z. mauritiana* and *O. biennis* might be due to several bioactive constituents such as terpenoids, flavonoids, tannins, alkaloids, and steroids which were detected in plant extracts. Flavonoids showed analgesic action by increasing the endogenous serotonin level or its interaction with 5-HT2A and 5-HT3 receptors. Acetic acid-induced writhing has been connected with upgraded levels of prostaglandin (PGE2 and PGF2α) in the peritoneal liquids and the lipoxygenase items [31]. The ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* possibly showed analgesic action by inhibiting the synthesis of the arachidonic acid metabolite. The hot-plate test has been used for the assessment of centrally mediated analgesic responses, which emphasizes mostly changes above the spinal cord level. The findings revealed that after 90 min at 200 mg/kg and 300 mg/kg, the ethyl acetate and ethanolic extract of *Z. mauritiana* and *O. biennis* significantly ($p<0.001$) increased in the reaction time (Tables 7 and 8). The effect increased with an increase in time and dose, and a greater effect was attained after 90 min at a higher dose. The ethanolic extract of *O. biennis* was more effective which showed a maximum percent increase (59.5%) in reaction time which was comparatively higher than the standard drug diclofenac sodium which showed a 57.2% percent increase in reaction time. These results are in line with [32] who reported similar results from the Moroccan medicinal plants. The *Z. mauritiana* and *O. biennis* showed antinociceptive activity in the hot-plate test by increasing the latency to discomfort. This action could be stimulating the periaqueductal gray matter to release endogenous peptides (endorphins or encephalins) [33]. These endogenous peptides run down the spinal cord and at the synapse in the dorsal horn and function as inhibitors of pain impulse transmission. *Z. mauritiana* and *O. biennis* showed central analgesic activity due to their action on the central opioid receptors or promoted release of endogenous opioid peptides. The analgesic activity of *Z. mauritiana* and *O. biennis* might be due to several secondary metabolites such as tannins, flavonoids, steroids, alkaloids, and terpenoids which were detected in plant extracts (Table 1). Flavonoids are involved in the management of pain by increasing the quantity of endogenous serotonin or by interacting with various receptors. Alkaloids have also been associated with the ability to inhibit pain perception [34, 35].

Anti-inflammatory drugs can be tested by the carrageenan-induced inflammation model [36]. Acute inflammation is

produced in the rat paw by the subcutaneous injection of carrageenan, and certain mediators including histamine, prostaglandin, and serotonin are released causing fever and pain. The findings indicated that ethyl acetate and ethanolic extract (100, 200, and 300 mg/kg) of *Z. mauritiana* and *O. biennis* at the 5th hour exhibited significant inhibition ($p<0.001$) in the volume of paw but less than the diclofenac sodium ($p<0.001$) (Tables 9 and 10). The high percent inhibition (73.3%) in paw edema was caused by ethanolic extract of *O. biennis* at the 5th hour at 300 mg/kg when compared to *Z. mauritiana* but less than the standard drug diclofenac sodium (75.4%). The ethanolic extract significantly suppressed edema in 1st phase, which might primarily be credited to the drop in the release and synthesis of serotonin and histamine. The anti-inflammatory activity shown by both plants was dose-dependent and time-dependent revealing similar dose-dependent and time-dependent anti-inflammatory activity from the ethanolic extract and ethyl acetate of *Albizia lebbeck* (L.) Benth. and *Senna sophera* (L.) Roxb. Several secondary metabolites such as steroids, tannins, flavonoids, terpenoids, and alkaloids might be responsible for the anti-inflammatory activity of *Z. mauritiana* and *O. biennis* (Table 1). Flavonoids inhibit significant enzymes involved in the biosynthesis of tissue activators, especially prostaglandins and arachidonic acid [37]. The triterpenes possess anti-inflammatory potentials and prevent the production of inflammatory mediators [38].

The effect of drugs on peristaltic movement can be tested by charcoal meal test [21]. The irritation and inflammation of intestinal mucosa can result from the hydrolysis of castor oil into ricinoleic acid which results in diarrhea. It results in the release of prostaglandins which provoke gastrointestinal motility and result in secretion of water and electrolytes [22]. The result showed that ethyl acetate ($p<0.001$) and ethanolic extract ($p<0.01$) of *Z. mauritiana* and *O. biennis* at a dose of 300 mg/kg showed a significant reduction in the distance travelled by charcoal meal when compared with atropine sulfate which also showed significant ($p<0.001$) reduction in charcoal meal movement (Table 11). The extract showed a dose-dependent inhibition of charcoal meal motility. Thus, 300 mg/kg doses of both plants' extracts had more antimotility effect. The ethyl acetate extract of *Z. mauritiana* and *O. biennis* was more effective and showed higher activity than atropine sulfate. However, the maximum antimotility effect was shown by the ethyl acetate extract of *O. biennis* than the ethyl acetate extract of *Z. mauritiana*. The inhibition in the peristaltic movement of the gastrointestinal tract might be due to the inhibition of acetylcholine by the plant extracts resulting in the absorption of water and electrolytes [39]. The antispasmodic activity of *Z. mauritiana* and *O. biennis* might be due to the presence of secondary metabolites such as flavonoids, alkaloids, steroids, phenol, terpenoids, phytosterol, and tannins which were detected in plant extracts (Table 1). Tannins present in the extract form protein tannates by precipitating the proteins in the intestinal mucosa which helps in the protection of intestinal mucosa by making it more resistant to certain chemicals [40]. Flavonoids and steroids help in absorbing electrolytes by inhibiting secretion induced by castor oil. Alkaloids and terpenoids are known to inhibit the secretion induced by castor oil by inhibiting the release of autocoids and prostaglandin [41, 42].

5. Conclusion

In the current research, both plants showed important phytochemicals and therapeutic potential. From the results, it can be concluded that *Z. mauritiana* and *O. biennis* contained important chemical constituents including alkaloids, tannins, saponins, flavonoids, steroids, and triterpenoids as determined by phytochemical screening. GCMS revealed the presence of pharmacologically active compounds such as hexadecanoic acid, 4-vinyl-2-methoxy-phenol, n-eicosane, and 2,3,6-trimethyldecane which may be responsible for the significant anti-inflammatory, analgesic, and antispasmodic activity. Hence, these plants can be used for alleviating pain and treating various inflammatory and diarrhoeal disorders. Both plants can be used in the future for drug development and various herbal formulations having fewer side effects.

Disclosure

The authors declare that they have no conflict of interest.

Authors' Contributions

A.A. administrated the project and proposed methodology, M.A. wrote the original draft, and M.N. edited, supervised, and visualized the study. F.Z.F. developed software, collected data, and provided funding.

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DETAIL

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Diversity of Production Techniques and Microbiology of African Cereal-Based Traditional Fermented Beverages

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ABSTRAK (ENGLISH)

Traditional fermented beverages are culturally and socially accepted products for consumption, drinking, entertainment, customary practices, and for religious purposes. The purpose of this review was to identify some cereal-based fermented beverages and determine the differences in their production technologies. There are many unique regional variations in the preparation of each of the identified fermented beverages. They are prepared from raw materials such as maize, millet, rice, and sorghum. Majority of the fermented alcoholic beverages (binuburan, amba beer, sake, dolo, pito, and tchoukoutou) were produced using spontaneous fermentation and industrial fermentation (use of starter cultures) techniques. The various microbial communities associated with the traditional fermentation processes were dominated by *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* for Lactic acid bacterial (LAB) species, *Saccharomyces cerevisiae* and *Candida mycoderma* for *Saccharomyces* and *Candida* species (yeasts), respectively; and *Aspergillus aceti* and *Rhizopus stolonifer* for *Aspergillus* and *Rhizopus* species (molds), respectively. *Acetobacter, Pseudomonas, Klebsiella, Weissella, Achromobacter, Flavobacterium, Micrococcus,* and *Bacillus* dominated other microbial genera. The involvement of lactic acid bacteria contributed to the safety and extension of the shelf life of the final products. Most of these beverages were found to be very rich in proteins, carbohydrates, calories, and B-group vitamins including thiamine, folic acid, riboflavin, and nicotinic acid. This article reviewed the available information, such as processing techniques of African traditional beverages, the raw materials used to producing them, and the microorganisms associated with the production processes.

TEKS LENGKAP

1. Introduction

Traditional fermented foods and beverages are culturally and socially accepted products for several reasons

including consumption, entertainment, customary practices, and for religious purposes [1]. Production and drinking of alcoholic and nonalcoholic beverages are widespread interest in enhancing the nutritional significance as well as impacting the pleasure of drinking [2]. Fermented alcoholic and nonalcoholic beverages include a wide range of fermented products including wine and beer [3]. These ethnic alcoholic beverages have a great ceremonial significance among African ethnic groups [4].

Several traditional fermented beverages, both alcoholic and nonalcoholic are produced at the household level in the world. Each fermented beverage has several regional differences in its production process. Therefore, there is a need to identify these beverages with the raw materials used for their production, various variations that exist among their production techniques, the diversity of microbial fermenters that were involved in each of the production techniques of these beverages, and their health benefits.

The majority of these beverages are prepared basically from cereals such as maize, millet, rice, and sorghum. Species of lactic acid bacteria [5] and yeasts [6] are the major microbial fermenters associated with the fermentation of these beverages. They serve to improve the taste, flavor, acidity, digestibility, and texture of both alcoholic and nonalcoholic beverages. The majority of these beverages are high in calories as well as B-group vitamins such as thiamine, folic acid, riboflavin, and nicotinic acid.

This study sought to review the available information such as the processing techniques involved in the production of these beverages, species of the various microorganisms involved in the fermentation processes, taxonomic tools used to identify these microbes, and the nutritional diversity of these traditional fermented beverages in the African countries.

2. Diversity of Traditional Fermented Beverages

Several types of cereal-based traditional fermented beverages are produced and consumed worldwide. Mostly, the preparation and fermentation of these beverages are done spontaneously; hence, they are characterized with different microbial communities. The involvement of this microflora determines the qualities of fermented products [7, 8]. The common processing stages characterizing them include cooking raw materials to gelatinize the starch, adding a source of enzymes to hydrolyze the gelatinized starch into fermentable sugars, and allowing them to go through fermentation [9]. In addition to microorganisms, the raw materials used, the production techniques adopted and the regions or countries where they are produced contribute to the variations. For example, *mahewu* (Zimbabwe) is produced from maize meal and basic ingredient, *ogi* (Nigeria) is manufactured from a three-day soaked maize grain [10]; and *Kwete* (Uganda) produced from a roasted sourdough for its unique golden-brown color and a typical Kwetey flavor [11]. According to Aka et al. [12] and Kouame et al. [13], tchapalo is also produced from two processing steps (i.e., a spontaneous lactic fermentation or backs-lopping is firstly initiated to obtain a sweet wort which is nonalcoholic, followed by alcoholic fermentation). These differences in the production process, microbial communities, raw materials, and the regions of production lead to variations in sensory characteristics and nutritional qualities of final products.

To iron out these differences, the use of defined starters of both lactic acid bacteria and yeasts or their combinations has been suggested [12, 14, 15]. These starters have the potential to rapidly acidify the fermenting products, eliminate or reduce considerable potential spoilage and pathogenic bacteria, improve organoleptic properties and above all, control the fermentation processes [12, 16, 17]. Some of the popular cereal-based traditional fermented beverages that are produced and consumed across the African continent are discussed below.

2.1. Oshikundu

Oshikundu is a popular daily traditional cereal-based fermented and very low alcoholic beverage made from pearl millet and malted sorghum meal produced and consumed in Namibia [18]. The ultimate product of Oshikundu is produced through the processing of raw materials, storage, and traditional milling of malted sorghum and Mahangu meal. In the first step, hot water is added to the Mahangu meal, which is then allowed to cool to room temperature while being stirred occasionally. After that, a malted sorghum meal is added to the mix. The mixture is then blended after adding warm water. Both techniques include the addition of bran, which is optional depending on availability and preference for bran in brewing. The mixture is diluted with water based on the amount of the starting material

used and the desired volume of the final product after previously fermented Oshikundu is added. After that, the mixture is then left to ferment spontaneously for one and a half hours [18]. Oshikundu, however, has a very short shelf life of less than six hours [19].

2.2. Finger Millet Slurries

Finger millet fermented slurries are mostly prepared and consumed by the people of Hwedza, Zimbabwe. The products are prepared from four different varieties of finger millet; red variety 1 (RV1), red variety 2 (RV2), white variety 1 (WV1), and white variety 2 (WV2). In short, fermented slurries are made by adding water to an aliquot of millet flour and allowing the entire mixture to ferment for 24 to 36 hours [20]. The presence of foam on the surface of the fermenting slurry indicated a successful fermentation. The fermenting wort is then cooked to make porridge (Figure 1).

[figure(s) omitted; refer to PDF]

2.3. Pito

Pito is an indigenous light brown alcoholic slightly bitter sweet-sour beverage with a fruity flavor made from malted, mashed maize, or sorghum fermentation. Kolawole et al. [21] indicate that the people of northern Nigeria and Ghana are the main producers of pito. Pito is made by soaking cereal millet grains in water for two days. The soaked grains are malted in baskets lined with moistened banana leaves for five days. The malted grains are crushed and cooked with water. After cooling, the mash is filtered through a fine-mesh basket, and the filtrate is left to ferment until it has a somewhat sour flavor. The mixture is then boiled to a concentrate. The cooled concentrate is mixed with a starter from the previous brew and fermented overnight again. Pito is made up of lactic acid, carbohydrates, and amino acids, with a 3% alcohol concentration [21].

2.4. Burukutu

Burukutu is a traditional fermented alcoholic beverage mainly by people of Northern Guinea Savannah of Nigeria, but brewed and consumed in other African countries such as Benin and Ghana. The beverage is brewed with varieties of sorghum and/or maize malts [22]. It has an acidic taste due to the action of *Lactobacillus* species in particular, and an opaque color due to suspended solids and yeast components with a thin consistency [23, 24]. Indigenous *burukutu* is produced by steeping sorghum grains in water overnight, washing the soaked grains and draining to remove excess water. The grains are then spread on banana leaves and watered while turning and waiting for germination. The malted grains are then sun dried, grinded, and then mixed with water and boiled for hours. After that, the mixture is allowed to ferment for 48 hours. *Burukutu* is a murky liquid with a vinegary flavor and odor. It has high calories, B vitamins, as well as vital amino acids contents [22].

2.5. Bantu Beer/Kaffir Beer

The Bantu tribe in South Africa produces and consumes Bantu beer (also known as kaffir beer). It is an alcoholic pinkish-brown effervescent beverage with a sour flavor, thin gruel viscous, and an opaque appearance [25]. Malted sorghum or maize grains are used for brewing *Bantu* beer. To speed up the amylolytic activities, the malt is pulverized, slurried to a thin gruel, boiled, and chilled, and a small amount of fresh, uncooked malt is added. The mixture is kept overnight, boiled, and allowed for alcoholic fermentation. More pulverized uncooked malt is added on the third and fourth day and then strained on the fifth day to remove the husks to get the beer ready for consumption [26].

2.6. Amgba

Amgba is a traditional fermented alcoholic beverage of some ethnic groups of Cameroons [27, 28]. It is brewed with either sorghum or millet malt. Two sequential phases of fermentation under ambient conditions are used in the manufacture of amgba. It has lactic acid fermentation and alcohol fermentation stages [29]. Typically, alcoholic fermentation begins by pitching wort with previously brewed or dried yeast obtained from bili bili [30]. The indigenous brewing process has been described by Nanadoum [30] in Figure 2.

[figure(s) omitted; refer to PDF]

2.7. Tchoukoutou

Tchoukoutou is a cereal-based opaque traditional alcoholic beverage produced in Benin and Togo. The principal

raw materials include sorghum, millet or maize malts [31]. The process involved in *tchoukoutou* production has been described by Kayodé et al. [31]. The malt of any of the raw materials preferred for the production is milled into a fined flour, mixed with water, and left for a few hours for enzymatic action. The mixture is gradually heated and finally boiled. After cooling, kpètè-kpètè is added for fermentation for about 14 h (Figure 3). It has about 4% (v/v) ethanol content (A. [31], and is very rich in iron, solid, and crude protein [32].

[figure(s) omitted; refer to PDF]

2.8. Dolo

Dolo is an important and a popular cereal-based traditional fermented beverage brewed and used in Burkina Faso. The traditional process (Figure 4) is similar to that of *ikigage* beer. Briefly, sorghum malt flour is mixed with water, decanted, and the precipitate is mixed with water and heated to gelatinize the starch, but the supernatant is kept unboiled [33]. After cooling, the previous supernatant is added and heated at 65−70°C for 12–16 h. Cooked wort is chilled and fermented overnight. The cooling wort is injected with a typical leaven to initiate fermentation, which results in dolo beer after 12–24 hours [34] fermentation. *Dolo* beer is opaque, with a red color, and an alcohol content of 2–4% [33].

[figure(s) omitted; refer to PDF]

2.9. Bushera

Bushera is the most widely consumed traditional cereal-based fermented alcoholic beverage in Uganda's Western highlands. Sorghum or millet flour is made by mixing boiling water with germinated sorghum and millet grains. The mixture is then allowed to cool to room temperature. After that, germinated millet or sorghum flour is added, and the mixture is allowed to ferment for 1–6 days at room temperature and *bushera* is ready for consumption [35].

2.10. Ogi

Ogi is a sour, white starchy beverage made from either fermented maize, sorghum, or millet. It is a common cuisine in the West African countries, and it is also used as a weaning food for babies. The traditional method of making *ogi* is soaking corn kernels in water for 1 to 3 days, then wet grinding and filtering to remove the bran, hulls, and germ [36]. The pomace is then retained on the filter and discarded as animal fodder, while the filtrate is then fermented for 48 to 72 hours to produce ogi. Prior to eating, ogi is diluted to an 8–10% solid content and boiled into a pap, or heated and converted into a stiff gel called "agidi" or "eko." [36].

2.11. Mahewu

Mahewu *(amahewu)* is a sour beverage prepared from corn flour that is popular in Africa. According to, mahewu is a fermented millet or sorghum beverage consumed in Zimbabwe. It is made from maize porridge that has been mixed with water. Thereafter, the sorghum, millet malt, or wheat flour is added, and the mixture is allowed to ferment. The natural flora of the malt performs the spontaneous fermentation process at room temperature [37]. Mahewu has a pH of roughly 3.5. It is a daily adult food that is also often used for weaning and as a preferred food for the sick due to its liquid condition and flavor. Traditional mahewu is produced in the same way as industrialized mahewu, with the exception that the latter uses a starting culture [38]. Mahewu is consumed between 24 and 48 h after preparation.

2.12. Kirario

Kirario is a lactic acid-fermented gruel made from maize and millet that is native to Kenya. It is frequently used by the natives as a low-cost meal. Different groups of people including adults who are recovering from circumcision are given as a special beverage. Kirario is traditionally processed by wet milling green maize, then mixing it with dried millet flour and water and grinding it to finer particles. At room temperature, the mixture is left to spontaneously ferment for two days. To produce porridge, the fermented slurry is cooked [39]. Traditional processed kirario has a high acidity level of about 3.0 to 3.5. When proper hygiene protocols are followed, kirario might have a shelf life of about one week or more [39].

2.13. Kunun-Zaki

Kunu-zaki is a native nonalcoholic fermented drink popular in northern Nigeria [40]. It is a millet-based beverage which is consumed within few hours of production [41]. *Kunu* is consumed in place of soft drinks [40]. According to Agarry et al. [4]; fermentation by chance inoculation and rudimentary equipment are used in *kunu* production

process (Figure 5). It has a short shelf life [2], however, hydrolytic enzymes have been used to increase the nutritional and sensory quality of the product as well as extend the shelf life [42]. According to Umoh et al. [43], *Kunun-zaki* produced through a traditional fermentation technique contains significant levels of spoilage and harmful microbes, which may account for its short shelf life. The beverage contains high amounts of carbohydrates, protein, fat, ash, zinc, calcium, iron, and manganese [44].

[figure(s) omitted; refer to PDF]

2.14. Ting

Ting is a traditional South African and Botswana fermented sorghum beverage. It is known as "*letting*" in South Africa. In preparation, sorghum flour is mixed with warm water to produce a slurry, which is then left to spontaneously ferment in a warm environment for 2-3 days. Alternatively, it can be inoculated with a previously fermented batch of ting. Depending on the starting material, the mixture can be fermented for about 6 to 24 h. While the soured slurry is used to make ting porridges of various consistencies, the soft porridge (motogo) is used for weaning or as breakfast for adults [38].

2.15. Aliha

Aliha is a beverage spontaneously fermented and used by several communities in Ghana. The name *Aliha* was derived from its native origins in the Ewe language of Ketu in Ghana, Volta region. [45]. It is produced using corn malt ("*hali*") as the raw material. The setup is then allowed to ferment which is known as "*aha*," hence, the name (*aliha*). During the production, maize is soaked overnight, and allowed to sun dry (malted). The malts are then ground or milled, mashed, and allowed to boil until no foam is found on the surface. The wort is allowed to ferment for three days after straining, and caramel or burnt sugar is added to obtain the unique brown color and flavor for consumption (Figure 6) [45]. Aliha has to be packaged and refrigerated immediately after production else fermentation continues to a level where it becomes too sour for human consumption [46]. Aliha is regarded as a refreshing drink by the community members, as well as a source of necessary nutrients and certain medicinal properties.

[figure(s) omitted; refer to PDF]

3. Classification of Traditional Fermented Beverages

Indigenous fermented beverages are consumed all over the world. Their production processes differ from place to place based on several factors including the raw materials used in the production, where the raw materials are cultivated (regions), the production techniques employed, the microbial composition whether exist naturally or added as defined starters and many more. This section of the review would take critical look at the different raw materials and fermentation techniques employed in manufacturing the traditional beverages across the African countries.

3.1. Classification by Raw Materials

The indigenous fermented beverages are unique to those who produce them in a particular geographical location using the raw materials readily available to them. The traditional recipes developed for processing fermented food and beverages are handed down from generation to generation and still considered by both developing and under-developed countries [47]. Most of these indigenous recipes were developed around cereals as the raw materials. However, as modernization and new technologies of food manufacturing begin to manifest, several other raw materials (Table 1) are been used to produce the same beverages probably with better qualities. Cereal utilization started in Neolithic era and still continues to be the most essential source of food worldwide [70]. Cereal-based beverages play many important roles in human life and are considered the major sources of energy for humans [71]. They form key components of human diets for several years and continue to be the main sources of nutrition in both developed, developing, and underdeveloped countries [72]. Like other sources of beverage, cereal-based fermented beverages are used as special vessels for nutrition enhancement [73]. Table 1 presents the common and important cereals that are regularly used for traditional fermented beverages.

Table 1

Type of cereals used for the production of African traditional fermented beverages.

Beverage	Raw material	Country	Reference
Aliha	Maize	Ghana	Madilo et al. [45]
Bushera	Sorghum, millet flour	Uganda	Marsh et al. [48]; Aka et al. [49]
Kwete	Maize, millet	Uganda	Enujiugha and Badejo [50]
Malwa	Finger millet	Uganda	Aka et al. [51]
Koko sour water	Cereal (pearl millet)	Ghana	Marsh et al. [48]
Koko	Maize	Ghana	Lei et al. [52]
Pito	Maize, sorghum, maize, sorghum	Ghana, Nigeria	Kolawole et al. [21]; François et al. [53]
Ice-kenkey	Maize	Ghana	Atter et al. [54]
Burukutu	Sorghum	Ghana	Blandino et al. [55]
Mahewu	Maize, sorghum/millet	Zimbabwe	Marsh et al. [48]
Doro	Finger and bulrush millet/sorghum	Zimbabwe	Gadaga et al. [56]; Jane et al. [57]
Mangisi	Millet	Zimbabwe	Aka et al. [49]
Togwa	Maize flour, finger millet malt,	Tanzania	Marsh et al. [48]
Ogi, akamu	Maize, sorghum, millet	Nigeria	Enujiugha and Badejo [58]
Kunun-zaki	Millet, sorghum	Nigeria	Oguntoyinbo et al. [59]; Enujiugha and Badejo [50]
Burukutu	Sorghum	Nigeria	Fadahunsi and Soremekun [60]
Oti-oka	Maize, millet, sorghum	Nigeria	Ogunbanwo and Ogunsanya [61]
Gowe	Sorghum	Benin Republic	Enujiugha and Badejo [58]
Tchoukoutou	Sorghum (and millet or maize)	Benin, Togo	Polycarpe Kayode et al. [62]

Mageu	Maize, wheat south	South Africa	Enujiugha and Badejo [50]
Bantu beer	Sorghum, maize malt	South Africa	Taylor [25]
Umqombothi	Sorghum, maize	South Africa	Shephard et al. [63]
Borde	Maize, finger millet, tef	Ethiopia	Enujiugha and Badejo [58]; Aka et al. [49]
Areki	Millet, sorghum, maize	Ethiopia	Tafere [64]
Keribo	Barley	Ethiopia	Tafere [64]
Tella	Barley, maize, millet, sorghum	Ethiopia	Tafere [64]
Bogobe	Sorghum	Botswana	Blandino et al. [55]
Dolo	Red sorghum	Burkina Faso	Lyumugabe et al. [22]
Bel-saalga	Pearl millet	Burkina Faso	Tou et al. [65]
Amgba	Sorghum (and millet)	Cameroon	Lyumugabe et al. [22]; Aka et al. [51]
Sha	Maize	Cameroon	Abia et al. [66]
Oshikundu	Rice flour+ginger	Namibia	Embashu et al. [18]; Embashu [19]
Oshikundu	Millet, sorghum	Namibia	Mu et al. [67]; Embashu [18]
Bouza	Wheat	Egypt	Blandino et al. [55]
Busa	Rice or millet	Egypt	Blandino et al. [55]
Kishk	Wheat	Egypt	Blandino et al. [55]
Busaa	Maize	Kenya	Katongole [68]; Aka et al. [49]
Tchapalo	Maize	Cote d'Ivoire	Aka et al. [69]; Aka et al. [51]

3.2. Classification by Fermentation Type

3.2.1. Malted Alcoholic Fermented Beverages

Malting according to MacLeod and Evans [74] and Taylor and Taylor [75] is "a limited controlled germination of grains in moist air, which results in the mobilization of amylases, proteases, and other enzymes which hydrolyze and modify grain components and its structure." The process has been used during the production of food and beverages to develop enzymes needed for fermentation, structural change in the endosperm of the grains into a form that is more readily utilized or extracted in the brewing process, and to develop distinctive malt colors, aromas and flavors [75]. Several African cereal-based alcoholic beverages are produced through malting. During malting

processes, hydrolytic enzyme production or activation is maximized leading to the degradation of cell wall and protein solubilization with a minimal starch breakdown [76], an essential component of fermentation.
Meanwhile, the processes involved in the production of malted alcoholic fermented beverages varied from one location to another. However, they are basically produced by subjection of the raw materials (cereals) to malting, mashing, souring, straining, boiling, and fermentation [51]. African malted alcoholic fermented beverages are manufactured by spontaneous fermentation. Spontaneous alcoholic fermentation involves species of LAB and yeasts [77]. It consists of lactic acid fermentation caused by a range of environmental microbes and alcoholic fermentation caused by dried yeast or a fraction of earlier brew [78]. As lactic acid fermentation results in production of nonalcoholic beverages, lactic acid and alcoholic fermentations are set out for alcoholic beverages [79]. According to N'Guessan et al. [80]; whereas lactic acid fermentation is caused by a complex population of environmental microbes as a source of souring taste and storage longevity, alcoholic fermentation is initiated by pitching the wort with a portion of previously fermented brew or dried yeast harvested from the previously fermented beverage. Men are the primary consumers of alcoholic beverages [51, 81]. They are consumed at several social gatherings including festivals, weddings, and funerals [77] in Africa and many developing countries which produce them.
African fermented malted beverages have prolonged shelf-lives due to the production of antimicrobial metabolites (e.g., carbon dioxide, ethanol, and hydrogen peroxide) by LAB; anti-inflammatory, antidiarrheal, antibacterial, antitumor, antispasmodic, laxative, antihemorrhoid, and antioxidant properties [3, 82, 83]. Table 2 presents the list of some malted traditional alcoholic beverages, the raw materials used for their preparations, their alcoholic compositions and fermentation periods.

Table 2

Summary of some African cereal-based malted alcoholic fermented beverages.

Main raw material	Name of beverage	Alcohol content	Fermentation period	Reference
Millet	Boza	Up to 1.5%	24 hours at 15−30°C	Tangüler [84]; Yegin; and Fernandez-Lahore [85]
Pearl millet	Oshikundu	2% or less	1 hour 30 mins	Embashu et al. [18]; Embashu [19]
Rice	Binuburan	18% or less	3-4 days at 35–37°C	Bhalla [86]
Millet	Finger millet slurries	2–4%	24–36 hour	Gabaza et al. [20];
Guinea corn	Burukutu	0.78 g/kg	48 hour at 25–30°C	François et al., [53]; Egemba and Etuk [87]
Maize	Pito	5% and above	2-3 days at 25–30°C	Kolawole et al. [21]
Sorghum/maize	Bantu/kaffir beer	Below 2%	5 days at 30°C	Altay et al. [25, 88]
Sorghum/millet	Amgba beer	4.5–7%	2-3 days at 30°C	Mbaiguinam et al.[30, 89]

Finger millet	Kodo ko jaanr or Chyang	4.8% or less	3-4 d (summer) and 5–7 d (winter)	Thapa and Tamang [90]
Sorghum	Tchoukoutou	4% or less	2-3 days at 30°C	Kayodé et al. [31, 32]
Rice	Sake	15–20%	2-3 d at 10° to 15°C	Yoshizawa and Ishikawa, [91]
Malted red sorghum	Merissa	Up to 6%	N/A	Lyumugabe et al. [22]
Red sorghum	Dolo	2–4%	12−24 h at room temp	Dicko et al. [33]; Nanadoum et al. [34]
Sorghum	Ikigage or Urwagwa	2.2%	12 to 24 h at room temp	Lyumugabe et al. [92];
Sorghum	Bushera	N/A	1–6 d at 27–30°C	Muyanja et al. [35]
Rye, barley	Kvass	1.5% or less	N/A	Marsh et al. [48]
Pearl millet	Oti-oka	1.56%	3 days at 30 C	Ogunbanwo and Ogunsanya [61]

3.2.2. Malted Nonalcoholic Fermented Beverages

Traditional cereal-based nonalcoholic fermented beverages are also prepared using spontaneous fermentation techniques [77] just like alcoholic fermented beverages. They are prepared from a single or combination of malted barley, maize, millet, oats, rye, sorghum, and wheat [93, 94] as essential sources of dietary proteins [95], energy, carbohydrates, vitamins, minerals, and fibre (arabinoxylan and β–glucan) [77]. They are however deficient in lysine, an essential amino acid [96, 97]. These beverages are drunk by individuals of all ages, particularly youngsters, pregnant women, the sick, and the elderly, and can be used to wean children [49]. They improve lactation in mothers and prevent coronary diseases and cancer.

Regulations and laws covering nonalcoholic beverages vary from country to country. For instance, EU regulation no. 1169/2011 states that an alcoholic beverage must have an alcoholic strength of 1.2% and above; in Great Britain, alcoholic content of nonalcoholic beverage should not be more than 0.05%; Germany has a limit of 0.5%; Spain, France, USA, China and Japan set a maximum of 1%; 1.2%; 0.5%, 0.5% [97], and 1% [98], respectively. Additionally, boza has an alcohol content of not more than 1% in Turkey, but up to 7% in Egypt [99]. The variations in alcoholic contents of cereal malted fermented beverages could be due to several factors including microflora compositions [99]. The combination of cereals with legumes, vegetables, fruits and spices can also lead to these variations.

Nonalcoholic cereal-based fermented beverages serve as alternatives to alcoholic beverages and are used to quench thirst, as nutrition-added value, and have cultural significance [100]. Through fermentation, nonalcoholic beverages have enhanced sensory characteristics, chemical properties, and some bioactive compounds and therapeutic agents significant for human health. Table 3 exhibits a few of these beverages, their raw materials, duration of fermentation, and functional compounds. Maize and millet dominated the cereals used as raw materials used for the production of the selected beverages (Table 3). Additionally, the uniqueness of the nonalcoholic beverages depends largely on the selection of the cereals, microbiota involved, fermentation duration and temperature, and other additional food matrices [109–111].

Table 3

Summary of some African cereal-based malted nonalcoholic fermented beverages.

Beverage	Raw material	Fermentation period	Functional compounds	Reference
Aliha	Maize	72 h at ambient temperature	Protein, iron, carbohydrates, ash, fats, calcium, phosphorus	Kwashie Felix et al. [46]
Sobia	Cereal malt	24 h at ambient temperature	Dietary fibre, amino acids, fatty acids, vitamins B1, B2	Gassem [101]
Koko	Millet	2−12 h at ambient temperature	Group B vitamins; dietary fibre	Lei and Jakobsen [102]
Mahewu	Maize	12−24 h at ambient temperature	Sodium, potassium, calcium, iron, zinc; fibre, carbohydrate, group B vitamins	Mugochi et al. [103]; Vasudha and Mishra [37]
Kirario	Green maize and millet	2 d at ambient temperature	Dietary fibres, amino acid, fatty acid, B1, and B2 vitamins	Kunyanga et al. [39]
Kununzaki	Millet	8 h at ambient temp	Minerals (Fe, Ca, Mg, and K)	Agarry et al. [4]; Obadina et al. [40]
Mawe (akassa)	Maize	1–3 days	N/A	Hounhouigan et al. [104, 105]
Pozol	Maize	0.5–4 d at ambient temperature	N/A	Wacher et al. [106]; Ampe et al. [107]
Ting	Sorghum	2-3 d/6−24 h at warm temperature	B1, B3, B2, and B6 vitamins; dietary fibres, zinc, copper, maltoses, maltotrioses, glucoses, fructose	Sekwati-Monang, [38]
Malwa	Millet	2–4 days	Dietary fibre, amino acids, fatty acids, vitamins B1, B2	Muyanja et al. [6]; Lyumugabe et al. [92]
Mangisi	Millet sweet-	8 h at ambient temp	N/A	Blandino et al. [55]
Togwa	Maize/millet	24 h at ambient temperature	Amino acids and some minerals (Fe, Ca, Zn, P)	Mugula et al. [108]

Borde	Millet	12 h at ambient temperature	N/A	Enujiugha and Badejo [50]
Gowe´	Maize/millet	6−24 h at ambient temperature	Amino acids and some minerals (Fe, Ca, Zn, P)	Aka et al. [49]
Koko sour water	Maize	12 h at ambient temperature	Group B vitamins; dietary fibre	Aka et al. [51]
Bushera	Sorghum	24 h at ambient temperature	Proteins, minerals, fibre	Muyanja et al. [35]

3.3. Classification by Fermentation Techniques

Fermentation is a biotechnology process which makes use of metabolic activities of microflora and their enzymes to breakdown the raw materials into a desired end product. It is a metabolic activity by which energy is given out by partly oxidizing carbohydrates and related compounds without the assistance of an external acceptor [112]. It is an ancient method used to produce fermented foods and beverages across the globe [113]. As an ancient technique, it was used for food preservation and still be used for same purpose since it has the ability to produce organic acids, ethanol and bacteriocins to either eliminate or reduce the pathogenic microflora from the final fermented products [114], hence, making them safe for human consumption.

Again, fermentation is a better and most cost-effective method of producing and storing food for a longer period of time [115, 116]. It has the ability to convert certain chemicals in the raw materials into physiologically active metabolites. For instance, LAB can synthesize phenolic substances such as flavonoids into active metabolites [117] which may enhance the nutrients and organoleptic characteristics of the final products [118]. Fermentation has the potential to eliminate digestive disorders, and reduce phytic stomach acid, and fermentable carbohydrate concentrations such as fermentable oligosaccharides, disaccharides, simple sugars, and polylactic acid, leading to the reduction of gastrointestinal illnesses [119].

Fermentation of foods and beverages could be attained using several techniques. It can be achieved by a spontaneous technique which is also referred to as the wild fermentation technique [120] where the microflora are naturally present in the raw materials, utensils used or the production environment; back-sloping, or defined starter cultures to produce several fermented products like *sauerkraut*, *kimchi*, *kefir*, *kombucha*, and *natto* [121]. Fermentation can also be achieved using either LAB (lactic acid fermentation), *Acetobacter* species (acetic acid fermentation), yeasts (alcoholic fermentation) or *bacillus* species (alkaline fermentation) [122, 123]. This section of the review is set to look at some of these techniques.

3.3.1. Spontaneous Fermentation

Fermentation is a metabolic process in which carbohydrates and other related chemicals are partially oxidized and energy is released in the absence of any external electron acceptors-organic substances created by carbohydrate breakdown [124]. The process usually uses living organisms or enzymes such as bacteria, yeast, or molds to produce a specific product. Traditional fermented foods and beverages have been a staple of the human diet since the dawn of time [125]. Traditional or indigenous fermentation aims at food preservation; to obtain inhibitory metabolites like organic acid, ethanol, and bacteriocins for the overall safety of the final products [126]. Traditional fermentation is classified into four categories which include alcoholic, lactic acid, acetic acid, and alkali fermentations. While lactic acid fermentation is characterized by lactic acid bacteria (e.g., *kimchi*, *sauerkraut*, and *gundruk*); yeasts are heavily involved in the alcoholic fermentation leading to alcohol production (e.g., wines, beers, vodka, whiskey, brandy, and bread); acetic acid fermentation engages acetic acid bacteria which converts alcohol to acetic acid in the presence of oxygen (e.g., vinegar); and *Bacillus* spp. are also majorly used for alkaline

fermentation during the fermentation of soybeans, fish, and seeds, mostly called condiments [125].
Generally, fermentation could be achieved through either spontaneous, back-sloping, or the use of starter cultures (industrial process). The competing actions of various microbial populations generally result in spontaneous fermentations. This type of fermentation depends largely on chance inoculation which involves mixed cultures. The microbiota involved in this method are from the raw materials, utensils, and the environment in which these beverages are processed, hence, the quality of the final products is difficult to predict or control, resulting in short shelf-life and quality diversity of the final beverage. Sáez et al. [127] reported that the microbial communities involved in spontaneous fermentation were LAB, enteric, and sporulated bacteria. They added that the dominant microflora associated with spontaneous fermentation includes species of LAB such as *Leuconostoc mesenteroides,* Brevilactobacillus *brevis,* and *Lactiplantibacillus plantarum.*
However, the length of spontaneous fermentation can be shortened by inoculation through back-slopping and starters. The use of small quantities of previously fermented raw materials in the new product or raw materials to be fermented is what is referred to as back-sloping [128]. It is a modified form of spontaneous fermentation [129]. It has been used to produce several food products including sauerkraut, sourdough, *koumiss,* and beverages. In contrast, the microbial qualities of these products have not been properly understood [124]. According to Holzapfel [130]; back-slopping fermentation technology has been widely utilized for generations due to its ease of application and high yields, despite the fact that its microbial ecology is unpredictable. Kim and Jazwinski [131] conducted a study in which they evaluated the microbiological, nutritional, physiochemical, and sensory qualities of innovative back-slopped fermented kefir to those of traditional fermented kefir. Kefir yields increased by 50%, and the microbiological, nutritional, and physicochemical features were not substantially different from those of spontaneously fermented kefir except for the amount of *Lactobacillus kefiri* and yeast, percentage carbohydrate, and pH.

3.3.2. Industrial Fermentation

Fermented foods and beverages have been one of the popular consumed foods in recent times [132]. The fermentation methods used have improved over the years to produce food products that are safer, free from synthetic chemicals, and have higher nutritional contents in order to meet the high demands of the consumers [133]. Specific microorganisms with special functional properties are been used to achieve these objectives. The most dominant bacterial general used heavily in industrial fermentation is *Limosilactobacillus* which has the ability to produce lactic acid from carbohydrates. Other bacteria include the acetic acid-producing *Acetobacter* for fruit and vegetable fermentation, and *Bacillus* species which are used for legume fermentation [132]. Food manufacturing industries also use beneficial yeasts such as *Saccharomyces cerevisiae* as they produce several enzymes to biochemically impact flavor and aroma in wine beer and ethanol, and leavening of bread [134].
Again, the effective application of various novel innovations such as co-culture, thermophilic fermentation, molecular tools, genetic engineering, mutant selection, and recombinant DNA technologies has enabled the design and construction of tailor-made defined microbes that outperform those found naturally [132]. Moreover, commercial or industrial processes of fermentation have employed several species of microorganisms to improve the qualities of fermented food and beverages. The most common products produced through the industrial processes includes but are not limited to wine (*Saccharomyces cerevisiae*; [135]; beer (*Saccharomyces cerevisiae, Saccharomyces pastorianus*; [136]; yogurt (*Streptococcus thermophilus, Lactobacillus delbrueckii*; [137]; cheese (*Lactococcus, Limosilactobacillus, Streptococcus* sp*., Penicillium roqueforti*; [135]; Acidophilus milk (*Lactobacillus acidophilus*; [138]; sauerkraut (*Leuconostoc* sp*., Brevilactobacillus brevis, Lactiplantibacillus plantarum*; [139]; fish sauce (Lactic acid bacteria (halophilic), *Halobacterium salinarum, Halobacterium cutirubrum, Bacillus* sp.; [140] and fermented meat (*Limosilactobacillus* sp.*,. Micrococcus* sp*., Staphylococcus* sp. [141].
As a result of industrial fermentation, the production of seasonal beverages, particularly wine and beers, with varying essential tastes and alcohol levels, such as strong beer brewed from caramelized malt in the winter and lighter beers with citrus flavor in the summer [142] are possible. Again, some yeast species are genetically modified to reduce fermentation time, produce desirable organoleptic properties in food and beverages. *Saccharomyces cerevisiae*

ML01, in particular, was genetically modified to reduce the generation of biogenic amines, which are harmful compounds produced during wine fermentation [143]. A different recombinant *S. cerevisiae* strain was employed to minimize the development of ethyl carbamate, a carcinogen formed during wine fermentation [144]. Biomolecular approaches are currently being utilized to investigate the fate of bacteria in alcoholic beverages.

3.3.3. Fermentation by Starter Cultures

Starter cultures are active and desirable microflora isolated from previously fermented products (food and beverages) and is intentionally added to the new raw materials at a high number to improve upon the qualities desired by the consumers in the fermented products [145]. They are purely used in manufacturing fermented food and beverages [146]. They could be single or combined pure cultures (defined as microorganisms) that are added to a raw or pasteurized product to start and accelerate its fermentation process [147]. They initiate and carry out the desired fermentation essentials in manufacturing food and beverages [148]. Their metabolic activities have desired effects on the final fermented products [149].

Lactic acid bacterial starters produce lactic acids which lead to a rapid decrease in the pH of the raw materials leading to assuring the safety of the final product. Moreover, the bacteriocins they produce also cause stability of the microbial content of the final products [150]. The starter cultures are normally seeded into the products to be fermented and are allowed to multiply under controlled conditions, which thereafter, impart the characteristic features such as acidity (pH), aroma, consistency, and flavor of the resultant products. During the process of inoculation, bacteria break down lactic acid in the substrates, resulting in increased acidity which imparts preservative effects, improving the nutritive and digestive qualities of the final products. Some of the well-known defined starter cultures that are used commercially for the production of fermented food and beverages includes the strains of *Limosilactobacillus* spp., *Bifidobacterium* spp., and *Propionibacterium* spp. *Lactobacillus acidophilus*, *Lacticaseibacillus casei, Limosilactobacillus reuteri, Lacticaseibacillus rhamnosus* and *Lactiplantibacillus plantarum* [151–153]. According to Holzapfel [154], several researchers have reported that *Brevilactobacillus brevis, Limosilactobacillus fermentum Lactiplantibacillus plantarum, Limosilactobacillus reuteri, Pediococcus pentosaceus* and *P. acidilactici* used as pure starter cultures either singly or in combinations show improved performance in lactic acid-fermented cereal and vegetable products in African countries [155–159]. However, Oyewole [160], Jespersen [161], Obilie et al. [162], and Dzogbefia et al. [163] also reported *Candida krusei, Candida tropicalis, Pichia saitoi, Pichia anomala, Saccharomyces cerevisiae, Zygosaccharomyces florentinus* and *Zygosaccharomyces* spp. as mold and yeast cultures used in fermentation of cassava tissues. Holzapfel [154] also revealed that yeast species such as *Saccharomyces, Candida, Torula,* and *Hansenula*, considered as starters improved the performance of food products produced by plant-based materials containing fermentable sugars. The lactic acid fungi produce alcohol to prevent bacterial and mold infection and improve storage qualities.

Furthermore, the starter cultures' primary and most important function is to hydrolyze carbohydrates and produce lactic acid in the final products. Coagulation, moisture ejection, texture formation, and flavor development are all secondary impacts of acid production. The lactic acid produced increases the coagulation of milk, strengthens the curd, and protects the finished product from infection. The enzymes of lactic acid bacteria starters also help the flavor development of cheeses during the ripening process by the activities of glycolysis, proteolysis, and lipolysis [164]. Again, the starters aid in pleasant taste, offer protection against potential pathogens and spoilage-causing microbes thereby extending the product's shelf life. Fermentative activities of LAB and yeasts during fermentation are thought to increase the quality, flavor, hygiene, and safety of fermented foods and beverages. Holzapfel [130] indicated that West Africa's cereal-based fermented foods and beverages are produced by spontaneous fermentation and on small industrial scales, hence, have varying quality and microbial stability. Therefore, the use of a preparation containing a large amount of known variable microflora is recommended to promote rapid acidification of the raw materials leading to final products with consistent quality and also preventing the growth and proliferation of spoilage and pathogenic bacteria, thereby prolonging the shelf life of the final products [58, 165].

Again, M'hir et al. [145] have it that though some *Enterococcus* species and strains are considered as pathogens, most of them contribute significantly to the improvement of the organoleptic properties of food and beverages. They

play important roles in the ripening of dairy products, mostly through proteolytic and lipolytic activities, exopolysaccharide production, and citrate breakdown, hence, giving a unique taste and flavor to the final products [166, 167]. The pH, color, titratable acidity, alcohol content, and some organoleptic characteristics of "*Pito*" produced by the use of starter preparations can be compared favorably to that produced using spontaneous fermentation techniques [168].

Additionally, LAB strains selected as starter cultures are able to proliferate during sourdough fermentation, acidify the dough and inhibit microbial pathogens [169]. According to Gallagher et al. [170], LAB starters such as *E. faecium* strains in particular, exhibited a functional role in the fermentation of *Hussuwa* through bacteriocin and other antimicrobial compounds production against *Listeria innocua, L. monocytogenes,* and *Staphylococcus aureus.* Moreover, they are also able to ferment indigestible oligosaccharides, leading to an improved nutritional quality of sorghum final products [169]. *E. faecalis* combined with *Leuconostoc mesenteroides* as starters are able to produce sufficient lactic acid, leaven the batter, and form a unique flavor in *idli* [32]. It was also reported that the bacteriocin and other antimicrobial compounds produced by LAB starter strains such as *faecium* ST62BZ and *faecium* BFE 900 isolated from *boza* were active against a number of food-borne pathogens including *Pseudomonas* spp., *Escherichia coli*, and *Klebsiella pneumoniae* [171]. The enterocins produced by *Enterococci* starter cultures promote inhibitory activity towards spoilage or food-borne pathogens such as *Listeria* spp. and *Clostridium* spp. [172, 173]. Their activities towards pathogens such as *Escherichia coli* and *Vibrio cholerae* have also been brought to light by Javed et al. [173], Khan et al. [174], and Vijayendra et al. [175]. Moreover, some of the defined starters used successfully in producing specific food products are summarized in Table 4. From the table, *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* dominated the lactic acid bacterial starters, while *Saccharomyces cerevisiae* dominated the yeast starters considered for the fermentation of the selected beverages.

Table 4

Starter cultures used in fermenting some cereal-based traditional fermented products.

Products	Defined starters	References
Sourdough	*Lactobacillus species including; brevis, hilgardii, sanfranciscensis, farciminis, fermentum, plantarum, amylovorus, reuteri, pontis, panis, alimentarius*	Palla et al. [176]
Kimchi	*Leuconostoc mesenteroides, Lactobacillus plantarum, Weissella kimchi and koreensis, Companilactobacillus kimchi and Lactobacillus sakei*	Choi et al. [177]; Lee et al. [178]; Muyanja et al. [35]
Busheera	*Lactobacillus species such as plantarum, paracasei* ssp. *paracasei, fermentum, brevis, delbrueckii* ssp. *delbrueckii and Streptococcus thermophilus*	Nuraida et al. [179]
Pozol	*Leuconostoc mesenteroides, Lactiplantibacillus plantarum, and W. confuses; Lactococcus lactis and raffinolactis*	Florou-Paneri et al. [180]
Bread	*Fructilactobacillus sanfranciscensis, Leuconostoc citreum and Weissella cibaria*	Alfonzo et al. [181]

Sato	*Rhizopus oligosporus, Mucor racemosus, Saccharomyces cerevisiae, Saccharomycopsis fibuligera, and Pichia anomala*	Gabaza et al. [20]
Enturire	*Lactiplantibacillus plantarum* MNC 21 combined with *Saccharomyces cerevisiae* MNC 21Y, and *L. plantarum* MNC 21 combined with *Weissella confusa* pH MNC 20 and *Saccharomyces cerevisiae* MNC 21Y	Mukisa et al. [182]
Busheria	*W. confuse, species of* (*Lacticaseibacillus paracasei, Limosilactobacillus fermentum, Brevilactobacillus brevis and Lactiplantibacillus plantarum*)	Muyanja et al. [183]
Togwa	*Brevilactobacillus brevis, L. cellobiosus, Limosilactobacillus fermentum,* (*Lactiplantibacillus plantarum*)*, and Pediococcus pentosaceus, Candida* (*pelliculosa and tropicalis*)*, and Saccharomyces cerevisiae*	Mugula et al. [184]
Gowe	*Lactobacillus fermentum and mucosae; W. confusa and kimchi; Pediococcus acidilactici and pentosaceus; Kluyveromyces marxianus, Pichia anomala; Candida krusei and tropicalis*	Vieira-Dalode et al. [185]
Ogi	*Lactobacillus fermentum, brevis, and plantarum; Yeast such as S. Cerevisiae, Rhodotorula graminis, Candida krusei and tropicalis, Geotrichum candidum, Geotrichum fermentum*	Omemu et al. [186]; Teniola and Odunfa [187]
Kenkey	*Lb. fermentum, Lb. brevis, C. krusei, S. Cerevisiae*	Olsen et al. [188]

4. Microbiology of Cereal-Based Traditional Fermented Beverages

The fermentation of the majority of cereal-based traditional fermented foods and beverages is spontaneous, hence, involves different species of microflora [189]. The association of different communities of microorganisms makes the production process difficult to control, standardize, safe and resulting in variable-end products. The qualities of the final fermented food and beverages are largely determined by the microbial properties involved in the fermentation process. Different production techniques, the raw materials employed, and hygiene and sanitation practices employed during production also contribute significantly to the microflora communities of the traditional fermented beverages. However, the acidity of the fermenting products increases when LAB dominates the initial stage through to the final stage of the fermentation, thereby eliminating the food spoilage and pathogenic microbiota and leading to the safety of the products [190].

4.1. Microbiota Associated with Cereal-Based Traditional Fermented Beverages

The fermentation processes of traditionally fermented food and beverages involve the hydrolysis of organic compounds into acids or alcohol through enzymatic actions of various microorganisms such as bacteria, yeasts, and molds [191]. The quality of the fermented products depends largely on the community of microbiota involved in the fermentation processes [7]. Which means the differences in the final-fermented products are as a result of the variations in the microbial community associated with the fermentation process. Tables 5 and 6 present the microbial communities associated with traditional fermented alcoholic and nonalcoholic beverages.

Table 5

Microorganisms involved in the fermentation processes of African traditional fermented alcoholic beverages.

Beverage	Microorganisms/taxonomic tool	Reference
Burukutu (sorghum)	*Aspergillus aceti*, *A. hansenii*, *A. pasteurianus*, *L.plantarum*, *L.brevis*, *L. fermentum; S. cerevisiae*, *B. licheniformis*, *Flavobacterium* spp., *Candida mycoderma*, *Hansenula anomala*, *S. diastaticus*, *and L. fermentum.* (API 50 CHL and 16S rRNA gene sequencing)	Sanni et al. [192]; Lyumugabe et al. [22]; Eze, et al. [193]; and Kolawole et al. [21]
Pito (maize/sorghum)	*Acetobacter aceti*, *Aspergillus hansenii*, *A. pasteurianus*, *Flavobacterium* spp., *Lactobacillus plantarum*, and *Lb. brevis, Saccharomyces cerevisiae*, *A. aceti, L. buchneri, Micrococcus varians, B. licheniformis L. fermentum, Candida* spp. *Lactococcus delbrueckii, Pediococcus. acidilactici, Lactobacillus lactis, and Leuconostoc lactis* (cultural, morphological, and biochemical characterization)	Sanni et al. [192]
Bantu beer (maize/sorghum)	*Saccharomyces cerevisiae*, *Candida* spp., *L. plantarum*, *L. fermentum*, *L. brevis*	Dirar [194]
Amgba (sorghum and millets)	*Cryptococcus albidius* var *albidius*, *C. melibiosica*, *Debaryomyces hansenii* var *hansenii*, *Dekkera bruxelensis*, *Rodotorula mucilaginosa* and *Torulaspora delbrueckii, Saccharomyces cerevisiae*, lactic acid bacteria (PCR/RFLP, partial sequencing of 26S of rDNA)	Nanadoum et al. [30]
Kodo ko jaanr (millets, barley)	*Saccharomycopsis fibuligera*, *Rhizopus* spp., *Mucor* spp., *Pediococcus pentosaceus and anomala, L. bifermentans, Mucor circinelloides, Rhizopus chinensis and stolonifer, Saccharomyces cerevisiae, Candida glabrata.* (Cultural, MBC)	Prakash Tamang and Thapa [195]
Bhaati jaanr (rice)	*Amylomyces rouxii, Rhizopus oryzae, Endomycopsis fibuligera, S. cerevisiae*, *Enterococcus faecalis, P. pentosaceus* (biochemical tests, API 50 CHL)	Nout [196]
Tchoukoutou (sorghum, millet, maize)	*Rhizopus, Mucor, Aspergillus* spp., *acetic acid bacteria, lactic acid bacteria, bacilli, Saccharomyces, Candida, Hansenula* spp.	Prakash et al. [31, 197]

Bouza (buza) (Wheat, maize, millet, sorghum)	*Saccharomycopsis fibuligera*, *Rhodotorula glutinis*, *Debaromyces hansenii*, *Candida parapsilosis*, *Trichosporon fennicum*, and LAB including *Leuconostoc* spp. (cultural, morphological and biochemical characterization)	Adegoke et al., [198];
Merissa (sorghum or millet)	*Lactococcus lactis, Aspergillus aceti*, *Aspergillus hansenii*, *Aspergillus pasteurianus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *L. lactis*, *L. delbrueckii*, *Alcaligenes*, *Saccharomyces cerevisiae*, *Micrococcus* spp. *Candida* spp. *Bacillus licheniformis*, *Flavobacterium* spp., *Candida mycoderma*, *Hansenula anomala*, *Saccharomyces diastaticus*, *Bacillus* spp. *Rhodotorula* spp. *Pediococcus acidilactici* (API 50 CHL, ITS-PCR/RFLP, (PFGE), 16S rRNA gene sequencing. MALDI-TOF)	Sanni et al. [192]; Jespersen [161]
Chibuku (sorghum)	*Lactococcus lactis, Aspergillus aceti*, *Aspergillus hansenii*, *Aspergillus pasteurianus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *L. lactis*, *L. delbrueckii*, *Alcaligenes*, *Saccharomyces cerevisiae*, *Micrococcus* spp. *Candida* spp., *Bacillus licheniformis*, *Flavobacterium* spp., *Candida mycoderma*, *Hansenula anomala*, *Saccharomyces diastaticus*, *Bacillus* spp. *Rhodotorula* spp. *Pediococcus acidilactici* (PCR-DGGE, PCR-RFLP, API 20 C kit, ABI 3130 genetic analyzer, API 50 CHL system)	Sanni et al. [192]; Jespersen [161]
Dolo (sorghum)	*Saccharomyces cerevisae*, *Candida inconspicua, Issatchenkia orientalis, Candida magnolia, Candida humilis, L. fermentum, Lactobacillus buchneri, Lactobacillus* sp., *Aspergillus niger, Fusarium* sp. and *Aspergillus* sp. (API 20 C kit, PCR -sequencing, ABI 3130 genetic analyzer, API 50 CHL system)	Jespersen [161]; Sawadogo-Lingani et a. [36, 199]

Ikigage (sorghum)	*Aspergillus aceti*, *Aspergillus hansenii*, *Aspergillus pasteurianus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *Alcaligenes*, *Saccharomyces cerevisiae*, *Micrococcus* spp., *Candida* spp., *Bacillus licheniformis*, *Flavobacterium* spp., *Candida mycoderma*, *Hansenula anomala*, *Saccharomyces diastaticus*, *Bacillus* spp. *Rhodotorula* spp.	Lyumugabe, et al. [92]
Tchapalo (sorghum)	*L. fermentum, L. brevis, L. plantarum, L. paracasei* subsp. *paracasei and L. delbrueckii* subsp. *delbrueckii; Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicun, Weissella confusa, and L. plantarum* (API 50 CHL and ID 32C)	Sanni et al. [192]; Muyanja et al. [35]
Bushera (sorghum, millet)	*Lactobacillus plantarum, Corynebacterium, Aerobacter, Candida mycoderma, Saccharomyces cerevisiae, Rhodotorula, Cephalosporium, Fusarium, Aspergillus, Penicillium, L. plantarum Corynebacterium, S. cerevisiae, Candida mycoderma. L. fermentum biotypecellobiosus, L. brevis,, L. curvatus, L. buchneri, L. delbrueckii* subsp. *bulgaricus, L. helveticus, L. pantheris, L. vaccinostercus, L. bifermentans, L. nantensis, Candida humicola, C. krusei, Geotrichum* spp., *Cryptococcus* spp., *Trichosporon* spp., *C. krusei, Clavispora lusitaniae, S. cerevisiae*. (API kit, PCR-DGGE and partial 26S rRNA gene sequencing, 16S clone library and PCR-DGGE)	Oguntoyinbo et al. [59]; Greppi et al. [200]
Ogi (maize, sorghum, millet)	*Cryptococcus albidius* var *albidius, Candida melibiosica, Debaryomyces hansenii* var *hansenii, Dekkera bruxellensis, Rhodotorula mucilaginosa, Torulaspora delbrueckii* (5). *S. cerevisiae and S. paradoxus* (PCR/RFLP, partial sequencing of 26S of rDNA and sequences)	Nanadoum et al. [34]

Bili bili (maize, sorghum, millet)	(*S. cerevisiae, S. carlsbergensis, C. tropicalis, C. pararugosa, C. diversa, C. boidinii, C. lactiscondes, C. lambica, C. norvegica, C. inconspicua, Pi. fermentans, Pi. norvegensis, R. mucilaginosa, R. araucariae and T. delbrueckii*) *and lactic acid bacteria, L. confusus, L. fermentum, L. plantarum, L. coryniformis, Lb. sanfrancisco, L. coprophilus, L. paracasei* subsp. *paracasei, L. brevis, L. acidophilus, L. rhamnosus, Leu. mesenteroides, Leu.oenos, Leu. raffinolactis, Lc. lactis, W. confusa* (RAPD PCR, partial 16S rRNA gene sequencing, API 50 CHL and API ZYM galleries)	Nanadoum et al. [34]
Boza (millet, maize, wheat, and rice)	*Lactobacillus plantarum, Lactococcus lactis* ssp. *lactis, Lactobacillus delbrueckii* ssp. *delbrueckii, L. fermentum, Lb. pentosus, and L. curvatus* ssp. *curvatus, Enterobacter cloacae, E. sakazakii, Pseudomonas luteola, P. aeruginosa, and Serratia ficaria.* (API 50 CH/CHL and API 20E media.sequences)	Heperkana et al. [201]; Hancıoğlu and Karapınar, [202]; Tamer et al. [203]; Botes et al. [204]
Oshikundu (millet, sorghum)	*Lactococcus, Weissella, Leuconostoc, Aeromonas, Enterococcus, Pseudomonas, Lactobacillus, and Acinetobacter* (Rep-PCR and 16S rRNA)	Embashu et al. [18]
Finger millet slurries (millet)	*Leuconostoc lactis, Lc. mesenteroides, L. plantarum, L. brevis, W. viridescens, E. casseliflavus, E. faecium, E. mundtii, E. durans, Pediococcus acidilactici and yeast species* (ISR-PCR fingerprinting, RAPD-PCR, 16S–23S ISR, RAPD-M13 and 16S rRNA)	Gabaza et al. [20]

Note. MBC, morphological and biochemical characterization.

Table 6

Microorganisms fermenting African traditional fermented nonalcoholic beverages.

Beverage	Microorganisms/taxonomic tools	Reference
Koko/akassa	*Lactobacillus fermentum,* (*Lb. cellobiosus, Lb. brevis, Lb. curvatus, Lb. buchneri,* and *Weissella confusa*), *pediococci and yeasts such as Candida krusei, Candida kefyr, Candida glabrata, Saccharomyces cerevisiae*	Nout [32]

Ben-saalga	*Lb. fermentum, Lb. plantarum, and Pediococcus pentosaceus Lb. plantarum*	Sifer et al. [205]
Togwa	*Lactobacillus* spp.*, Saccharomyces cerevisiae, Candida* spp. *L. plantarum, L. brevis, L. fermentum, L. cellobiosus, P. pentosaceus, W. confusa Issatchenkia orientalis, C. pelliculosa, C. tropicalis.* (biochemical tests, API tests)	Vasudha and Mishra [37]; Mugula et al. [108]
Amazake	*Aspergillus* spp.	Yamamoto et al. [206]
Mahewu (amahewu)	*Lactococcus lactis* subsp. *lactis, Lactobacillus, Streptococcus, Enterococcu*s (cultural, morphological, and biochemical characterization)	Blandino et al. [55]; Gadaga et al. [207];
Kunnu zaki	*Lactobacillus fermentum and Lactobacillus leichmannii, Leuconostoc* spp.*, Lactococcus* spp.	Akoma et al. [208]; Agarry et al. [4]
Mageu	*Saccharomyces cerevisiae, Issatchenkia orientalis, Pichia fabianii, Aureobasidium pullulans, Candida glabrata, Pichia ciferrii, Saccharomycopsis fibuligera, Hanseniaspora opuntiae, Zygoascus hellenicus, Cryptococcus flavus, Cryptococcus magnus, Candida parapsilosis, Candida pyralidae and Rhodotorula mucilaginosa, Lactobacillus agilis, L. minor, L. Confuses and L. fructosus. L. Minor, L. divergens L. agilis and L. plantarum, L. bifermentans, L. divergens, L. fermentum, L. hilgardii, L. minor, Streptococcus* spp.	Nyanga et al. [209]; Fleet [210]
Pozol	*Streptococcus, L. fermentum, L. plantarum, L. casei, L. delbrueckii Streptococcus bovis, S. macedonicus, L. lactis,* and *Enterococcus sulfureus.* (Cultural, morphological, and biochemical characterization, RT-PCR)	Muyanja et al. [6]
Malwa	*Lactobacillus* spp.*, Lactococcus, coliforms*	Muyanja et al. [6]
Sobia	*Lactobacillus cellobiosus, L. buchneri, L. plantarum, L. brevis, L. delbrueckii. delbrueckii, Leuconostoc lactis, Pediococcus pentosaceus, Klebsiella pneumonia, Enterobacter aerogenes, E. sakazakii, E. cloacae, Serratia liquefaciens, Saccharomyces cerevisiae, Candida tropicalis, C. ciferrii, C. guilliermondii, C. lipolytica, Kloeckera japonica, Rhodotorula rubra, Penicillium* spp. API 50 CHL system, API 20 system, API 20C AUX system	Mavhungu [211]

Kirario	*Leuconostoc mesenteroides* ssp., *mesenteroides/detranicum, Leuconostoc citreum, Lactococcus lactis* ssp. *lactis, Lactococcus raffinolactis, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus collinoides,* and *Lactobacillus coprophilus* (API 50 CH strips, API 50 CHL medium)	Kunyanga [212]
Ting	*Lactobacillus pentosaceus, Lact. plantarum, Lact. pentosaceus, Lact. cellobisus, Leuconostoc mesenteroides, Lact. collinoides, Lact. brevis, Lact. fermentum,* and *Lact. curvatus* (PAGE, API 50CHL medium and API 50CH strips)	Mavhungu [211]

Furthermore, Tables 5 and 6 reveal that bacteria, yeasts, and molds were the major microbial compositions of the selected traditional beverages. The lactic acid bacteria were dominated *Lactobacilli* (*Lactiplantibacillus fermentum*, *Brevilactobacillus brevis*, *Lactiplantibacillus plantarum*, and *delbrueckii*), followed by *Lactococci, Leuconostoc,* and *Pediococci* with the least being *Streptococci* (*Streptococcus bovis*). *Lactobacillus* and other LAB species are known and are very important in food technology. Zalán et al. [213] reported that LAB in cheese, yoghurt/fresh dairy products industries, and production of probiotics represent a market of 55 billion Euros, 25 billion Euros, and 20 billion Euros, respectively. Again, while *Saccharomyces cerevisiae* and *Candida mycoderma* dominated *Saccharomyces* and *Candida* species (yeasts) respectively; *Aspergillus aceti* and *Rhizopus stolonifer* dominated *Aspergillus* and *Rhizopus* species (molds) respectively. Other microbial dominants found to be associated with the selected local fermented beverages include *Acetobacter, Pseudomonas, Klebsiella, Weissella, Achromobacter, Flavobacterium, Micrococcus*, and *Bacillus*.

4.1.1. Lactic Acid Bacteria (LAB)

Lactic acid bacteria are classified according to their morphology, glucose fermentation potentials, temperature tolerance, lactic acid synthesis, ability to thrive at high salt concentrations, and acid or alkaline tolerance [214, 215]. *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Leuconostocaceae, Lactobacillacea,* and *Streptococcaceae* are the six families defined by Parte [216]. Out of the six families, *Enterococcaceae, Leuconostocaceae, Lactobacillacea,* and *Streptococcaceae* account for the majority of the genera and species involved in fermentation. The LAB genera in the other two families are more closely linked to food deterioration. Among the four families, seven genera such as *Enterococcus, Oenococcus, and Leuconostoc, Lactobacillus, Pediococcus, Lactococcus,* and *Streptococcus* from each of the four families, respectively, were associated with food and beverage fermentation [216]. Studies have established that the appearance of microorganisms, particularly LAB in fermented food and beverages depend largely on their geographical locations [217]. Fujimoto et al. [218] isolated *Lactobacillus* species such as *brevis, alimentarius, pentosus, vaccinostercus, sanfranciscensis,* and *sakei* from fermented wheat and corn sourdough; Fujimoto et al. [218] and Liu et al. [219] reported species of *Lactobacillus, Pediococcus,* and *Leuconostoc* in wheat sourdough. While Liu et al. [219] and Zhao et al. [220] identified 217 strains of *Lactiplantibacillus plantarum, Lacticaseibacillus. pantheris, L. raffinolactis, Leu. mesenteroides, Leuconostoc citreum, Leu. pseudomesenteroides, Weissella viridescens,* and *Lactococcus lactis* from wheat sourdough from Ya'an city of China, Zhang et al. [221] and Yan et al. [222] reported *Lactobacillus* spp., *L. brevis, Latilactobacillus curvatus, L. lactis* ssp. *lactis, Lactococcus lactis* ssp. *lactis*, *E. casseliflavus, E. durans, E. faecium, S. constellatus*, and *S. equinus* as the predominant LAB species in corn and rye sourdoughs.

The LABs are safe microbes playing very important roles in food fermentation and preservation through natural fermentation or added as defined starters [223]. The preservative ability of LAB is actually as a result of antimicrobial compounds production which include hydrogen peroxide, ethanol, diacetyl, *γ*-aminobutyric acid, propionic acid, benzoic acid, fatty acids, bacteriocins, and bacteriocin-like inhibitory substances [224]. Again, these compounds are produced to prevent the development of undesirable microbes thereby improving the shelf life and the overall safety

of the final beverage [225]. The organic compounds produced could also improve food and beverage functionalities [226, 227].

4.1.2. Fungi

Fungi are a broad group of microorganisms that live in a variety of environments, including soil, plant parts, water, food, and beverage sources [228–230]. Temperature, pH, moisture, degree of aeration, and the amount and kind of nutrients are all elements that influence their growth and distribution [231]. Fungi are a family of yeast and molds naturally found in fermented food and beverages or added as defined starter cultures.

(1) Yeasts. Yeasts are considered to be the primary spoilage microbes. The various spoilage-causing yeast genera that are found in low-alcoholic and nonalcoholic beverages include *Zygosaccharomyces bailii, Saccharomyces, Brettanomyces, Hanseniaspora, Hansenula,* and *Pichia.* Several researchers have also identified *Schizosaccharomyces pombe, S. japonicus, Candida castellii, C. fructus, C. intermedia, C. krusei, C. tropicalis, Geotrichum candidum, Hansenula anomala, Kloeckera apiculata* [122], *Pichia membranifaciens, P. ohmeri, Saccharomyces chevalieri, S. uvarum, Kluyveromyces africanus, Torulaspora delbrueckii* and *Rhodutorula graminis* as the predominant species associated with most of the African fermented beverages [114, 232, 233].

Like bacteria, yeasts have advantageous and disadvantageous effects in food fermentations. They can be applied in the production of ethanol, single-cell protein (SCP), feeds, industrial enzymes, and metabolites [114]. During the fermentation of traditional food and beverages, they ferment carbohydrates leading to the formation of alcohols and other aroma compounds [114]. Yeasts like *Pichia* are seen as food spoilage organisms while *Candida* spp. is utilized for single-cell protein production [234]. The most beneficial yeast in terms of desirable food fermentations are from the family of *Saccharomyces*, especially *S. cerevisiae* which is widely associated with bread making and alcohol in wine fermentations. *Saccharomyces cerevisiae* var. *ellipsoideus* is employed extensively in beverage (wine) production [235]. For beverages produced with maize and millets, *Schizosaccharomyces pombe* and *S. boulderi* have been identified as the most dominant yeasts in the fermentation of the substrates [236]. While *Saccharomyces cerevisiae* var. *carlbergenisis* is associated with beer production, *Schizosaccharomyces pombe* has been found to degrade malic acid into ethanol and carbon dioxide and has been used successfully to lower the acidity in the grape and plum musts [237].

(2) Molds. Molds are white, delicate, fluffy, cottony masses suspended in alcoholic and nonalcoholic beverages. Their spores cannot grow in carbonated beverages but can survive. The most beverage contaminating molds found to be associated with alcoholic and nonalcoholic beverages are *Aspergillus ochraceus, A. tamarii, A. flavus, Byssochlamys nivea, B. fulva, Paecilomyces variotii, Neosartorya fischeri, Eupenicillium brefeldianum, Phialophora mustea, Talaromyces flavus, T. trachyspermus,* and *T. aurantiacum.* Others include *Penicillium notatum, P. roqueforti, Rhizopus, Fusarium,* and *Cladosporium* spp. [232, 233].

Molds are equally essential microorganisms in food processing, preservation, and spoilage. The majority of the species have the ability to produce enzymes of commercial importance such as pectinase by *Aspergillus niger* [238]. *Aspergillus* species have been linked to the generation of citric acid from waste materials such as apple pomace [238, 239]. These species are frequently responsible for undesired changes in foods that lead to spoiling, whereas *Penicillum* species are involved in cheese ripening and flavor development. While *Ceratocystis* species are important in fruit flavor synthesis, *Penicillium* is the causal agent for toxin formation such as patulin [240].

4.1.3. Pathogenic and Spoilage Microorganisms

Beverages have high water activity and are often rich in nutrients including vitamins and minerals so they are highly susceptible to microbial contamination and spoilage. Potential microbes that can contaminate foods and beverages could include species of bacteria and fungi. Bacterial species could be *E. coli, Salmonella, Shigella,* and *Staphylococcus* [241], while fungal contaminants could be several species of *Aspergillus* and *Saccharomyces* [242]. These spoilage microbes are well reported to be identified with fermented food and beverages [243, 244]. Microbial analysis of “Ikigage,” a traditional fermented beverage of Rwanda revealed several species of spoilage microbes including lactic acid bacteria, *E. coli, fecal streptococci, Staphylococcus aureus*, yeast, and molds [92]. In “pito,” Minamo et al. [245] reported *Staphylococcus aureus*, *E. coli, B. subtilis, Streptococcus* species, *Proteus* species,

Rhizopus stolonifer, Aspergillus flavus, Aspergillus niger, Saccharomyces cerevisiae, and *Mucor* species as the predominant spoilage microorganisms contaminated the beverage. Their presence in the beverage was as a result of improper handling during the production [245]. Even in the acid environment, yeasts could contaminate foods and beverages leading to spoilage by producing film, causing color changes and off-flavor [88]. *Candida krusei, Candida pelliculosa*, and *Candida lipolytica* were the main causes of *shalgam* beverage spoilage [88]. *E. coli* [246] and *Salmonella typhi* [247] were also recorded as the major spoilage bacteria in shalgam.

Pathogens are disease-causing organisms found in food. They may include bacteria, viruses, and parasites and have the potential to cause illnesses and even death [248]. *E. coli* 0157:H7, *Salmonella enterica, Listeria monocytogenes, Yersinia enterocolitica,* and *Staphylococcus aureus* were found to be the most predominant food pathogens contaminating several beverages [249–253]. The traditional techniques of production and marketing by the local producers or traders expose the beverage to pathogenic and spoilage microbes. Specifically, species of pathogenic microbes such as *E. coli, S. typhimurium, S. aureus, L. monocytogenes C. albicans,* and other *Enterobacteriaceae* were found to be associated with several fermented beverages [254, 255].

Again, several factors could make food and beverages liable to microbial contamination and spoilage. Food and beverages are easily contaminated by spoilage microorganisms during the production process, by the production environment, raw materials for production, water and other additives, processing equipment, poor hygienic handling, packaging materials, and storage conditions [256–258]. It is however required that food handlers must observe strict hygiene protocols in the food value chain, particularly, during food preparations.

5. Quality Issues of Cereal-Based Traditional Fermented Beverages

Traditional cereal-based fermented foods and beverages have been produced and consumed since the inception of civilization [259]. Traditional foods and beverages produced through fermentation serve several purposes. However, it was majorly and currently used for preservation and safety through the production of inhibitory metabolites by microbial communities associated with the fermentation process [260]. When LAB and yeasts are added as starters or dominate the spontaneous fermentation process, bacteriocins, and other organic acids are produced to eliminate the pathogenic and spoilage organisms to ensure the safety of the final products [125]. There have been several concerns about food safety due to microbial contaminations leading to outbreaks of food-borne illnesses. LABs are capable of acidifying fermented products and are also able to produce acetic aroma compounds, bacteriocins, enzymes, and exopolysaccharides when considered for controlled fermentation as one of the natural surest ways of addressing all safety issues regarding fermented products [125, 261]. Therefore, this section is to review all the safety issues concerning the fermented beverages.

5.1. Microbiological Safety Issues

Factors not limited to the use of different raw materials, production techniques, microbial community, and fermentation conditions have greatly influenced the final traditional fermented beverages. However, consumers are currently aware of maintaining strong immune systems to avoid diseases and are seriously searching for food products which could assist them maintain their health status and preventing health-related problems [100]. There are different types of traditional fermented beverages produced and used worldwide, particularly in Africa. The fermentation of these beverages is spontaneously done which involves diverse microbial communities (bacteria, yeasts, and molds) thereby determining the qualities of the final beverages [7]. The diversity of microbial communities in cereal-based fermented alcoholic and nonalcoholic beverages in Africa are summarized in Tables 5 and 6, respectively. For this reason, the safety of traditional fermented beverages raises so much concerns. However, the initial development of the acidifying bacteria plays a key role of regulating the microbial communities in the beverage. The involvement of species of LAB either by natural process or added as starters prevents the growth and existence of microbial pathogens in the final beverage [262, 263]. The food pathogens that are aerobes and facultative anaerobes and ferment simple sugars can grow at pH between 4.3 and 9, but combining growth factors such as pH and water activity can prevent the growth of food-borne pathogens [190] to ensure the safety of the products. Moreover, the major problems observed with the production of traditional fermented beverages include unhygienic processing environments coupled with highly variable production techniques. Since the soaking and the

malting parameters vary within and between processors [45], the grains can easily be infected by fungi with aflatoxin contamination potentials (aflatoxins) [264].

Nonetheless, to ensure that traditional fermented beverages are safe by all standards, several studies have been conducted to keenly select microorganisms with probiotic potential as starter cultures for control fermentation [259, 265]. Examination of enterocin-producing *Enterococcus faecium* YT52 pose low or no risk to the health of the consumers; hence, it could be used as starters to inhibit the growth of food pathogens, thereby making the spontaneous fermented products safe for consumption [100]. To buttress this point, Arslan-Tontul and Erbas [266] used *Enterococcus faecium* YT52 to completely prevent the growth of *Listeria monocytogenes* and *Bacillus cereus.* The LAB strains such as *Lactiplantibacillus plantarum* IL4I1, *L. plantarum* A1MM10, *Lactococcus lactis* IL5I1, *Leuconostoc lactis* A1MS3, *Lc. pseudomesenteroides* IL5I2 and *Pediococcus pentosaceus* S0I10 were also used to inhibit the growth of several species of *Enterobacteriaceae* in Atole *agrio*, a traditional Mexican fermented beverage [267]. Studies have published the effectiveness of low pH caused by LAB against food pathogens [262, 267]. *Lactococcus lactis* A1MS3 and *P. pentosaceus* S0I10 were used to ferment *Atole agrio* and a plant-based fermented food, respectively, due to their strong antimicrobial properties against *Enterobacteriaceae* [267, 268].

Furthermore, due to proven probiotic effects and disease prevention abilities of the traditional fermented beverages, they are receiving much attentions by both researchers and consumers [269]. These health benefits are strongly linked to high probiotic microbial contents in the fermented beverages. As a result of the presence of probiotic LAB, the traditional fermented beverages have the potentials to improve gastrointestinal health status of the consumers [48]. Consumption of these beverages improves liver function, levels of *Lactobacilli* and *bifidobacteria* in the intestinal microbiota, a balanced gut microbiota, and the avoidance of bacterial translocation, which leads to a reduction in nosocomial infections [269]. They also have the potentials to remove antinutrient compounds, mycotoxins, endogenous toxins and cyanogenic compounds and enhance bioavailability making these beverages safe for consumption [270]. Moreover, due to the presence of the varieties of lactic acid bacteria metabolites, the consumption of these beverages confers bactericidal, bacteriolytic, and bacteriostatic properties, resulting in therapeutic effects at a digestive level. These antimicrobial compounds found in the fermented beverages exhibited activities against several species of bacteria including pathogenic yeasts and molds [269, 271].

5.2. Nutritional Issues

The nutritional properties of cereal-based traditional fermented beverages depend largely on the raw materials and other ingredients used in the production [272]. They then reported carbohydrates, protein, potassium, magnesium, and phosphorus as the key nutritional contents of *amahewu*. Similar studies by Fadahunsi and Soremekun [60], Olusanya et al. [273], and Qaku et al. [274] identified improved proximate and mineral compositions in maize and sorghum-fortified *amahewu*. Varying the production processes such as the periods of soaking, fermentation times, and terminating fermentation at different pH values revealed different nutritional compositions of *amahewu* [274]. According to Fernandes et al. [275], Mckevith [276] and Brennan and Cleary [277] cereals are the major sources of macronutrients and minerals, phytochemicals, and antioxidants in cereal-based fermented beverages. These beverages are able to exert probiotic effects due to the water-soluble fibre in the raw materials [277]. They are excellent media for the transportation of nutrients and bioactive compounds into the human body (the consumer) [278]. Vitamins B &E and many minerals (Ca, Mg, Mg, Fe, and Zn) are required for proper functioning of the body and are found in cereals [279].

Additionally, apart from improved digestibility, functional and sensory properties, fermentation is largely used for nutritional enhancement, particularly in cereal-based fermented products [269, 280–282]. Most African-fermented beverages are basically fermented and produced from cereals [109]. Traditional fermented beverages are important to the human body because they play key roles in human health and for their nutritional, nutraceutical, and pharmaceutical properties [125, 263, 283, 284]. Consumption of cereal-based fermented beverages improves the bioavailability of both macro and micronutrients [109]. They are rich sources of vitamins of all kinds, fibre, flavonoids, phenolic compounds, antioxidants, omega-3 fatty acids, amino acids, and biopeptides [285].

Again, the proximate composition of *borde* includes high amounts of ash, fat, protein, and carbohydrate [286]; *tej*

and *grawa* contain equally high amounts of protein, carbohydrate, fat, and few minerals [287]. *Mabisi* and *Munkoyo* (Zambia) have high values of vitamins B1, B2, B3, calcium, protein, and zinc [288].

Furthermore, cereal grains are essential in transporting organs for nutrients and bioactive compounds into the bodies of consumers and also facilitate the availability of these compounds. These bioactive compounds are phytochemicals (phytoestrogens, phenolic compounds, flavonoids, and carotenoids), dietary fibre, vitamins, fatty acids, probiotics, and minerals which are readily available in cereal-based fermented beverages and essential compounds for disease control [100].

Furthermore, LAB in fermented cereal-based meals and beverages release various B vitamins, including niacin (B3), pantothenic acid (B5), folic acid (B9), as well as vitamins B1, B2, B6, and B12 [3]. Folates, for example, prevent neural tube abnormalities in infants and protect against cardiovascular disease and various malignancies by acting as co-factors in metabolic events [3]. Hence, the consumption of fermented beverages is of great important to human.

5.3. Sensory Issues

The purpose of fermentation is not only to preserve, and improve the nutritional values of the products, and make the products safe for consumption but rather also to enhance the organoleptic qualities of the final products desired by the consumers. The sensory characteristics of fermented beverages are equally important as the nutritional values are essential from the consumers' point of view since they determine whether or not the consumers will patronize a particular food product despite their nutritional values [272].

For instance, consumers reject *amahewu* enriched with *Aloe vera* leaf powder due to its bitterness [271]. Despite the nutritional compositions of *amahewu* fortified with *Moringa oleifera* leaf powder, it was poorly rated by the sensory panelists as compared to the conventional *amahewu* [273]. Again, Awobusuyi and Siwela [289] and Awobusuyi et al. [290] discovered that adding processed bambara groundnut flour to *amahewu* manufactured from provitamin A biofortified maize and white maize samples enhanced sensory characteristics when compared to *amahewu* made without bambara groundnut flour. Oyeyinka and Oyeyinka [291] added that in the move to improve the nutritional properties of fermented food and beverages, the organoleptic characteristics must be highly upheld. The characteristics of traditional fermented food and beverages including organoleptic properties are impacted by a number of factors not limited to production technologies and metabolic reactions of microbiota associated with the products [292].

Moreover, during cereal fermentation, different microbial metabolites such as lactic, acetic, oleic, and linoleic acids, esters, higher alcohols and aldehydes, ethyl acetate, and diacetyl are synthesized which were identified to have had a significant influence on the shelf-life and the sensorial properties of fermented products [293]. The color, flavor, aroma, appearance, taste, and texture differ from beverage to beverage depending on the raw materials or qualities of the raw materials involved and are essential for their acceptability [294]. For instance, the sensory properties such as taste, aroma and color of *gowe* (a sorghum-based beverage) fermented with only *Limosilactobacillus fermentum* and in combination with *Kluyveromyces marxianus* rated far higher than the spontaneous fermented *gowe* [295]. The aroma and taste of *Brevilactobacillus brevis* and *Saccharomyces cerevisiae* fermented *obushera* were more acceptable than the naturally fermented *obushera* [296]. However, there was no significant difference in the aroma, texture, color and appearance of *ogi* fermented with *Lactiplantibacillus plantarum, Kluyveromyces marxianus* singly and spontaneously fermented *ogi* [297]. Similarly, the sensory panelists also rated the taste and flavor of spontaneously fermented *akamu* and *L. plantarum* fermented *akamu* above *akamu* fermented with both *Lactiplantibacillus plantarum* and *Kluyveromyces marxianus* [297]. These incidences might be as a result of over fermentation leading to over acidification.

Salmeron [93] indicated that volatile compounds produced by microbial communities involved in fermentation have considerable impacts on the sensory properties of food products. He therefore concluded in his report that the starter organism used during the fermentation affected the aroma profile of the grains significantly, but each grain substrate has a unique aroma profile. Again, the organic compounds such as aroma and flavor synthesized after barley and malt substrates which were fermented by single starters of *L. acidophilus*, *Limosilactobacillus reuteri, and*

Lactiplantibacillus plantarum were uniquely enhanced for each of them as compared to the spontaneously fermented substrates [93]. Hence, the amount and the types of organic compounds produced by the microbial communities involved in the fermentation together with the types of raw materials used and the duration of fermentation determine the organoleptic qualities of the final traditional fermented beverages.

6. Conclusion

Despite the fact that the same raw materials are used during the production of traditional fermented beverages, the preparation varies significantly from ethnic group to ethnic group. The review of these beverages revealed maize, millet, and rice were the major cereals used as the raw materials for the production of these traditional beverages. *Binuburan, amgba beer, tchoukoutou, sake, dolo*, and *pito* were identified as the major alcoholic traditional fermented beverage; while *aliha, mahewu, kunun-zaki, ting, borde,* and *bushera* were traditional fermented nonalcoholic beverage. The fermentation of these beverages was achieved through spontaneous fermentation (*burukutu, mahewu, sake, kirario, mawe, ikegage,* and *ikivunde*), back-slopping (*amba beer, tchoukoutou,* and *dolo*), and the use of industrial techniques (*pito, Bantu beer,* and *Bhaati Jaanr*). Moreover, the dominant microbial species typical of the traditional fermented beverages identified in this review so far were *Limosilactobacillus fermentum, Brevilactobacillus brevis, Lactiplantibacillus plantarum, L. delbrueckii*; *Lactococcus* (*Lact. lactis, Lact. curvatus,* and *Lact. pantheris*), *Leuconostoc* (*Leuc. mesenteroides* and *Leuc. paracasei*); and *Pediococcus* (*P. pentosaceus, P. acidilactici*); fungi (*Saccharomyces, Candida, Aspergillus,* and *Rhizopus* spp.); and other bacterial species (*Acetobacter, Pseudomonas, Klebsiella, Weissella, Achromobacter, Flavobacterium, Micrococcus,* and *Bacillus* spp.). However, the nutritional composition of these beverages cannot be overemphasized. They were found to have sensory properties such as good taste, flavor, acidity, digestibility, and texture in both alcoholic and nonalcoholic beverages. Most of these beverages were found to be rich in calories, and B-group vitamins including thiamine, folic acid, riboflavin, and nicotinic acid. However, due to microbial quality issues associated with spontaneously fermented beverages, defined starter cultures or industrial processing techniques are recommended for the production of these beverages, since they improve the microbial, sensory, and nutritional qualities of the final products [298, 299].

Additional Points

Highlights. (1) The major production technique used was spontaneous fermentation. (2) The dominant raw materials identified for the production were maize and millet. (3) LAB and fungi were the major microbiota involved in the fermentation. (4) These beverages are rich in proteins, carbohydrates, calories, and B-group vitamins.

Authors' Contributions

FKM designed the topic and wrote the manuscript. APHK, KTD, and FKS approved the topic, supervised, and reviewed the manuscript. FKM, APHK, and KU formatted and revised the manuscript.

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DETAIL

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Nutritional and Microbial Qualities of Fermented Cereal-Based Porridges Produced in Northern Benin

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ABSTRAK (ENGLISH)

Fermentation has been used for centuries to enhance the sensory and nutritional qualities and the antioxidant content of plant-based foods, making them beneficial for health. This study aims to investigate the microbiological and nutritional qualities of fermented porridges produced in northern Benin. Various nutritional tests and the identification of different microorganisms have gained insights into eight porridges produced in 9 localities of northern Benin. Lactic acid bacteria have the highest proportion among all microorganisms in fermented porridges, followed by the total mesophyll aerobic flora. *E. coli*, thermotolerant coliforms, and molds are not present in all porridges analyzed. Recorded data suggested that porridges have a variable microbial load depending on the collection municipalities. The dry matter of the eight types of porridge varies greatly, with akloui having 27.03 ± 3.83 g/100 g and fourra having 48.63 ± 3.83 g/100 g. The total ashes also differ significantly, with bita having 39.36 ± 4.67 g/100 g and sagagnèga having 63.19 ± 4.67 g/100 g. It is worth noting that all fermented porridges have a pH lower than 5, and the titratable acidity ranges from 0.01 ± 0.00 g to 0.02 ± 0.00 g. The brix degree varies from 0.46 ± 0.54 to 4.4 ± 0.54. The beta-carotene values of the 8 types of porridge vary from 0.037 ± 0.018 mg/g to 0.138 ± 0.018 mg/g, while the total sugars range from 1.926 ± 0.877 to 5.773 ± 0.877 g/100 g. The lipid content, when present, varies from 0.226 ± 0.029% to 0.408 ± 0.029%. Finally, the protein percentage of the porridge ranges from 7.061 ± 0.779 to 12.419 ± 0.779.

TEKS LENGKAP

1. Introduction

Fermented cereal-based foods are a significant cultural heritage in sub-Saharan Africa, where some of the world’s most decadent fermented foods can be found [1]. These cereal-based foods are staples, complementary, and

weaning for infants and young children [2]. These products are crafted using equipment and raw materials that are readily available in the local area. However, the products and materials used vary from region to region [2], as with fermented porridges [3]. Traditional food fermentation processes rely on a wide range of microorganisms and their enzymes to achieve the desired characteristics [2], which are often uncontrolled due to the preparation process. Fermented products are known to have health-promoting effects due to the presence of functional microorganisms. These microorganisms, such as Lacticaseibacillus, Lactobacillus, Levilactobacillus, and Bifidobacterium, can occur naturally and/or be added to various products [4]. Lactic acid fermentation has gained attention because it reduces contamination by pathogenic microbes by producing lactic acid and other antimicrobial metabolites, thereby decreasing the pH of fermented food products [5]. These spontaneously fermented foods have multiple health benefits [6] and help extend food availability beyond the production area and season, contributing to national and household food security [1]. Microorganisms in fermented products can have a multidirectional beneficial effect [4] and temporarily affect the gut microbiome [7]. This allows for modifying and modulating intestinal function, improving health, or reducing the risk of dysbiosis-related diseases.

In Benin, spontaneously fermented porridge, the manufacturing process of which remains empirical, constitutes an essential part of the daily diet. The significant variability of production conditions leads to fermented porridges of low and variable technological quality and, consequently, to fermented porridges of equally variable nutritional value. Indeed, the safety of these products is not always guaranteed because the cereals and oilseeds used to produce complementary foods could be contaminated with microorganisms and/or mycotoxins [8]. Undesirable microorganisms, toxins, and chemicals can cause food poisoning [8]. Pathogenic bacteria and viruses can cause many eating disorders. These pathogens include *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Staphylococcus aureus*, *Vibrio cholerae*, *Streptococcus* spp., *Bacillus cereus*, *Yersinia enterocolitica*, *Campylobacter* spp., *Listeria monocytogenes,* and *Clostridium perfringens* [9]. A good understanding of the nutritional quality and microbial diversity of cereal-based fermented porridges in northern Benin would be a prerequisite for developing and implementing evidence-based policies to improve food and nutrition security and have a standardized process. Therefore, this work aims to evaluate the nutritional, physicochemical, and microbiological parameters of microorganisms from fermented porridges produced and consumed in northern Benin.

2. Material and Methods

2.1. Sample Collection

A total of 147 samples of fermented porridges were collected for microbiological analysis. Three samples from each kind of porridge from the 49 producers and each sample were placed in sterile bags and kept in coolers with storage batteries. The samples were transported, stored at 4°C in the laboratory, and analyzed about 48 hours after collection. Forty-nine collected samples were used for physicochemical and nutritional analysis, taking one sample per type of porridge.

2.2. Microbial Analysis of Fermented Porridges

In the laboratory, 10 ml of each fermented porridge sample was mixed with 90 ml of sterile bacteriological peptone (Oxoid, Hampshire, England). The microbiological analysis focused on staphylococci, *E. coli*, *Salmonella* spp., coliforms, yeasts, molds, and total mesophilic aerobic flora. Decimal dilutions were made with peptone water (Bio-Rad, Paris, France) from the incubated suspension and used to count the bacteria. Baird–Parker agar (Biovar Diagnostics, France) with egg yolk [10, 11] was used for Gram-positive cocci. The enumeration of *E. coli* was carried out on TBX culture medium (Tryptone Bile X-Glucuronide) according to ISO standards. Total coliforms and thermotolerant coliforms were counted according to standard NF V08-050 and NF V08-060. Lactic flora was counted using MRS, and the fungal flora was counted using Dichloran Rose Bengal Chloramphenicol (DRBC) agar (BD, France). *Salmonella* spp. was identified according to ISO 6579-1 [12]. The frequency of contamination was calculated as the ratio of contaminated products to all products, and the prevalence was obtained as the ratio of strains isolated to all biological products tested according to the standard.

2.3. Physicochemical Characterization of Porridges

The dry matter content, pH level using the HI 8418 pH meter, titratable acidity, brix degree, and total minerals of the

different types of porridge collected were detected by using the adaptation of a method previously described by Nout et al. [13].

2.4. Determination of Dry Matter (DM) Content

To determine the amount of dry matter, samples were placed in an oven at 105°C for 24 hours [14]. After that, they were weighed using a differential method. The dry matter content was determined by using the following formula: dry matter content (DM) (%) = $(P2 - P0)/(P1 - P0) \times 100$, where $P0$ is the weight of the empty crucible, $P1$ is the weight of the fresh sample, and $P2$ is the weight of the dried sample.

2.5. Determination of pH and Titratable Acidity

The modified method of Nout et al. [13] was used, and the tests were duplicated. A sample mix (10 g) and water (20 ml) were used to measure the pH. The titratable acidity was determined by titrating the suspension with 0.1N NaOH until the pH stabilized at 8.2. The results are expressed as a percentage of lactic acid on a dry basis. To calculate the percentage of lactic acid (b.s), the following formula was used: %lactic acid (b.s) = $V/Ma \times 0.9$, where V is the volume of 0.1 N NaOH in ml, M is the mass of the sample in g, and a is the dry matter content of the sample.

2.6. Assay of Ash Content

To determine a porridge sample's raw ash content, 5 g was carbonized and incinerated at 550°C for 24 hours [14]. The resulting substance is weighed after being cooled in a glass desiccator. The ash content is then calculated as a percentage of dry flour.

2.7. Determination of the Brix Degree

A refractometer (Sopelem 9596, France) calibrated with a pH 7 buffer solution was used to measure the brix degree. The measurement involved placing a drop of the wet sample on the lens of the refractometer and taking a direct reading after exposure to light.

2.8. Lipid Assay

Free lipids were assayed using the automated Soxhlet extraction apparatus (E-812/E-816HE, Buchi AG, Switzerland) [14]. It consists of extracting the free lipids from the sample for 4 hours with petroleum ether. Extraction is followed by drying in an oven at 105°C for one hour. The flasks are cooled in a desiccator and then weighed. The lipid content is expressed as a percentage on a dry basis.

2.9. Crude Protein Determination

The total nitrogen was measured by using the Kjeldahl method to analyze the crude proteins in porridge [15]. This involves mineralizing the sample, distilling the mineralized product, and titrating it. The resulting nitrogen content is multiplied by a conventional factor of 6.25 to determine the total protein content.

2.10. Distillation and Titration

After the mineralization process, excess soda is used to neutralize and alkalize the material. During this process, all ammonium ions are converted into ammonia, resulting in NH_3 being the only nitrogen present. The ammonia is then extracted through hydrodistillation with water vapor, and the vapors are collected in an adequately acidic medium containing boric acid. Subsequently, a sulfuric acid solution of known strength is added to the ammonia to determine the equivalence point through an indicator's color change. The ammoniacal nitrogen content is calculated by using the following formula: $N(\%) = ((V1 - V0) * T * 0.014 * 100)/m$, where $V0$ is the volume of acid poured into the blank, $V1$ is the volume of acid poured into the sample, T is the titer of sulfuric acid (0.5 Mol/l), and m is the test portion of the sample.

The crude protein content of the product can be determined by multiplying the nitrogen content value obtained by 6.25 for animal feed and 6.38 for dairy products. Furthermore, the nitrogen content equals N (%) multiplied by the protein factor.

2.11. Data Analysis and Processing

Minitab 16 software was used to analyze variance (ANOVA) to compare the means of chemical and microbiological variables in different study areas. To structure the means, Fisher's grouping test was used. The correlations between various chemical and microbiological variables were also conducted by using the same software. Statistical differences were determined using R software version 4.2.2 [16] with a probability value of less than 5% ($p<0.05$).

3. Results

3.1. Microbiological Characterization of Fermented Porridges

3.1.1. Distribution of Microorganisms by Region

Figure 1 displays the distribution of microorganisms by region. The data indicate that lactic acid bacteria are the most prevalent in this study's porridge samples collected from municipalities. The total mesophilic aerobic flora (TMAF) is the second most prevalent. However, small amounts of *Escherichia coli*, total coliforms, and thermotolerant coliforms are present. The highest proportion of lactic acid bacteria (70%) was found in Matéri's porridges, followed by Cobly (64%) and Djougou (45%). Porridge from Parakou had a higher staphylococci prevalence (55%), followed by N'Dali (36%) and Banikoara (35%). Although *E. coli* was present in small amounts in all municipalities, the highest contamination was in Djougou's (7%) and Ouaké's (5%) porridges. However, *E. coli* was not found in Parakou, N'Dali, Copargo, Cobly, and Banikoara porridges.

[figure(s) omitted; refer to PDF]

3.1.2. Distribution of Microorganisms according to the Type of Porridge

The study found that different types of porridge contain varying numbers of microorganisms, including lactic acid bacteria, TMAF, staphylococci, yeasts, and coliforms (Figure 2). Lactic acid bacteria were the most prevalent, followed by TMAF, and not all porridges contained microorganisms such as *E. coli* and thermotolerant coliform molds. Overall, lactic acid bacteria were found in higher proportions than other microorganisms in all eight types of porridge. The contamination rates were highest in bobossou at 50%, followed by sagagnèga at 40%, koko at 36%, apkan at 34%, gbangba at 32%, akloui at 31%, fourra at 30%, and bita at 26%. TMAF was most frequently found in bobossou at a rate of 40%, followed by sagagnèga at 33% and apkan at 31%. Bita had the highest proportion of *E. coli* contamination at 5%, while akloui, bobossou, koko, and sagagnèga contained none. Yeasts were more prevalent in bobossou (6%), and staphylococci were more commonly encountered in gbangba (30%), followed by akloui (29%), koko (27%), apkan (24%), bita (20%), sagagnèga (20%), fourra (17%), and bobossou (2%).

[figure(s) omitted; refer to PDF]

3.1.3. Correlation Matrix of Germs Depending on the Product and Locality

The correlation matrix of microorganisms found in the porridges is shown in Figure 3. This matrix indicates a correlation between different microorganisms. Strong correlations exist between thermotolerant coliforms and *E. coli* (0.95), total mesophilic aerobic flora and lactic acid bacteria (0.97), and total aerobic mesophilic flora and yeast (0.87), which suggests that the population of these microorganisms increases proportionally in the porridges. However, weak correlations are observed between *E. coli* and lactic acid bacteria (0.34), yeast and staphylococci (0.39), yeast and *E. coli* (0.28), mold and thermotolerant coliforms (0.21), and *E. coli* and mold (0).

[figure(s) omitted; refer to PDF]

Based on the visited municipalities, positive and negative correlations were discovered between microorganisms found in the porridges (Figure 4). Specifically, it was observed that strains of *Escherichia coli* have a negative correlation with staphylococci (−0.45) and total coliforms (−0.19). Moreover, molds and yeasts negatively correlate with coliforms and lactic acid bacteria with staphylococci. However, it was also found that yeasts and total coliforms positively correlate with molds and thermotolerant coliforms.

[figure(s) omitted; refer to PDF]

3.1.4. Isolation of *Salmonella* spp. according to Porridges

According to Table 1, *Salmonella* spp. was isolated from different types of porridge. Koko porridge had the highest percentage of *Salmonella* spp. at 21%, followed by bita and akloui porridge at 19%. Bobossou porridge had the least amount of *Salmonella* spp. at 3%. Fermented porridges had a higher presence of *Salmonella* spp. at 94% than nonfermented porridges at 53%.

Table 1

Isolation of *Salmonella* spp. according to the porridges.

Porridges	*Salmonella* spp.		Total	X^2	P value
Presence	Absence	Koko	21 (22.30)	6 (11.30)	27 (18.40)
14.929739	0.037	Fourra	8 (8.50)	10 (18.90)	18 (12.20)
Bobossou	3 (3.20)	6 (11.30)	9 (6.10)	Bita	19 (20.20)
5 (9.40)	24 (16.30)	Sagagnega	6 (6.40)	6 (11.30)	12 (8.20)
Apkan	9 (9.60)	9 (17)	18 (12.20)	Akloui	19 (20.20)
8 (15.10)	27 (18.40)	Gbangba	9 (9.60)	3 (5.70)	12 (8.20)
–					
Total	94 (100)	53 (100)	147 (100)		

3.1.5. Characterization of Porridges

Based on the analysis of the eigenvalues of the correlation matrix (Table 2) and the principal component analysis, it was found that the first two dimensions account for 68.02% of the variability of the microorganisms. This is a significant amount of information, exceeding the 50% threshold, which means that the first three dimensions can be effectively used to interpret the results of the PCA.

Table 2

Evolution of the cumulative percentage of explained variance according to the first 6 factorial axes.

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6
Variance	2.693	1.388	0.811	0.589	0.313	0.206
% of variance	44.885	23.135	13.513	9.824	5.214	3.429
Cumulative % of the variance	44.885	68.020	81.533	91.357	96.571	100.000

Based on Table 3 and Figure 5, the correlation between the three dimensions and initial variables was studied. The variables TMAF, lactic acid bacteria, and thermotolerant coliforms are positively correlated with axis 1, which explains 44.89% of the variability.

Table 3

Correlation between the starting variables and the factorial axes.

Variables	Dim.1	ctr	cos2	Dim.2	ctr	cos2	Dim.3	ctr	cos2
TMAF	0.851	26.873	0.724	−0.032	0.074	0.001	0.163	3.274	0.027
Lactic acid bacteria	0.698	18.102	0.488	−0.440	13.922	0.193	−0.269	8.911	0.072

Total coliform	0.511	9.688	0.261	0.560	22.619	0.314	−0.586	42.387	0.344
Thermotolerant coliforms	0.855	27.126	0.731	0.130	1.220	0.017	−0.029	0.102	0.001
S. aureus	0.438	7.120	0.192	0.665	31.862	0.442	0.559	38.519	0.312
Yeast	0.547	11.090	0.299	−0.649	30.303	0.421	0.235	6.807	0.055

[figure(s) omitted; refer to PDF]

Therefore, in the porridges sold in the municipalities of this axis, TMAF, lactic acid bacteria, and thermotolerant coliforms were found simultaneously (Figure 5). Axis 2 explains 23.13% of the variability of microorganisms in the porridges. It is positively correlated with the “total coliform (TC)” variable and “staphylococci (Staph)” and negatively correlated with the “yeast” variable. Axis 2 suggests that the presence of “total coliforms” in the porridges is positively linked to that of “staphylococci.” However, the presence of yeasts in the porridges is linked to the absence of total coliforms and staphylococci (Figure 5).

3.1.6. Porridge Typology

Three distinct categories of porridges were identified based on the ascending hierarchical classification dendrogram shown in Figure 6. Each category corresponds to a specific profile of porridges, determined by carefully selected criteria.

[figure(s) omitted; refer to PDF]

Three distinct porridge categories can be identified by analyzing the dendrogram of the ascending hierarchical classification (Figures 6 and 7). Each category corresponds to a specific porridge profile that meets precise and carefully chosen criteria.

[figure(s) omitted; refer to PDF]

There are 25 samples in group 1, which comprise 50% of the porridges. This group is characterized by staphylococci, TMAF, and lactic acid bacteria found in porridges. The porridges representing this group are koko from Djougou, N’Dali, and Ouaké, gbangba from Djougou, and akloui from Ouaké.

There are six porridge samples in group 2, which comprise 12% of the total sample. This group shows a significant association with total and thermotolerant coliforms in porridges. The representative porridges from this group are bita from Kandi and Copargo, apkan from N’Dali, koko from Kandi, and akloui from Parakou.

Out of the 19 participants, 38% belonged to group 3. This group mainly consumes porridge from Djougou, Banikoara, and Copargo communes. Their porridge contains microorganisms such as total aerobic mesophyll flora, lactic acid bacteria, yeasts, thermotolerant coliforms, molds, staphylococci, and *E. coli*. These microorganisms are present in bita porridge from Djougou, apkan from Banikoara, sagagnèga from Copargo, bita from Banikoara, and apkan from Copargo.

Table 4 shows the different microorganisms present in each group and their specific characteristics. Upon analysis, it was observed that the total mesophyll aerobic flora, lactic acid bacteria, and type 1 thermotolerant coliforms are closely associated with types 2 and 3. However, there is no apparent connection between staphylococci, *E. coli*, and molds. Furthermore, there is a relationship between types 1 and 2 regarding total coliforms, but not with type 3. Regarding yeasts, there is a correlation between types 1 and 2 compared to type 3.

Table 4

Microbiological characteristics of each group.

Cluster	Type 1 (50%)	Type 2 (12%)	Type 3 (38%)
TMAF	6.90±0.06a	7.27±0.10b	7.43±0.02b

Lactic acid bacteria	6.98±0.09a	7.23±0.12ab	7.40±0.03b
Total coliforms	4±0.40a	7.06±0.06b	5.67±0.36a
Thermotolerant coliforms	3.04±0.56a	7.10±0.10b	6.24±0.51b
S. aureus	6.02±0.23a	7.07±0.14a	6.95±0.18a
Yeast	5.67±0.09a	5.58±0.15a	6.30±0.08b
E. coli	1.03±0.43a	2.86±1.30a	3.73±0.61a
Mold	4.09±0.30a	4.89±0.16a	3.99±0.59a

The different letters a, b, and c present on the means of the same line indicate that these means are significantly different ($p<0.5\%$).

3.2. Physicochemical and Nutritional Parameters of Fermented Porridges

Table 5 displays the physicochemical parameters based on the different types of fermented porridge and shows that the dry matter of the eight different types of porridge ranges from 27.03±3.83 g/100 g for akloui to 48.63±3.83 g/100 g for fourra. In all porridges, the water content is higher than the dry matter. Akloui's dry matter is significantly different from apkan and fourra, but not significantly different from bita, bobossou, gbangba, koko, and sagagnèga ($p<0.0001$). The total ashes vary from 39.36±4.67 g/100 g for bita to 63.19±4.67 g/100 g for sagagnèga. Sagagnèga, akloui, bobossou, apkan, and fourra porridges have more than 50% ash in the 100 g sample. However, bita, koko, and gbangba have less than 50% ash in the 100 g sample. There is a significant difference between bita and sagagnèga ($p=0.028$). All fermented porridges have an acidic pH of less than 5, ranging from 3.57±0.09 for apkan to 4.44±0.09 for bita. There is a significant difference ($p=0.0001$) between akloui and apkan and between apkan and bita, fourra, gbangba, and koko. The titratable acidity ranges from 0.01±0.00 g of lactic acid for akloui, apkan, bita, bobossou, fourra, gbangba, and koko to 0.02±0.00 g of lactic acid for sagagnèga. There is a significant difference between akloui and sagagnèga ($p<0.0001$), but no difference was observed between the other types of porridge. The brix degree ranges from 0.46±0.54 for akloui to 4.4±0.54 for sagagnèga. A significant difference exists between akloui, fourra, gbangba, and sagagnèga ($p=0.0001$).

Table 5

Physicochemical parameters according to the types of porridge.

Porridge	Akloui	Apkan	Bita	Bobossou	Fourra	Gbangba	Koko	Sagagnèga	SEM	pvalue
Dry matter	27.49a	46.86bc	27.03a	30.10ab	48.63c	34.21abc	30.86ab	33.64abc	3.83	0.0001
Total ash	55.46ab	54.84ab	39.36a	55.04ab	50.60ab	46.95ab	47.50ab	63.19b	4.67	0.028
pH	4.21b	3.57a	4.44b	3.98ab	4.33b	4.29b	4.34b	4.04ab	0.09	0.0001

Titratable acidity	0.01a	0.01ab	0.01a	0.01a	0.01ab	0.01a	0.01a	0.02b	0.00	0.0001
Brix degree	0.46a	1.65ab	2.00abc	1.73ab	3.97bc	3.85bc	1.01a	4.43c	0.54	0.0001

The letters a, b, and c on the means of the same line indicate that these means are significantly different. SEM, standard error of the mean.

The nutritional parameters for different types of porridge are presented in Table 6. The beta-carotene values vary from 0.037 ± 0.018 mg/g to 0.138 ± 0.018 mg/g among the eight types of porridge. The highest value is for sagagnèga, followed by bobossou, gbangba, and fourra. Akloui has the lowest value. There is a significant difference between akloui, bobossou, and sagagnèga (p value 0.001), but no difference was observed between the other types of porridges. Total sugars range from 1.926 ± 0.877 to 5.773 ± 0.877 g/100 g, with bobossou having the highest value, followed by sagagnèga and gbangba. Akloui has the lowest value. Presently, the lipid content of the porridges varies from 0.226 ± 0.029% for fourra to 0.408 ± 0.029% for bita. The protein percentage ranges from 7.061 ± 0.779 to 12.419 ± 0.779. Gbangba has the highest protein value, followed by bita, bobossou, and akloui. The smallest value is observed in apkan. A significant difference exists between apkan, bita, bobossou, and gbangba porridges ($p<0.0001$).

Table 6

Nutritional parameters according to the types of porridge.

Porridge	Beta-carotene	Total sugars	Lipid content	Protein
Akloui	0.037a	1.926a	0.322	10.168ab
Apkan	0.045ab	3.096ab	0.269	7.061a
Bita	0.042a	2.535ab	0.408	12.419b
Bobossou	0.126bc	5.773b	0.308	11.051ab
Fourra	0.061ab	4.745ab	0.226	8.049a
Gbangba	0.062ab	5.074ab	0.265	12.812b
Koko	0.041a	2.005a	0.251	9.411ab
Sagagnèga	0.138c	5.700ab	0.235	9.469ab
SEM	0.018	0.877	0.029	0.779
P value	0.001	0.001	0.054	0.0001

The letters a, b, and c on the means of the same line indicate that these means are significantly different. SEM, standard error of the mean.

3.3. Distribution of Microorganisms in Different Porridges

Three different colors represent three groups of interactions. The size of each group represents its importance. At

the same time, the thickness of the line indicates the strength of the connection between the different groups. Bobossou is the least encountered porridge, and *E. coli* is the least identified bacterium in porridge. The most robust links are “TMAF” followed by “lactic acid bacteria,” “yeast,” “*S. aureus,*” and “*Salmonella* spp.,” respectively (Figure 8).

[figure(s) omitted; refer to PDF]

4. Discussion

This study focuses on fermented cereal-based porridges produced and consumed in northern Benin. The study includes microbiological, physicochemical, and nutritional analyses. The results indicate a significant variation in the microbial composition and porridge characteristics across different communes. The porridges in Matéri have the highest proportion of lactic acid bacteria, followed by Cobly. Bobossou’s fermented porridge has many lactic acid bacteria, followed by sagagnèga and koko. These porridges are recommended for consumption. However, porridges in Djougou and Ouaké (porridge bita, fourra, and apkan) are heavily contaminated by *Escherichia coli*. This is due to manual handling and the use of raw water after cooking. The fourra is left in the open air for preservation, diluted by hand, and mainly consumed without reheating, which increases the risk of contamination. The staphylococcal contamination of gbangba, akloui, koko, apkan, bita, sagagnèga, fourra, and bobossou porridges shows that although desirable microorganisms such as lactic acid bacteria are present, there are also potentially pathogenic microorganisms. This could be due to producers’ lack of knowledge of HACCP, especially after cooking the porridge. In addition, lactic acid bacteria dominate fermented porridges in northern Benin with an average load of 6.7309 log CFU/g/ml, followed by total aerobic mesophilic flora with an average load of 6.7, and yeasts and molds with an average load of 7.9771. These microorganisms are responsible for the fermentation process of the porridges. According to Kagambega et al. [17], fermented cereal-based foods frequently contain lactic acid bacteria, yeasts, molds, and some *Bacillus* and *Escherichia coli* species. Lactic acid bacteria and yeasts are generally the predominant microorganisms found in most fermented products from cereals and cassava in West Africa, as proven by studies conducted by N’Tcha et al. [18]. The development of lactic acid bacteria is accompanied by the development of yeasts, which results from a symbiotic relationship between the two microorganisms. Recent studies conducted by Ponomarova et al. [19] have shown that yeasts allow the development of lactic acid bacteria through endogenous cross-feeding, resulting in a quickly established community. Lactic acid bacteria (LAB) mainly perform lactic acid fermentation, which is essential for the preservation and safety of fermented foods, as proven by Awobusuyi et al. [20]. These microbes can produce and respond to neurochemicals, which are potentially helpful in treating anxiety and depressive disorders [21]. Thus, consuming fermented cereal-based porridges can positively impact the oral microbiota by lowering the pH and producing antioxidants that inhibit plaque growth, thereby reducing the risk of gum disease, tooth decay, and oral inflammation. Fermented products can also treat halitosis, metabolizing volatile sulfur compounds that cause unpleasant mouth odor [22, 23]. According to N’Tcha et al. [18], the presence of LAB in a medium creates an acidic environment that promotes the growth of yeasts, which produce vitamins and other compounds favorable for the proliferation of yeasts and bacteria. This acidity also inhibits the proliferation of pathogenic microorganisms, such as *E. coli*, which are absent in some collected porridges.

This study found a strong correlation between thermotolerant coliforms and *E. coli* ($r = 0.95$), as well as between total mesophyll aerobic flora and lactic acid bacteria ($r = 0.97$) and between total mesophyll aerobic flora and yeast ($r = 0.87$). However, there was a weak correlation between *E. coli* and lactic acid bacteria ($r = 0.34$), yeast and staphylococci ($r = 0.39$), yeast and *E. coli* ($r = 0.28$), mold and thermotolerant coliforms ($r = 0.21$), and *E. coli* and mold ($r = 0$).

It has been observed that certain strains of *Escherichia coli* have a negative correlation with staphylococci ($r = -0.45$) and total coliforms ($r = -0.19$). In addition, molds and yeasts are also negatively correlated with strains of total coliforms and lactic acid bacteria with staphylococci. This implies that the growth of one of these microorganisms can hinder or eliminate the growth of the other. However, yeasts and total coliforms positively correlate with molds and thermotolerant coliforms. Total coliforms and thermotolerant coliforms in food indicate fecal contamination [24]. This study found total and thermotolerant coliforms in fermented porridges, which suggests potential contamination

by pathogenic *E. coli*. This is supported by the correlation between thermotolerant coliforms, *E. coli*, and total coliforms.

Koko porridge has the highest percentage of *Salmonella* spp. at 22.3%, followed by bita and akloui porridge at 20.2%. Conversely, bobossou porridge has the lowest amount of *Salmonella* spp. at only 3.2%. Most porridge samples (94%) were contaminated with *Enterobacteriaceae*, compared to only 54% of the porridges. The primary source of contamination may be unclean water used for washing dishes, utensils, and hands.

It is important to note that TMAF is an indicator of food quality, not safety, and cannot directly contribute to food safety assessment. However, it can give helpful information about the remaining shelf life of foods. In addition, the presence of *Enterobacteriaceae* in ready-to-eat prepared foods may be due to the safety of the environment in which the food is served. These findings align with the research conducted in Ethiopia by Bolaji et al. [25] on ready-to-eat foods contaminated with microorganisms such as *Salmonella* spp. and *E. coli*.

Staphylococci in the porridge indicate that humans have contaminated it. This contamination may be due to poor hygiene practices during production or consumption. In addition, concerns about the quality of the raw material should also not be neglected. Indeed, contaminated raw materials will not produce a safe product, especially fermented ones, without heat treatment. Hence, to minimize contamination, it is necessary to ensure appropriate harvesting dates, pay attention to the weather during harvesting, and reject batches of raw material whose visual quality deviates from the expected. The present research found staphylococci in all fermented tested porridges. This is likely due to the salespeople not wearing nose masks and gloves while handling the products. Through principal component analysis, fermented porridges could be grouped based on similarity. Three large groups could be observed: group 1 comprised of 50% of the porridge, group 2 comprised of 38% of the porridge, and group 3 comprised of 12% of the porridge. These findings differ from a study performed by N'Tcha et al. [18] on kpètè-kpètè, a traditional beer fermented in Benin. The variation in parameters measured from municipality and producer to producer explains the significant difference in groupings. Therefore, it is essential to define parameters to ensure the quality of fermented porridges for consumers.

The eight types of porridge have varying physicochemical and nutritional parameters. Dry matter, for instance, ranges from 27.03 ± 3.83 g/100 g for akloui to 48.63 ± 3.83 g/100 g for fourra. All porridges have higher water content than dry matter. Akloui's dry matter significantly differs from apkan and fourra, but not from bita, bobossou, gbangba, koko, and sagagnèga ($p=0.0001$). These results are better than those of Kagambèga et al. [17], who found traditional porridge dry matter values to be between 7 and 10 g/100 g and added sugar to increase values.

Total ashes ranged from 39.36 ± 4.67 g/100 g for bita to 63.19 ± 4.67 g/100 g for sagagnèga. Sagagnèga, akloui, bobossou, apkan, and fourra have over 50% ash in the 100 g sample, while bita, koko, and gbangba have less than 50%. The obtained results are higher than those of *Fatoumata* et al. [26], who found 2.81 ± 0.06 to 4.93 ± 0.08% ash in soumbala of soya sold in Côte d'Ivoire. The difference in raw materials used could explain this variation.

The pH of all fermented porridges is below 5. The pH varies from 3.57 ± 0.09 for apkan to 4.44 ± 0.09 for bita. So, all porridges have an acidic pH. A significant difference ($p=0.0001$) exists between akloui and apkan and apkan and bita, fourra, gbangba, and koko. The recorded results are superior to those obtained by Coulibaly et al. [27]. Several authors [28, 29] believe that fermentation by lowering the pH of products to values below 4.0 limits the development of *Enterobacteriaceae* and other Gram-negative bacteria. The titratable acidity at akloui, apkan, bita, bobossou, fourra, gbangba, and koko is 0.01 ± 0.00% lactic acid and at sagagnèga is 0.02 ± 0.00% lactic acid. A significant difference exists between akloui and sagagnèga ($p=0.0001$). On the other hand, no difference was observed among the other types of porridge. The results of this study are, on the other hand, lower than the 0.495 ± 0.01–0.405 ± 0.01% obtained by Coulibaly et al. [27] on the identification of non-Saccharomyces yeast strains isolated from traditional beer in the district of Abidjan (Côte d'Ivoire) and their ability to carry out alcoholic fermentation. The brix degree values vary between 0.46 ± 0.54 for akloui to 4.4 ± 0.54 for sagagnèga. A significant difference exists between akloui, fourra, gbangba, and sagagnèga ($p=0.0001$). At this level, the results are much lower than the 11.7 ± 0.14° brix obtained by the authors in [25].

The beta-carotene values of the eight types of porridge vary from 0.037 ± 0.018 mg/g to 0.138 ± 0.018 mg/g.

Sagagnèga has the greatest value, followed by bobossou, gbangba, and fourra. Total sugars vary from 1.926 ± 0.877 to 5.773 ± 0.877 g/100 g, with bobossou porridge having the highest value, followed by sagagnèga and gbangba. The lowest value was observed for akloui porridge. These values are much lower than those of Amoin et al. [30] on germinated and fermented compound flour (69.20 ± 0.8% and 67.80 ± 0.3%). This could be explained by these flours made from germinated and fermented cereals.

The protein levels (7.061 ± 0.779 and 12.419 ± 0.779%) found in the analyzed samples are much lower than those (33 ± 2.4 to 34.6 ± 2.6%) reported by *Fatoumata* et al. [26] in their study of fermented *Hibiscus sabdariffa* L. seeds, a condiment commonly used in West Africa. This difference in protein content could be attributed to the variation in raw materials and the lower dry matter content of the porridge-tested samples. Nonetheless, the protein levels are significantly higher than the reported 6.74 g/100 g DM and 2.34 g/100 g DM found in sorghum porridge [17]. Kagambèga et al. [17] also noted that traditional porridges usually contain less than 2.6 g/100 g DM of protein and less than 0.8 g/100 g DM of lipids. In comparison, the lipid content of the eight types of porridge tested in this study (ranging from 0.226 ± 0.029% to 0.408 ± 0.029% in fourra) is lower than that found in traditional porridge, as reported by Kagambèga et al. [17] in their study.

5. Conclusion

The porridge also contained beneficial microorganisms such as lactic acid bacteria, yeasts, and molds responsible for their fermentation and total mesophyll aerobic flora. Fermentation processes that involve various microorganisms have many benefits. However, if good production practices are not followed, there may be a risk of microbiological contamination in cereal-based porridge. Unfortunately, our research found potentially harmful microorganisms such as *Escherichia coli*, staphylococci, and *Salmonella* spp. in such porridges. This means that consuming such porridge can lead to food-borne infections. Therefore, it is crucial to inform food handlers about the risks associated with consuming contaminated food, including fungi, bacteria, and the toxins they can produce. This will make individual producers want to pay more attention to the sanitary safety of their food.

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DETAIL

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Detection of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus in Inactivated Fermented Milk Using Fluorescence

Quantitative Loop-Mediated Isothermal Amplification

Zhou, Shuaikang; Hu, Lianxia; Xue, Yuling; Zhang, Dong; Song, Baokuo; dkk.

ABSTRAK (ENGLISH)

Currently, no effective method exists to detect and monitor fermentation probiotics and evaluate the quality of inactivated fermented milk. Therefore, in this study, a fluorescence quantitative loop-mediated isothermal amplification (FQ-LAMP) method was developed to detect *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The specificity of LAMP primers for *L. bulgaricus* and *S. thermophilus* was verified using S-type amplification curves and a single peak at approximately 88.568°C and 83.704°C of the melting curves, respectively. The lowest quantification limits of FQ-LAMP for the two strains in inactivated fermented milk were 8.1×10^3 CFU/g (170 fg/μL) and 6.8×10^3 CFU/g (170 fg/μL), respectively. FQ-LAMP was used to analyse 40 inactivated fermented milk samples from six randomly selected brands. The logarithmic concentration of *S. thermophilus* in all products was between 7.482 and 8.936. The logarithmic concentration of *L. bulgaricus* ranged from 4.590 to 8.277, with no detectable *L. bulgaricus* in three samples. FQ-LAMP has the potential as a rapid, specific, and accurate method for detecting and monitoring *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk during their shelf life.

TEKS LENGKAP

1. Introduction

Fermented milk is made from raw cow (goat) milk or milk powder that has a reduced pH, achieved through processing procedures such as sterilisation and fermentation [1, 2]. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus,* two fermentation bacteria, are beneficial for the intestinal tract [3, 4]. They can maintain the intestinal microecological balance [5], inhibit the growth and reproduction of harmful intestinal bacteria [6, 7], regulate intestinal immune function [8], and improve intestinal barrier function [9], among other health functions. As most of the lactose in fermented milk is degraded by lactic acid bacteria, their administration is more suitable for people with lactose intolerance, especially Asian populations [10, 11]. Fermented milk can be classified as sterilised (inactivated) and nonsterilised (activated), depending on whether it is sterilised at the end of fermentation [12]. However, the shelf life of activated bacteria-fermented milk is short (generally 21 days), and the acid structures tend to be easily altered, resulting in the deterioration of the milk. Therefore, a cold chain system is required throughout the storage, transportation, and sales process, which is an inconvenience for manufacturers and dealers [13]. Inactivated fermented milk and its products have several advantages over activated milk, such as a long shelf life (generally 6 months), easy storage and transportation, and no risk of infection for susceptible people [14]. Inactivated probiotics have many beneficial effects on the human body; for example, heat-inactivated *Lactobacillus brevis* can enhance the nervous system and memory [15] and alleviate specific dermatitis symptoms [16]. Moreover, long-term use of products containing inactivated lactic acid bacteria can improve the intestinal environment and intestinal function of the consumers, aiding the treatment of gastrointestinal diseases [17–19]. Given these advantages, several inactivated fermented milk products have been introduced in the market, and the quantity of lactic acid bacteria is the core parameter for quantifying probiotic function [20]. Currently, the method for detecting lactic acid bacteria in inactivated fermented milk is primarily based on the traditional culture method after fermentation and thermal inactivation. However, applying this method is tedious, and it cannot specifically detect mixed fermentation

bacteria or achieve real-time monitoring of the number of inactivated lactic acid bacteria during storage [20]. Therefore, establishing a rapid and quantitative method to detect commonly used fermentation bacteria in inactivated fermented milk is important to evaluate the quality of inactivated fermented milk.

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification method developed in 2000 [21]. This method uses four specific primers to identify six specific target gene regions that can be amplified under isothermal conditions. Gene amplification and product detection can be completed in one step with high amplification efficiency (10^9–$l0^{10}$-fold) in 15–60 min. Furthermore, a fluorescent dye (SYBR Green I) can be optimised and added to the LAMP reaction system to produce fluorescence quantitative LAMP (FQ-LAMP) [22]. SYBR Green I binds only to double-stranded DNA grooves, resulting in a fluorescence that is 800–1,000 times stronger than the original. The fluorescence intensity represents the number of double-stranded DNA molecules. During nucleic acid synthesis, SYBR Green I can be used to automatically add double-stranded DNA, and the cycle threshold (Ct) value is obtained by detecting the fluorescence intensity. According to the standard curve, the initial concentration of the bacterial template solution can be determined for quantification. This method has the advantages of simple operation, strong specificity, high sensitivity, good repeatability, low pollution, fast running time, and automatic quantitative analysis and has thus become an important method for probiotic detection [23].

However, effective methods to detect and monitor fermentation probiotics in inactivated fermented milk have not yet been developed. Thus, in this study, we developed an FQ-LAMP method for detecting and monitoring two commonly used fermentation bacteria, *L. bulgaricus* and *S. thermophilus*. In addition, this study analysed the quantities of *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk purchased from Shijiazhuang Supermarket, Hebei Province, China, to provide a basis for ensuring the quality of inactivated milk.

2. Materials and Methods

2.1. Strains and Culture Conditions

Four strains of *L. bulgaricus* and *S. thermophilus* and thirteen common strains usually present in raw milk were used in this study for FQ-LAMP-specific detection (Table 1). All strains were preserved in the R &D Laboratory of Jun Le Bao, Shijiazhuang, China. *Listeria monocytogenes* (ATCC19111), *Cronobacter sakazakii* (ATCC29544), and *Pseudomonas fluorescens* (CICC23246) were cultured in a brain heart infusion broth medium (BHI, Beijing Land Bridge Technology Co. Ltd., Beijing, China) at 37°C for 24 h. *L. bulgaricus* (CICC6097, CICC6047, CGMCC14425, and CGMCC14427), *S. thermophilus* (CICC6063, CICC6222, CICC20174, and CGMCC11672), *Lactobacillus acidophilus* (CICC6081), *Lactobacillus rhamnosus* (CICC6001), *Lactobacillus plantarum* (CGMCC1.1856), *Lactobacillus plantarum* (CICC6009), *Lactobacillus casei* (CICC6117), *Lacticaseibacillus paracasei* (CGMCC4691), *Bifidobacterium animalis* (CICC6250), *Bifidobacterium breve* (CICC6185), *Bifidobacterium adolescentis* (CICC6180), and *Bifidobacterium bifidum* (CICC6173) were cultured separately in a Man, Rogosa, and Sharpe (MRS, Beijing Land Bridge Technology Co., Ltd., Beijing, China) liquid medium at 37°C for 24 h. The four *Bifidobacterium* strains were cultured in an anaerobic environment (Anaerobic gas bag, BioMerieux Company, Lyon, French). The medium and culture conditions for plate counting of *L. bulgaricus* and *S. thermophilus* included incubation in an MRS agar medium at 37°C for 48 h.

Table 1

Strains used in the study.

Order number	Strain name	Strain number	Results of FQ-LAMP detection	
L. bulgaricus gene	*S. thermophilus* gene	1	*L. bulgaricus*	CICC6097
+	−	2	*L. bulgaricus*	CICC6047

+	−	3	*L. bulgaricus*	CGMC C14425
+	−	4	*L. bulgaricus*	CGMC C14427
+	−	5	*S. thermophilus*	CICC6 063
−	+	6	*S. thermophilus*	CICC6 222
−	+	7	*S. thermophilus*	CICC2 0174
−	+	8	*S. thermophilus*	CGMC C11672
−	+	9	*L. acidophilus*	CICC6 081
−	−	10	*L. rhamnosus*	CICC6 001
−	−	11	*L. plantarum*	CGMC C1.185 6
−	−	12	*L. plantarum*	CICC6 009
−	−	13	*L. casei*	CICC6 117
−	−	14	*L. paracasei*	CGMC C4691
−	−	15	*B. animalis*	CICC6 250
−	−	16	*B. adolescentis*	CICC6 180
−	−	17	*B. breve*	CICC6 185

−	−	18	*B. bifidum*	CICC6173
−	−	19	*P. fluorescens*	CICC23246
−	−	20	*L. monocytogenes*	ATCC19111
−	−	21	*C. sakazakii*	ATCC29544

Note. CICC strains were purchased from the China Center of Industrial Culture Collection, Beijing, China. CGMCC strains were purchased from the China General Microbiological Culture Collection Center, Beijing, China. ATCC strains were purchased from the American Type Culture Collection, Rockefeller, Maryland, USA.

2.2. Sample Pretreatment and DNA Extraction

To extract the DNA of *L. bulgaricus* and *S. thermophilus* from inactivated or activated fermented milk, the fermented milk sample was pretreated [24]. Then, the DNA of the pretreated samples was extracted using a bacterial DNA extraction kit(Tiangen Biotech Co., Ltd., Beijing, China). Briefly, 0.2 g inactivated fermented milk was added to a 2-mL centrifuge tube with 1,500 μL deionised water, 200 μL 18% sodium citrate, and 100 μL 1 mol/L sodium hydroxide. Thereafter, the mixture was centrifuged at 4°C for 5 min at 13,523 × g, then the supernatant was discarded, and the precipitate was retained. Next, the genomic DNA of the precipitate was extracted using a bacterial DNA extraction kit.

2.3. Design of Specific FQ-LAMP Primers for *L. bulgaricus* and *S. thermophilus*

Sequence data for the *L. bulgaricus recA* gene [25] sequence (LC685718.1) and *S. thermophilus thioredoxin reductase* (NADPH) gene sequence (GenBank Acc. No: AGFN01000211.1) were obtained from the National Center for Biotechnology Information microbial genome database (https://www.ncbi.nlm.gov), and the gene sequences were aligned, followed by primer design. Conserved sequences were determined and used to design primers. Primers were designed using PrimerExplorer V5 (https://primerexplorer.jp/lampv5e/index.html). Information on the specific primers used is listed in Table 2. The two sets of primers included the forward and backward inner primers (FIP/BIP), the forward and backward loop primers (FL/BL), and the forward and backward outer primers (F3/B3).

Table 2

Information on specific primers.

Target strain	Primer name	Sequence (5′3′)	Primer sequence length (nt)	Amplified region length (bp)
L. bulgaricus (GenBank Acc. No: LC685718)	FIP	TGGAGATCAAGGTGTCCGCGAATCCTGTCTCAGCCAAACAC	41	174
BIP	CCATCGACATCGTCGTGGTCGTTCACCTTCGATTTCGGCC	40	F3	GCGTGGACATCGACCAATT

19	B3	TCCAACGTGGGAGTCACC	18	BL
TTTGCAGCCCTTCTTCCCCA	20	-		
S. thermophilus (GenBank Acc. No: AGFN01000211.1)	FIP	TCGAAATTAAGGGTGAAAATGGTCACTTCATCCGACTTACTCTCTG	46	215
BIP	ACTGATGATTGATAAAGAAGCTCCAGATTCACCGTCGTGATGC	43	F3	AGCTAACAATGAGGGCATC
19	B3	GTGTTGCTGAGAGTGTGA	18	FL

2.4. Reaction System and Reaction Conditions of FQ-LAMP

The volume and concentration of the optimised FQ-LAMP reaction system for *L. bulgaricus* and *S. thermophilus* are listed in Table 3. A 25-μL reaction system, as described in Table 3, was placed in a polymerase chain reaction (PCR) tube, gently vibrated for mixing, and instantaneously centrifuged (25°C). Next, mineral oil (20 μL) was added to the reaction system to cover the reaction surface and prevent cross-contamination between samples, which can reduce the accuracy of the results.

Table 3

Optimised FQ-LAMP reaction system for *L. bulgaricus* and *S. thermophilus*.

Reagent name	Volume (μL)		Reagent sources
L. bulgaricus	*S. thermophilus*	10× ThermoPol reaction buffer	2.5
2.5	New England Biolabs Inc., USA	$MgSO_4$ (50 mmol/L)	1.0
1.3	8 U Bst DNA polymerase	1.2	1.0
–			
dNTPs (10 mmol/L)	1.5	1.5	Sigma-Aldrich, St. Louis, MO, USA
–			

1/400 dilution 10,000× SYBR green I	0.3	0.3	Coolaber Science and Technology Co., Ltd., Beijing, China
–			
Betaine (5 mol/L)	2.0	2.0	Leagene Co., Ltd., Beijing, China
–			
F3/B3 (10 μmol/L)	0.5	0.7	Tsingke Biotechnology Co., Ltd., Beijing, China
FIP/BIP (10 μmol/L)	3.5	3.5	Loop (10 μmol/L)
3.5	3.5	-	
DNA template	1.0	1.0	Bacterial DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China)
–			
Sterile water	4.0	3.5	Tiangen Biotech Co., Ltd., Beijing, China

Amplification reactions were performed using Applied Biosystems QuantStudio 3 (Applied Biosystems, Waltham, MA, USA) at 63°C for 40 min. Melting-curve analysis was performed at the end of FQ-LAMP assays by heating the reaction mixtures to 95°C for 15 s, cooling to 60°C for 60 s, and then increasing the temperature to 95°C for 15 s.

2.5. Specificity of FQ-LAMP

FQ-LAMP was used to detect the DNA of 21 strains of common lactic acid fermentative bacteria in fermented milk. The specificity of the primers used was verified by assessing whether there was an S-type amplification curve and whether the melting curve had a single peak. Distilled water was used as a blank control. All experiments were repeated five times. Positive results are indicated by the "+" symbol, and negative results are indicated by the "−"symbol as shown in Table 1.

2.6. Evaluation of the Effect of Pasteurisation and Storage Time on FQ-LAMP

Fermented milk was prepared by adding *L. bulgaricus* and *S. thermophilus* to raw milk, and the prepared fermented milk was divided into four groups, with three samples in each group. In Group A, the samples were sterilised at 65°C for 10 min; in Group B, samples were sterilised at 75°C for 10 min; in Group C, samples were sterilised at 85°C for 10 min; and in Group D, samples were not sterilised. Samples of the four groups were pretreated, and DNA was extracted for FQ-LAMP detection.

Four randomly selected brands of new date-inactivated fermented milk products were purchased from supermarkets in Shijiazhuang and stored at room temperature in the laboratory (samples of each brand were produced in the same batch). On days 3, 7, 14, 30, 60, 90, 120, and 150 of the shelf life, three samples from each brand were obtained for FQ-LAMP.

2.7. FQ-LAMP Detection Limit for *L. bulgaricus* and *S. thermophilus* in Inactivated Fermented Milk and Drawing Standard Curves

L. bulgaricus and *S. thermophilus* were inoculated separately in an MRS liquid medium and incubated at 37°C for 24 h. The *L. bulgaricus* suspension was added to sterilised raw milk in a 5% ratio for fermentation, and then, plate counting of *L. bulgaricus* in the fermented milk was performed, resulting in a count of 8.1×10^8 CFU/g. Subsequently, 0.2 g of fermented milk was obtained, and the DNA was extracted and diluted with a 10-fold gradient to achieve a final concentration of 21 fg/μL to 21 ng/μL. The corresponding concentration of *L. bulgaricus* was 8.1×10^2 to 8.1×10^8

CFU/g. The *S. thermophilus* suspension was added at a 5% ratio to sterilised raw milk for fermentation, and then, plate counting of *S. thermophilus* in the fermented milk was performed, resulting in a count of 6.8×10^8 CFU/g. Subsequently, 0.2 g of fermented milk was obtained, and the DNA was extracted. The extracted DNA was diluted using a 10-fold gradient to achieve a final concentration of 17 fg/μL to 17 ng/μL. The corresponding concentration of *S. thermophilus* was 6.8×10^2 to 6.8×10^8 CFU/g. Subsequently, FQ-LAMP analysis of different concentrations of DNA from *L. bulgaricus* and *S. thermophilus* was performed, with three analyses per concentration gradient, and the average Ct value was calculated. Thereafter, a standard curve was constructed with the logarithm of the corresponding bacterial concentration as the *x*-axis and the corresponding Ct value as the *y*-axis.

Five measurements were obtained under the same conditions, and the mean, standard deviation (SD), and coefficient of variance (CV) of the peak time were calculated.

2.8. Comparison of the Accuracy of the FQ-LAMP and Plate Count Methods

L. bulgaricus and *S. thermophilus* were inoculated separately in an MRS liquid medium and incubated at 37°C for 24 h, and then different concentrations of each species were inoculated into sterilised milk for fermentation. The prepared fermented milk was divided into two groups; in one group, the number of bacteria was counted using the plate count method, while in the other group, the FQ-LAMP method was used for counting after heat sterilisation treatment.

2.9. Quantitative Detection of *L. bulgaricus* and *S. thermophilus* in Inactivated Fermented Milk Samples Using FQ-LAMP

Forty inactivated fermented milk samples claiming to be fortified with *L. bulgaricus* and *S. thermophilus* were randomly selected from three supermarkets in Shijiazhuang, Hebei Province, China, including different batches of the six brands. During the shelf life, each of the six brands was randomly sampled. After sample pretreatment, DNA was extracted, and quantitative detection (two parallels) and analyses of *L. bulgaricus* and *S. thermophilus* in the inactivated fermented milk samples were performed.

2.10. Statistical Analysis

SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft Corporation, Redmond, WA, USA) were used to analyse the mean, SD, CV, and scatter distribution. Comparisons between the two data groups were analysed using an independent sample *t*-test, and the significance level was set at $p<0.05$.

3. Results

3.1. FQ-LAMP Specificity

The specificity of the FQ-LAMP primers of *L. bulgaricus* and *S. thermophilus* was evaluated using four strains of *L. bulgaricus* and *S. thermophilus* and thirteen strains of common lactic acid fermentation bacteria in fermented milk. The fluorescence intensity (delta Rn) of the four strains of *L. bulgaricus* (Figure 1(A)) and *S. thermophilus* (Figure 1(B)) showed continuous amplification compared with that of other strains and the blank control. In addition, the melting curves (Figure 1(A, B)) revealed that the melting temperatures of the amplified products were almost identical; they were approximately 88.568°C (*L. bulgaricus*) and 83.704°C (*S. thermophilus*), indicating that the FQ-LAMP assay was highly specific and no nonspecific amplification occurred.

[figure(s) omitted; refer to PDF]

3.2. The Effect of Pasteurisation and Storage Time on FQ-LAMP

FQ-LAMP detection of inactivated fermented milk (groups A, B, and C) and noninactivated fermented milk (group D) was conducted. The results (Figure S1) showed no significant differences in the Ct values between groups A, B, C, and D ($p>0.05$), indicating that FQ-LAMP detection is not affected by the pasteurisation of fermented milk.

FQ-LAMP detection was conducted on four brands of inactivated fermented milk with different storage times, and the results (Figures S2 and S3) showed no significant difference in the Ct values of the four brands of inactivated fermented milk on days 3, 7, 14, 30, 60, 90, 120, and 150 ($p>0.05$). Therefore, there was no significant change in the accuracy of FQ-LAMP detection of inactivated fermented milk with different storage times.

3.3. Detection Limit and Standard Curves of FQ-LAMP for *L. bulgaricus* and *S. thermophilus* in Fermented Milk

The average Ct values of inactivated fermented milk with seven 10-fold serial dilutions of *L. bulgaricus* were 11.277,

13.428, 15.777, 18.640, 20.651, 22.335, and 26.582. The CV values of the peak emergence time of inactivated *L. bulgaricus* in fermented milk ranged between 2.93 and 5.29% (Table 4). The average Ct values of inactivated fermented milk with 10-fold serial dilutions of *S. thermophilus* were 11.429, 12.917, 15.466, 17.984, 19.693, 21.918, and 26.312. The CV values of the peak emergence time of inactivated *S. thermophilus* in fermented milk ranged between 2.96 and 5.04% (Table 5).

Table 4

Reproducibility results of the limit of detection of *L. bulgaricus* in fermented milk using FQ-LAMP.

Order number	Concentration (CFU/g)	Times number	Mean Ct	SD	CV (%)
1	8.1×10^{8}	5	11.277	0.425	3.78
2	8.1×10^{7}	5	13.428	0.393	2.93
3	8.1×10^{6}	5	15.777	0.532	3.38
4	8.1×10^{5}	5	18.640	0.723	3.88
5	8.1×10^{4}	5	20.651	0.957	4.64
6	8.1×10^{3}	5	22.334	1.182	5.29
7	8.1×10^{2}	5	26.582	1.36	5.12

Note. 1–7: these 10-fold serial dilutions of *L. bulgaricus* were analysed using FQ-LAMP.

Table 5

Reproducibility results of the limit of detection of *S. thermophilus* in fermented milk using FQ-LAMP.

Order number	Concentration (CFU/g)	Times number	Mean Ct	SD	CV (%)
1	6.8×10^{8}	5	11.428	0.361	3.16
2	6.8×10^{7}	5	12.917	0.382	2.96
3	6.8×10^{6}	5	15.466	0.585	3.78
4	6.8×10^{5}	5	17.984	0.686	3.81
5	6.8×10^{4}	5	19.693	0.954	4.85
6	6.8×10^{3}	5	21.918	1.104	5.04
7	6.8×10^{2}	5	26.312	1.270	4.83

Note. 1–7: these 10-fold serial dilutions of *S. thermophilus* were detected using FQ-LAMP.

A standard curve was drawn using the average Ct values as the ordinate and the logarithm of the concentration of

inactivated *L. bulgaricus* (log_{10}CFU/g) corresponding to the DNA template as the abscissa (Figure 2(a)). The resulting equation is as follows:(1)y=–2.2805x+31.6318,which describes a linear relationship of the standard curve (R2 = 0.9949) between the Ct values in the 11.277–22.335 min range and the logarithm of the concentrations of inactivated *L. bulgaricus* in the 8.908–3.908 range. This finding indicates that the lowest detection limit for FQ-LAMP quantification of *L. bulgaricus* is 8.1×10^3 CFU/g (210 fg/μL)

[figure(s) omitted; refer to PDF]

A standard curve was drawn using the average Ct values of inactivated *S. thermophilus* as the ordinate and the logarithm of the concentrations of inactivated *S. thermophilus* (log_{10}CFU/g) corresponding to the DNA template as the abscissa (Figure 2(b)). The resulting equation is as follows:(2)y=–2.1513x+30.192,which is a linear relationship of the standard curve (R2 = 0.9955) between the Ct values in the 11.429–21.918 min range and the logarithm of the concentrations of inactivated *S. thermophilus* in the 8.833–3.833 range, indicating that the lowest detection limit for FQ-LAMP quantification of *L. bulgaricus* is 6.8×10^3 CFU/g (170 fg/μL).

3.4. Comparison of the Accuracy of the FQ-LAMP and Plate Count Methods

The inoculation ratios of *L. bulgaricus* and *S. thermophilus* in fermented milk were 3 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 3. The detection results of the plate count and FQ-LAMP methods are shown in Table 6. The results show that there was no significant difference in the numbers of *L. bulgaricus* and *S. thermophilus* between the two methods. Compared with that of the plate count method ($p>0.05$), the FQ-LAMP produced a higher quantitative error of 0.16 log_{10}CFU/g. Therefore, the FQ-LAMP can quantify the number of *L. bulgaricus* and *S. thermophilus* in sterilised fermented milk more quickly. Moreover, the counting error of the plate count method was between 0.021 and 0.054, while the counting error of the FQ-LAMP was between 0.085 and 0.178, indicating that the FQ-LAMP method has worse stability and accuracy compared to those of the plate count method.

Table 6

Detection results of manually prepared samples using the plate count method and FQ-LAMP.

Ratio of *S. thermophilus* and *L. bulgaricus*	Counting results (Log_{10}CFU/g)			
S. thermophilus		*L. bulgaricus*		Plate count
FQ-LAMP	Plate count	FQ-LAMP	3 : 1	8.374 ± 0.054
8.332 ± 0.159	8.069 ± 0.041	8.219 ± 0.159	2 : 1	8.290 ± 0.022
8.422 ± 0.150	8.249 ± 0.023	8.352 ± 0.127	1 : 1	8.348 ± 0.035
8.510 ± 0.120	8.234 ± 0.022	8.300 ± 0.149	1 : 2	8.505 ± 0.038
8.444 ± 0.085	7.277 ± 0.040	8.324 ± 0.097	1 : 3	8.111 ± 0.024

3.5. Quantitative Detection of *L. bulgaricus* and *S. thermophilus* in Inactivated Fermented Milk Samples Using FQ-LAMP

Table 7 shows the FQ-LAMP results for 40 samples of inactivated fermented milk from six brands. Samples beyond the detection range were diluted 10 times and tested again. As shown in Table 7, the total number of *L. bulgaricus* and *S. thermophilus* across all inactivated fermented milk samples was $>10^6$ CFU/g, and the logarithmic concentration of *S. thermophilus* in all products was between 7.482 and 8.936. There was little difference in the concentrations of *S. thermophilus* between the different batches of samples from each brand. The logarithmic concentrations of *L. bulgaricus* ranged from 4.590 to 8.277, and three samples had no detectable *L. bulgaricus*. The

number of *L. bulgaricus* in the products was generally lower than that of *S. thermophilus*, and the concentration of *L. bulgaricus* between different batches of brands A and D varied by up to 100 times.

Table 7

FQ-LAMP detection results of *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk that was randomly purchased from the market.

Sample	*L. bulgaricus* (Log_{10}CFU/g)	*S. thermophilus* (Log_{10}CFU/g)
A1	0	8.227
A2	6.193	8.508
A3	6.899	8.036
A4	5.646	8.139
A5	4.876	8.548
A6	5.780	8.892
B1	7.179	8.296
B2	7.486	8.02
B3	6.479	8.266
B4	7.598	7.96
B5	7.321	8.277
B6	7.166	8.437
B7	7.519	8.412
C1	7.412	8.137
C2	7.095	7.756
C3	6.687	8.358
C4	7.935	8.383
C5	6.549	7.865
C6	5.915	8.358

C7	6.241	8.257
D1	4.59	8.936
D2	0	8.528
D3	4.778	8.476
D4	0	8.802
D5	6.691	8.359
E1	8.276	7.482
E2	7.734	8.058
E3	7.335	7.699
E4	7.522	8.125
E5	7.742	7.701
E6	8.182	7.526
E7	7.350	7.985
E8	8.125	8.011
F1	6.211	7.976
F2	6.934	7.898
F3	7.036	8.253
F4	6.147	8.125
F5	6.327	8.256
F6	6.765	8.223
F7	6.2456	7.99

Note. A, B, C, D, E, and F are six brands of inactivated fermented milk.

4. Discussion

In recent years, fermented milk consumption and sales have rapidly increased in China [20]. Inactivated fermented milk has many advantages over activated fermented milk and a market growth rate as high as 50% [20]. Owing to

the rapid increase in the popularity of inactivated fermented milk and its products, detecting fermentation bacteria is a concern for consumers and poses a crucial quality issue. For such products, the number and strain of the fermentation bacteria are important indicators for determining their quality. Different businesses use different fermentation strains for fermented milk. Fermented milk is mainly prepared through the mixed fermentation of *L. bulgaricus* and *S. thermophilus*, which are highly sensitive to pH and bile; thus, it is difficult for these bacteria to reach the intestinal tract in an active state. Live probiotics are thought to enter the intestine to exert their probiotic effects, but metabolites produced by lactic acid bacteria during fermentation, such as organic acids, bacteriocins, enzymes, extracellular polysaccharides, and short-chain fatty acids, also have beneficial effects [26]. Moreover, bacterial cell components contain peptidoglycan, teichoic acid, lipoteichoic acid, and acetal phospholipid, which have been shown to have beneficial functions [27]. Therefore, *L. bulgaricus* and *S. thermophilus* have beneficial properties in inactivated fermented milk.

The traditional cultivation method is largely used for quantitative analysis of bacteria; however, it is cumbersome and cannot determine specific species and the quantity of dead bacteria, making it impossible to monitor sterilised products in circulation to ensure their quality. In this study, FQ-LAMP specifically amplified *L. bulgaricus* and *S. thermophilus* from 17 common probiotic and pathogenic bacteria in fermented milk, which indicated that the method had strong primer specificity. The FQ-LAMP detection results of fermented milk before and after sterilisation showed no significant difference in Ct values between *L. bulgaricus* and *S. thermophilus* indicating that the FQ-LAMP method can accurately quantify inactivated bacteria. Notably, in the FQ-LAMP analysis of four randomly selected brands of inactivated fermented milk during the 5-month storage period, there was no significant change in the Ct values of *L. bulgaricus* and *S. thermophilus*. This finding suggests that the method is sufficient to analyse product quality during the 5-month shelf life. This may be because short-term pasteurisation reduces the enzyme activity in fermented milk but does not completely destroy the cell structure of Gram-positive bacteria, resulting in little degradation of bacterial DNA. However, this avenue requires further research.

In this study, the FQ-LAMP limit of quantitation of *L. bulgaricus* and *S. thermophilus* in fermented milk was 8.1×10^3 CFU/g and 6.8×10^3 CFU/g, respectively. Similarly, Wang et al. [9] used qPCR to detect *S. thermophilus* with a detection limit of 10^3 CFU/mL, which was in the same magnitude order. The CV range of Ct values for detecting *L. bulgaricus* and *S. thermophilus* using the FQ-LAMP method was 2.93–5.29% and 2.96–5.04%, respectively. In comparison, Achilleos and Berthier [28] used qPCR to quantify lactic acid bacteria in cheese, which had a CV range of 2.16–3.56%. The FQ-LAMP method has a fast amplification speed, and the reaction system has many components that are easily affected by human factors; therefore, the stability of FQ-LAMP and qPCR is relatively poor, especially when the concentration of bacteria is less than 10^5 CFU/g. However, the bacterial count in fermented milk is generally greater than 10^6 CFU/g and does not require precise counting; thus, this method can be used for quantifying inactive *L. bulgaricus* and *S. thermophilus* in milk. Furthermore, FQ-LAMP has a faster amplification speed than qPCR and does not require a thermal cycling device, making it less expensive.

Yamamoto et al. [29] found that *L. bulgaricus* and *S. thermophilus* can be cofermented at certain concentrations, which results in a faster fermentation speed and better flavour. In the process of collaborative fermentation, *S. thermophilus* initially decomposes lactose and produces organic acids, which promotes the growth of *L. bulgaricus*. Subsequently, some amino acids and valine produced by *L. bulgaricus* metabolism contribute to the growth of *S. thermophilus* [2, 30]. A starter with *S. thermophilus* as the dominant strain presented superior fermentation in terms of acid production, butanedione production, and texture characteristics. By contrast, a starter with *L. bulgaricus* as the dominant bacteria showed high acetaldehyde production and a strong protein hydrolysis ability [31]. *S. thermophilus* is generally more abundant than *L. bulgaricus* in fermented milk products as it produces a good flavour and controls postacidification. In a survey of 40 inactivated fermented milk products on the market, it was found that the concentration of *S. thermophilus* was above 10^7 CFU/g, while the concentration of *L. bulgaricus* varied greatly and even varied by more than 100 times between different batches of the same brand. This may be because the manufacturer has made improvements to the formula of the product, or it may be due to unstable product quality. In this study, *L. bulgaricus* was not detected in three samples, which may be due to the number of bacteria being below

the detection limit or the product itself being substandard. Alternatively, some factors may have degraded the DNA of *L. bulgaricus* during storage, but this needs to be further studied.

FQ-LAMP can also be used to quantify *L. bulgaricus* and *S. thermophilus* in other fermented products and other lactic acid bacteria with the generation of appropriate primers. Therefore, with continued development, FQ-LAMP, a reliable and rapid detection method, can be applied to a wider range of fields. Compared with that of the plate count method, FQ-LAMP has a larger error, is, thus, only suitable for rapid counting of products with high concentrations of bacteria, such as fermented milk, and is not suitable for accurate enumeration of bacteria. In the future, further research is needed to improve the accuracy of this method.

5. Conclusions

The FQ-LAMP method has high specificity and sensitivity for detecting *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk. It can accurately quantify *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk in the range of 8.1×10^8 to 8.1×10^3 CFU/g and 6.8×10^8 to 6.8×10^3 CFU/g, respectively. If the bacterial count in fermented milk exceeds the upper limit of quantification, the sample can be diluted before testing. Using the scatter distribution of FQ-LAMP detection, 40 samples of inactivated fermented milk from six brands that were randomly selected from supermarkets were analysed. The concentration logarithm of *L. bulgaricus* was lower than that of *S. thermophilus*, and the concentration of *S. thermophilus* in all samples was above 10^7 CFU/g. By contrast, there was a significant difference in the concentration of *L. bulgaricus*, and three samples did not contain *L. bulgaricus*. Thus, FQ-LAMP is a specific, sensitive, accurate, and reliable detection method for *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk. This method can be used to monitor the number of fermentation bacteria in inactivated fermented milk during storage in real time and provide a basis for evaluating the quality of inactivated fermented milk.

Authors' Contributions

Shuaikang Zhou, Lianxia Hu, and Yuling Xue contributed equally to this work and shared the first authorship.

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DETAIL

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Effect of Semolina Replacement with Amaranth Flour on Quality Characteristics of Functional Pasta

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ABSTRAK (ENGLISH)

Amaranth is one of the gluten-free pseudocereals and is suitable for celiac disease. This research aimed to investigate the effect of amaranth flour in concentrations of 0 (C), 10 (A_{10}), 15 (A_{15}), and 20% (A_{20}) on the rheological, physicochemical, and sensory characteristics of functional pasta during 3 months. The prepared pasta was analyzed for its quality characteristics such as chemical composition, cooking loss, texture, color, and amylose leach out and was subjected to farinograph and extensograph tests and scanning electron microscopy (SEM). The results showed that the addition of amaranth flour weakens the rheological characteristics of pasta dough. Amaranth flour had a significant effect on the physicochemical properties of pasta ($p<0.05$). The lowest moisture content, fat, protein, and fiber were observed in the control sample, and the highest amounts were observed in the A_{20} sample. The moisture content decreased during storage. The control and A_{20} samples recorded the lowest and highest cooking loss, respectively. The addition of amaranth decreased the hardness, lightness (L), and yellowness (b^*) and increased the redness (a^*) of pasta. The amylose leach out in the cooking water of all amaranth pasta ranged from 2.03 g to 3.38 g/100 g which was lower than that of the control (4.95 g/100 g). The structure of control pasta is an interwoven network of gluten with many holes and swollen starch granules. A more uniform structure with fewer holes and a looser gluten network was observed in the microstructure of amaranth pasta. A_{10} treatment obtained the highest score of sensory evaluation. In conclusion, the amaranth flour has a good potential to be used in functional foods and gluten-free pasta. A_{20} was favorable in terms of physicochemical characteristics, and A_{10} was the best in terms of sensory characteristics.

TEKS LENGKAP

1. Introduction

Functional foods have nutritional value and beneficial effects on human health and reduce the risk of various diseases [1]. They include probiotics, prebiotics, polyunsaturated fatty acids, omega-3, conjugated linoleic acid, antioxidants, vitamins, minerals, some proteins, peptides, and amino acids [1].

Today, the consumer demand for cereal-based functional foods, i.e., pasta is increasing. Pasta is made from semolina or durum wheat and is known as a cheap food due to its simple formulation and production process [2]. Semolina is the starting material for pasta manufacture and is high in protein and fiber, both of which slow digestion. It contains various vitamins such as B complex and minerals such as iron [3]. Considering that most cereal products are made from wheat flour and wheat protein is deficient in some amino acids such as lysine, so in recent years, many studies related to the enrichment of these products have been undertaken [2].

Moreover, gluten enteropathy or celiac is caused by an inappropriate immune response to wheat gluten. Patients are unable to consume some of the most common products including breads, baked goods, and other food products made from wheat flour. The gluten-free diet is highly effective in alleviating the symptoms of the disease, i.e., small bowel villous atrophy and crypt hyperplasia. In recent years, there has been an increase in consumer interest in

gluten-free foods, and researchers have been working more on the development of gluten-free products, prepared with nonwheat flours such as rice, maize, soya, guar, and amaranth [4].

Amaranth belongs to pseudocereals and does not contain gluten and with a high glycemic index, and it is suitable for celiac disease. It is a good source of carbohydrates (73–77%), protein (15.5−12.5%), lipids (7–8.1%), dietary fiber (19.5–49.3), and minerals (3.5−3%). The bran fraction is proportionally higher in amaranth seeds than in common grains such as corn and wheat, which explains the higher levels of protein and fat content in these seeds. Starch (62–65%) is the most abundant carbohydrate in amaranth. Moreover, unlike cereal grains, there is a high content of lysine in the amino acid profile of amaranth. Amaranth starch generally produces more stable pastes than cereals and legumes, due to its small size and low amylose content. Amaranth contains various minerals such as calcium (134–370 mg/100 g), magnesium (230–387 mg/100 g), phosphorus, iron, potassium, and zinc and E and B vitamins, riboflavin, ascorbic acid, polyphenols, and flavonoids. Caffeic, p-hydroxybenzoic, and ferulic acids are the main phenolic compounds in amaranth. Its oxalate content is between 178 and 278 mg/100 g. Although oxalates are a potential risk factor for kidney stone formation and reduce the availability of calcium and magnesium, most of the oxalates in amaranth seeds are in insoluble form, and their absorption may be low. Amaranth contains higher lipids than most cereals, and its oil composition is quite similar to other cereals with high unsaturated fatty acids (approximately 77%) such as linoleic acid. Amaranth oil mainly contains tocotrienols, which are associated with cholesterol-lowering activity, and squalene, which exhibits anticancer and hypocholesterolemic effects [5].

Some studies evaluated the enrichment of cereal products with amaranth, such as bread [6–12], cake [13], and pasta [3, 14]. So far, very few studies have reported on the technological evaluation of pasta [3, 14]. The purpose of this research was to investigate the possibility of amaranth flour as a food supplement to replace semolina in pasta making and improve the quality properties of pasta with enriched protein content and determine the highest proportion of the amaranth flour that provides the required functionality to produce acceptable pasta.

2. Materials and Methods

2.1. Materials

Amaranth (*Amaranthus hypochondriacus* L.) grains were obtained from Jam Noor Co. (Tehran, Iran), manually cleaned, and milled separately into flour using a blender (Philips Mexicana, Mexico, D.F.). Semolina was purchased from Zar Macaron Industrial Co. (Tehran, Iran). All chemicals (hexane, potassium sulfate, copper sulfate, and sulfuric acid) were obtained from Merck (Germany).

2.2. Chemical Composition of Semolina, Amaranth Flour, and Pasta

Moisture content (AACC, 16–44), fiber (AACC, 32–10), fat (AACC, 10–30), and protein (AACC, 46–12) were measured according to AACC methods (2005) [15].

The pasta was characterized for moisture content (AACC, 16–44) during 3 months, protein (AACC-10-30), fat (AACC-46-13), and fiber (AACC-32-10) after cooking [15].

2.3. Pasta Production

Pasta was prepared in a pasta machine (Zar Macaron, Iran) with amaranth flour to semolina ratios of 0 : 100, 10 : 90, 15 : 85, and 20 : 80. Semolina pasta was also prepared as a control. All batches (280 g each, 14 g of water/100 g) were mixed at room temperature with 1.5 g of distilled monoglycerides/100 g and 10 g of egg white powder/100 g in a mixer (Moulinex, France) at a low speed for 2 min, and 50 g of warm distilled water/100 g was slowly added and mixed for 10–15 min. Afterward, the dough was allowed to rest for 35 min in a proofing chamber at 30°C and 95% RH. First, the proofed dough was laminated in the pasta machine at setting 1 and finally at setting 3. The pasta was hand-cut into strips of approximately 25 cm long (fresh pasta) using a scissor and dried at 75°C and 91% RH for 3 h (dried pasta) in an oven (Behdad, Iran). The four pasta samples were allowed to cool, placed in individual sealed containers to avoid moisture exchange, and stored at room temperature for 3 months [3].

2.4. Dough's Rheological Properties

The water absorption, dough development (DT), dough stability (DS), consistency, degree of dough softening (DOS), and the farinograph quality number (FQN) were evaluated using the Farinograph analyzer (Brabender, Version 1.1.8), according to the AACC-54-21 method [15].

The dough resistance to constant deformation (stretching) after 50 mm (R_{50}), extensibility (E), maximum resistance (R_{max}), the ratio of these parameters (R_{50}/E) and (R_{max}/E), and test area (A) were determined using the extensograph (Brabender, Version 1.1.8), according to the AACC-54-10 method [15].

2.5. Physical Characteristics of Pasta

The cooking loss was measured after 10, 20, and 30 min according to the Iranian National Standard No. 213 (2010). The hardness of the pasta was analyzed by using a texture analyzer (Brookfield, USA). The color was checked using HunterLab (FMS Jansen GmbH and Co.KG, USA), and L, a*, and b* values were determined [3].

2.6. Amylose Leach Out in the Cooking Water

Pasta (6 g) was cooked in 120 ml of water until optimum cooking time. Cooked water was drained thoroughly into a 100 ml volumetric flask and made up to 100 ml. After mixing, this was filtered using suction. 1 ml of the filtrate was mixed with 1 ml of iodine solution and made up to 25 ml. The color developed was read at 650 nm. The amylose content was estimated by linear regression analysis [4].

2.7. Microstructure Analysis

The morphology of the pasta after coating with gold and immersion in liquid nitrogen was analyzed using the scanning electron microscope (SEM, LEO 435VP, UK) at a voltage of 5–15 kV.

2.8. Sensory Characteristics of Pasta

The samples were coded before the test, and sensory characteristics of cooked pasta such as flavor, odor, color, texture, and overall acceptability were evaluated by 12 trained panelists from the Food Science and Technology Department, using the 5-point hedonic method. Samples were evaluated after cooking and served in porcelain cups coded with random numbers. Panelists were asked to rinse the mouth with water between each sample. Three samples were assessed per session according to a completely randomized design [4].

2.9. Statistical Analysis

All experiments were conducted in a completely randomized design and with three replications. The results were expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed by SPSS software (ver. 21) at a significance level of 0.05 ($p<0.05$) [13].

3. Results and Discussion

3.1. Chemical Characteristics of Flour

Amaranth flour has a significantly higher moisture content, fat, protein, and fiber than the semolina flour ($p<0.05$) (Table 1).

Table 1

Chemical characteristics of raw materials (dwb).

%	Semolina	Amaranth
Moisture	11.72 ± 0.01^{b}	14.98 ± 0.08^{a}
Fat	0.86 ± 0.29^{b}	6.58 ± 0.04^{a}
Protein	12.72 ± 0.04^{b}	15.79 ± 0.08^{a}
Crude fiber	1.07 ± 0.4^{b}	13.32 ± 0.05^{a}

The mean ± SD (standard deviation) within rows with different small letters differs significantly ($p<0.05$).

The chemical composition of amaranth flour was consistent with other researchers [9, 13, 16].

3.2. Dough's Rheological Properties

Farinograph and extensograph results are summarized in Tables 2 and 3.

Table 2

Farinography characteristics of pasta dough.

Sample	Water absorption (%)	Dough consistency (BU)	Dough development time (min)	Dough stability (min)	Degree of softening after 10 min (BU)	Degree of softening after 12 min (BU)	Farinograph quality number
C	58.4 ± 0.01^{d}	495 ± 0.12^{d}	1.48 ± 0.15^{d}	14.50 ± 0.11^{a}	13 ± 1.1^{d}	19 ± 1.05^{d}	174 ± 1.78^{a}
A_{10}	59.7 ± 0.14^{c}	503 ± 0.12^{c}	6.04 ± 0.16^{a}	8.35 ± 0.16^{b}	19 ± 1.12^{c}	51 ± 1.09^{c}	120 ± 1.56^{b}
A_{15}	62.4 ± 0.11^{a}	517 ± 0.22^{a}	5.10 ± 0.15^{c}	8.52 ± 0.17^{b}	23 ± 1.14^{b}	60 ± 1.11^{b}	114 ± 1.44^{c}
A_{20}	61.4 ± 0.01^{b}	514 ± 0.15^{b}	5.55 ± 0.21^{b}	6.54 ± 0.16^{c}	25 ± 1.16^{a}	75 ± 1.08^{a}	106 ± 1.23^{d}

The mean ± SD (standard deviation) within rows with different small letters differs significantly ($p<0.05$). C, control (without amaranth); A_{10}, pasta with 10% amaranth; A_{15}, pasta with 15% amaranth; A_{20}, pasta with 20% amaranth.

Table 3

Extensograph characteristics of pasta dough.

Sample	Resistance (BU)	Maximum resistance (BU)	Extensibility (mm)	Resistance/extensibility	Maximum resistance/extensibility	Energy (cm^2)
C	476 ± 1.32^{d}	636 ± 2.05^{a}	138 ± 1.17^{a}	3.61 ± 0.02^{d}	3.91 ± 0.08^{d}	114 ± 1.07^{a}
A_{10}	498 ± 1.11^{c}	476 ± 2.09^{d}	122 ± 1.12^{b}	3.91 ± 0.01^{c}	4.41 ± 0.03^{c}	84 ± 0.95^{b}
A_{15}	521 ± 1.19^{b}	528 ± 2.03^{c}	120 ± 1.16^{c}	4.35 ± 0.15^{b}	4.61 ± 0.06^{b}	81 ± 1.01^{c}
A_{20}	571 ± 1.16^{a}	602 ± 2.06^{b}	104 ± 1.15^{d}	5.50 ± 0.13^{a}	5.79 ± 0.03^{a}	80 ± 0.91^{c}

The mean ± SD (standard deviation) within rows with different small letters differs significantly ($p<0.05$). C, control (without amaranth); A_{10}, pasta with 10% amaranth; A_{15}, pasta with 15% amaranth; A_{20}, pasta with 20% amaranth.

Amaranth increased water absorption, dough consistency, and development time. The water absorption increase is probably related to the hydrophilic compounds (fibers) that reacted with water. Amaranth reduced the dough's stability. The highest and the lowest DS were related to the control and A_{20} samples, respectively, because of the dilution effect of amaranth on the gluten network formation [17]. On the other hand, amaranth contains polysaccharides (insoluble fiber) with a weakening effect on gluten [7].

The degree of softening (DOS) increased with the increase in the concentration of amaranth, which is due to the dilution of the gluten network and the reaction between fiber and gluten, which causes the softening of dough [17]. The farinograph quality number (FQN) is a standard that describes the overall quality of the flour. Weak flours show low FQN, and strong flours show high FQN. As can be seen from Table 2, the control treatment showed the highest FQN and the increase in the amaranth concentration decreased FQN.

With the increase of the amaranth substitution, the elastic behavior (R_m: maximum resistance) is strengthened and the viscous behavior (E: extensibility) is weakened. During the dough rest, these components are recovered and form a uniform gluten network due to glutenin changes. Therefore, R_m and E improve. The elastic properties are related to the presence of glutenins, and the viscous properties are related to gliadins. The dilution of gluten changes the ratio of gliadin to glutenin [18]. Amaranth treatments had lower Rm and *E* and higher R_{50} than the control sample. This is probably related to the coarser size of the amaranth particles compared to wheat particles, which causes the rupture of gluten during stretching [11].

Considering the different effects of amaranth on R_{50} (resistance), Rm (maximum resistance), and *E* (extensibility), the evaluation of energy required for dough stretching (A: energy) can be a better explanation for the rheological behavior. A larger value indicates high dough strength. Amaranth decreased A in comparison with the control sample.

3.3. Chemical Characteristics of Pasta

The moisture content of pasta samples decreased during 3 months of storage (Figure 1(a)). The lowest and the highest moisture contents were observed in the control and A_{20} samples, respectively, with a significant difference ($p < 0.05$). The ANOVA results showed that amaranth had a significant effect on the contents of fat, protein, and fiber ($p < 0.05$). The semolina pasta (control) showed the lowest amount of fat, protein, and fiber, and the highest amounts of these parameters belong to the A_{20} sample (Figures 1(b)–1(d)).

[figure(s) omitted; refer to PDF]

The presence of hygroscopic compounds such as soluble and insoluble fibers, cellulose, hemicellulose, and lignin in amaranth preserves the moisture content after cooking and increases water absorption [7]. The results of moisture content confirmed the results of water absorption in farinograph tests.

The higher fat and protein content of pasta samples containing amaranth can be attributed to the difference between fat and protein contents of amaranth and semolina (Table 1).

Sanz-Penella et al. [7] showed that replacing wheat flour with amaranth increases the amounts of protein, fat, ash, dietary fiber, and minerals. According to the findings of Hamzehpour and Dastgerdi [13], the addition of amaranth flour significantly increases the fiber and protein content of the cake.

According to the national standard of Iran (no. 213; 2010), the permissible limit of moisture content, protein, and fiber is maximum 12%, 10%, and 2-3%, respectively, in regard to moisture, all pasta samples were within the standard range, and in regard to protein and fiber, A_{20} treatment was in the standard range [19].

The microbial growth, chemical reactions, and enzyme activities that can affect the stability of products are influenced by the moisture content and water activity. The lower the water activity, the more the stability increases. A lower moisture content is essential for safe storage. However, a very low moisture content leads to dry and brittle pasta [3].

3.4. Cooking Loss

The results of ANOVA revealed that amaranth had a significant effect on the cooking loss of different pasta types during 30 min ($p<0.05$). The lowest and highest cooking loss was observed in the control (semolina pasta) and A_{20} samples, respectively (Figure 2). As the time increased from 10 to 30 min, the cooking loss increased ($p<0.05$).

[figure(s) omitted; refer to PDF]

The amounts of cooking loss (4.14–5.37%) in all samples are in accordance with the national standard of Iran (no. 213; 2010), in which the cooking loss has been determined at a maximum of 11% [19].

3.5. Texture

Amaranth reduced the hardness of pasta. The lowest hardness was observed in the A_{20} sample, and the highest hardness belonged to the semolina pasta (Figure 3).

[figure(s) omitted; refer to PDF]

Regarding the reduction of hardness of some samples, it can be said that the preservation of moisture due to the high amount of fiber is one of the important factors [20]. It seems that fat and hydrocolloids in amaranth flour were also effective in reducing hardness (Table 1). The hardness reduction of cereal products as a result of adding dietary

fibers has been reported in other studies [21]. Islas-Rabio et al. [3] showed that replacing semolina with amaranth reduces the hardness of pasta.

The hardness of pasta samples increased during storage ($p<0.05$). Staling or hardening of the baked products during storage is a complex process that involves several factors including the recrystallization of gelatinized starch, especially amylopectins, retrogradation of amylose, binding of amylose and amylopectin to each other, moisture migration after crystallization of starch, and reduction of moisture content or distribution of moisture between amorphous and crystalline regions. The staling of bakery products is related to the moisture content, so there is an inverse relationship between the moisture content and its staling. Water can be effective in reducing hardness by playing the role of a plasticizer. Also, the tendency of fibrous compounds to absorb water causes less gelatinization and recrystallization during storage, which ultimately leads to a reduction in hardness [13].

3.6. Color

By adding amaranth flour to pasta, the *L* and b* values decreased and the a* value increased (Figure 4). During storage, the L* value of all samples decreased and a*and b* values increased ($p<0.05$).

[figure(s) omitted; refer to PDF]

The difference in color parameters can be due to the natural pigments of the amaranth and the hydrophilic role of fibers, which decreased the *L* and b* values and increased the a* values in the amaranth pasta [7]. The color values in the present study are similar to those found for other gluten-free products containing amaranth [13, 22–24].

The color can be attributed to the interaction of fibers with amylose. Sugar and protein compounds can affect the color parameters. During the cooking process, fiber compounds cause a migration of moisture from the crumb to the crust. Another cause of color change is the browning reactions [13].

3.7. Amylose Content in Cooking Water

The amylose content of all amaranth pasta ranged from 2.03 g to 3.38 g/100 g, which was lower than that of the semolina pasta (4.95 g/100 g), as shown in Figure 5.

[figure(s) omitted; refer to PDF]

A higher amylose content is associated with higher hardness and less surface stickiness. Pasta surface stickiness is influenced by both the surface structure of the strand and starch exuded onto the strand surface during cooking. Amylose decrease increases the stickiness of the pasta. The higher protein content and lower amylose leach out into cooking water may be the reason for the less stickiness of gluten-free pasta [14].

These results are in agreement with the results of Martinez et al. [25]. They reported that 2–5 g/100 g of amylose leach out in cooking water of commercial spaghetti.

3.8. SEM Analyses

The microstructure of the functional pasta is shown in Figure 6. The structure of control pasta is an interwoven network of gluten with many holes and swollen starch granules (Figure 6(a)). The microstructures of pasta-containing amaranth are slightly different (Figure 6(b)). A more uniform structure with fewer holes and a looser gluten network can be seen.

[figure(s) omitted; refer to PDF]

SEM images of gluten-free pasta showed a protein matrix distinct from the gluten network. This may be due to the high protein content, fiber, and starch of the flour used in pasta. The microstructure showed a fibrous and protein network but not similar to the gluten network. Amaranth pasta showed a smooth but an intact structure when observed in cross section of the pasta which clearly showed the starch molecules that may be the reasons for less starch leaching out during cooking. The control pasta revealed the gluten protein network with wheat starch molecules embedded in it [14].

3.9. Sensory Characteristics

A_{10} obtained the highest score for flavor. The highest score of odor belonged to A10 and A15 samples and the control sample obtained the lowest score. The semolina pasta (control) and A10 samples showed the highest scores of color and texture. The highest overall acceptability belonged to the A10 sample, followed by the semolina pasta (Figure 7).

[figure(s) omitted; refer to PDF]

The higher scores in the A_{10} treatment are due to the high ability of the fiber compounds in amaranth to retain moisture and its more uniform release of moisture during the cooking process [9, 10]. However, the decrease in sensory characteristics of A_{20} treatment is due to the increasing amaranth flour substitution and the insufficient cohesion of the gluten network, the reduction of the chewiness, and the aftertaste of amaranth [8].

Buresova et al. [6] showed that the overall acceptability of bread-containing amaranth was negatively influenced by the flavor of amaranth. In the research of Nasir et al. [10], the bread prepared by substituting 5% and 10% of amaranth flour is acceptable from the nutritional and sensory points. Derkanosova et al. [8] showed that amaranth bread was similar to traditional samples. Also, amaranth improved the nutritional, sensory, and technological quality of some products [9, 13].

4. Conclusion

The formulation of gluten-free bakery products presents a challenge to food technologists. The present study aims at the development of functional pasta mainly covering the evaluation of product quality.

The results showed that increasing amaranth flour weakened the rheological properties of the dough and improved the physicochemical properties of the final product. With the increase of amaranth flour, the moisture content increased and hardness decreased. Although the cooking loss of enriched pasta was higher than that of the control sample, it was in accordance with the standard limit. Amaranth also reduced amylose leach out compared to the control sample. Amaranth (even in small levels) darkened the final product. Amaranth increased the fat, protein, and fiber contents of pasta. The increase in protein and fiber can be seen as a reason for the higher nutritional value of pasta products. Thus, amaranth can be used in the formulation of functional baking goods. However, the industrialization of this product requires more studies on increasing shelf life and preventing microbial growth.

Authors' Contributions

S. Alizadeh designed the experiment and data collection. S. Tahriri analyzed the data. M. Zokaei and F.Ebrahimi Tirtashi helped in the design and contributed to the compilation of the manuscript. A. Ahmadi Dastgerdi read and approved the final version of the manuscript.

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Comparison of Chemically Treated, Pasteurized, and Microwave-Treated (at Different Time Durations) Chia Seeds Added To Mango-Whey Beverage, during Different Storage Periods, for Physicochemical and Sensory Parameters

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ABSTRAK (ENGLISH)

Foods that are widely consumed and accepted have had a lot of ingredients added to them which may enhance consumer’s health. Whey is a byproduct in the manufacture of cheese or curd and is widely used in different beverage formulations, due to its nutritional importance. In this context, a mango-based functional whey drink, added with omega-3-rich chia seeds, was de veloped. Different treatments of beverage were as follows: untreated control beverage (T0), application of chemicals (T1), pasteurization (T2), and microwave heating (at 4 different time lengths: T3, T4, T5, and T6). Beverages were analyzed for physiochemical, microbiological, and sensorial changes, during the 90 days of storage. Chemical analysis of chia seeds before incorporation in beverages revealed a high nutritional profile of chia seeds, especially the presence of essential fatty acids. Significant variation in color parameters of the beverage was observed as a result of treatments and storage, with optimum values observed for T3. A decrease in pH and an increase in acidity during storage for all treatments were evident, with the most significant results for microwave treatment for longer time periods. The total plate count of the beverage was the highest (2.36 ± 0.1

CFU/ml) for control, followed by chemically treated (2.17 ± 0.1 CFU/ml) and lowest in microwave-treated (0.98 ± 0.1 CFU/ml) at the start of the experiment, and this total plate count was found to be increasing in all treatments during storage. Total solids were increased and soluble solids were decreased during longer microwave treatments and also during storage of all treatments. The most acceptable treatment T3 (85 ml whey, 2 g chia seed, and microwave heating for 30 sec) was further subjected to a storage study and it was observed that the sensory scores gradually decreased during the 90 days of storage. From the outcomes of the study, it was concluded that microwave treatment of the beverage for 30 sec, as compared to longer durations and chemical preservatives, was proved helpful for optimum quality retention of the formulated beverage, with minimum deteriorating effects. Thus, proper treatments of different beverage formulations, following the necessary protocols, could provide the consumers safe, healthy, and nutritious beverages.

TEKS LENGKAP

1. Introduction

In the food sector, there is now considerably more emphasis on the production of food products with various bioactive components. The creation of these multifunctional meals satisfies the expectations of the targeted customers. Given the ubiquity of whey as the most palatable drink with nutritional components, the creation of such a drink with functional food ingredients appears to be fascinating [1]. Application of different novel processing technologies has been proven helpful in enhancing the functionality and nutritional contents of beverages [2]. Whey, a byproduct of the cheese-making process, is the primary component of whey drinks and has been employed for product development for the past 20 years. Whey provides several nutritional benefits since it contains a variety of bioactive substances [3]. After preparation, whey still contains lactose, minerals, water-soluble vitamins, and bioactive whey proteins [4]. Although whey has been utilized in the food sector for approximately twenty years, attention to its utilization has lately grown due to its strong nutritional and functional qualities [5]. Milk sugars, proteins, minerals, fat-soluble and water-soluble vitamins, polyphenols, and water, each component of whey have different medical advantages [6].

The phytochemistry of fruits and vegetables gives us the foundation for combining their constituent parts to create value-added products that are brimming with antioxidants and antimicrobial elements that prevent pathogen invasion and free radical production, respectively [7, 8]. Mango (*Mangifera indica* L.), a tropical fruit, has been well known among the most eaten fruits on the planet. It swiftly and significantly softens as the fruit gets closer to becoming fully ripe [9]. Mango is one of the greatest fruits, because of its alluring scent, delectable taste, superb flavor, and high nutritional content [10]. Mango juice, nectar, and squash are the three significant refreshments of mango fruit, which contain nutritional components [11], and thus, consumption of mango-based beverages could prove helpful in mediating the normal body functions.

Fruit components such as seeds, peels, pulp, concentrate, and juices serve as the foundation for creating food formulations that increase the nutritional content of finished goods [7, 12]. *Salvia hispanica* L. is the scientific name for chia, an herb plant that is a member of the *Lamiaceae* family. The health advantages of chia seeds to people are thought to be related to their greater levels of alpha-linolenic acid [13]. Some common food products such as cakes, pasta, biscuits, and bread which have been developed by using chia seeds can be found in the markets [14]. Stirred yogurt was prepared by using different doses of chia seeds [15]. Kowaleski et al. [16] used chia seeds and strawberries for the formation of yogurt. Chia seed extract in whey drink were found in the studies of Kwon et al. [17].

Large-scale foods and beverages frequently contain benzoates and sorbates as preservatives since they work as antibacterial and antifungal agents and have very low toxicity for mammals. They are universally acknowledged to be inherently free of carcinogenicity. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has investigated the safety of these compounds [18, 19]. However, ultrasonic and microwave processing have lately

garnered significant potential in food applications as green processing technologies [20]. Microwaves are electromagnetic waves that range in frequency from 300 MHz to 1000 GHz. Microwaves heat up quickly and produce better results [21]. The use of microwaves has been shown to be beneficial in preserving the optimum amount of nutritious ingredients and eliminating any bacteria found in treated juices [22, 23]. Microwave processing could be applied as a good technique that utilizes less energy and time [24]. Although the reviewed literature provided a number of processing and preservation experiments, conducted on fruit juices and beverages, there is a lack of study about the whey beverage formulations added with chia seeds and mango pulp. This unique and novel recipe has been tested in the current study, which could enrich the market with a brand-new product, the consumers wish to take. Microwaved preservation of this beverage could minimize the food safety concerns of the food business operators. Keeping in view the valorization potential of whey, the nutrition and delicacy of mango, and the health-promoting potential of chia seeds, the present research project was designed to develop innovative and functional whey beverage formulations, enriched with mango pulp and chia seeds, and to investigate the different physicochemical, sensory, microbial, and storage parameters of beverage under different processing and preservation treatments.

2. Materials and Methods

2.1. Procurement of Raw Materials

Raw materials for beverage development were chia seeds and mangoes, which were purchased from the local market of district Sargodha, Pakistan. Other ingredients of the beverage such as sugar and guar gum were purchased from Imtiaz Super Market, Sargodha district, Pakistan, whereas two chemical preservatives used (sodium benzoate and potassium metabisulfite) were purchased from Sigma Chemical Store, Islamabad, Pakistan. Whey was collected from Fauji Foods Ltd., Bhalwal, Pakistan, and mango-whey beverage was prepared in laboratories of the Institute of Food Science and Nutrition, University of Sargodha. Before the onset of the experimental study, whey and mango pulp were handled under aseptic conditions to avoid any microbial contamination and spoilage.

2.2. Recipe Development and Treatment Plans

A recipe with different ingredients, of mango-whey beverage, was adopted from the earlier experiments of Alane et al. [25] and is presented in Table 1. Different treatments were T0, T1, T2, T3, T4, T5, and T6, and these treatments, except control, were applied in chemical preservation, pasteurization, and different times of microwave heating as presented in Table 1. A microwave oven (MRO-AV200E, Hitachi, Japan) was used to microwave the beverages (200 ml in a 500 ml beaker) at 90°C and 400 W, as was reported earlier by Zia et al. [26]. The abovementioned microwave treatment was employed to treat juices because of its special features, including thermostatic control, temperature resistance up to 120°C, a working temperature range of 25°C to 100°C with an accuracy of 0.3°C, and a 220 Volt/50 Hz electric supply. A double wall pasteurizer (Harvest Hi-Tech Equipment Pvt. Ltd, Coimbatore, India) was used for the pasteurization process, and it was heated to a maximum temperature of 85°C using water as the heating medium. The temperature probe was used to keep an eye on the beverage's temperature when it was moved to the pasteurizer once it had reached equilibrium. Pasteurization was performed by following the procedure of Prithviraj et al. [27]. By following the guidelines of Pandiselvam et al. [28], to find out the impact of different preservation techniques on the physicochemical and microbiological qualities during storage duration, aseptically packed beverage samples were kept at a refrigeration temperature of 5 ± 2°C for 3 months.

Table 1

Treatment plan with recipe and preservation techniques.

Treatment	Whey (mL)	Chia seeds (g)	Sugar (g)	Mango pulp (g)	Guar gum (g)	Variables
T0	85	2	8	5	0.1	N/A

T1	85	2	8	5	0.1	Sodium benzoate 0.25 g and potassium metabisulfite 0.75 g
T2	85	2	8	5	0.1	Pasteurization (85°C for 15 min)
T3	85	2	8	5	0.1	Microwave heat (30 sec)
T4	85	2	8	5	0.1	Microwave heat (60 sec)
T5	85	2	8	5	0.1	Microwave heat (90 sec)
T6	85	2	8	5	0.1	Microwave heat (120 sec)

2.3. Preparation of the Mango-Based Whey Beverage Added with Chia Seeds

For incorporation in mango-based whey beverage, first of all, chia seeds were soaked in water. After that, whey and mango pulp were mixed together in a juice blinder, and sugar was added to the mixture and ground again. At the end, chia seeds were added to the beverage. Then, the beverage was subjected to different microwave heat treatments, pasteurization, and chemical preservation. The beverage prepared was filled in bottles with labels and kept chilled at 5 ± 2°C. For the development of the formulated beverage, guidelines from the studies of Tanwar et al. [29] were taken and adjusted accordingly.

2.4. Analysis of Chia Seeds

2.4.1. Proximate Composition

Using the Association of Official Analytical Chemists' procedures, the crude fiber, moisture, ash, fat, nitrogen-free extract (NFE), and protein contents of chia seeds were calculated by following their respective methods of AOAC [30], with required modifications. The NFE percentage of chia seeds was analyzed by subtracting the percentage of crude fiber, moisture, fat, crude protein, and ash from 100, as indicated in the NFE value given as(1)NFE%=100−moisture%+ash%+crudeprotein%+crudefiber%+crudefat%.

Before incorporation in mango-whey beverage, the proximate composition of chia seeds was determined, which is shown in Table 2. These results revealed that the percentage of fiber, fat, and protein was high in chia seeds. The high value of fat was attributed to the higher percentage of fatty acids in chia seeds, while the lower amount of moisture showed the lower perishability of chia seeds. These findings were found in line with those of Otondi et al. [31] Rodríguez Lara et al. [32], and Aamer et al. [33].

Table 2

Proximate analysis of chia seeds.

Chemical parameters	Quantity (%)
Moisture	5.67 ± 0.08
Ash	3.98 ± 0.12
Fat	35.66 ± 0.09
Protein	25.01 ± 1.25

Fiber	26.92 ± 2.32
NFE	2.78 ± 0.05

Values are presented as means of triplicate analysis, along with standard deviations. NFE, nitrogen-free extract.

2.4.2. Fatty Acid Composition

The method used by Aamer et al. [33] was adjusted to ascertain the fatty acid composition of chia seeds. After being ground to 100 g, the chia seeds were extracted three times using 0.5 L of n-hexane at 60°C for four hours. A rotavapor apparatus was then used to mix and concentrate the extracted materials. By mixing 1 mL of Hex and 1 mL of a 2 N methanolic potassium hydroxide solution with 100 μL of the resultant oil, stirring for 15 seconds, and letting the mixture sit at room temperature for five minutes, fatty acid methyl esters were produced. The upper layer, which included methyl esters of fatty acids, was removed and kept at −20°C for additional examination of fatty acids. The fatty acid composition of chia seeds was investigated by gas chromatography (GC) (7820A, Agilent, Santa Clara, CA, USA), coupled with a FID and a Trace TR-FAME capillary column (i.d. 0.25 μm, 60 m × 0.25 mm, Thermo Fisher Scientific, Grand Island, NY, USA). The analysis condition was programmed as follows: the injector and FID temperatures were kept at 250°C and the initial oven temperature was held at 80°C for 3 min. It was then increased to 215°C (15°C/min) and finally up to 215°C (20 min hold time). Nitrogen was used as carrier gas at 1 mL/min, with a split ratio of 1 : 20. The chromatogram of the authentic fatty acids was used to characterize the fatty acids according to their retention times. The fatty acid composition was expressed as a percentage of the total fatty acids.

The results of the analysis of the fatty acid composition of chia seeds are shown in Table 3, from where it was revealed that the major unsaturated fatty acids that were present in chia seeds were linolenic acid, linoleic acid, and oleic acid. Some long-chain saturated fatty acids were also present in chia seeds, and these were palmitic and myristic acids. This fatty acid composition of chia seeds was also supported by earlier studies of Kulczynski et al. [34], Hernandez-Pérez et al. [35], and Aamer et al. [33].

Table 3

Fatty acid composition of chia seeds.

Fatty acid compounds	Values (%)
Linolenic acid	51.15 ± 0.70
Linoleic acid	25.42 ± 0.30
Oleic acid	7.88 ± 0.40
Palmitic acid	7.63 ± 0.30
Mysteric acid	8.11 ± 0.19

Values are presented as means of triplicate analysis, along with standard deviations.

2.5. Analysis of the Formulated Beverage

2.5.1. Color Analysis

Using a ColorQuest XE (HunterLab), color assessments of all the treatments of mango-whey beverages were carried out. The colorimeter has a 10° angle of view with specular reflection and a D65 illuminant. The values of color parameters, including a^*, b^*, and L^*, were established by following the procedures adopted by Siefarth et al. [36], with slight modifications. Briefly explaining, before sample measurement, the equipment was first standardized against white and black tiles. The color properties were then determined in triplicate, and the averages of the results

were recorded.

2.5.2. pH Analysis

Using an electronic digital pH meter (Inolab pH 720, WTW 82362), the mango-whey beverage's pH was estimated by following the protocols adopted by Siefarth et al. [36]. First of all, the pH meter was calibrated with a buffer solution of pH 4 and 7. Then, an appropriate amount of beverage was taken as a sample and was put in the beaker. The electrodes of the pH meter were placed in the sample to check the pH, and all analyses were repeated in triplicate to find out the mean values.

2.5.3. Determination of Titratable Acidity

The titratable acidity of mango-whey beverage was determined by following the procedure used by Dong et al. [37], with slight changes. Briefly explaining, a sample of 9 ml of mango-whey beverage was placed in a titration flask and then phenolphthalein (2-3 drops) was added to it. This mixed solution was titrated against 0.1 N NaOH until it turned into a light pink color. The titratable acidity of mango-whey beverage was determined with the volume of NaOH used, and the below given formula was used to calculate the acidity %.(2)% Titrable acidity=0.009×Vol. of NaOH usedweight of the sample×100.

2.5.4. Determination of Total Solids

To determine the total solids of formulated beverages, a known amount of mango-whey beverage samples was placed in a crucible and relocated to a hot air oven for drying. The content of total solids in mango-whey beverage was determined by drying the samples to a constant weight at 105°C, overnight, using a heating oven by adopting the procedure given in AOAC [36], with slight modifications.(3)Total solids%=Weight of dried samplegWeight of sample×100.

2.5.5. Analysis of Viscosity

The samples of the mango-whey beverage were conditioned at 4°C before the viscosity was measured. The viscosity of the beverage samples was measured by using a Lamy Rheology Instruments' rotator-type viscometer. All mango-whey beverages with chia added had their apparent viscosity measured, with minor modifications, using the technique outlined by Cheong et al. [38]. Spindle number 5 was used to measure viscosity, and 50 rpm was the speed setting. The viscosity data were given in centipoises (cP).

2.5.6. Calculation of Brix

Since the lab refractometer had an upper number of 30°, the refractometer was first calibrated to 0° Brix using 1 ml of distilled water, and then, each sample (5 ml) was diluted with 10 ml of water to get a ratio of 1 : 2 before measuring the brix, and values were recorded by adopting the procedure used by Nduko et al. [39]. Briefly describing, with the help of a micropipette, a drop of the sample was placed on the detector of the refractometer and the value shown on the screen was noted. Calibrating the refractometer after each step, the same process was repeated thrice to find the mean value.

2.6. Total Plate Count

Thermo Fisher Scientific Inc.'s Oxoid™ plate count agar was used for the microbiological study of beverage samples. The inoculum was mixed into the plate using the pour plate technique, and the agar and inoculum were then united by gently rotating the plate. After that, incubation was performed at 37°C for 48 hours. The total plate count testing was employed for bacterial evaluation for the safe storage duration criteria; however, it was examined every hour for the next eight hours. Colony-forming units per milliliter (CFU/ml) would be the definition of total plate count, and mold and yeast count, and guidelines were taken from the experiment of Amelia et al. [40].(4)Total Plate CountTPC=Average numbers of colonies×dilution factorvolume TPC was expressed as CFU/ml.

2.7. Sensory Evaluation of Different Treatments of Formulated Beverages

Sensory evaluation of all treatments of mango-whey beverage was first carried out before storage, to find out the most acceptable treatment, which was then further analyzed through a sensory panel of experts, at the end of each month, during the 90 days of storage. The Institute's assessors used a nine-point hedonic scale to evaluate the sensory qualities such as color, flavor, texture, and taste and overall acceptability. The sensory assessment was planned for 80 participants, as was earlier reported by Lawless and Heymann [41], as they suggested 70 to 100

expert people for the sensory evaluation test. Participants were given 150 ml of iced beverage in transparent cups during the test, which was conducted in separate booths. Scores were calculated by using a nine-point hedonic rating system. The means and standard deviations of the data were displayed.

2.8. Statistical Analysis

The current study's treatments were all administered a total of three times before a statistical analysis was conducted. The data were translated into means and standard deviations using a statistical analysis. The experimental data were processed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and SigmaPlot 12.0 Statistical Analysis software (Systat Software, Inc., San Jose, CA, USA) to indicate the variables with statistically significant effects ($P<0.05$). Every experiment was run in a different order, and the model was able to fit the data from the experiments. Following Steel and Torrie's [42] conventional techniques, an analysis of variance (ANOVA) was carried out.

3. Results and Discussion

3.1. Color Profile of the Mango-Whey Beverage

3.1.1. L* Value

The L* value indicates the darkness to the whiteness of any food material. The mean values of L* for treatments and storage are shown in Table 4, and the values were found to be decreasing with increasing the time of microwave heat treatment for formulated beverages. A decrease in the values of L* may be due to the Maillard reaction that might have occurred during the heat treatment [43]. In addition, polyphenols also polymerize during heat treatments, producing new compounds with dark colors, which are similar to Maillard reaction products [44]. Another reason could be the addition of chia seeds, which might have shifted the color of the beverage towards dark. The whiteness of beverages also decreased during the storage, as there was a decrease in the L* value at the time of storage. The decrease in the whiteness during storage may be due to the changes in the composition of the drink [22]. So the treatments and storage time have a highly significant effect on the L* value of the whey drink.

Table 4

Mean value for L* parameter of mango-whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	79.10 ± 0.2^{a}
77.39 ± 0.4^{b}	75.02 ± 0.2^{c}	70.02 ± 0.1^{e}	77.38 ± 0.2^{A}	T1	77.39 ± 0.4^{b}
75.61 ± 0.4^{c}	75.47 ± 0.3^{c}	71.21 ± 0.3^{de}	75.96 ± 0.3^{B}	T2	75.38 ± 0.1^{b}
74.89 ± 0.2^{b}	73.15 ± 0.1^{c}	72.29 ± 0.1^{cd}	73.93 ± 0.1^{C}	T3	73.16 ± 0.2^{c}
72.53 ± 0.2^{cd}	71.82 ± 0.1^{de}	72.13 ± 0.2^{cd}	72.41 ± 0.2^{D}	T4	70.93 ± 0.2^{e}
68.86 ± 0.2^{f}	67.56 ± 0.1^{g}	63.34 ± 0.2^{i}	67.67 ± 0.2^{E}	T5	64.82 ± 0.2^{h}
61.34 ± 0.1^{j}	59.21 ± 0.1^{k}	58.86 ± 0.2^{k}	61.06 ± 0.2^{E}	T6	59.87 ± 0.6^{k}
58.72 ± 0.4^{l}	57.76 ± 0.3^{m}	56.67 ± 0.2^{mn}	58.87 ± 0.2^{F}	Mean ± SE	72.32 ± 0.2^{A}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia

seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

Comparing current experimental results with previous findings, it was observed that there was a decrease in the color of the formulated foods during storage and upon heat treatment. The study was conducted to evaluate the effects of radio frequency heating on the properties of stirred yogurt [36], and high frequency and prolonged treatment times were found involved in decreasing the L* value of the yogurt. Results of the current study were also compared with the color values provided by another study of Buse et al. [45], which showed similar findings, witnessing the significant effect of different microwave heat durations on the colors of the treated beverages. A slight decrease in the L* value in all treatments of chia seeds added to mango beverage was also reported by Aamer et al. [33], and these results agreed with the present ones.

3.1.2. a* Value

The a* value indicates the redness to the greenness of the food items, and the mean values of the a* parameter of mango-based chia seeds-added whey beverage processed under different conditions are shown in Table 5. From the experimental results, it was observed that beverages having a short time of microwave exposure showed higher positive values of a*, which were also significantly affected during the storage study, and were found in a decreasing mode, with the passage of storage time. This decrease in values of a* may be attributed to the heat treatment by microwave and pasteurization, which might have produced the brown-colored compounds [22]. Experimental outcomes witnessed that a* values were significantly affected by treatments and storage time. When the results of the current study were compared with previous findings of Siefarth et al. [36], a similar decreasing pattern of a* parameter was observed during storage of yogurt treated with different radio frequencies and durations. Current experimental findings about color parameters affected by processing and storage conditions were also compared with the results of the study by Buse et al. [45], conducted on drinks added with powdered chia seeds and processed under different conditions. Gehlot et al. [13] reported that during the three months of storage, a decline in chlorophyll of chia seeds added to the mango drink was significant, and this may have been caused by the thermal breakdown of chlorophyll, which produced the yellow pigments known as pheophytins, as well as the oxidation and isomerization of carotenoids during heating.

Table 5

Mean values for a* parameter of mango-whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	2.31 ± 0.2^{j}
2.09 ± 0.1^{mn}	1.98 ± 0.1^{n}	1.87 ± 0.1^{no}	2.06 ± 0.1^{G}	T1	2.54 ± 0.2^{j}
2.27 ± 0.1^{k}	2.19 ± 0.2^{m}	2.14 ± 0.1^{mn}	2.17 ± 0.2^{F}	T2	2.60 ± 0.2^{i}
2.34 ± 0.2^{j}	2.30 ± 0.3^{j}	2.29 ± 0.2^{k}	2.38 ± 0.2^{E}	T3	2.83 ± 0.1^{ghi}
2.80 ± 0.1^{ghi}	2.73 ± 0.2^{hi}	2.69 ± 0.1^{hi}	2.76 ± 0.1^{D}	T4	3.37 ± 0.1^{e}
3.07 ± 0.1^{f}	2.99 ± 0.2^{fg}	2.91 ± 0.2^{fgh}	3.09 ± 0.2^{C}	T5	4.14 ± 0.1^{bcd}
4.09 ± 0.1^{cd}	3.93 ± 0.2^{d}	3.51 ± 0.2^{e}	3.92 ± 0.2^{B}	T6	4.67 ± 0.2^{a}

4.57±0.3[a]	4.36±0.3[b]	4.22±0.3[bc]	4.46±0.3[A]	Mean±SE	3.51±0.1[A]

*Each mean value within the same column, followed by different letters is highly significant at P<0.05. T0=control; T1=85ml whey, 2g chia seed, 0.25g sodium benzoate, and 0.75g potassium metabisulfite; T2=85ml whey, 2g chia seed, and pasteurization; T3=85ml whey, 2g chia seed, and microwave for 30sec; T4=85ml whey, 2g chia seed, and microwave for 60sec; T5=85ml whey, 2g chia seed, and microwave for 90sec; T6=85ml whey, 2g chia seed, and microwave for 120sec.

3.1.3. b* Value

The mean values of the b* parameter of color analysis of formulated beverages are shown in Table 6. The present study's results showed that there was a decrease in values of b* during storage of all treatments. Values of b* shifted from yellowness to blueness, which showed that the yellowness of the whey drink was due to the composition of milk, which produced the colored components in the whey beverage. Visible changes in the color of whey beverage added with chia seeds were significantly dependent upon microwave heat treatment durations and storage days. For the values of b*, all treatments had higher values than the control; however, a change in the time of microwave treatment had a significant effect on a* and b* values in all studied samples, and these results are in agreement with those of Aamer et al. [33].

Table 6

Mean value for b* parameter of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean±SE
0	30	60	90	T0	14.56±0.1[ij]
13.59±0.1[k]	13.06±0.1[m]	12.95±0.1[m]	13.59±0.1[G]	T1	16.67±0.2[j]
14.55±0.3[ij]	13.12±0.1[k]	12.98±0.1[m]	14.21±0.2[F]	T2	17.51±0.3[j]
15.70±0.4[k]	15.68±0.4[k]	15.53±0.3[k]	16.11±0.3[E]	T3	19.61±0.1[h]
18.70±0.3[l]	18.39±0.2[i]	17.71±0.1[j]	18.60±0.2[D]	T4	21.20±0.2[f]
20.34±0.3[g]	20.11±0.3[gh]	19.86±0.3[gh]	20.38±0.3[C]	T5	24.19±0.2[cd]
23.77±0.2[de]	23.48±0.1[e]	23.11±0.2[e]	23.64±0.2[B]	T6	25.54±0.3[a]
25.47±0.2[ab]	24.85±0.3[bc]	24.66±0.3[c]	25.13±0.3[A]	Mean±SE	21.61±0.3[A]

*Each mean value within the same column, followed by different letters is highly significant at P<0.05. T0=control; T1=85ml whey, 2g chia seed, 0.25g sodium benzoate, and 0.75g potassium metabisulfite; T2=85ml whey, 2g chia seed, and pasteurization; T3=85ml whey, 2g chia seed, and microwave for 30sec; T4=85ml whey, 2g chia seed, and microwave for 60sec; T5=85ml whey, 2g chia seed, and microwave for 90sec; T6=85ml whey, 2g chia seed, and microwave for 120sec.

Present findings were also paralleled with the color values of chia seeds-added drink developed in another similar study by Buse et al. [45], which showed a decrement of b* value in drinks during storage. The consequences of the microwave heat treatments on the color values of beverages, observed in the current study were also strongly related with previous results presented by Siefarth et al. [36], as the b* values were decreased during the storage of

drinks.

3.2. pH of the Mango-Whey Beverage

The mean values of pH for different treatments of whey beverage are presented in Table 7, where pH value relates to the concentration of hydrogen ions. The pH value of the mango-whey beverage decreased as a result of pasteurization and an increase in time of heat exposure by microwave. The decrease in the pH of the whey drink with the passage of storage time probably was due to the production of acids during the storage, as an increase in the percentage of acids may lead to an increase in the concentration of hydrogen ions as well as a decrease in pH. Second, evaporation of moisture during heat treatment and storage might also have resulted in the concentration of acids, causing a decrease in the pH of the beverages, as microwave and pasteurization resulted in a more significant decrease in pH, possibly due to the greater moisture loss [34]. Another opinion could also be included in reasoning the drop in pH, which may be attributed to the destruction of lactose owing to the production of lactic acid [22].

Table 7

Mean value for pH of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	4.62 ± 0.1^{a}
4.57 ± 0.2^{ab}	4.47 ± 0.2^{bc}	4.32 ± 0.2^{de}	4.49 ± 0.2^{A}	T1	4.54 ± 0.3^{ab}
4.43 ± 0.3^{bcd}	4.34 ± 0.2^{cde}	4.22 ± 0.3^{e}	4.38 ± 0.3^{B}	T2	4.48 ± 0.2^{abc}
4.38 ± 0.2^{cd}	4.22 ± 0.1^{e}	3.96 ± 0.2 fg	4.26 ± 0.2^{C}	T3	3.97 ± 0.2^{fg}
3.83 ± 0.3^{ghi}	3.77 ± 0.1^{hij}	3.68 ± 0.2^{j}	3.91 ± 0.2^{D}	T4	3.89 ± 0.4^{fgh}
4.03 ± 0.4^{f}	3.96 ± 0.5^{fg}	3.74 ± 0.3^{ij}	3.81 ± 0.4^{E}	T5	3.67 ± 0.2^{i}
3.59 ± 0.2^{j}	3.49 ± 0.3^{k}	3.39 ± 0.2^{l}	3.59 ± 0.2^{F}	T6	3.01 ± 0.1^{k}
2.58 ± 0.2^{l}	2.51 ± 0.1^{i}	2.45 ± 0.1^{m}	2.65 ± 0.1^{G}	Mean ± SE	4.30 ± 0.3^{A}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

Results of the previous similar findings from the experiments of Siefarth et al. [36] can be correlated with the current study's results of pH, as the pH of stirred yogurt was decreased with the increase in radio wave frequency and storage days. Another previous research on yogurt showed a similar decrease in pH, recorded with the increase in storage time and heat treatment [46]. Results of a latest study from Pinky et al. (2023) were also in line with the current findings, suggesting the use of novel processing techniques as useful for drinks processing.

Similar research was conducted by Adulvitayakorn et al. [47], when they evaluated microwave and conventional thermal processing methods for processing sugarcane juice. They found that the sugarcane juice processed at 700 W using microwave technology had a minor pH reduction. In addition, they connected the temperature variation of the juice samples being treated with the pH variation, as in current experiments, longer microwave treatments might

have raised the temperature of the beverage, resulting in loss of moisture. Yikmis [48] extracted the juice from red and yellow watermelons and subjected them to various processing methods in order to evaluate the physicochemical characteristics of both the processed and raw juices. Juice samples that were left unprocessed, pasteurized, and ultrasonic treated at shorter times did not differ in pH from one another, and this contradiction of results could be justified by considering the ultrasound as a nonthermal processing method, which did not rise the temperature of the beverage to such extent as the microwave could raise. Malik et al. [49] looked into the effects of microwave treatments and storage on the pH of lemon cordial. They found that, when treated for 120 seconds, the pH of the cordial increased nonsignificantly when compared to untreated samples, but that, over the course of a 90-day storage period, the pH of the cordial gradually decreased, which they associated with the treatment's decrease in organic acid content.

3.3. Titratable Acidity of the Mango-Whey Beverage

The mean values of titratable acidity of chia seeds added to mango-whey beverage are shown in Table 8. Titratable acidity is directly related to the pH and acid concentration within the food material. The titratable acidity of the beverage increased with the increase in microwave heating time, as well as the storage days. Increased acidity of beverages, due to heat treatments and longer storage durations, could develop a sour taste in the beverages. Prolonged storage time resulted in the production of acids due to enzymatic and nonenzymatic chemical reactions, which was probably due to the presence of acid-producing bacteria. These bacterial colonies might have utilized the sugars and other components present in chia-based whey drinks, which may have led to the higher production of acids. This phenomenon resulted in an increase in acidity, as well as a drop in pH. The acids of the beverage were also increased due to the evaporation of moisture and concentration of whey drink with an increase in time of microwave heating [34, 34].

Table 8

Mean value for titratable acidity (%) of whey drink developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	0.78 ± 0.1^{r}
0.81 ± 0.1^{q}	0.89 ± 0.2^{p}	0.91 ± 0.2^{n}	0.84 ± 0.1^{G}	T1	0.87 ± 0.2^{op}
0.92 ± 0.1^{n}	0.98 ± 0.2^{lm}	0.87 ± 0.2^{p}	0.91 ± 0.2^{F}	T2	0.92 ± 0.4^{no}
0.95 ± 0.3^{mn}	1.02 ± 0.3^{l}	1.09 ± 0.2^{k}	0.99 ± 0.3^{E}	T3	1.26 ± 0.3j
1.32 ± 0.2^{hi}	1.34 ± 0.2^{ghi}	1.40 ± 0.2^{de}	1.33 ± 0.2^{D}	T4	1.30 ± 0.1^{ij}
1.35 ± 0.2^{fgh}	1.37 ± 0.1^{efg}	1.39 ± 0.2^{def}	1.35 ± 0.1^{C}	T5	1.43 ± 0.2^{d}
1.49 ± 0.2^{c}	1.57 ± 0.3^{b}	1.63 ± 0.1^{a}	1.53 ± 0.2^{B}	T6	1.79 ± 0.2^{def}
1.77 ± 0.1^{efg}	1.75 ± 0.1^{fgh}	1.62 ± 0.1^{hi}	1.76 ± 0.1^{A}	Mean ± SE	1.15 ± 0.3^{D}

∗Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

In the trials of Gehlot et al. [13], the acidity of the mango-mint RTS drink versions prepared without and with 2% chia seeds increased significantly over the course of three months of storage. Results regarding titratable acidity of a similar previous study reported by the authors in [46] were in agreement with the current findings. Another previous finding showed similar results, as an increase in acidity was recorded with the increase in storage time and time of heat treatment of the drink. Another study conducted on the manufacturing and storage of stirred yogurt, processed with different heat treatments, showed that titratable acidity was increased with the increase in heating time and storage days [37]. Moussa et al. [15] reported that the postacidification effect of yogurt was decreased with the addition of chia seeds and the minimum value of postacidification was shown by the treatment having 3% chia seeds, and these results could be correlated with current experiments in a positive way that chia seeds might have contributed towards maintaining the acidity and pH of the formulated beverage.

Findings of Yikmis [48], upon different treatments including microwave and pasteurization of juices, presented nonsignificant findings about the effects of pasteurization and sonication at various times on titratable acidity. As sonication has been known as a nonthermal technique for food processing, that is why the temperature of the juices might not have been raised, due to which variation in titratable acidity and pH was nonsignificant. Similar reports were also present in the findings of Yikmis [50]. Adulvitayakorn et al. [47] discovered a considerable rise in the titratable acidity of sugarcane juice processed at 700 W. This effect on titratable acidity was shown to be connected with loss of temperature, which is in line with the current findings. Their titratable acidity was estimated by comparing juice samples that were thermosonicated and microwave-treated with sugarcane juice that had been heat-treated traditionally.

Malik et al. [49] looked into how storage and microwave treatments affected the titratable acidity of lemon cordial and found that it gradually decreased over time. They postulated that longer microwave treatments and higher temperatures may have destroyed fermenting microorganisms, which may have led to a decrease in the generation of organic acids. Titratable acidity reduces as a result of the acid's use in the hydrolysis of polysaccharides, which turns nonreducing sugars into reducing sugars [51]. Titratable acidity is one of the important parameters defining the taste, shelf life, and microbial deterioration of fruit juices; therefore, the impact of processing conditions on titratable acidity and optimizing the process protocols for maintaining the pH and acidity of juices is very crucial.

3.4. Total Plate Count of the Mango-Whey Beverage

Statistical analysis of the results obtained has shown that microwave heat treatment significantly influenced the total plate count of mango-whey beverages, as microwave treatment caused a significant reduction in total plate count, with increased exposure. The mean values of the total plate count of the whey mango drink are provided in Table 9. The highest value was found for T1 and the lowest for T6 at 90 days of refrigerated storage. As the duration of microwave heating was increased, a significant decrease in the total plate count of the beverage was noticed, whereas control presented the highest total plate count, as there was no treatment applied for the preservation of the beverage. Pasteurization also exhibited a relatively higher total plate count of the beverage than the microwave, indicating that the microwave is an effective approach for juice treatment. Results of the total plate count analysis of current experiments were supported by the findings of Abdul Alim et al. [52], where they reported that the total plate count of the mulberry-whey beverage was found to increase during storage, unless proper heat treatment was applied. Microbes require a specific water activity and moisture content for their growth, and as the temperature increases, it increases the solid content of the beverage, thus reducing the pH and moisture contents, which ultimately results in the lowering of the microbial count [53]. When the time duration of microwave heating was increased in current experiments, a significant decrease in the total plate count of the beverage was evident. Jacob et al. [43] reported that the microbial load of untreated juices significantly increases, both during atmospheric or cold storage.

Table 9

Mean value for total plate count (log_{10} CFU/ml) of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	2.36 ± 0.1^{b}
2.39 ± 0.2^{b}	2.43 ± 0.2^{ab}	2.48 ± 0.2^{a}	2.41 ± 0.2^{A}	T1	2.17 ± 0.1^{cde}
2.18 ± 0.1^{cd}	2.21 ± 0.1^{c}	2.25 ± 0.1^{c}	2.20 ± 0.1^{B}	T2	1.77 ± 0.2^{h}
1.86 ± 0.2^{g}	1.88 ± 0.2^{g}	1.93 ± 0.2^{g}	1.86 ± 0.2^{C}	T3	1.48 ± 0.1^{j}
1.63 ± 0.2^{i}	1.69 ± 0.1^{hi}	1.73 ± 0.1^{h}	1.63 ± 0.1^{D}	T4	1.38 ± 0.2^{dfg}
1.35 ± 0.1^{def}	1.32 ± 0.2^{fgh}	1.30 ± 0.1^{hi}	1.37 ± 0.2^{E}	T5	1.04 ± 0.1^{f}
1.05 ± 0.1^{f}	1.09 ± 0.1^{ef}	1.18 ± 0.2^{def}	1.07 ± 0.1^{F}	T6	0.98 ± 0.1^{g}
0.85 ± 0.1^{h}	0.78 ± 0.1^{i}	0.71 ± 0.1^{j}	0.83 ± 0.1^{G}	Mean ± SE	1.96 ± 0.1^{C}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

Storage duration also had a significant influence on the total plate count of whey drink, as with the increase in the storage period, the growth of microbes was significantly increased in all treatments. Similarly, treatment during storage also had a significant influence on the total plate count of the drink. With the increase in storage period, the microbial count also increased due to variations in pH, acidity, and moisture contents of the beverage, as well as increased enzymatic activity also increased the microbial count of whey drinks [54]. Moussa et al. [15] conducted a study in which stirred yogurt was prepared by using different doses of chia seeds and found that the bacteria present in yogurt have maximum viability as chia seeds added to yogurt exhibited maximum antioxidant and antimicrobial activities. The addition of chia seeds in mango-whey beverages might have provided the favorable environment for the growth of microorganisms, but at the same time microwave heat treatment caused a significant reduction in the growth and survival of microorganisms.

Kowaleski et al. [16] used chia seeds and strawberries for the formation of yogurt, and the acceptability of yogurt was up to 70 percent for the formulation, having 12 percent strawberry and 6 percent chia seeds. There were 107 colony-forming units of lactic acid bacteria during storage. Moreover, 106 colony-forming units of *Bifidobacteria* were also produced during storage, and these microorganisms could be considered as probiotics. Similarly in another study, the addition of chia seed extracts increased the growth of lactic acid bacteria and fermentation rate of set-type yogurt, as was reported by Kwon et al. [17]. Gupta et al. [55] calculated complete bacterial counts and yeast and mold counts of whey-based dairy drinks, which were expanded from 3.14 to 6.48 log CFU/ml and 1.14 to 2.10 log CFU/ml, respectively, during refrigeration storage. A similar fashion study by Moussa et al. [56] revealed that storage of juices without any treatments results in an increase in microbial counts as in the guava-whey drink, whose complete counts were high going from 1120 to 2500 CFU/ml, and mold and yeast counts changed between 0.0 and 18 CFU/ml, during storage. Thus, microwave heat treatment used in comparison to untreated and pasteurized samples, in current experiments, was capable of controlling the microbial growth of the beverage samples, the basic agents responsible for food safety concerns.

3.5. Total Solid Content of the Mango-Whey Beverage

The mean values of total solid contents of different treatments of mango-whey beverage, during different storage days, are presented in Table 10. Analysis of results has shown that microwave heat treatment had significant influences on the total solid content of whey drink, and as the microwave time was increased, the rise in total solids was more significant. Similarly, the storage period also had a significant influence on the total solid content of the whey drink, and as the storage days increased, total solids were decreased for all treatments. The highest value of total solid content was found for T6 and the lowest for T0. Increased temperature as a result of pasteurization and microwave treatment for longer periods caused an increase in the total solid content of the whey drink by evaporating the moisture content. The results of the total solid content analysis of current experiments were in line with the findings of Panghal et al. [57], where papaya-based whey drink was developed and total solid contents were decreased during storage.

Table 10

Mean value for total solid content of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	12.11 ± 0.1^{k}
11.15 ± 0.1^{l}	10.65 ± 0.3^{m}	9.21 ± 0.1^{n}	10.78 ± 0.1^{G}	T1	14.42 ± 0.1^{j}
13.24 ± 0.2^{k}	12.55 ± 0.2^{k}	11.73 ± 0.2^{l}	12.98 ± 0.2^{F}	T2	18.39 ± 0.3^{f}
17.58 ± 0.1^{g}	16.25 ± 0.2^{hi}	15.63 ± 0.2^{i}	16.96 ± 0.2^{E}	T3	19.65 ± 0.1^{de}
18.58 ± 0.1^{f}	17.59 ± 0.2^{g}	16.70 ± 0.1^{h}	18.13 ± 0.1^{D}	T4	21.53 ± 0.2^{b}
20.34 ± 0.3^{cd}	19.55 ± 0.4^{e}	17.45 ± 0.2^{f}	19.35 ± 0.3^{C}	T5	23.0 ± 0.2^{b}
20.37 ± 0.2^{c}	20.14 ± 0.3^{fg}	18.88 ± 0.2^{i}	19.97 ± 0.2^{B}	T6	25.21 ± 0.1^{a}
22.23 ± 0.2^{c}	21.19 ± 0.1^{d}	$19,12 \pm 0.1^{i}$	20.12 ± 0.1^{A}	Mean ± SE	19.40 ± 0.2^{A}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T 1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

Total solid contents and moisture showed an inversely proportional relationship with each other. Reduction in the moisture content as a result of microwave treatments for longer time periods reduced the total solid content of whey beverages, whereas, during the storage period, viscosity decreased due to the increase in syneresis and enzymatic activity, which decreased the solid content of the whey beverage [58]. The results of total solids of mango-whey beverages were in accordance with the research outcomes of Tanwar et al. [35], in which the total solids of mango-whey beverages were decreased during storage. As total solids of microwave-treated beverages were decreased during storage in the current study, these results exhibited a close resemblance to those of Sattar et al. [59]. Yikmis [48] observed nonsignificant results for total solids of the juice when comparing raw melon juice with pasteurized and ultrasonicated juice, offering comparable findings as have been reported in the current study. Similar results were also seen in the studies conducted by Bora et al. [60], who found that shorter durations of

sonication and microwave had no discernible impact on the solid components of banana juice. Similar findings were also reported by Yikmis, [50] during a comparison of thermal, nonthermal, and chemical treatment of juices with untreated juice.

3.6. Total Soluble Solids (Brix) of the Mango-Whey Beverage

Brix represents the sugar-to-acid ratio of food commodities. Perishable food commodities possess a higher content of water, and as vitamins, sugars, and amino acids are soluble in water, this high water content causes an increase in the sugar-to-acid ratio in food commodities. The values of the brix of different treatments during different storage days are presented in Table 11. Statistical analysis has shown that microwave heat treatments have significantly influenced the brix of whey drinks. The highest value was found for T0 and the lowest for T6, at the start of the study, and during storage, the brix of all treatments was found in decreasing mode. Pasteurization and prolonged microwave time decreased the brix of whey beverage. Similarly, the storage period also had a significant influence on the brix of the whey drink, and as the storage period increased, the acidity level increased, and it decreased the brix of the whey beverage. Results of brix of current analyses were in the range of findings of Kaur et al. [61], where fruit juice-added whey beverage exhibited a decrease in brix during storage. The beverage's lengthy storage caused the monosaccharide and other sugars to break down, which resulted in a decrease in brix. This decrease may also be due to the conversion of insoluble polysaccharides into reducing sugars. The level of reducing sugar might also have been increased due to the acid hydrolysis of sugars, which may have resulted in the breakdown of disaccharides into monosaccharides [13]. According to Nadeem et al. [62], total soluble solids (TSS) were decreased in carrot and grape juice, when sonication treatment was applied. It is possible that the fermentation of carbohydrates into ethyl alcohol, carbon dioxide, and water led to a decrease in TSS during storage. Findings of Pandiselvam et al. [63] and Pandiselvam et al. [22] were also in line with the current ones, where microwave processing of coconut juice was carried out in a similar fashion study.

Table 11

Mean value for Brix of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	10.81 ± 0.1^{a}
10.35 ± 0.2^{b}	10.12 ± 0.1^{c}	9.25 ± 0.1^{d}	10.44 ± 0.1^{A}	T1	10.23 ± 0.2^{b}
10.18 ± 0.2^{b}	10.01 ± 0.3^{b}	9.10 ± 0.2^{b}	10.17 ± 0.2^{B}	T2	9.74 ± 0.1^{c}
9.34 ± 0.1^{d}	9.26 ± 0.1^{de}	9.08 ± 0.2^{de}	9.35 ± 0.1^{C}	T3	8.94 ± 0.2^{ef}
8.71 ± 0.2^{fg}	8.45 ± 0.3^{g}	8.10 ± 0.2^{h}	8.55 ± 0.3^{D}	T4	7.60 ± 0.2^{i}
7.52 ± 0.3^{i}	7.47 ± 0.1^{i}	7.06 ± 0.3^{j}	7.41 ± 0.3^{E}	T5	6.90 ± 0.1^{j}
6.45 ± 0.2^{k}	6.21 ± 0.2^{l}	6.01 ± 0.2^{m}	6.45 ± 0.2^{F}	T6	6.51 ± 0.2^{k}
6.34 ± 0.2^{j}	6.02 ± 0.2^{m}	5.98 ± 0.2^{n}	6.21 ± 0.2^{G}	Mean ± SE	9.46 ± 0.1^{A}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed,

and microwave for 120 sec.

When compared to individual microwave, sonication, and untreated treatments, the synergy of ultrasonics and microwaves, as reported by Zia et al. [36], showed no change in the TSS of the sugarcane juice samples. This was likely because there was a lower temperature rise than with current microwave processing for longer periods of time. In melon juice processing, Liu et al. [64] evaluated ultrasonic and ultrahigh temperature treatments, along with a control group. The results showed that there was no significant difference in the juice's TSS. These differences in results were possibly due to a higher power and longer time durations of microwave used in previous studies, when compared with the current one. Malik et al. [49] detected a significant decrease in the TSS of juice over storage periods in both the control and microwave-treated samples, which provided supportive data. They connected the rise in internal temperature of the treated samples, which caused the water to evaporate, with the reduction in TSS brought about by microwave treatment. Juices' TSS decreases during storage as a result of yeast and lactic acid bacteria converting carbohydrates into their corresponding acids and alcohols [36].

3.7. Viscosity of the Mango-Whey Beverage

The viscosity of dairy commodities is directly proportional to lactic acid production. When the synthesis of lactic acid is enhanced, it increases the protein content that produces a viscous gel structure in the dairy product. The analysis of the viscosity values of all treatments of mango-whey beverage is presented in Table 12. The highest value was observed for T6 and the lowest for T0. The microwave heating time had significantly influenced the viscosity of the whey drink. Similarly, the storage period had a significant effect on the viscosity of whey beverage, and as the storage period increased, it reduced the viscosity of whey drink, by increasing the enzymatic activity, microbial reactions, and other chemical reactions. Another reason might be the rise of temperature, as it results in an increase in solid content and a decrease in moisture content of the beverage, which ultimately increases the viscosity [34]. Heating increases the viscosity of whey drink due to the interaction of phenolic content and protein that produced the gel-like structure, which improves the texture of whey drink and reduces the syneresis [65]. The addition of chia seed extracts increased the water-holding capacity, syneresis, and viscosity of set-type yogurt, as reported by Kwon et al. [17], just as chia seeds-added mango-whey beverage showed high values of viscosity. In order to study the changes that occurred in physicochemical properties and functional components, Sattar et al. [59] chosen to pasteurize peach-based functional beverage for 10 min at 90°C, microwave for 1.5 min at 850 W, and sonicate for 90 min at 20 kHz of frequency. They then stored the beverage in the refrigerator for up to 30 days. TSS almost always remained stable, even in samples that have undergone microwave and ultrasound treatment, whereas the cloud values of all processed juice samples decreased with storage time.

Table 12

Mean value for viscosity (cP) of whey beverage developed with different processing and preservation techniques.

Treatments	Storage days				Mean ± SE
0	30	60	90	T0	0.87 ± 0.2^{j}
0.81 ± 0.2^{ij}	$0.79 \pm \pm 0.1^{k}$	$0.71 \pm \pm 0.2^{i}$	0.79 ± 0.1^{G}	T1	0.96 ± 0.1^{i}
0.85 ± 0.1^{j}	0.72 ± 0.2^{k}	0.68 ± 0.2^{k}	0.80 ± 0.1^{F}	T2	1.16 ± 0.1^{f}
1.05 ± 0.1^{g}	0.98 ± 0.2^{hi}	0.87 ± 0.1^{j}	1.01 ± 0.2^{E}	T3	1.28 ± 0.2^{d}
1.27 ± 0.1de	1.16 ± 0.1^{f}	1.02 ± 0.2^{gh}	1.18 ± 0.2^{D}	T4	1.37 ± 0.2^{bc}
1.26 ± 0.2^{de}	1.13 ± 0.1^{f}	1.05 ± 0.2^{g}	1.20 ± 0.2^{C}	T5	1.45 ± 0.1^{b}

1.38±0.1^{d}	1.23±0.2^{e}	1.17±0.2^{e}	1.35±0.2^{B}	T6	1.51±0.1^{a}
1.43±0.2^{c}	1.38±0.1^{d}	1.38±0.1^{e}	1.49±0.1^{A}	Mean±SE	1.24±0.1^{A}

*Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

To evaluate the effects of chia seed in ice cream stabilization, Feizi et al. [66] replaced a commercial stabilizer with chia seed gum, and the analysis of ice cream including fat globule size distribution, the viscosity of the ice cream mix, destabilized fat index, overrun, hardness of ice cream, and meltdown rate was performed, with conclusions that the ice cream was highly viscous due to chia seeds' gum. Thus, the chia seeds-added mango-whey beverage developed in the current study was an ideal recipe for food lovers, because the trio of chia seeds, mangoes, and whey contributed positively in developing a beverage with optimum quality.

3.8. Sensory Evaluation of the Mango-Whey Beverage

Sensory evaluation of the product was based on the five parameters, which included color, texture, taste, flavor, and overall acceptability. Statistical analysis of the sensory evaluation scores of all treatments of whey drink is presented in Table 13. The highest scores for all parameters were observed for T3 and the lowest for T6. The color profile of the whey drink was significantly influenced by the microwave heat treatment duration. The color of dairy products is one of the significant factors that influence the consumer acceptance. As the microwave duration was increased, it increased the yellowness of the whey drink, reducing the lightness, due to which consumers might have not preferred treatments with longer microwave times. The heating of the drink results in the caramelization that imparts a dark color to the drink. The flavor is a combination of taste and aroma, and the evaluation of flavor and taste was based on the taste buds such as bitter, sweet, umami, sour, and salty. Results indicated that T3 obtained higher consumer acceptance for taste and flavor. As the temperature was increased, it increased solid contents, by decreasing the moisture and pH, which resulted in the bitterness and reduced the acceptance level of consumers. The appearance of dairy commodities has been found to be directly proportional to the lactic acid production. When the synthesis of lactic acid is enhanced, it results in increased protein content, which produces a viscous gel structure in dairy products. The texture profile of the whey drink was significantly influenced by the addition of chia seeds. Increased microwave heating duration increased the viscosity of the whey drink due to the interaction of the phenolic content and protein that produced a gel-like structure, which improves the texture of the whey drink and reduces the syneresis [58]. For overall acceptability, the highest score was observed for T3 and the lowest for T6. Microwave heat treatment durations significantly influenced the overall acceptability of whey drink.

Table 13

Mean value for sensory evaluation of whey beverage developed with different processing and preservation techniques.

Treatment	Color	Flavor	Texture	Taste	Overall acceptability
T0	7.92±0.2^{b}	7.50±0.3^{c}	6.98±0.2^{d}	6.5±0.2^{d}	7.12±0.3^{c}
T1	7.14±0.2^{c}	7.43±0.3^{ab}	7.60±0.3^{b}	7.60±0.1^{b}	7.14±0.3^{b}
T2	7.13±0.3^{c}	7.39±0.4^{ab}	7.54±0.4^{b}	7.11±0.4^{bc}	6.51±0.4^{d}

T3	7.65 ± 0.2^{a}	7.76 ± 0.2^{a}	7.70 ± 0.2^{a}	7.70 ± 0.2^{a}	7.41 ± 0.3^{a}
T4	7.21 ± 0.3^{c}	7.26 ± 0.4^{ab}	7.08 ± 0.2^{b}	7.03 ± 0.3^{bc}	5.99 ± 0.3^{e}
T5	6.26 ± 0.2^{d}	7.04 ± 0.1^{b}	5.63 ± 0.2^{c}	6.69 ± 0.2^{c}	5.89 ± 0.2^{e}
T6	6.12 ± 0.2^{f}	6.08 ± 0.2^{e}	5.41 ± 0.2^{e}	5.32 ± 0.1^{d}	5.54 ± 0.2^{f}

∗Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T0 = control; T1 = 85 ml whey, 2 g chia seed, 0.25 g sodium benzoate, and 0.75 g potassium metabisulfite; T2 = 85 ml whey, 2 g chia seed, and pasteurization; T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec; T4 = 85 ml whey, 2 g chia seed, and microwave for 60 sec; T5 = 85 ml whey, 2 g chia seed, and microwave for 90 sec; T6 = 85 ml whey, 2 g chia seed, and microwave for 120 sec.

Shrestha and Dahal [67] developed a drink by utilizing natural whey with magnificent wholesome characteristics and blended flavors, alongside banana juice and the necessary measure of sugar. A huge variety in taste, color, flavor, and overall acceptability was seen by differing the organization of whey and banana juice. In a similar study, Yonis et al. [68] uncovered that the drink developed with 75% whey and 25% guava juice scored the greatest for practically all sensorial quality credits such as the taste, color, flavor, texture, and overall acceptability. An ice cream formulation was evaluated by Feizi et al. [66], and a sensory evaluation by a focused group showed that the ice cream was highly acceptable as it had a cream texture, smooth texture, and hardness was desirable without any crystallization.

The development of innovative thermal and nonthermal food processing techniques has made it feasible to produce food products that are safer, healthier, and have acceptable sensory qualities. According to Ozkan et al. [69], the use of nonthermal processing was proven to be an efficient strategy in maintaining maximum antioxidant chemicals and acceptable sensory qualities in natural fruits and their juices. The length of the beverage's microwave treatment and storage time had a significant impact on its sensory qualities [63].

The use of microwave heat treatments for 30 sec was proved effective in maintaining the optimum quality of the formulated beverage; that is why consumers preferred this treatment over untreated and pasteurized beverage samples. The most acceptable treatment T3 (85 ml whey, 2 g chia seed, and microwave heat treatment for 30 sec) was further subjected to a storage study, to find out the influence of storage on the sensory aspects and consumer acceptance level.

3.9. Storage Study of the Most Acceptable Treatment

Whey beverage supplemented with 2 g chia seed that had a microwave treatment for 30 sec (T3) was most acceptable for consumers in terms of taste, color, flavor, texture, and overall acceptability. The three-month storage period had a significant influence on the sensory aspects of T3. The means of sensory scores are shown in Table 14. An increase in the storage period significantly decreased the acceptance level of consumers, as was evident from the decreased scores obtained during increased storage days. As the storage period increased, the enzymatic activity, chemical reaction, and microbial growth also increased, which would possibly have influenced the taste, color, and flavor of whey beverages. From the storage study results, it was found that formulated beverage got good scores during the 60 days of storage, but a further increase in storage time caused a reduction in consumer preferences, so the chia seeds-added mango-whey beverage microwaved for 30 sec was recommended for consumption during the 60 days of refrigerated storage.

Table 14

Mean values for sensory evaluation of most acceptable treatment of whey beverage (T3).

Treatment T3	Storage days

0	30	60	90	Color
7.90 ± 0.1^{a}	7.37 ± 0.2^{b}	6.26 ± 0.2^{c}	4.78 ± 0.2^{d}	Taste
7.48 ± 0.2^{a}	6.37 ± 0.3^{b}	5.40 ± 0.3^{c}	2.95 ± 0.3^{d}	Flavor
7.91 ± 0.1^{a}	6.99 ± 0.2^{ab}	6.15 ± 0.2^{b}	3.48 ± 0.2^{c}	Texture
7.34 ± 0.4^{a}	6.55 ± 0.2^{b}	5.95 ± 0.3^{b}	4.28 ± 0.4^{c}	Overall acceptability

⁎Each mean value within the same column, followed by different letters is highly significant at $P<0.05$. T3 = 85 ml whey, 2 g chia seed, and microwave for 30 sec.

Gupta et al. [55] conducted experiments for the exploration of microbial and physicochemical properties of cocoa and whey protein-advanced utilitarian dairy drink, that was exposed to storage at a refrigeration temperature of 4 ± 1°C, and these findings proposed that the drink could be stored for up to 18 days at 4 ± 1°C. Experimental investigation of Shrestha and Dahal [67] showed that refreshments arranged with 85% fluid whey and 15% banana juice could be stored for 30 days under refrigerated conditions, without the expansion of additives. Incorporation of chia in the form of gel was performed to avoid the competition of starch and ground chia, by Zettel et al. [70], and it was found that the volume yield of bread loaf as well as the stability of dough during fermentation was increased by the addition of chia gel, providing the high consumer acceptance attributed to chia seeds, just as our product got good preferences. During the three months of storage, a significant decline in the sensory scores for the mango-mint drink with chia seeds added was observed in the areas of color and appearance, taste, mouthfeel, flavour, and overall acceptability [13]. Whey beverages developed in the current study were initially pleasing in color and appearance with high sensory scores, but changes were evident as three months of storage progressed. It might be because mango pulp and chia seeds initially had attractive colors during storage, but those colors started to fade as ascorbic acid, carotenoids, and total phenols degraded with time. The Maillard process, which occurs when sugars and amino acids are mixed in an acidic environment, may be the cause of the beverage colors deteriorating after storage.

Tangerine juice samples were processed and preserved using microwave treatments by Demirok and Yikmis [71], who also produced comparable findings for storage trials. In a related study, the antioxidant capacity of untreated and pasteurized juices reported a decreasing trend during storage, following a first-order kinetics [72], supporting the findings of the current work regarding the decrement of nutritional potential of blend juices during storage. Correlating these experiments with the current one, it could be argued that either untreated, or pasteurized and microwaved, all treatments of beverages start deteriorating after a limited storage duration under controlled refrigerated conditions. Microwave and ultrasonic as green processing technologies work chemically (free radicals) and mechanically (cavitation and shock waves). Juices have higher antioxidant capacities and retain more bioactive compounds during storage under controlled circumstances, therefore processing them using ultrasound and microwaves separately or in combination yields better results [73].

4. Conclusion

In the current experiments, a novel beverage formulation containing whey, mango pulp, and chia seeds was developed, which had undergone different preservation techniques, to study their effect on chemical, physical, microbial, and sensory attributes. The chemical composition of chia seeds revealed the high nutritional profile of chia seeds, before incorporation into beverages. The color of chia seeds added to mango-whey beverages was significantly affected by pasteurization and microwave treatments. There was a significant increase in the titratable acidity of the beverage with the increase in time of storage and microwave heating, whereas the pH value significantly decreased. Microwave heat treatment significantly influenced the total plate count of whey beverages,

as the highest value of total plate count was found for T1 and lowest for T6. Storage duration also had a significant influence on the total plate count of beverages as with the increase in the storage period, the growth of microbes was significantly increased, but as the microwave treatment time was increased the total plate count decreased. An increase in the microwave heating caused an increase in total solids. On the other hand, brix was decreased, both for increased microwave time and storage. Sensory analysis showed that the best results were shown by the treatment, which had 2 g of chia seeds, and was applied 30 sec of microwave heat treatment (T3). The conclusive result of the sensory evaluation of T3 at different days of storage showed that good scores were found at 0 and 30 days of storage, scores at 60 days of storage were also acceptable, but were highly decreased at 90 days. In the end, it could be concluded that microwave treatment for 30 sec, of formulated beverage, provided the best results to obtain the optimum parameters for physicochemical, microbial, and sensory characteristics during storage. The food processing industry can employ the microwaved mango-whey beverage with chia seeds added as an effective and premium functional food.

5. Recommendations

Fruit extracts and juices can be used in functional beverages in a variety of ways because fruits are valuable dietary sources and are recognized for being rich in phenolic and antioxidant components. These beverages are becoming more and more popular worldwide. Consumers are becoming more conscious of the nutritional content and possible health advantages of food. Whey is a byproduct of cheese production that can be used to make useful food items. Chia seed is a rich source of omega-3 fatty acids and is recommended for patients suffering from cardiovascular disease and for intended consumers for fiber intake. Mango-whey beverage enriched with chia seeds is recommended for the sportsmen and adults as a potent source of energy and valuable nutrients. This beverage has been also recommended for the school-going children suffering from protein deficiency and is particularly significant for athletes and bodybuilders for mass gain. Further phytochemical and antioxidant studies are required on these formulated beverages processed and preserved under different conditions for different time periods, in order to commercialize these value-added functional food formulations.

Additional Points

Highlights. (i) Mango-based whey beverage, added with chia seeds, is an exotic and nutritious drink. (ii) Comparison of different processing and preservation techniques for beverage formulation. (iii) Significant changes in pH, acidity, brix, viscosity, phenolics and sensory parameters during the 90 days of storage. (iv) Microwave treatment had a significant influence on the physiochemical, nutritional and sensory profiles of beverages. (v) Microwave treatment for 30 sec provided the best results as compared to other treatments.

Authors' Contributions

Ashiq Hussain conceptualized the study. Mansoor Hassan and Farzana Siddique curated the data and performed the formal analysis. Haya Fatima acquired the funding. Amer Ali Mahdi and Sameh A. Korma investigated the study. Syeda Ayesha Batool developed the methodology. Mansoor Hassan administered the project. Saima Noreen collected the resources. Faiza Iftikhar Gorsi developed the software. Farzana Siddique and Shazia Yaqub supervised the study. Tahira Siddique and Amer Ali Mahdi validated the study. Samina Kauser and Sameh A. Korma visualized the study. Ashiq Hussain wrote the original draft of the manuscript. Ashiq Hussain, Amer Ali Mahdi, and Sameh A. Korma wrote, reviewed, and edited the manuscript. All authors contributed equally in the preparation of this manuscript and gave their consent for publication.

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DETAIL

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Determination Methods and Influencing Factors of Grain Mechanical Properties

Gao, Peng; Tian, Shuangqi; Xing'ao Xue; Lu, Jing.

ABSTRAK (ENGLISH)

Grain is extremely vulnerable to external loads during production and processing, resulting in the deterioration of grain quality. Deteriorated grain not only affects the economic value of grain but also affects the safety of storage. This has a very important relationship with the biomechanical properties of grains. It is of great significance to explore the mechanical properties of grain under different conditions and analyze the relationship between its physical and chemical properties and mechanical properties for improving its processing and eating quality. In this paper, the research methods of the mechanical properties of grains are reviewed. Various factors influencing the mechanical properties of cereals were analyzed. The relationship between the internal organizational structure of grain and its mechanical properties was discussed. This paper puts forward the shortcomings in the current research on the mechanical properties of grains and puts forward the prospect and analysis of its importance in future development in order to provide a reference for reducing crushing in the grain processing process.

TEKS LENGKAP

1. Introduction

Grain refers to the seeds of gramineous plants, including rice, wheat, maize, millet, sorghum, and other grains. The staple foods processed from grains provide most of the energy and protein and are widely cultivated around the world [1]. However, in today's highly mechanized popularization, grain will be subjected to various mechanical forces in production and processing. These forces can cause irreversible damage to the grain, causing the grain to deform and even leading to the fracture of the grain [2]. According to the nature of the force, it can be roughly divided into the following categories: the friction between threshing and the machine, the impact force between harvesting and the machine, and the extrusion force [3–5]. When these external forces exceed the limit that the grain can withstand, the grain breaks. Ruptured grains not only affect the physical and chemical properties of grains but also increase the difficulty of grain storage [6]. These biomechanical problems in the processing process will directly affect the quality and economic effects of grain. Therefore, the mechanical parameters such as rupture force and rupture energy of grain under external load are clarified, and the relationship between the organizational structure and mechanical properties of grain is studied. It can provide technical parameters for mechanical processing equipment, which is of great significance in reducing the loss of grain in harvesting, transportation, storage, and processing. For example, based on the theoretical and mechanical properties of buckwheat, the threshing unit of the thresher can be optimized to improve the cleaning efficiency of the buckwheat thresher [7]. By combining the compression tests, compression-relaxation tests, insertion-relaxation tests, and stretch tests, the threshing rate of maize kernels was obtained. By establishing an artificial neural network model, the mechanical properties of maize kernels were used to predict the breakage rate [8]. The study of the differences in mechanical properties of different threshing directions of maize provides a theoretical basis for the design of mechanized threshers [9]. These are inseparable from the study of the mechanical properties of grains.

The research on the mechanical properties of grain can be divided into dynamic mechanical and static mechanical properties. Dynamic mechanical research refers to the mechanical response of materials under alternating stress (alternating strain), that is, the relationship between mechanical properties (modulus and internal friction) and temperature and frequency. The static load's mechanical properties refer to the changes in the mechanical behavior of the grain after the slow loading of the external force in the static state. At present, the research on the mechanical properties of grain is mainly based on static load mechanics. The changes in the mechanical behavior of grains

under compression, shear, and other forces were explored [2, 10]. The values used to characterize the static mechanical properties of cereals include elastic modulus, contact stiffness, damage strength, shear strength, yield strength, crushing force, and crushing energy [11]. In recent years, studies have found that the mechanical properties of grains were affected by many factors, and the antidamage ability of different grains was different [12]. In this paper, the factors affecting the mechanical properties of grain were summarized by summarizing the current research methods on the grain's mechanical properties. The relationship between the mechanical properties of grain and its processing quality was analyzed, and the research status of the mechanical properties of grain was expounded.

2. Research Methods of Mechanical Properties of Grain

The study of the grain's mechanical properties can provide data support for the design and parameter optimization of processing machinery, transportation, and storage, as well as the evaluation of grain quality. As early as the 1970s, the mechanical properties of grain had been studied. The results of the mechanical behavior of rice under quasi-static compressive load showed that the moisture content had a great influence on the mechanical properties of rice. The yield point and maximum compressive strength of the sample decreased with the increase in moisture content [13]. With the continuous development of science and technology, the research on the mechanical properties of grains is becoming more and more extensive, and the methods are becoming more and more perfect. In order to explore the mechanical properties of grains, the methods used by researchers mainly include experimental methods, mathematical calculation methods, and finite element analysis methods.

2.1. Experimental Method

The experimental method was a commonly used method for studying the mechanical properties of grains. The materials testing machine or texture analyzer can be used to simulate the different loads that grains are subjected to during production and processing and to analyze the changes in their mechanical properties. The mechanical indexes of grain, such as rupture force, rupture energy, elastic modulus, and compression displacement, were measured directly. The experimental method has a strong applicability and can be used to measure the mechanical properties of most grains, such as maize, wheat, and rice [14–16]. Appropriate experimental methods can be selected for research needs and research objects. Table 1 lists several test methods for measuring the mechanical properties of the grain. Using the experimental method to determine the mechanical properties of grain is convenient and fast, and data accuracy is high. However, due to the large differences between grain individuals, the repeatability of the data obtained by the experimental method is poor, and a large number of experimental results are often needed to ensure the stability of the data.

Table 1

Experimental method to explore the mechanical properties of grains.

Research object	Instrument	Experimental method	Factor	Reference
Maize	Tension/compression testing machine	Compression test	Moisture content	[17]
–				
Wheat	Texture analyzer	Shear test	Loading rate	[18]
–				
Rice	Self-made machine	Impact test	Impact velocity	[19]
Texture analyzer	Three-point bending	Drying condition	[20]	

Millet	Assembled apparatus	Wear test	Friction media	[21]
–				
Soybean	Universal testing machine	Compression test	Moisture content	[22]
–				
Sorghum	Grain hardness tester	Compression test	Moisture content	[23]

2.2. Finite Element Analysis

The finite element method (FEM) is an efficient and discrete numerical method, which is widely used in engineering design and scientific research [24]. It is widely used in granaries. In addition to analyzing the transfer of grain heat, airflow, and water in silos, it can also study the bulk density of grain in silos. As a key factor in predicting grain pressure in silos, it plays a vital role in food security storage [25, 26]. By establishing a finite element model to analyze the mechanical properties of grains, the internal stress-strain distribution of grains under different ballasts can be obtained [27]. Based on the mechanical properties of compression, the finite element analysis of soybeans was carried out. The results showed that there were differences in the internal stress distribution of soybean grains when soybeans were compressed under different placement methods. This is greatly related to the contact area of the compression plate. The finite element simulation results are consistent with the compression test results, which can better explain the crack distribution of soybean when it is compressed [28]. The finite element model of wheat grain can better simulate the stress distribution inside wheat grain during storage. Studies have shown that the stress on the side of wheat grain during storage is about three times to that on the bottom [29]. Compared with other experimental methods, the microscopic mechanical properties of grains under external loads can be obtained by establishing a finite element model, which reflects the distribution law of the internal stress of grains. Combined with the results of the experimental method, the mechanical behavior of grains under different conditions can be better explained.

2.3. Mathematical Algorithm

In addition to the two experimental methods mentioned above, researchers often use mathematical calculations to obtain mechanical indicators such as the elastic modulus and failure stress of grains and further analyze the relationship between grain deformation and its mechanical properties. When the grain is compressed, the contact area between the indenter and the grain is elliptical, and the failure stress of the grain can be calculated according to the contact area and the magnitude of its rupture force [30]. Taking maize as an example, when it is compressed, the semimajor axis (a) and semiminor axis (b) of the contact surface area can be calculated by using the following equations:(1)a=m3FK1+K221RU+1RU′+1RL+1RL′−11/3,(2)b=n3FK1+K221RU+1RU′+1RL+1RL′−11/3,where *m* and *n* are constants, and K1 and K2 are calculated by using the following formula:(3)K1=1−μ2E,K2=1−μ22E2,where *μ* and μ1 are Poisson's ratio of maize and compression plate and *E* and E2 are the elastic modulus of maize and compression plate, respectively [31]. In general, the elastic modulus of the plate is much higher than that of the grain, so K2=0 can be used in the calculation process. Now, the contact area can be calculated according to the elliptic area formula as follows:(4)A=πab.

The contact surface stress is calculated by using the following formula [32]:(5)S=1.5FA.

As a physical quantity describing the elasticity of an object, elastic modulus (also known as Young's modulus) can well reflect the difficulty of object deformation. The general definition of elastic modulus is the ratio of stress to strain in this direction. From a macroperspective, elastic modulus is a measure of the ability of an object to resist elastic deformation. From a microperspective, it is a reflection of the bonding strength between atoms, ions, or molecules [33]. The elastic modulus of wheat grains can be determined by the stress-strain curve under lower deformation. The specific calculation formula is as follows:(6)E=σε=P/ADe/H,where *E* (MPa) is the elastic modulus, *P* (*N*) is the pressure on wheat, *A* (mm^2) is the size of the contact area, De (mm) is the elastic deformation of wheat grain during

compression, and H (mm) is the initial height of wheat [34]. In addition to calculating the ratio of stress to strain, the calculation of the elastic modulus of grain can be carried out according to the following formula:(7)E=0.338F1−μ2D3/2KU1RU+1RU′1/3+KL1RL+1RL′1/33/2,where E is the apparent elastic modulus, F is the compression force, μ is Poisson's ratio, KU and KL are the constant of 1.3531, D is the compression displacement, RU and RU′ are the maximum and minimum curvature radius of the maize kernel, and RL and RL′ are the maximum and minimum radius of the curvature of the indenter. In the compression test, the calculation method of elastic modulus is also different due to the different loading modes and contact areas of the indenter, including single plate contact, parallel plate contact, and spherical indenter on a curved surface [35]. The uniaxial compression test is the most commonly used method to calculate the elastic modulus of grain, but some researchers use other methods to determine the elastic modulus of the grain. For example, the elastic modulus of grain was measured by an acoustic wave experiment. The results show that the elastic modulus of grain can be predicted within a certain range of water content. The experiment provides a new idea for the determination of the elastic modulus of grain.

3. Mechanical Properties of Grain

3.1. Characterization of Static Mechanical Properties of Grain

The static mechanical properties of cereals are usually studied by using a material universal testing machine or a texture analyzer. During the grain static loading mechanics experiment, the grains are fixed on the plate and remain motionless, and the slowly descending indenter exerts a force on the specimen. As the loading indenter descends, the force acting on the grain increases, and when the force exceeds the limit that the grain can stand, the grain ruptures. By analyzing the force-displacement curve from the time the indenter touches the grain until the grain breaks, the amount of rupture force and energy that need to be absorbed by the grain to break can be obtained [18]. Rupture force and rupture energy are the most intuitive physical quantities to represent the damage resistance of grains, so these two mechanical indexes are often used in the research process to further analyze the relationship between the mechanical properties of grains and their quality traits. In addition, the indicators used to characterize the mechanical properties of grains also include shear force, bending stress, elastic modulus, yield strength, and crushing stress [36].

3.1.1. Compression Mechanical Properties

The compression force is one of the most common external loads on grain. Under pressure, the grains are prone to deformation and structural changes. When the pressure exceeds the limit that the grain can withstand, the grain is broken, which affects the safety of grain storage [37, 38]. The experimental device of grain compression mechanical properties is shown in Figure 1. The grain was fixed on the stage to keep still, the upper-pressure indenter decreased slowly at a fixed rate, and the force-displacement curve was obtained under the action of pressure. Mechanical indexes such as rupture force, rupture energy, and compressive displacement can be obtained by analyzing the force-displacement curve [39, 40]. During the compression process, the loading rate of the indenter would affect the mechanical properties of the grain. Therefore, a constant loading rate should be ensured during the experiment [18].

[figure(s) omitted; refer to PDF]

There are differences in the compressive mechanical properties of different grains. Table 2 shows the magnitudes of compressive rupture force and rupture energy of several common grains. The antidamage ability of grains was maize > soybean > rice > wheat > sorghum. The maximum compressive rupture force of maize was in the range of 374.18 N~629.72 N. The compressive rupture force of rice was the smallest in the range of 23.54 N~38.19 N. The ability of grains to resist damage varies depending on the orientation. Taking maize kernels as an example, the compression rupture force was horizontal > lateral > vertical. This was due to the difference in the contact area between the indenter and the grain under different compression orientations. When placed horizontally, the contact area between the grain and the indenter is large, and the force is dispersed on the surface of the grain, so the grain has a high compressive rupture force at this time [44]. On the side of maize kernel, the proportion of the horny endosperm was higher than that of the floury endosperm. The horny endosperm tissue had a high bonding strength and hardness and was not easy to be destroyed. Therefore, the side compression rupture force was larger. When

the top surface is compressed, the indenter first contacts the embryo of the maize kernel. This part was the main part of the life activities of maize kernels, and it is also the weakest part of the antidamage ability of kernels. At the same time, the proportion of the floury endosperm at the top of the maize kernel was higher than that of the horny endosperm, so the maize kernel had the lowest compressive rupture force when standing [45, 46]. The difference in the compressive mechanical behavior of grains was closely related to their physical and chemical properties. The relationship between the physical and chemical properties of grains and their mechanical properties will be further discussed in the following sections.

Table 2

Different grains' compression rupture force.

Variety	Placement	Rupture force (N)	Rupture energy (mJ)	Reference
Maize	Horizontal	374.18~629.72	31.42~154.72	[41]
Lateral	92.54~144.70	54.19~304.33	Vertical	69.84~163.05
342.99~778.83	–			
Wheat	Horizontal	48.51~88.96	36.33~50.19	[42]
Horizontal	154.62~287.98	—	-	
Soybean	Lateral	114.71±197.67	—	[28]
Vertical	90.80±172.05	—	-	
Rice	Horizontal	99.15~154.42	15.57~31.81	[40]
Vertical	23.54~38.8	12.42~24.13	-	
Sorghum	Horizontal	50~75.26	—	[43]

3.1.2. Bending Mechanical Properties

The determination of grain bending mechanical properties can be used to characterize the ability of grain to resist bending loads during production and processing. The commonly used experimental method of bending mechanical properties is the three-point bending test, which can be used to test the bending strength, fracture energy, and other indicators of materials [47]. At the beginning of the experiment, the material was placed on two fulcrums at a certain distance, and a downward load was applied from the center above the material. Two equal moments were formed between the three contact points of the material, so that the material breaks at the midpoint [20]. The experimental device is shown in Figure 2. The results of the multipoint bending test of intact brown rice and rice with cracks in the husk showed that the flexural strength and failure energy of intact rice were much higher than those of damaged rice. As drying continued, the rice grains became stronger and tougher. Under lower moisture content conditions, intact brown rice had a higher apparent elastic modulus, flexural strength, and fracture energy [48]. In addition, the three-point bending breaking force of brown rice increases with the increase in rice maturity, and the yield of the first rice has a certain correlation with the breaking force of rice grains [49]. Although there was no relevant research, this should be related to the change of protein and starch content in rice during maturation. Compared with other

mechanical indexes, the bending crushing force of rice can better reflect its crushing characteristics, which provides an important basis for reducing the breakage rate and rice cracks in rice processing.

[figure(s) omitted; refer to PDF]

3.1.3. Shear Mechanical Properties

The shear characteristics of grain are very important engineering data in the study of grain crushing, threshing, and antibreaking ability under seed harvest [50]. The determination of the mechanical properties of materials under shear force is one of the basic experimental methods for the mechanical property test of materials. The grain was placed on a central suspended-loading platform before the start of the shear test of the grain. The upper indenter was a blade indenter. When the indenter was in contact with the grain, the force-displacement curve was recorded. The experimental device is shown in Figure 3. The shear mechanical test results of rye grains showed that when the water content of rye grains increased from 10% to 20%, the average shear force decreased from 60.8 N to 31.4 N. This is due to the swelling of the endosperm after absorbing water, resulting in a decrease in the shear strength of the grain [51]. There are some differences in the shear resistance of different wheat varieties. The research results of hard wheat varieties and soft wheat varieties showed that the shear resistance of hard wheat was significantly higher than that of soft wheat, but the water content had no significant effect on the shear energy of the two varieties of wheat [52]. When wheat grains were sheared at lower moisture content, the shear deformation of wheat was small. In the case of high water content, the wheat grains gradually ruptured during the shearing process, and the deformation was large. The shear energy of wheat depends on the shear force and shear deformation. Although the high moisture content led to the decrease in wheat shear force, the shear deformation increased with the increase in water content, so the moisture content had no significant effect on the shear energy of wheat [18].

[figure(s) omitted; refer to PDF]

3.2. Grain Dynamic Mechanics

Dynamic mechanical properties are used to study the law of stress and strain changes of materials under the action of changing forces. An important direction of grains' dynamic mechanics research was the influence of the impact force on grain's mechanical properties. The impact force refers to the force that suddenly increases and then disappears rapidly between two objects during a collision. It is characterized by short action time and large force value. The study of impact mechanical properties is of great significance for reducing the crushing rate of grain in the process of warehousing.[53]. When the grain is put into the warehouse, the higher the warehouse, the greater the impact of grain contact with the ground, and the higher the grain crushing rate. The research on the impact mechanical properties of grain was often combined with the experimental results of compression mechanics.. Based on the results of the compression test and impact test, a mathematical model for predicting seed breakage was established [54]. The experimental results on the impact force of rice show that there was a velocity threshold when the grain was broken by the impact force, and the grain will not break below the velocity threshold. The probability of grain rupture tends to be stable with the increase in impact velocity [19, 55]. According to the size of the impact rupture force, the appropriate adjustment of the grain storage height can effectively reduce the grain crushing rate and improve the utilization rate of grain resources.

3.3. Grain Viscoelasticity

The viscoelasticity of food refers to the characteristics of both viscosity and elasticity when food materials are subjected to force. It mainly studies the distribution law of internal stress and strain of materials and the relationship between them and external forces. Common food materials such as bread and dough are the research objects of food viscoelasticity [56, 57]. Due to the complex deformation of food during chewing, it is usually subjected to the shear and compression of the teeth and tongue at the same time. Viscosity and elasticity will affect the taste of food. Therefore, it is of great significance to explore the viscoelasticity of food for understanding the changes in food's physical properties and human chewing bionics. The research on grain viscoelasticity can be divided into two categories: dynamic viscoelasticity and static viscoelasticity. Dynamic viscoelasticity refers to the phenomenon that the strain and stress of the material change with time under the action of alternating stress or alternating strain. Static viscoelasticity studies the stress relaxation and creep properties of materials under constant stress or strain.

Thus, we explored the compression deformation of the object under the action of external force [58].

3.3.1. Static Viscoelasticity

The study of the static viscoelasticity of cereal is widely used in the study of the rheological properties of wheat dough and starch gel. The differences in physical properties of grains were analyzed by compression or shear tests combined with mechanical indexes such as grain elastic modulus, shear modulus, and loss modulus [59]. In addition to these studies on the viscoelasticity of starch gel or dough, there is also a certain relationship between the rupture behavior of grains and their viscoelasticity [60, 61]. The maximum rupture force of wheat and rye showed a strong correlation with their viscoelasticity. The viscoelasticity of grains is strongly related to their composition, especially the water content. Moisture content affects the viscoelasticity of grain to a great extent, the most important of which is the elastic modulus of grain [62]. The uniaxial compression experiment of wheat further verified this result. With the increase in water content, the elastic work of wheat grain decreased during compression, and the plastic work increased with the increase in water content. The total work in the compression process was reduced by 80% [63]. Molenda and Stasiak reported that the elastic constants (elastic modulus E and Poisson's ratio) of wheat, rye, barley, oats, and rapeseed are determined by the linear phase of the sample loading curve. The increase in moisture content will lead to a decrease in the elastic modulus of the grain [64]. The size of the elastic modulus reflects the strength of the material's resistance to deformation, so it is feasible to analyze the fracture behavior of grains by grain viscoelasticity.

3.3.2. Dynamic Viscoelasticity

Dynamic mechanical analyzer (DMA) is widely used in the study of the dynamic viscoelasticity of materials. During the measurement, the sample will be affected and controlled by the periodically changing mechanical stress. The relationship between the mechanical properties of viscoelastic materials and time, temperature, or frequency is obtained. Dynamic mechanical analysis is of great significance for evaluating the tensile properties, viscosity, and elasticity of wheat dough and can better predict the fermentation stability of dough [65, 66]. Through the study of the dynamic viscoelasticity of grain, the dynamic storage modulus, loss modulus, and loss tangent can be obtained. The experimental results of stress relaxation and frequency scanning of different varieties of maize showed that the varieties had no significant effect on the viscoelasticity of maize kernels [41]. The moisture content affects the stress relaxation behavior of maize kernels to a certain extent. The loss modulus and loss tangent under high moisture content are higher than those under low moisture content. The storage modulus of maize kernels decreased with the increase in moisture content [67, 68]. The experimental results of the creep properties of highland barley kernels show that the creep strain increases with the increase in moisture content. For the dynamic viscoelastic analysis of grains, the generalized Maxwell model can better fit the experimental data of stress relaxation and the curve of relaxation modulus [69, 70]. However, how to combine the viscoelasticity of grains in actual production and how to dynamically analyze the changes in the mechanical behavior of grains under external loads are still important problems that researchers need to solve urgently.

3.4. Other Mechanical Properties

The research on the mechanical behavior of grain is not limited as abovementioned. The friction coefficient of grain varies greatly on the surface of different materials. Generally speaking, the coefficient of static friction on different surfaces is manifested in concrete > galvanized steel > wood [71]. Low surface roughness could greatly reduce the wear of the grain surface and reduce the energy consumption of grain during processing. As an important index to characterize the mechanical properties of grain, hardness plays an important role in the processing and grinding processes. There were many methods for measuring grain hardness, but it was a common method to obtain grain hardness through a puncture test. Hardness is closely related to the grinding performance of the grain, which is of great significance to determine the final processing performance [72, 73].

Although research on the mechanical properties of grain has been greatly developed, there were few studies on the comprehensive consideration of the mechanical properties of grain. Considering the mechanical properties of grains in many aspects, a comprehensive consideration can be made for grain from harvest to final sale, so that the loss of grain in the middle of each link can be minimized, and its economic value can be better maintained.

4. Influencing Factors of Grain Mechanical Properties

4.1. Raw Materials and Geometric Properties

The internal organizational structure of grain is complex, the material composition of each part is different, and the mechanical strength is also different. The structural composition and density of cells are important factors affecting the mechanical behavior of grains [74]. Common grain's mechanical strength is usually expressed as maize > soybean > wheat > rice [75, 76]. The maize kernel is composed of three parts: seed coat, endosperm, and embryo. Compared with other grains, maize has a larger geometric size. The maize kernels have a larger surface flatness than other grains. Therefore, when subjected to external loads, the force required for maize to break is greater than that of other grains. Soybean has a higher oil content and viscosity than other grains, which increases its fracture energy. The shape characteristics of wheat and rice lead to low mechanical strength [40]. In addition to its own shape, the triaxial size also affects the mechanical properties of grains. There is a strong correlation between the thickness and grain hardness of different varieties of wheat, and the change in size will cause a change in wheat hardness. The greater the thickness, the higher the hardness of wheat [15]. The increase in thickness will reduce the flatness of wheat, and similar findings are found in rice. The rupture force of rice is affected by the combined action of three-axis dimensions. The rice particles with a higher flattening ratio and smaller elongation ratio seem to have a greater compression rupture force (flattening ratio: thickness/width; elongation ratio: width/length) [55].

4.2. Grain Endosperm

Endosperm is the main component of grains, and the bonding force between its parts has a great influence on the mechanical properties of endosperm [77]. Endosperm is essentially a complex mixture of starch granules and protein groups. According to their different structures, they can be divided into the horny endosperm and floury endosperm. The horny endosperm contains high amylose content, and the starch granules exist in the form of polyhedral. It is closely combined with the protein group, the internal cavity of the tissue is small, and it has high hardness. The floury endosperm's starch granules are mainly spherical, not closely arranged, and have a large cavity volume inside. Therefore, its hardness is lower than that of the horny endosperm [45]. Grains can be divided into cutin grains and powder grains according to the proportion of the horny endosperm and floury endosperm. Due to the high content of the horny endosperm, cutin grains often have high grain hardness. In dry wheat grains, there was a positive correlation between grain hardness and cutin rate. Wheat with a higher cutin rate had higher hardness [78, 79]. Maize kernels also have similar experimental results. According to the proportion of the horny endosperm and floury endosperm, maize can be divided into flint corn, floury corn, sweet corn, dent corn, popcorn, and so on [80]. The force required for the rupture of maize kernels is proportional to the content of the horny endosperm. Maize kernels with high horny endosperm content tend to have higher mechanical strength [81]. The proportion of horny endosperm of flint corn is higher than that of other kinds of maize, so flint corn has higher damage resistance than other varieties [82].

4.2.1. Protein

Although protein does not account for a high proportion of the total composition of some grains, it also has a certain effect on the mechanical properties of grains. The protein content and composition are different in different endosperms. The contents of total protein and insoluble protein in the horny endosperm were higher than those in the floury endosperm and sorghum grain. The content of α-zein in the horny endosperm was higher than that in the floury endosperm in maize grain [83, 84]. The mechanical properties of grains are also affected by the endosperm structure during the test, and compared with the protein composition, the mechanical properties of grains have a strong correlation with the total protein content [85]. A large number of studies have shown a positive correlation between wheat hardness and protein content. The higher the protein content, the harder the wheat grain [41, 86]. The degree of vitreous affects the hardness of wheat grains. In addition to environmental conditions, vitreous is also affected by the genes that control grain hardness. Vitreous was positively correlated with protein content, so the hardness of wheat grains showed a strong correlation with protein content [87]. This phenomenon that protein content is affected by gene regulation and ultimately affects the mechanical properties of grains has similar experimental results to maize grains. The higher kernel hardness of flint corn is also related to its protein content

[88]. The microstructure of the grain fracture form verified the contribution of high protein content to grain hardness. The microstructure of the broken grains was characterized by the fracture of the connection between the starch granules and the protein matrix or the fracture of the protein matrix or the fracture along the boundary of the starch granules [45]. This fracture pattern indicates that the thicker the protein matrix covering the surface of the starch granules is, the less likely it is to break.

4.2.2. Starch

Similar to protein, starch, as one of the main components of cereal endosperm, affects the mechanical properties of cereals by affecting the hardness of the endosperm. According to the different molecular structures, starch can be divided into amylose and amylopectin. Although both of them are polymers of glucose, their physical and chemical properties are quite different. Previous studies have shown that the content of amylose is positively correlated with the hardness of maize endosperm. The higher the amylose content, the higher the hardness of the endosperm [89, 90]. The shape and size of starch granules in different cereals were different. The starch granules of wheat, rye, and barley were spherical and discoid. In addition to the starch structure, the morphology of starch granules has also been proven to be related to the mechanical properties of cereals [82, 91]. The starch granule morphology of the maize horny endosperm is different from that of the floury endosperm. The starch granules in the floury endosperm are mostly spherical and loosely packed in the protein matrix. The starch granules in the horny endosperm showed a dense accumulation of polyhedral. The starch granules in the corn horny endosperm bind more tightly than the starch granules in the floury endosperm after being squeezed by the protein matrix. This compactness increases the density of the endosperm and thus the firmness of the endosperm. The observation results of the microstructure of maize endosperm also confirmed this conclusion. The starch granules in the cuticle endosperm were more tightly bound to the protein matrix than those in the silty endosperm, so the hardness of the horny endosperm was higher than that of the floury endosperm [92]. There was a positive correlation between amylose content and grain rupture force in grains [93]. However, some researchers have shown that the amylose/starch ratio is more representative than the amylose content itself in explaining the effect of starch composition on grain hardness [94]. This has a very important guiding significance for evaluating the change in the mechanical behavior of the grain grinding process.

4.3. Moisture Content

The difference in water content will not only affect the basic physical properties of grain and the safety of grain storage but also affect the mechanical properties of grain and the damage resistance [9, 95]. Studies have shown that under conditions of high moisture content, the mechanical strength of grains is low, and they are more likely to deform or even rupture when subjected to external forces. The compression test results of broad bean under different water content and compression directions show that the rupture force of any axis along the three axes is highly dependent on the water content. There is a significant negative correlation between water content and rupture force [96]. This is because, under conditions of high moisture content, the endosperm cells in the grain will absorb water and fill the internal voids, making the grain structure expand. This not only affects the physical properties of grains, such as 1000-grain weight, bulk density, and true density, but also makes the endosperm texture softer, which in turn reduces the mechanical strength of the grain [97]. The microstructure of oats was different under different water contents. When the water content was low, the starch granules in oat grains were closely arranged. At this time, the grains have higher mechanical strength. With the increase in water content, starch and protein in grains will swell to different degrees. Due to the presence of -NH_3 and -COOH groups in the protein, its water absorption capacity is stronger than that of starch granules. Therefore, when the moisture content of the grains increases, the internal starch-protein network system becomes irregular, and this irregular starch-protein system weakens the mechanical properties of the grains [98]. Therefore, the grains should be dried in time after harvest. Reducing the moisture content can not only prolong the storage time and reduce the harm of microorganisms in the storage process but also prevent mildew [99]. Higher grain mechanical strength under low moisture content is of great significance for reducing the damage of grain during processing..

4.4. Other Influencing Factors

In addition to the differences in the grain itself, external conditions such as storage and processing methods will also

affect the mechanical properties of the grain. In order to ensure the safety of stored grain, it is often necessary to reduce the moisture content by drying before the grain is put into storage. However, drying temperature, drying time, and drying method also have an impact on the mechanical properties of grains. This is mainly due to the influence of different drying processes on the internal stress cracks of grains [100]. Different drying conditions will cause changes in the internal stress of grains, which will affect the mechanical properties such as fracture force and fracture energy [101]. High-temperature drying can make rice obtain higher bending strength and fracture energy and make the grains stronger and tougher, while thelower-temperature drying conditions will reduce the bending strength of rice grains and have a certain impact on the yield [16]. In addition, the mechanical behavior of grains will also change during the storage process. The storage time and pressure will also affect the mechanical properties of rice grains. The mechanical properties of rice grains are linearly related to the storage pressure. The compressive capacity decreases with the increase of storage pressure, and the length of storage time will also affect the damage resistance of grains [32]. The reason for this phenomenon is due to the grain after-ripening phenomenon under long-term storage conditions. In this process, the contents of protein, starch, and free fatty acids in grains will change, which leads to the change in grain mechanical behavior. In addition, during the storage process, the grains are squeezed by the surrounding grains, and the closer they are to the bottom of the granary, the greater the extrusion pressure. Compared with the compression of a single grain, the mechanical properties of stacked maize grains are different from those of a single grain [39]. Therefore, it is of great significance to analyze the mechanical properties of grains by using a triaxial test to explore the change of stress in grain piles under pressure.

5. Conclusion and Prospect

So far, the research on the mechanical properties of grain still needs further development. The mechanical properties of grain have a great influence on its processing quality. Although there are various research methods on the mechanical properties of grain, the mechanical properties of grain under different conditions are simulated by shear, compression, bending, and other experimental methods, and the mechanical indexes such as rupture force, rupture energy, and elastic modulus of grain are obtained. However, the relationship between the mechanical properties of grain and its physical and chemical properties and even the unified mechanical determination method of grain has not been standardized. Most research on the mechanical properties of grain is limited to the static force change, while the research on the mechanical properties of grain in the process of circulation is still limited. How to apply the experimental mechanical indexes to solve practical problems, reduce the loss in the process of grain processing, and reduce mechanical damage is an urgent problem to be solved. At the same time, the systematic discussion of many factors affecting the mechanical properties of grains and the use of various experimental methods are of great significance for reducing the grain breakage rate and improving the grain utilization rate.

Authors' Contributions

Peng Gao conceptualised the study, developed the methodology, wrote the original draft, and validated the study. Shuangqi Tian administered the project and wrote, reviewed, and edited the study. Xing'ao Xue investigated and supervised the study. Jing Lu supervised and wrote, reviewed, and edited the study. All the authors have read and agreed to the published version of the manuscript.

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DETAIL

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A Comprehensive Narrative Review on the Hazards of Bee Honey Adulteration and Contamination

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ABSTRAK (ENGLISH)

Honey bees are renowned for producing a remarkable substance known as bee honey, which stands as a functional food celebrated for its numerous health benefits. This natural wonder possesses a spectrum of advantageous properties, including anti-inflammatory, antioxidant, analgesic, antibacterial, and wound-healing qualities. However, in our modern era of heightened utilization of bee products, a new and pressing global health concern has emerged—the contamination of honey with pesticides, antibiotics, microorganisms, and heavy metals. The consumption of beekeeping products containing pesticide residues has been linked to a range of health issues, including genetic malformations, cellular degradation, allergic reactions, and even potential carcinogenic effects. Troublingly, documented cases exist of botulism in newborns resulting from the ingestion of contaminated honey. Additionally, the use of antibiotics in beekeeping practices has been associated with the concerning emergence of antibiotic resistance. This comprehensive review sheds light on the substantial consequences of honey contamination for human health. It underscores the urgent need for the establishment of a rigorous monitoring system, the validation of minimum acceptable pollutant levels, and, at the very least, the regulation of maximum residue limits for bee products, with a particular emphasis on bee honey.

TEKS LENGKAP

1. Introduction

Functional foods offer the potential for substantial health benefits, serving not only as a source of essential nutrients but also as a preventive measure or therapeutic intervention for specific medical conditions. Functional foods may have positive health benefits because they contain bioactive components with specific biological substance properties [1]. Even though the functional food business is one of the fastest-growing industries in Europe, those foods are not specifically regulated in the region [2].

Honey is naturally produced by bees (*Apis mellifera* L.), and it is described as a sweet substance derived from plant

nectar or secretions of living plant components that have been dried. It has long been recognized as food with functional qualities that have been demonstrated through studies and clinical trials to promote a healthy lifestyle [1, 3].

Since there is a substantial honey trade among the European Union (EU) nations, a special regulation that addresses the unique properties of honey is in place to govern the quality of honey. Therefore, honey from EU countries or sold on its territories must meet certain specific compositional requirements related to its carbohydrate content, humidity, acidity, electrical conductivity, diastase activity, and hydroxymethylfurfural content (HMF) to protect the authenticity, safety, and quality of honey [4].

Unfortunately, throughout production, honey can be falsified, for instance, by adding glucose solutions, sucrose syrups, corn syrup, high fructose corn syrup, inverted sugar syrup, or sugar cane juice, or by deploying improper beekeeping techniques [4–6]. Anthropogenic contaminants from the environment (e.g., metal traces, polychlorobiphenyls, and pesticides from doused or rotating crops), as well as improper beekeeper's management, can contaminate honey (e.g., medicines and insecticides used to treat the colony) [4].

As a result, the consumption of honey raises concerns about the health of the population and the safety and quality of the product in terms of the number of contaminants it contains, especially harmful trace elements [4].

Consumers can obtain valuable information from the conclusions of the analysis of the extraordinary therapeutic properties of bee honey and the risk to which they are exposed following the consumption of contaminated or adulterated honey [4].

Given the rise in interest in honey's elemental analysis over the past few years, it is unsurprising that honey and its quality are the subjects of several research publications. This review highlights the importance of analyzing honey's quality and identifies possible contamination causes.

2. Strategies and Methods Used to Collect Data from the Literature

The data presented in this review were obtained through literature analysis (MEDLINE, PubMed, Google Scholar, OMIM, and MedGen databases) using the following keywords: honey adulteration, honey contamination, pesticides, insecticides, antibiotics, xenobiotics, microorganism, pathogens, heavy metals, or *Apis mellifera*.

3. Honey: A Functional Food

3.1. Composition of Honey

Honey is a nutrient-rich, liquid food. Its composition includes fructose and glucose (≅80%), water (≅16%), ash (0.2%), and amino acids (<0.1%), with trace amounts of enzymes, antioxidants, vitamins (e.g., B_1, B_2, B_3, B_5, B_6, B_9, C, and K), phenolic compounds, minerals (e.g., Na, Ca, K, Mg, P, Se, Cu, Fe, Mn, Cr, and Zn), and other chemicals compounds [7]. The botanical and geographic sources of the product significantly influence its chemical composition and health benefits [8].

Honey contains flavonoids from nectar, pollen, and plant resins collected by honey bees. Flavonoids and phenolic acid are the main polyphenolic components of honey, and they are responsible for preventing oxidation due to their capacity to decrease free radical production and scavenge radicals. Also, flavonoids significantly improve honey products' antibacterial and antifungal value, being responsible for the anti-inflammatory, antioxidant, and antimutagenic effects. Bee salivary enzymes (e.g., glucose oxidase) and peptides (e.g., defensin-1) also contribute to the antibacterial properties of honey, and they ensure its microbiological stability [1, 8, 9]. Despite the complexity of honey's chemical composition, hydrogen peroxide remains a crucial compound responsible for its antimicrobial action [10, 11].

3.2. Health Benefits

Honey was the first sweetener used by humans. Ancient civilizations universally acknowledged several advantages of frequent honey intake, such as treating cardiovascular and gastrointestinal illnesses and pleasant organoleptic characteristics of honey [12–15]. As a result, honey has been established as one of the most essential treatments in natural medicine [1].

Bee honey's medical value has been studied in both humans and animals. Based on the *in vivo* results on rats, those who consumed altered honey underwent significant changes, including weight growth and increased levels of

circulating glucose, triglycerides, and cholesterol. Over time, that could lead to liver and renal disease [16, 17].
Bee honey has been used for centuries to treat colds, sore throats, and coughs due to its anti-inflammatory and immune-boosting characteristics. Specialized literature places a strong emphasis on honey's anti-inflammatory [18], antioxidant [19], and anticarcinogenic capabilities against breast and colon cancer [20]. Either oral ingestion or topical use of honey can provide a therapeutic effect on human health. When consumed orally, linden honey relieves fevers and stomach pains and prevents migraines, whereas lavender honey treats coughs and sore throats; acacia honey is an excellent sedative and tonic; mint honey is a great analgesic, antihemorrhagic, and tonic; wildflower honey has a strong antibacterial effect; fir honey and sunflower honey are helpful in respiratory disorders for fluidizing bronchial secretions; mountain honey provides potential advantages in allergies and pulmonary diseases [21]. Honey is excellent for healing nocturnal cough brought on by an upper respiratory tract infection by reducing its frequency and intensity and improving both children's and parents' sleep quality [9]. But even so, due to the newborns' weakened immunity against *Clostridium botulinum*, a potential honey contaminant, the consumption of honey in infants under 12 months is banned. Complex natural substances represent an innovative approach to cough management. They create a film that covers the oropharynx, thus acting as a mucoadhesive physical barrier instead of suppressing cough by engaging with specific receptors [22]. When consumed orally, Manuka honey has been shown to have a range of health benefits, including soothing sore throats, aiding in digestion, and boosting the immune system. Additionally, Manuka honey has been found to have anti-inflammatory properties, making it a potential treatment option for conditions such as inflammatory bowel disease and arthritis. When applied topically, Manuka honey has been shown to have wound-healing properties, making it a popular choice for treating burns, cuts, and other skin conditions [23, 24].
Bee honey is formulated in syrups in combination with extracts of certain plants such as *Grindelia robusta*, *Plantago lanceolata*, and *Helichrysum italicum*, which have protective, demulcent, anti-inflammatory, and adjuvant cytoprotective properties [9].
At the same time, the benefits of topical therapy with honey have been noticed in the treatment of athlete's foot, vaginal lesions, burns, lip injuries, and eczema [3].
Due to its anti-inflammatory and antioxidant properties, honey benefits metabolic syndrome. Six months of daily honey consumption of 15 g was associated with weight loss, improved lipid metabolism, and reduced levels of triglycerides and cholesterol [1, 23].
Furthermore, it has been suggested that honey can relieve digestive issues such as gastroenteritis-related diarrhea. Due to its bactericidal properties (reduced colonization of Enterobacteriaceae and improved colonization with probiotic bacteria (*Bifidobacterium* and *Lactobacillus*)), orally ingested honey appears to be a proper adjuvant therapy in acute diarrhea in children and adults, reducing the duration of diarrhea episodes [1].

3.3. Potential Health Risks of Consuming Honey

Currently, there are multiple honey varieties, either monofloral or polyfloral. Among the types of monofloral honey, we mention acacia honey from *Robinia pseudoacacia* L., chestnut honey from *Castanea sativa* Mill., clover honey from *Trifolium pratense* L., dandelion honey from *Taraxacum officinale*, eucalyptus from *Eucalyptus* spp., lavender honey from *Lavandula angustifolia* Mill., linden tree honey from *Tilia cordata* Mill., orange honey from *Citrus* spp., pine honey from *Pinus* spp., raspberry honey from *Rubus idaeus* L., rhododendron honey from *Rhododendron* spp., rosemary honey from *Rosmarinus officinalis* L., strawberry tree honey from *Arbutus unedo* L., sunflower honey from *Helianthus annuus* L., thyme honey from *Thymus* spp., etc. [25].
Regarding the bee species used by beekeepers, only two of the *Apis* species (*A. mellifera* and *Apis cerana*) are used for commercial reasons. That is a consequence of the characteristics of some species, such as giant and dwarf bees, which prefer to nest outside and cannot be housed in artificial hives. The species *A. cerana* is more productive in bee honey production than other species [18].
Flowers are the bee's main sources of nutrition. Traces of unsuitable metals and insecticides can be identified on their surface, which the bees subsequently introduce into the hive through nectar and pollen they collect and transport on their bodies. Contaminants are concentrated during the maturation process of honey (enzymatic

conversion of sucrose and reduction of water). Due to pest control treatments of hives or through the transfer of contaminated beeswax, veterinary medicinal drugs (such as antibiotics banned in beekeeping or legal acaricides) can be found in honey [26–30]. At the same time, honey must not contain traces of metals or the heavy metal content should be minimal, as defined by EU food regulations [8, 31–33].

Among the samples of bee honey monitored annually by the EU, cadmium (Cd) is the residue that was detected in the highest concentration, followed by lead (Pb), copper (Cu), numerous antibiotics, organophosphates organochlorines, and other pesticides [8].

By implementing ecological agriculture standards, employing appropriate beekeeping techniques, keeping the hives far enough from potential sources of contamination, and limiting treatment against mite pests, honey residues can be kept to a minimum [8].

4. Honey Adulteration

One of Europe's top ten most adulterated food products is honey, which is also placed third on the list of food fraud victims in the United States Pharmacopoeia food fraud database, after milk and olive oil. Adulteration is incorporating foreign substances into food, often done to increase quantity at the expense of quality [34–36]. Honey falsification is the strategy of decreasing manufacturing costs while increasing profits. Adulterated honey leads to economic effects by decreasing its price [37].

Bee honey can be altered by using store-bought syrups and inexpensive sweeteners. Cane sugar, beet sugar, glucose syrup, fructose syrup, corn syrup, inverted syrup, and high fructose inulin syrup are the most widely recognized adulterants. Figure 1 provides an overview of the chemical structures of some frequently reported sugar adulterants.

[figure(s) omitted; refer to PDF]

Adding those sweeteners affects bee honey's chemical and biochemical activity, including its enzymatic activity, electrical conductivity, and special component concentration [38, 39]. It has been noticed that the choice of adulterants relies on the geographic area, the financial benefits, and the accessibility of acquiring them. For instance, producers from Turkey and France use wheat and rice syrup as adulterants, unlike other EU producers who adulterate honey with high fructose inulin syrup [39, 40].

There are three types of honey adulteration: direct, indirect, and blending. Direct adulteration is a postproduction process that involves adding sweeteners to honey in varying amounts (7%, 15%, and 30%) to raise sweetness. Indirect adulteration entails overfeeding bees with pesticides and synthetic sweeteners to extract additional honey from the hives. Another method of altering honey is blending, which is achieved by diluting pure, high-quality honey with cheaper, low-quality honey [3].

The adulteration process reduces the antibacterial effects of pure honey and increases blood glucose levels, followed by insulin release, abdominal weight, and blood cholesterol levels [41, 42]. In addition to producing H_2O_2 and fructose, insulin also causes a rise in uric acid by activating the plasma membrane enzyme system with NADPH-oxidase characteristics. Atherosclerosis, diabetes, obesity, high blood pressure, coronary heart disease, and even heart failure are chronic diseases that are brought on by the simultaneous production of reactive oxygen species by glucose and fructose from sugar [39, 43].

Notwithstanding, it might be challenging to identify those kinds of adulterants because some studies have found sugar or syrup residue quantities that are equal to those found in pure honey [44]. The authenticity of the honey samples was assessed using multiple analyses: principal component analysis (PCA) and linear discriminant analysis (LDA) for physicochemical and rheological examination, high-performance liquid chromatography (HPLC), gas chromatography with mass spectrometry (GC-MS), micellar electrokinetic capillary chromatography (MEKC), and voltammetry [45–47]. Isotope ratio mass spectrometry (IRMS) is the most commonly employed approach for honey analysis, although nuclear magnetic resonance (NMR) is more reliable because it involves less sample preparation and delivers results faster. It is useful to carry out a 1H NMR investigation with statistical analysis to distinguish honey from various botanical and geographic origins. The NMR approach includes magnetic resonance to identify the molecule's structure [37].

5. Honey Contamination

Honey contamination refers to foreign substances or contaminants that are not naturally part of the honey or are present in quantities exceeding acceptable limits. Honey can become contaminated at various stages, including during production, processing, transportation, and storage. Contaminants can come from environmental sources, beekeeping practices, or external factors. Contamination of honey can negatively affect human health, mainly if it involves harmful substances. Adulteration or contamination of honey with other substances may trigger allergic reactions in sensitive individuals. Also, long-term consumption of contaminated honey can pose health risks and cause acute or chronic health issues [48–51].

Honey and other bee products are contaminated by a wide range of pollutants, including pesticides, heavy metals, pathogens, and radioactive elements, as represented in Figure 2 and Table 1 [42, 49, 51, 52].

[figure(s) omitted; refer to PDF]

Table 1

Bibliographical references corresponding to Figure 2 referring to the main sources of bee honey contamination.

Contaminants	References
Pesticides	[8, 33, 42, 52–54]
Antibiotics	[42, 52, 53, 55–57]
Microorganisms	[22, 42, 52, 53, 58, 59]
Heavy metals	[6, 42, 52, 60–62]

According to EU legislation, honey must not contain chemicals because it is a natural product. Insecticide usage harms wildlife by reducing the number of bees, decreasing honey quantity, damaging plant ecosystems, and generating pesticide residues in food [53].

5.1. Pesticides

One of the most significant threats originating from honey contamination is the presence of pesticide residues. Bees, as pollinators, may inadvertently collect nectar from plants exposed to pesticides, transferring those harmful chemicals into the honey they produce. Long-term consumption of pesticide-contaminated honey may lead to a range of health issues, including neurological disorders, hormonal imbalances, and an increased risk of certain cancers. As those pesticides accumulate in the human body over time, their effects can be insidious, emphasizing the urgency of monitoring and controlling pesticide use in beekeeping practices [27, 31, 33, 53, 54].

To avoid bee diseases and pests, pesticides are employed worldwide; for the most part, their application is unregulated and performed without following recognized procedures. Their use is supposed to protect crops and boost agricultural output [53]. Nevertheless, uncontrollable applications can contaminate humans, animals, and the environment. Acaricides, organic acids, insecticides, fungicides, herbicides, and bactericides are a few pesticide residues that may have a carcinogenic effect on individuals. The contamination of honey and other hive products is a danger associated with using pesticides within hives [53].

Pesticides can be potentially hazardous to individuals depending on the chemical's toxicity, the length of exposure, and the severity of the effects. Chemical compounds can bioaccumulate, and their effects may amplify in the body, generating bioconcentrations. Due to their underdevelopment and small size, children are most susceptible to pesticide contamination. Pesticide exposure can cause issues ranging from a minor skin rash to congenital malformations, neoplasm, genetic mutations, hematological disorders, and sometimes even coma or death. The endocrine, reproductive, and immune systems can be harmed by many persistent organic pollutants (POPs), including aldrin, dihedral, heptachlor, chlordane, and hexachlorobenzene. POPs are prohibited due to the severe

complications they cause after chronic exposure, though some are still in use [53, 63].
Varroacides accumulate in beeswax and pollen and are the main sources of pesticide residues. Maximum residue limits (MRLs) have been established at levels expressed as parts per billion for several pollutants [52]. Various national authorities have set MRLs in honey, but the absence of consistent regulation affects international marketing and commerce [53]. For instance, the MRLs for amitraz, bromopropylate, coumaphos, cyamizole, flumethrin, and fluvalinate established by Switzerland, Germany, and Italy differ from those set by the EU, which regulated MRLs for amitraz ($0.2\,mg{\cdot}kg^{-1}$), coumaphos ($0.1\,mg{\cdot}kg^{-1}$), and cyamizole ($1\,mg{\cdot}kg^{-1}$). On the other hand, the US Environmental Protection Agency has implemented MRLs for amitraz ($1\,mg{\cdot}kg^{-1}$), coumaphos ($0.1\,mg{\cdot}kg^{-1}$), and fluvalinate ($0.05\,mg{\cdot}kg^{-1}$) [53].
The most popular extraction and purification approach used to detect honey pesticides is liquid-liquid extraction (LLE). Nevertheless, LLE requires a lot of sample handling steps, big sample volumes, and hazardous organic solvents. Moreover, it often allows the extraction of analytes from a single chemical class. Notwithstanding the drawbacks mentioned above, LLE is still used to investigate pesticides in honey. Organochlorine pesticides are extracted from honey using ethyl acetate, acetonitrile, and methanol as organic solvents. Another innovation in that direction was using low-temperature liquid-liquid extraction (LLE-LTP) in detecting deltamethrin and cypermethrin in dairy. Studies employ that technique to extract chlorpyrifos, cypermethrin, deltamethrin, and k-cyhalothrin from honey [54].
Solid phase extraction (SPE) is used to retain certain analytes on absorbents and subsequent elution of those analytes with the appropriate solvents. That technique combines the extraction and cleanup operations into a single process, resulting in clean extracts that can be analyzed rapidly through GC or LC. That method is attractive for detecting pesticides in bee honey because of its simplicity, precision, and minimal solvent usage [54].
The principle behind magnetic solid phase extraction (MSPE) is using magnetic or magnetizable adsorbents. MSPE extracts the analyte by introducing a magnetic adsorbent to a suspension or solution. The adsorbed analyte is then recovered using a suitable magnetic separator. Magnetic nanoparticles are frequently utilized as adsorbents in MSPE because, compared to conventional SPE adsorbents, they have a bigger surface area and distinct magnetic characteristics [54].
Gas chromatography is an effective method for assessing contaminant levels in complex matrices. It is frequently used to analyze pesticides in honey, in conjunction with several detection methods, including MS, MS/MS, nitrogen phosphorus detector (NPD), electron capture detector (ECD), atomic emission detector (AED), and flame photometric detector (FPD). Mass spectrometry is the most efficient pesticide detection method because it offers structural details that provide exhaustive confirmation, which is necessary for a multi-residue investigation [54].
For pesticide identification in honey, liquid chromatography (LC) is frequently employed, especially for thermally labile chemicals. Pesticides can be detected in complex matrices in low quantities using LC-MS. That system adds structural information, enhances sensitivity, and lessens matrix interference. In recent years, LC-MS/MS methods have been successfully used to determine if residues are present in honey [54].
To identify several classes of pesticides (pesticides, biopesticides, and other veterinary drugs) in honey, ultra-high-performance liquid chromatography (UHPLC-MS/MS) can be performed [54].

5.2. Antibiotics

In some cases, beekeepers use antibiotics to treat diseases in bee colonies, inadvertently contaminating the honey [53, 56]. The consumption of honey contaminated with antibiotic residues poses a concerning challenge to public health: the emergence of antibiotic-resistant bacteria [30, 56]. Regular intake of antibiotics through contaminated honey can contribute to the development of antibiotic-resistant strains, rendering those vital medications less effective in treating bacterial infections in humans [30, 53, 55, 56, 64]. The widespread use of antibiotics leads to an accumulation of antibiotic residues in honey, thus leading to decreased quality and challenging marketing [53, 65, 66]. Skin rashes, dermatitis, gastrointestinal symptoms, and anaphylaxis are side effects of lactam antibiotics, even in the relatively low levels that may be found in honey [64, 65]. Also, individuals are more susceptible to developing cancer after exposure to nitrofurans and nitroimidazoles [67, 68].

The *Paenibacillus (Bacillus) larvae* and *Melissococcus plutonius* bacteria that cause European and American foulbrood in honeybees are typically treated with oxytetracycline [66]. Tetracycline resistance has been reported in those bacteria, widely believed to result from its widespread usage [69].

Streptomycin, erythromycin, lincomycin, sulfonamide, and chloramphenicol are other antibiotics beekeepers use [57, 70–72].

An aminoglycoside called streptomycin is utilized in several countries, especially in Central and South America, for treating both American foulbrood (caused by *P. larvae*) and European foulbrood (caused by *Melissococcus plutonius*) [66]. Streptomycin residues were detected in those areas and in various dietary items such as milk, viscera, and meat [69, 73]. Streptomycin is currently prohibited in many countries due to its increased toxicity, even though it is frequently recommended in bee forums and beekeeping guides [57, 73].

Erythromycin is a prevalent chemotherapeutic antibacterial drug used in human and veterinary medicine, along with clarithromycin and azithromycin, which is recognized for its prolonged effects. Due to the allergic responses that macrolide residues might produce because of their metabolites, consumers have an elevated risk. Another macrolide, tylosin, is used by beekeepers worldwide to treat American foulbrood, but it is not recommended as a preventative measure in healthy colonies. Tylosin usage must be stopped at least 4 weeks before honey is extracted [30, 53, 55].

Sulfonamides are among the most frequently utilized medications in veterinary medicine due to their low cost, extended antibacterial spectrum, and acknowledged therapeutic efficacy in various infectious diseases. Sulfonamides are frequently used in beekeeping to treat nosemosis and European and American foulbrood [55]. Consuming honey with sulfonamide residues might be dangerous for human health. Anaphylaxis, urticaria, and pruritus are adverse responses to food sulfonamide residues. Studies on rats revealed that the sulfamethazine in honey induces tumors with various localizations, and it is highly toxic to the thyroid gland. The most frequently utilized sulfonamides in veterinary medicine, including beekeeping, are sulfadimethoxine, sulfamethoxazole, sulfaquinoxaline, and sulfadiazines. Honey can get contaminated with those residues if the period between treatments of the bees and honey extraction is not recommended [74]. Sulfonamides have a moderate toxicological profile, which includes gastrointestinal problems (nausea, vomiting, and diarrhea), hypersensitivity (allergic) reactions that include skin rashes, eosinophilia, rarely anaphylactic shock, and the possibility of developing hemolytic anemia in individuals who have a genetic deficiency in glucose-6-phosphate dehydrogenase. Sulfamides should not be given to infants or pregnant women in the third trimester since they compete with bilirubin for the same binding sites. Unbound bilirubin can accumulate in the subthalamus and basal ganglia of the brain, leading to toxic encephalopathy.

Moreover, sulfonamides have been linked to liver toxicity (including necrosis), drug fever, serum sickness, and systemic lupus erythematosus (type III hypersensitivity mediated by immunoglobulin G) [67, 72, 75, 76]. In addition to dermatological toxicity, sulfonamides can also cause severe allergic responses and hepatic and hematological destruction. Sulfonamides can cause a variety of allergic reactions (hypersensitivity), ranging from modest skin rashes to severe or occasionally life-threatening reactions, including erythema multiforme, Stevens-Johnson syndrome, and toxic epidermal necrolysis [77].

Quinolones are a group of synthetic antimicrobial medications that treat various infectious disorders caused by bacteria in both human and veterinary medicine [57]. They take effect by decreasing the activity of the enzymes DNA gyrase and topoisomerase IV. The determination of residues in honey demonstrated the presence of enrofloxacin, ciprofloxacin, and norfloxacin. Because they can trigger allergic reactions or the development of drug resistance in humans, quinolones used in veterinary medicine may pose a risk to human health [65, 71, 78].

Cephalosporins are a subclass of beta-lactam antibiotics that beekeepers frequently use to treat Gram-negative bacteria. Ceftiofur, a third-generation cephalosporin exclusively utilized in veterinary medicine, has a bactericidal action by inhibiting the growth of bacteria’s cell walls. Even though it has been recommended not to be used in beekeeping in recent decades, it can still be identified in honey [53, 55, 68].

Tetracycline, oxytetracycline, dimethyl-chlortetracycline, doxycycline, minocycline, and Vibramycin are standard

antibacterial chemotherapeutics used in veterinary medicine due to their broad bacteriostatic spectrum. Research has demonstrated that utilizing tetracycline in powder form leads to smaller residues than in liquid form. The FDA in the United States has authorized a variety of drugs, including oxytetracycline, to be utilized in beekeeping. But, at least six weeks before honey is extracted, its administration should be stopped [56, 65, 69].

The EU legislation has not yet established a minimum performance limit for antibiotics except for chloramphenicol ($0.3\,\mu g \cdot kg^{-1}$). Additionally, Belgium has imposed restricted levels for several antibiotics in honey, including tetracyclines and the total amount of sulfonamides ($20\,\mu g \cdot kg^{-1}$). The United Kingdom has regulated the total amount of sulfonamides as low as $50\,\mu g \cdot kg^{-1}$, Switzerland has set it as low as $20\,\mu g \cdot kg^{-1}$, and France has set it as low as $15\,\mu g \cdot kg^{-1}$. Italy established a detection limit of approx. $1.5\,\mu g \cdot kg^{-1}$ for aminoglycosides and $5\,\mu g \cdot kg^{-1}$ for tetracyclines, sulfonamides, and macrolides [30].

The most widely used techniques for identifying antibiotics in bee honey involve screening tests (microbiological and immunological enzyme testing) and confirmatory approaches (mass spectrometric detection and chromatographic methods). Rapid tests are generally quick and cheap, but they can potentially provide false positive results. The Biochip immunochemical method has lately been compared to the LC-MS/MS method, and the results show selectivity and accuracy for quantifying drug residues in honey at very low levels [70]. Due to the uncontrolled usage of drugs, those analysis results can contribute to a greater understanding of environmental concerns and should impose preventative measures [30].

5.3. Microorganism

Bacteria, molds, and yeasts can be found in honey and honeycomb and come from bees, nectar, or other external sources such as dust, pollen, humans, tools, containers, equipment, and wind [53].

The first source of the microorganisms located in the bees' intestines might have originated from pollen. Bee's intestinal microbiota contains 27% Gram-positive bacteria (including *Bacillus, Bacteridium, Clostridium,* and *Streptococcus* spp.), 70% Gram-negative bacteria (including *Citrobacter, Enterobacter, Escherichia coli, Klebsiella, Proteus,* and *Pseudomonas*), and 1% yeast [53, 59].

Due to its antibacterial properties, most bacteria and microorganisms will not grow or reproduce in honey. Furthermore, honey has a relatively small amount of water, which limits bacterial development and survival. Honey has not been associated with many infections; thus, the high amount of vegetative bacteria may only result from recent contamination [64].

Many countries have reported finding *Clostridium botulinum* type F spores in various honey product containers. Nevertheless, there was no distinction between contaminated honey and sterilized honey regarding pH, HMF concentration, or diastase activity. *Bacillus alvei* may have influenced the growth of *C. botulinum* in honey because it stimulated the production of toxins by *C. botulinum* type F [53, 64, 79].

In countries like Argentina, 1.12% of samples from rural producers were found to be contaminated with *C. botulinum* type A, while 7% of samples from producers in Brazil were found to be contaminated with *C. botulinum* type A, B, and D. In the United States, 10% of samples are contaminated with the same bacteria. In Japan, 5% of samples were contaminated with *C. botulinum*, while in Finland, 8% of samples from local sources and 12% from imported honey were contaminated similarly [53, 64].

Ingestion of *C. botulinum* spores has been related to infantile botulism, the most prevalent type of disease in children. Spores ingested by newborns and children proliferate in the digestive tract and release botulinum toxin. In 15% of infant botulism cases reported to the Centers for Disease Control and Prevention, honey consumption was associated. Gamma radiation is an effective way to sterilize honey used in therapeutic practice to prevent the growth of botulinum spores or other potential contaminants. Gamma radiation does not affect honey's antibacterial properties [53, 64, 79].

Candida parapsilosis, *Rhodotorula mucilaginosa, Meyerozyma caribbica*, *Occultifur* aff. *externus*, *Vishniacozyma victoriae,* and *Aureobasidium* sp. are a few yeast species related to *A. mellifera* that have been identified in the United States and Brazilian environments. *Aureobasidium* sp. was the most prevalent on the body surface of nurse bees and forager bees in Brazil. *Aureobasidium pullulans* is a ubiquitous species that lives on the surface of fresh

fruit, in water, and soil. *Rhodotorula* sp. and *Candida* sp. are frequently identified on leaves, flowers, and fruit. The genus *Candida* was linked to bees in the form of 4 species*: C. hawaiiana*, *C. oleophila*, *C. parapsilosis*, and *C. orthopsilosis*, the last being the most predominantly reported. The ubiquitous yeast *Rhodotorula mucilaginosa* has been found in various natural habitats, including flower pollen, nectar, decaying plant material, insects, and stingless bees [59].

The conventional approaches to identifying microorganisms isolated from food are focused on phenotypic techniques, including observing the morphology and growth of microbes on media, Gram staining of cells, carrying out catalase and oxidase tests, microscopically evaluating cell morphology and the type of hemolysis, and carrying out biochemical tests such as tests for indole, urease, or aminopeptidase. However, those procedures need a spectrometer operator to analyze data, which is time-consuming and highly laborious [72].

Other methods for identifying microorganisms in bee honey include immunological tests (antibody-based assays) such as enzyme-linked immunosorbent assay (ELISA) and molecular methods together with bioinformatic tools such as polymerase chain reaction (PCR), polymerase chain reaction based on repetitive sequences (rep-PCR), random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), DNA fingerprinting techniques, intron splice site priming, 16S rRNA gene sequencing, PCR in combination with sequencing, real-time PCR (quantitative PCR, qPCR), denaturing gradient gel electrophoresis (DGGE), PCR-DGGE fingerprinting, time-temperature gradient gel electrophoresis (TTGE), temperature gradient gel electrophoresis PCR (PCR-TGGE), multilocus sequence analysis (MLSA), Fourier transform infrared spectroscopy (FT-IR), and pyrolysis mass spectrometry (PyMS). Although those techniques provide accurate identification, most are complex, laborious, expensive, and time-consuming. Because of that, it is challenging to perform routine analysis utilizing any of those techniques [80].

To identify microorganisms, it is necessary to develop new techniques that are fast, accurate, reliable, highly specific, uniform for the analysis of various microorganism groups, accessible, and simple to use [80].

In that sense, proteomic methods appear to be advantageous. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is a chemotaxonomic technique that involves the analysis of ribosomal proteins characteristic of a certain family, genus, species, or even a strain of microorganism [26, 80, 81]. Due to its simplicity of use, extremely high sensitivity, accuracy, reproducibility, and low cost, the MALDI-TOF method is becoming more and more attractive for use in microbiological diagnostics [80, 82, 83]. Using MALDI-TOF MS and 16S rDNA, comparative investigations were conducted to identify the microorganisms present in several varieties of honey from various geographical and botanical sources (such as honeydew honey, multiflower honey, and sunflower honey) [58, 80, 82]. *Bacillus* spp., *Micrococcus* sp., *Staphylococcus* spp., and *Lysinibacillus* spp. in honey samples were all confirmed by the results of both study methods. The MALDI-TOF method, in contrast to the 16S rDNA methodology, enabled a clear differentiation between species such as *B. subtilis* and *Bacillus cereus*, a significant discovery for study because those two species are the most frequently discovered in honey [80].

The presence of those species in bee substrates suggests that bees serve as vectors, spreading the yeast throughout the environment and carrying it into the substrates of the colony [59].

5.4. Heavy Metals

Heavy metals in honey refer to the presence of metallic elements with high atomic weights that exceed the acceptable limits in the honey. Those metals can contaminate honey through various sources, including polluted soil, water, air, and industrial activities. The magnitude of honey contamination with heavy metals is substantially associated with the degree of environmental pollution, as shown in Figure 3. As a result, in highly populated and industrial areas, all bee products seemed to have concentrations over the acceptable limits for most examined metals. One indication for identifying the extent of environmental contamination is the detection of heavy metals in samples of honey [60]. On the other hand, phytosanitary treatments are one of the major issues contributing to the rise in bee family mortality nowadays [84].

[figure(s) omitted; refer to PDF]

It is necessary to fully understand those factors since heavy metals enter the food chain through soil, plants, and animals. Since heavy metals persist and accumulate over time, becoming a danger to both human health and the

ecosystem, their discharge into the environment in large amounts has multiple consequences. Uncontrollable exposure to heavy metals has mutagenic and carcinogenic tendencies that can cause irreversible effects. Some of those consequences can even be transferred from mother to fetus [84]. Even in instances with limited exposure to heavy metals, children are susceptible to neurological problems such as attention, memory, and cognitive deficiencies [85]. Heavy metals impact all organs in the environment and in foods [86].

According to investigations, heavy metals including aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), mercury (Hg), nickel (Ni), vanadium (V), and zinc (Zn) may also be detected in honey. They can be divided into many classes based on how carcinogenic they are. As a result, they were classified into four groups: group 1—carcinogenic (Al, As, Cd, Cr, and Ni), group 2A—probably carcinogenic (Pb), group 2B—possibly carcinogenic (Co, Hg, and V), group 3—carcinogenicity not classifiable (Cr, Cu, and Se), and group 4—probably not carcinogenic (Ag, Mg, and Zn), by the International Agency for Research on Cancer (IARC) [60]. Concerns about the impact on ecosystems of increasingly high concentrations from anthropogenic sources (e.g., fuel combustion, mining, industrial industries, agricultural wastewater, and solid waste) have been highlighted [62]. The amount of heavy metals in the body and their toxicological consequences are influenced by many components, including metal intake or inhalation, metal entry rate, tissue distribution, concentration attained, and metal excretion rate [62].

Enzyme inhibition, protein synthesis inhibition, nucleic acid function variations, and cell membrane permeability alterations are just a few of the mechanisms of toxicity. Hepatotoxicity, nephrotoxicity, and neurotoxicity are some significant consequences of heavy metals [60].

Heavy metal concentration in honey varies from 0.02 to 1.03 g/100 g [6, 61, 84, 87].

Various techniques, including atomic absorption spectroscopy (AAS), atomic emission spectroscopy (ICP-AES), total reflection X-ray fluorescence (TXRF), inductively coupled plasma spectroscopy (ICP-AES), and spectrometry (ICP-OES), have been utilized to measure the concentration of heavy metals in honey, in the last two decades.

One of the most widespread environmental pollutants is Pb. Lead contamination in honey can occur due to human activities such as industrial emissions, lead-based pesticides, or lead-containing paints. Ingesting honey contaminated with lead can be particularly harmful, as lead is a neurotoxic metal that affects the nervous system and brain development, especially in children [60, 87].

Cd is one of the heavy metals recognized as bioindicators for contaminated honey and lead. Cadmium contamination often results from industrial activities, waste incineration, wastewater, and fertilizers. Long-term consumption of honey contaminated with cadmium can lead to kidney disease and increase the risk of various health problems, including bone disorders [60, 88].

Fe is another metal found in honey from anthropogenic sources. Drilling, digging, and metal corrosion constitute the most common sources of Fe [60, 61, 88].

Even in extremely low quantities, public health is severely threatened by Hg, one of the most hazardous heavy metals. The agricultural area surrounding the hive, mines, combustion, and industrial and municipal sewage systems are the sources of Hg toxicity. Mercury is a potent neurotoxin and can cause severe neurological issues, particularly in developing fetuses and young children [60, 89].

Nevertheless, given its high acidity, bee honey can also corrode stainless steel and/or galvanized steel containers, releasing heavy metals such as Pb, Cr, Al, Ni, and Sn during harvesting, processing, preparation, and storage [60].

Based on the formula THQ = EDI/RfD, the carcinogenic risk related to the presence of heavy metals in honey is estimated. THQ refers to the target hazard quotient, where EDI is the dose of heavy metals ingested (μg/kg-d), and RfD or TDI is the oral reference dosage (mg/kg-d) [60, 90]. THQ values greater than 1 are harmful to human health [91]. Following the THQ value, Pb is the most dangerous element, followed by Cd, Mn, Fe, Ni, As, Cu, Hg, and Cr. THQ levels in honey have reportedly increased in Turkey, Iran, Bulgaria, Bangladesh, Poland, Austria, Mexico, Italy, Nigeria, France, Argentina, Pakistan, Spain, United States, Brazil, Kosovo, Canada, Australia, Switzerland, Germany, Greece, China, Slovakia, Lithuania, Bosnia, Herzegovina, Algeria, and Macedonia, among other countries. Consequently, consumers in Turkey, Iran, and Bulgaria seem more vulnerable to illness than those in

other countries [3, 60, 91].

Thus, beekeepers must regularly check the soil in agricultural areas and agriculturally utilized waterways and avoid placing beehives in places with a lot of industrial activity to prevent the buildup of contaminants in their products [60]. Regulatory authorities in different countries have established maximum permissible limits for heavy metals in food, including honey, to safeguard public health. Monitoring and controlling heavy metal levels in honey are essential to ensure its safety for consumption. Regular testing of honey for heavy metal contaminants and enforcing stringent quality control measures in beekeeping practices and honey processing can help prevent heavy metal contamination.

Consumers can also play a role in reducing the risk of heavy metal exposure from honey by purchasing products from reputable sources with proper labeling and certifications. Being aware of potential environmental sources of heavy metals and supporting sustainable practices that reduce pollution can also minimize heavy metal contamination in honey and protect our health.

6. Final Considerations

The desirable physical, chemical, sensory, and healing properties of bee honey have positioned it as an appealing and commercially viable functional food. It also emphasizes the dangers of consuming honey that has been tampered with or contaminated by pesticides, antibiotics, microorganisms, and heavy metals. Bee honey plays a significant role in assessing environmental pollution, acting as a mirror of the current environmental condition. Consuming adulterated and contaminated honey threatens safety, food security, and environmental sustainability. Contaminated honey can lead to genetic abnormalities, allergic reactions, and carcinogenic effects while also contributing to the development of antibiotic resistance in certain microorganisms. Various analytical techniques have been employed over time to identify residues in honey. Unfortunately, there is currently no established regulation determining the minimum acceptable levels of contaminants for honey.

In light of those concerns, honey producers and processors must be well-informed about honey contaminants and adhere to the regulations that ensure the safety and authenticity of their honey products. Furthermore, consumers can make informed decisions by scrutinizing product labels for pertinent information and seeking certifications that confirm adherence to quality and safety standards. It is worth noticing that the specific laws and regulatory bodies governing honey may vary from country to country, highlighting the importance of consulting the relevant local or national authorities for the most current information.

Authors' Contributions

L.A., M.V., V.V.L., I.I., B.-A.M., A.L., P.-C.M., O.-L.P., V.M.B., I.M.S., and L.T. contributed equally with I.-D.M. to this article. All authors have read and agreed to the published version of the manuscript.

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Application of Slightly Acidic Electrolyzed Water as a Potential Sanitizer in the Food Industry

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ABSTRAK (ENGLISH)

The food industry has extensively explored postharvest microbial control, seeking viable technologies to ensure food safety. Although numerous chlorine-based commercial sanitizers serve this purpose, many are plagued by constraints such as instability and diminished disinfectant efficacy. These issues arise from exposure to organic matter in wash water, light, or air. As an innovative and promising alternative, slightly acidic electrolyzed water (SAEW) has emerged, captivating attention for its robust sterilization potential and eco-friendliness in agricultural and food sectors. SAEW generated via electrolysis of a diluted hydrochloric acid (HCl) solution with concentrations ranging from 2 to 6% or aqueous solution of sodium chloride (NaCl) in a nonmembrane electrolytic chamber is reported to possess equivalent antimicrobial properties as strong acidic electrolyzed water (StAEW). In contrast to traditional chlorine sanitizers, SAEW leaves less chlorine residue on sanitized foods such fresh-cut fruit and vegetables, meat, poultry, and aquatic products due to its low available chlorine concentration (ACC). Its near neutral pH of 5 to 6.5 not only renders it environmentally benign but also mitigates the production of chlorine gas, a contrast to low pH conditions seen in StAEW generation. The bactericidal effect of SAEW against various strains of foodborne pathogens is widely believed and accepted to be due to the combined action of high oxidation-reduction-potential (ORP) reactions and undissociated hypochlorite/hypochlorous acid (HOCl). Consequently, a burgeoning

interest surrounds the potential of SAEW for sanitation in the food industry, offering an alternative to address shortcomings in sodium hypochlorite solutions and even StAEW. It has been hypothesized from a number of studies that SAEW treatment can increase the quality and nutritional value of harvested fruits, which in turn may enhance their ability to be stored. Therefore, SAEW is not only a promising sanitizer in the food industry but also has the potential to be an efficient strategy for encouraging the accumulation of bioactive chemicals in plants, especially if it is used extensively. This review encapsulates the latest insights concerning SAEW, encompassing its antimicrobial effectiveness, sanitization mechanism, advantages vis-à-vis other sanitizers, and plausible applications across the food industry.

TEKS LENGKAP

1. Introduction

Ensuring food safety remains a paramount concern within the food industry, necessitating the utilization of an effective and appropriate sanitization method. Among the array of disinfection techniques, a burgeoning approach has emerged, centered on the implementation of electrolyzed water (EW) as a novel and alternative means of sanitation in the food sector, characterized by its dual benefits of environmental safety and user well-being [1]. The inception of electrolyzed water dates back to its initial application in food processing within the Japanese soda industry in 1980 [1–3].

Through electrolytic processes, a dilute sodium chloride solution undergoes dissociation, resulting in the formation of acidic electrolyzed water (AEW). AEW is of two distinct acidic variants, namely, strong acidic electrolyzed water (StAEW, pH 2-3) and slightly acidic electrolyzed water (SAEW, pH 5–6.5). The latter, slightly acidic electrolyzed water (SAEW), has been refined over two decades by Japanese enterprises [4], obtaining official recognition as a food additive by the Japanese Ministry of Health, Labor, and Welfare in 2002, as well as securing authorization as a control agent from the Ministry of Agriculture, Forestry, and Fisheries and the Ministry of the Environment in 2014 [4]. In contrast to StAEW, which arises from the electrolysis of a 2% sodium chloride aqueous solution, SAEW is derived from electrolyzing a diluted hydrochloric acid (HCl) solution, typically ranging from 2% to 6%, within an electrolytic cell devoid of a diaphragm [5–7].

The ascendancy of SAEW in Japan's realm of food sanitation is swiftly advancing, positioning it as a prospective alternative to antimicrobial detergents and an ecologically friendly disinfecting agent [8]. The distinguishing attribute of SAEW is its capacity to mitigate the deleterious implications of chlorine residuals on human and environmental well-being [9]. While StAEW finds application across various agricultural domains such as vegetable sterilization, food processing, and domestic kitchens for food material and utensil disinfection, SAEW's industry-level utilization has remained comparatively limited. Unlike StAEW, with its pH within the range of 2.0 to 3.0, SAEW boasts a pH of 5 to 6.5 and exhibits substantial antibacterial potency attributed to its elevated hypochlorous acid (HOCl) concentration, concurrently resulting in reduced negative impacts on human health and the environment [7, 10]. SAEW's superiority over StAEW is multifaceted, including heightened preservative efficacy during storage [11–13], minimal pH, color, and appearance alteration of fresh-cut vegetables [14], as well as reduced contributions to equipment corrosion and skin irritation in comparison to StAEW [15]. In addition, SAEW sidesteps issues such as phytotoxicity in plants and safety concerns stemming from chlorine gas emissions [16], positioning it as a promising avenue for application, particularly within the fresh and ready-to-eat fruit and vegetable sector. Consequently, SAEW has gained substantial traction as a preservative within the food industry [3, 17, 18].

Although StAEW has limitations such as loss of its bactericidal activity due to chlorine (Cl_2) loss [19] hindering its postproduction storage which necessitates the on-site production and application, the fact remains, however, that StAEW exhibits potent bactericidal and virucidal effects, as well as moderate fungicidal properties [1]. In addition to StAEW, chemical sanitizers such as chlorine and its derivatives are commonly utilized in the food industry to produce food that is both high-quality and microbiologically safe for human consumption [20], hydrogen peroxide

[21], ozone [22], and organic acids [23]. However, due to the possibility for the development of carcinogenic halogenated disinfection byproducts, which pose a threat to human and environmental health, certain chemical sanitizers are prohibited in a number of European nations and other countries [24–26]. Although chlorine and chlorine derivatives are frequently employed as sanitizers in the fresh-cut food business to reduce microbial contamination, they pose a health concern and should be avoided. Furthermore, the chlorine solution can cause irritation to the skin and lungs. In addition, its effectiveness diminishes when exposed to air, light, and metals [27]. Trihalomethanes (THMs), chloroform, and chlorophenols are all byproducts that pose a health risk due to their potential mutagenicity and classification as probable human carcinogens by the US Environmental Protection Agency (EPA) [28].

On the other hand, unlike StAEW that is reported for its storage unstability, SAEW is known to exhibit greater stability during storage due to a considerable reduction in chlorine loss at pH 5–6.5 5. As a result, the factor responsible for the bactericidal effect of SAEW is more stable compared to the similar factor in StAEW [7, 10, 29, 30]. The fundamental properties and effectiveness against microorganisms of electrolyzed water (StAEW and SAEW) are significantly affected by the storage conditions. This is due to the degradation of the main antimicrobial component (HOCl) and the evaporation of Cl_2, which can be greatly influenced by the storage conditions, especially when exposed to open or light conditions. For instance, Rahman et al. [31] compared the ACC of low-concentration electrolyzed water (LcSAEW: 10 mg/L, pH range of 6.8–7.4) exposed to open and closed conditions and found that under open-dark settings, the ACC of LcSAEW dropped from 10 to 0 mg/L in just 7 days, compared to 21 days under closed-dark conditions. SAEW (ACC:20 mg/L) was evaluated for changes in fundamental properties (pH, ORP, and ACC) during storage in open and closed glass bottles at room temperature in both light and dark settings [32]. The findings indicated that storing EW in a closed-dark container was a more favorable setting. According to a study conducted by Len et al. [33], the depletion of chlorine in electrolyzed water (EW) when exposed to an open environment can be attributed to the evaporation of chlorine and its subsequent interaction with the atmosphere. This finding was further supported by the research conducted by Xuan and Ling [32]. The chlorine underwent self-decomposition at a significantly greater rate when held under open conditions, as opposed to closed ones.

While the efficacy of SAEW in microbial inactivation in foods has been demonstrated, its utilization is vulnerable to interference caused by organic matter, leading to a reduction in disinfection effectiveness [34]. Hence, the recent investigation conducted by Zhao et al. [35] has explored the combination of SAEW with other disinfection techniques as a potential solution to address this issue. Among them, combination of SAEW with ultraviolet (UV) light (SAEW + UV) has demonstrated a remarkably potent germicidal action. An investigation by Zhang et al. [36] showed that the combination treatment of SAEW and UV has shown a greater antimicrobial efficacy against *Staphylococcus aureus* than the individual treatments of UV or SAEW alone. Furthermore, the antimicrobial impact was observed to intensify with both time and ACC. Recent reports have highlighted several limitations of SAEW, such as water hardness, which is thought to have a significant impact on its physical qualities and sanitization performance. Nevertheless, SAEW is still being proposed as a potential novel sanitizer for use in agriculture and food processing [37]. Therefore, the agriculture and food industries should pay attention to SAEW as a revolutionary technology with significant potential for sterilizing.

2. Production of Slightly Acidic Electrolyzed Water

Slightly acidic electrolyzed water (SAEW) is created from the electrolysis of a combination of diluted hydrochloric acid solution HCl (2–6%) in a chamber cell without a membrane and tap water in an electrolytic cell containing both cathode (Ti) and anode (IrO2) [38]. SAEW can also be made by electrolysis of dilute electrolyte of NaCl in an electrolysis chamber without the diaphragm [37, 39, 40]. The SAEW generator comprises an electrolytic cell with anode and cathode electrodes that are without a separating membrane between them [41–43]. Figure 1 shows a schematic depiction of the SAEW generating equipment and the compounds that are generated through electrolysis.

[figure(s) omitted; refer to PDF]

The basic chemical reactions at the anode and cathode can be summarized below. At the anode side,(1)2Cl− Cl2 ↑ + 2e−(2)Cl2+H2O HOCl+H++Cl−

At the cathode side,(3)H++2e− ↑H2

Electrolysis causes the dissociation of diluted hydrochloric acid in water, resulting in the formation of negatively charged chlorine ions (Cl^-) and positively charged hydrogen ions (H^+). The negatively charged ions (Cl^-) migrate towards the anode to give up electrons and become chlorine gas (Cl_2) that dissolves in water to create hypochlorous acid also known as undissociated hypochlorite (HOCl), H^+, and Cl^- ions. Positively charged hydrogen (H^+) ions move to the cathode to take up electrons and become hydrogen gas (H_2) that evaporates. As a result of this process, unlike StAEW, this solution dissociates into only one type of solution (SAEW), with near neutral pH (5.0–6.5) and high oxidation–reduction potential (ORP, 800–900 mV), and contains available chlorine concentration (ACC) of 10–30 mg/L in the form of hypochlorous acid. In StAEW generation, a solution of sodium hydroxide has two forms of electrolyzed water: acidic electrolyzed water (AEW) and basic electrolyzed water (BEW) [32, 44].

3. SAEW-Producing Equipment

Currently, there are various SAEW-producing devices accessible in the market, and generators manufactured by well-known companies have been widely embraced by consumers. Such devices include MIOX (Albuquerque, NM, United States), AQUACIDO NDX-250KMS (OSG Company Ltd., Osaka, Japan), Water God HD-240L (Shanghai, China), and APIA series (Japan) [1, 29, 45]. Currently, Japan is widely recognized as the principal manufacturer of such machines with the most common machines being the HOCL series (Institute of Slightly Acidic Electrolyzed Water, Inc., Kanagawa, Japan), Purestar series (Morinaga Engineering Co., Ltd, Kanagawa, Japan), and Apia series (Hokuty Co., Ltd, Kanagawa, JAPAN). Other common SAEW generators manufactured by reputable companies in different countries are summarized in Table 1.

Table 1

Common SAEW generators and their respective available chlorine concentration (ACC: mg/L).

SAEW model	Country of origin	ACC (mg/L)	References
Apia60	Japan	10–30	[46]
Apia210	Japan	10–30	Unpublished
AQUACIDO NDX-250KMS	Japan	60	[42]
Apia5000H	Japan	10–30	Unpublished
Water God HD-240L	China	10–40	[47]
Anywhere-320W	China	30–35	[41, 48]
ORPWG	China	30	[49]
Purester MP-600T	Japan	20–25	[50]
Purester MP-240	Japan	10–30	Unpublished
Purester MP-2000T	Japan	10–30	Unpublished
Apia1000H	Japan	10–30	Unpublished

HOCL series (e.g 2.5t/5t)	Japan	10–30	Unpublished
ecoTree	Korea	10–30	[43]
BD-600L	China	30–80	[36]

The properties of SAEW can be significantly influenced by the chlorine concentration, amperage, voltage, flow rate, and water hardness [51], and each of these generators is made with specific technical parameters to meet the requirements. For research purposes, the SAEW generator could be self-developed and used to study the effect of SAEW [10, 52, 53]. For instance, Forghani and Oh [54] used a self-developed device to generate SAEW at a setting of 2.9 A and 24 V. The production capacity ranges from 60 l/h to 10,000 l/h indicating that the technology can be used at the laboratory, household, and industrial levels to produce safe food. Slightly acidic electrolyzed water of the desired 10–30 mg/L ACC or even more is produced by suitably adjusting the amount of supply of hydrochloric acid, amount of supply of water, and current, which is performed automatically in most models, and could be manually preset by an operator in some models.

4. Antimicrobial Properties of Slightly Acidic Electrolyzed Water

The prominent properties (features) of SAEW are shown in Table 2. Physicochemical properties of SAEW as reported by most researchers include a near-neutral/slightly acidic pH of 5–6.5, high oxidation-reduction potential (ORP; ≥800 mV), and low available chlorine concentration (ACC; 10–30 mg/l) [16, 61, 72]. Hypochlorous acid (HOCl), also referred to as undissociated hypochlorite, exhibits the most potent bactericidal activity against a wide spectrum of microbes among all forms of free available chlorine. In aqueous solutions, the equilibrium between HOCl and the hypochlorite ion (OCl-) is pH dependent with the concentration of HOCl increasing as pH decreases (Figure 1). At the pH 5.0–6.5, the active form of chlorine compounds in SAEW is mainly HOC (>95%) and 5% is OCl- and traces of Cl_2 as shown in Figure 2 [56, 73]. Both HOCl (undissociated hypochlorite/electrically neutral) and dissociated hypochlorite (OCl-/electrically negative) have microbial disinfecting behavior. However, undissociated hypochlorite (HOCl) is considered the most microbicidal form of chlorine. The cell wall of pathogenic microbes has an inherent negative charge. As such, the uncharged species (HOCl) is freely permeable across the plasma membrane of microorganisms and thus can enter the cell. HOCl is important in sanitization more than other forms because the chlorine in the Cl_2 form can volatilize as pointed out by Cui et al. [55], and the efficacy against microorganisms can be lost. Therefore, a neutral pH is an advantageous property for preventing the evaporation of chlorine and keeping the concentration of HOCl stable. With this property and at the same concentrations, SAEW's sanitizing activity is 80 times more than that of OCl-, making it the most effective germicide form of chlorine in solutions [57].

Table 2

Important features of slightly acidic electrolyzed water.

Properties (features) of SAEW	Details	References
The active ingredient for sanitization	Hypochlorous acid (HOCl), >95% of ACC is in this form	[16, 55, 56]
Acidity	Slightly acidic, it has a near-neutral pH of 5–6.5	[16, 55, 56]
Available chlorine concentration (ACC)	10–30 mg/l	[16, 55, 56]

Oxidation-reduction potential (ORP)	800–900 mV	[16, 55, 56]
Microbial inactivation power	80 to 150 times than that of hypochlorite ion (OCl^-)	[3, 57, 58]
Safety	Designated as a food additive by the Japanese Government and does not produce chlorine gas when applied in food sanitization	[59]
Effects on sensory quality of foods	Does not alter the flavor, hue, aroma, or nutritional content of food	[36, 45, 60]
Antimicrobial effect	Effective against bacteria, fungi, yeast, virus, bacteria spores	[42, 57, 60–62]
Effect on environment	Can be used the same as tap water and disposed of as it is immediately after use and it is environmentally friendly as it does not produce trihalomethanes (THMs)	[63, 64]
Sterilization effect	Possesses a strong effect on bacteria, fungi, yeast and molds, virus, bacterial spores, etc.	[3, 7, 57, 58, 65]
Quick effect	Can sterilize most bacteria in the first few seconds of treatment	[43, 66–68]
Rinsing not required	Can be used in the same way as tap water and be disposed of as is after use	[69, 70]
Inexpensive	Can run at a cost only a little higher than tap water after the purchase of the generator	[5, 71]
Application	Multiple uses in every field, food and agricultural industry, medical, household sanitary, dental	
Stability on storage	Exhibits greater stability under storage conditions and poses less risks to worker health	[57]

[figure(s) omitted; refer to PDF]

Hence, the application of SAEW with a pH close to neutral holds great potential due to its ability to mitigate human health and safety concerns arising from the release of Cl_2 gas, minimize surface corrosion, and restrict phototoxic side effects, while maximizing the efficacy of hypochlorous acid species [16]. In recent years, SAEW has drawn attention from scientists and processing companies due to its nearly neutral pH (5.0–6.5) and lower ACC (10–30 mg/L), and its strong sanitization efficacy on food materials and food-contact surfaces has been widely acknowledged [12, 42].

Sanitizing efficacy of EW is directly affected by its fundamental properties, such as ACC (Cl_2, OCl^-, and HOCl), pH, and ORP. In addition, several electrolytic parameters, including electrolyte composition, flow rate, current, salt concentration, electrode materials, water temperature, water hardness, and storage conditions, have been revealed to directly influence the properties of EW [32].

Pangloli and Hung [74] found that water hardness had a positive correlation with the levels of ACC and ORP and a

negative correlation with the pH of EW. Similarly, it has been documented that the physical characteristics of SAEW and its ability to deactivate pathogens are influenced by various conditions, including water hardness. Forghani et al. [75] evaluated the effect of water hardness on the properties of SAEW, and it was observed that an increase in water hardness was accompanied by a drop in pH. While water hardness was shown to increase ACC and ORP levels, it decreased the pH of EW. Moreover, the rise in ACC (mg/L) can be attributed to an increase in the concentration of electrolytes and conductivity, both of which were induced by the existence of water hardness [74]. The typical use of SAEW is the inactivation of bacteria in food matrices that contain organic components. Organic compounds may undergo a chemical reaction with chlorine present in SAEW, resulting in the formation of combined chlorine. Hence, SAEW's anticipated antibacterial activity in organic material-containing environments is not directly proportional to the available chlorine concentration, which comprises both free and mixed chlorine [52]. The significance of water hardness as a raw material becomes even more crucial for the manufacturing of SAEW due to the strict requirements such as pH (5.0–6.5) [7]. In addition, the storage of SAEW can potentially impact the physicochemical properties and bactericidal efficacy of SAEW [76].

Forghani et al. [75] and Pangloli and Hung [74] observed that EW's fundamental properties are affected by water hardness and temperature. After its production, heating EW to 40°C could reduce its inactivation efficacy since some free chlorine is lost during heating [75].

The basic properties of EW are furthermore impacted by the type and flow rate of electrolyte, along with the electrode settings and materials [32]. Hsu [77] revealed a direct relationship between the ACC and the concentration of the electrolyte. Augmenting the concentration of electrolytes can elevate conductivity, potentially increasing chlorine production and bolstering its bactericidal efficacy. Furthermore, Forghani et al. [75] revealed that the increase in pH was triggered by the increase of electrolyte concentration. According to Martínez-Huitle and Brillas [78], the production of oxidants and other species is mostly influenced by the choice of electrode material, rather than factors such as current, temperature, and type of electrolysis. Typically, platinum serves as the anode in the EW generator. Rahman et al. [3] ordered several electrode materials to assess their effectiveness in producing free chlorine. The order of electrocatalytic activity, from highest to lowest, is Ti/IrO2, Ti/RuO2, Ti/Pt-IrO2, BDD, and Pt. Furthermore, it has been found that altering the size or gap of the electrode has a notable impact on chlorine production and electric current, while not influencing its electric efficiency and current efficiency [77]. In addition to the basic properties of SAEW, such as ACC (Cl_2, OCl^-, and HOCl), pH, and ORP, which impact its disinfecting effectiveness, several electrolytic factors, such as flow rate, salt concentration, current, electrolyte, water temperature, electrode materials, hardness, and storage conditions, must be considered and monitored during the production and use of SAEW [77]. Therefore, it is crucial to take into account and closely observe various electrolytic parameters, including current, flow rate, salt concentration, electrolyte, electrode materials, water temperature, hardness, and storage environments, in addition to the basic properties of SAEW. These parameters possess a significant impact on the sanitizing effectiveness of SAEW.

5. Microbial Inactivation Mechanisms of Slightly Acidic Electrolyzed Water

Similar to strong acidic acid electrolyzed water type, SAEW has been reported to possess a strong antimicrobial activity against different food pathogens, yeast and molds, and virus. However, action mechanisms of slightly electrolyzed water are not consensus, but a lot of theories exist. Several mechanisms are interconnected, and the effect was based on one or more ways evolving chlorine species, ORP, and others. These mechanisms specifically target bacterial enzymes, causing membrane damage and other effects [72]. Slightly acidic electrolyzed water has a pH value of 5.0–6.5 and contains a high concentration of HOCl and high ORP values. The antibacterial action of SAEW is primarily attributed to the presence of HOCl and high ORP, which has been thoroughly investigated and demonstrated to be highly effective [31, 57, 79]. In their previous study, Al-Haq et al. [1] asserted that the main factors contributing to the bactericidal activity of SAEW are a high concentration of HOCl, a high ORP, a significant amount of free available chlorine, and the presence of hypochlorite ions (OCl^-). At a pH range of 5.5–6.5, the majority of available chlorine exists as HOCl (~97%), is the primary active form of chlorine compounds, and is believed to be responsible for killing microorganisms [56]. The bactericidal efficacy of chlorine-related compounds is

more pronounced in the form of nondissociated HOCl compared to dissociated OCl^-. Studies have demonstrated that HOCl is 80–150 times more efficient than an equivalent concentration of OCl^- as a sanitizing agent [3, 57, 58]. According to Schaik [80], electrochemically activated HOCl in SAEW is more effective than chemically produced HOCl in bleach by more than 400%. It is generally believed and acknowledged that the bactericidal effect of SAEW at a pH close to neutral is caused by the combination of high ORP reactions with hydrochloric acid (HOCl). Initially, the bacteria' defensive mechanism is rendered inactive by ORP reactions that occur at the cell membrane, which damage both the outer and inner membranes. According to Fabrizio and Cutter [81], one way that ORP can kill microbes is by making their cell membranes more permeable, which allows antimicrobial agents to disrupt their metabolism and ultimately render them inactive. The study conducted by Liao et al. [82] provided additional evidence that the high oxidation-reduction potential (ORP) of SAEW can harm cell membranes, oxidize sulfhydryl compounds on cell surfaces, and disturb cell metabolic processes. Consequently, this leads to the inactivation of bacterial cells. Nan et al. [79] hold a similar argument that SAEW with high ORP effectively damaged, destroyed, or caused deformation of the outer membrane of foodborne pathogenic bacteria, such as *S. aureus* and *E. coli O157:H7*, inactivation of these pathogens being as a result of its high ORP (mV). In the same vein, the research conducted by Ding et al. [49] revealed that the disinfection mechanism of SAEW involved the disruption of the cell membrane's permeability and the cytoplasmic ultrastructures in *S. aureus* cells. The SAEW solution has an oxidation-reduction potential (ORP) ranging from 800 to 900 mV, as previously stated. This ORP has a direct and irreversible detrimental effect on the microbial cell wall. Furthermore, the configuration of water molecules undergoes electrochemical modifications, enhancing the capacity of microbicidal ions to penetrate and interact. This property is absent in traditional disinfectants [80].

The second active species of SAEW, HOCl, has indirect antimicrobial effects due to the generation of the radical OH- after HOCl permeates bacterial cells [83]. The bactericidal effect of HOCl is ascribed to its ability to infiltrate microbial cells by traversing the cell walls and membranes, as depicted in Figure 3 of the model proposed by Fukuzaki [84]. This model illustrates that the bactericidal action of EW is determined by the capacity of HOCl and −OCl to penetrate the microbial cell membrane. The presence of the lipid bilayer, a hydrophobic layer in the microbial cell membrane, prevents the penetration of ionized −OCl. At times, certain components of the microbial cell wall provide protection against the penetration of −OCl. The ionized form of hypochlorite (-OCl) is unable to pass through the microbial membrane and has demonstrated little effectiveness in killing bacteria. -OCl specifically targets the external membrane of the cell (circle A). The active agent responsible for the bactericidal effect is therefore HOCl, since it is electrically neutral and capable of permeating the cell membrane. HOCl has the ability to target both the outside membrane (circle A′) and the interior of the cell (circles B and C) [84].

[figure(s) omitted; refer to PDF]

After cell membrane disruption by ORP activity, HOCl can then penetrate slime layers, cell walls, and protective layers of microorganisms and oxidize it leading to the death of microorganisms [80]. In their study, Ding et al. [49] discovered that SAEW led to a decrease in TCC-dehydrogenase activity in *S. aureus*. This drop was attributed to the reaction between the HOCl present in SAEW and the enzymes, resulting in the formation of N–Cl linkages. In addition, the researchers noted the presence of protein leakage and the degradation of the bacterial ultrastructure in *S. aureus* following treatment with SAEW.

Assessing the bactericidal mechanism of SAEW on *E. coli*, Suzuki et al. [85] suggested that SAEW killed *E. coli* by first changing the porin-proteins and channels-proteins of the outer and inner membrane, causing them to open and remain open. Then, ATP immediately is ejected into the *E. coli* suspension by the inner pressure. HOCl then enters the *E. coli* cell through the channel holes as a result of the molecular concentration gradient. HOCl neutralizes the ATP-ase and other enzymes, destroying *E. coli*. Previous studies suggested that HOCl (undissociated) produces hydroxyl radicals after penetrating cell membranes, which in turn exert their antimicrobial activity through the oxidation of key metabolic systems [83, 84, 86]. According to Hati et al. [87], HOCl in SAEW is demonstrated to kill microbes by blocking the sulfhydryl groups of enzymes involved in carbohydrate metabolism that oxidize glucose. Kurahashi et al. [4] established that HOCl in SAEW is an essential sterilizing component in slightly acidic

electrolyzed water, in contrast to hypochlorous acid solutions produced by mixing acids with sodium hypochlorite. The third antimicrobial agent of SAEW is its available chlorine concentration in mg/L (ACC). Some studies on the antimicrobial mechanism of SAEW attribute ACC as a main factor affecting the disinfection efficacy of SAEW [16, 55, 56]. According to Li et al. [53], SAEW with an available chlorine concentration (ACC) above 12 mg/L was able to kill all *L. monocytogenes* strains in just 30 seconds, demonstrating that ACC is the main factor affecting SAEW's disinfection efficacy. As a result of its high ORP value, SAEW could disrupt or break the intracellular reactive oxygen species (ROS) balance of *L. monocytogenes* by inhibiting the antioxidant enzyme activity, thus promoting the death of *L. monocytogenes* [36, 53]. In a study by Liu et al. [88], it was observed that the intracellular ROS generated by SAEW was strengthened significantly with the increase of ACC, and the cells were injured to death accordingly. The disinfection efficacy of SAEW on spores depended primarily on ACC and treatment duration, according to Zhang et al. [65], who explored the inactivation mechanism of slightly acidic electrolyzed water on *Bacillus*. This finding is in line with a prior study [89]. Evidently, the bactericidal effect of SAEW at a pH close to neutral against various bacterial strains is caused by the synergistic effect of high ORP reactions and high hypochlorous acid (HOCl). When ORPs are activated, membranes burst, hydrochloric acid (HOCl) goes through microbial cell walls, and protective layers of microorganisms to oxidize it, leading to the death of microorganisms.

6. Advantages of SAEW over Other Food Antimicrobial Sanitizers

Slightly acidic electrolyzed water, a novel disinfectant with a strong and broad-spectrum action, is colorless, odorless, and nontoxic to both humans and the environment. It is presently applied directly to food surfaces in Japan, China, Korea, and America [69]. SAEW demonstrates potent bactericidal action within a narrow pH range of 5–6.5 in a high concentration of HOCl (roughly 95%) and a low concentration of available chlorine (10–30 mg/L) [7, 10, 12]. Similarly, a previous study has demonstrated that SAEW, at this low ACC (10–30 mg/L), exhibits comparable or greater bactericidal effectiveness than NaOCl solution (100–200 mg/L) [57, 90, 91]. This could allow the food industry to reduce the amount of chlorine used and would help to improve the safety of both products and workers. In another study, SAEW has proven to demonstrate a higher antibacterial efficacy in comparison to sodium hypochlorite water disinfection [92]. Furthermore, studies have demonstrated that SAEW exhibits an equivalent or superior capacity to inactivate all types of aerobic bacteria, molds, and yeast in freshly cut cabbage when compared to the use of sodium hypochlorite disinfectant treatment [60].

Due to its neutral pH and low chlorine concentration, studies by Abadias et al. [15], Possas et al. [67], Yan et al. [93], and Zhao et al. [94] have shown that SAEW has a lower effect on the corrosion of processing equipment and is less likely to cause irritation to the hands compared to StAEW. Furthermore, studies have demonstrated that SAEW exhibits less phytotoxicity in plants and presents fewer safety risks associated with the release of Cl_2 gas [16]. SAEW, which has a pH range of 2.0 to 3.0, is a cost-effective and safe approach for preserving fresh produces after harvest, as demonstrated by Song et al. [5] and Sun et al. [71]. Compared with StAEW ($2.0 \leq pH \leq 3.0$), SAEW is a low-cost and safe postharvest approach for fresh produces [5, 71]. SAEW has greater advantages and fewer drawbacks than StAEW, mostly due to its pH level and the kind of chlorine it contains. These factors contribute to its enhanced efficacy and reduced corrosiveness. Operators and employees are at a reduced risk as it does not produce chlorine gas and may be transformed into ordinary water after use, without emitting hazardous gasses [95]. The advantage of the use of electrolyzed water at neutral pH in comparison with strong acidic pH is that it does not affect the pH, surface color, or general appearance of fresh-cut vegetables [14]. With SAEW, there is no residue of sodium chloride after packaging and no denaturing of the products due to sodium chloride. It is nearly odorless, and no smell remains behind after application on food and agricultural products. SAEW can be used the same as tap water and disposed of as it is immediately after use, and it is environmentally friendly as it does not produce trihalomethanes (THMs) [64].

Recently, SAEW has been found to have greater stability during storage due to a considerable reduction in chlorine loss at pH levels between 5 and 6.5. Consequently, the factor responsible for the bactericidal effect of SAEW is more stable than the similar factor in StAEW [11, 57, 96]. A previous study has additionally shown that SAEW can preserve the factors that contribute to its ability to kill bacteria, such as ACC, pH, and ORP, for an extended period

of time when stored in tightly sealed containers [12]. This characteristic makes it particularly suitable for use in situations where on-site production and application are not feasible, such as in rural areas of developing countries with unreliable power supply. SAEW is extensively advocated in the field of food preservation due to its exceptional antibacterial properties, cost-effectiveness, ease of application, and environmentally benign nature [13, 18, 97]. With these, SAEW is particularly attractive for practical applications in the food industry elsewhere.

7. Application of Slightly Acidic Electrolyzed Water (SAEW) in the Food Industry

Slightly acidic electrolyzed water, a recently discovered sanitizer, has demonstrated promising and secure outcomes in several prior investigations. It is regarded as a versatile and very effective bactericide that is increasingly being used in the food industry. Studies have shown that SAEW exhibits potent antimicrobial properties against various types of bacteria, including both pathogenic and nonpathogenic bacteria, as well as spores, viruses, and fungi. These effects have been observed both *in vitro* and on different food and agricultural items [42, 57, 60, 62, 98]. SAEW's mild pH makes it more suitable for usage in the food industry [16, 55, 56]. The key feature of SAEW is its ability to inactivate microorganisms at low chlorine levels, resulting in reduced residual chlorine on vegetables following disinfection treatment, as compared to other chlorine sanitizers [99]. A previous study has shown that SAEW exhibits comparable bactericidal activity to StAEW against various bacteria and fungi found on fruits and vegetables, including *Listeria spp.* [10, 100], *Salmonella spp.* [46], *Coliform populations* [101, 102], *Bacillus spp.* [103], and *Escherichia coli* [46]. Ding et al. [104], Hao et al. [102], Issa-Zacharia et al. [46], Koide et al. [12], and Tango et al. [10] also observed that SAEW had a noteworthy antibacterial effect on a variety of fruits and sprouts, including apples, cherry tomatoes, celery, strawberries, cilantro, cabbage, and lettuce.

7.1. Inactivation of Foodborne Pathogens in Suspension (*In Vitro*) Using SAEW

Several studies have reported a strong bactericidal effect of SAEW on most pathogenic and nonpathogenic bacteria *in vitro* [16, 57, 61]. The antibacterial activity of SAEW against several pure cultures of foodborne pathogens that pose a significant public health issue is summarized in Table 3. After subjecting a pure culture of *Salmonella enteritidis* to a 2-minute SAEW (with a pH range of 6.3–6.5 and an ACC concentration of 2 mg/L) treatment at temperatures of 4, 20, and 45°C, the population decreased to less than 1.0 $\log_{10}$ CFU/ml [57]. When a solution containing SAEW with a chlorine concentration exceeding 4 mg/L was used at temperatures of 4, 20, and 45°C, there was an estimated decrease of 8.2 $\log_{10}$ CFU/ml. It was found that the bactericidal efficacy of SAEW was enhanced with increased chlorine availability, irrespective of temperature [57]. In another study, Guentzel et al. [16] reported a complete inactivation (>6.0 $\log_{10}$CFU/ml reduction) of pure cultures of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*. Likewise, Issa-Zacharia et al. [98] demonstrated that SAEW (pH 5.8, 21 mg/L ACC) successfully decreased the population of *Escherichia coli* and *Staphylococcus aureus* (*in vitro*) by >5 $\log_{10}$CFU/ml after only 1.5 min of exposure, while the treatment of pure culture of *Salmonella* spp. using SAEW (pH 5.6, 23 mg/L ACC) for 1 min resulted in >5 $\log_{10}$CFU/ml of their population [98]. An in vitro assessment was conducted to examine the impact of ACC and pH of SAEW on the inactivation of pathogenic microorganisms. Kim et al. [47] have provided more evidence that SAEW has the ability to completely eradicate pure cultures of *Escherichia coli, Salmonella enterica, Typhimurium, Staphylococcus aureus*, and *Bacillus cereus* spores within a treatment time of 1 minute. The study showed that when the pH value is 6.0 and the concentration of free chlorine is 20 ppm, a 1-minute treatment with SAEW is effective in killing roughly 8-9 log CFU/mL of all foodborne pathogens tested [47]. This further confirms that SAEW can effectively reduce or kill food pathogens in vitro. In the disinfection test on pure culture, exposure of SAEW (ACC 33 mg/L, pH 6.4, and ORP of 834.9 mV) significantly reduced *S. aureus* by 5.8 log CFU/mL in 1 min [49]. In a separate experiment, Li et al. [109] found that exposing *B. subtilis* and *B. cereus* spores to a 5-minute treatment of SAEW with an ACC concentration of 60 mg/L, a pH of 5.89, and an ORP of 930 mV resulted in a substantial decrease in spore levels. The reduction was around 4.94 log CFU/mL for *B. subtilis* and 6.22 log CFU/mL for *B. cereus*. In a similar vein, Kurahashi et al. [4] observed that SAEW with around 30 mg/L of available chlorine exhibited potent sterilizing action, effectively eliminating *Bacillus* spores within 15 minutes. It was found that a concentration of 30 mg/L ACC was able to destroy pure cultures of *E. coli, P. aeruginosa, S. enterica, S. aureus,* and *S. epidermidis* within 15 seconds. In addition, *C.*

cladosporioides was removed within 60 seconds of exposure to the same concentration of SAEW [4]. As recognized by many studies that SAEW has shown strong bactericidal efficacy on foodborne pathogens, therefore SAEW is a promising sanitizer in the food industry.

Table 3

Antimicrobial activity of slightly acidic electrolyzed water against microorganisms in suspension (*in vitro*).

Foodborne pathogen	Time (min)	pH	Physicochemical properties of SAEW			References
ORP (mV)	ACC (mg/L)	Log red. (CFU/ml)	*B. subtilis* and *B. cereus spores*	5	5.89	930
60	>5.0	[105]	*Salmonella enteritidis*	2	6.3	850–900
15	>5.0	[57]	*Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Listeria monocytogenes, Escherichia faecalis*	≥5	6.5	800
20	>6.0	[16]	*P. aeruginosa, S. enterica, S. aureus,* and *E. coli (O157:H7)*	0.25	5.9	800
30	>5.0	[4]	*Staphylococcus aureus, Escherichia coli 0157:H7, Listeria monocytogenes*	≥5	6.3	500
5	>5.0	[106]	*C. cladosporioides*	1	5.9	800
30	>5.0	[4]	*S. enteritidis, E.coli O157:H7,* and *S.aureus*	≥5	5.74	902
60	>3.0	[91]	*Escherichia coli* and *Staphylococcus aureus*	1.5	5.8	948
21	>5.0	[98]	*Pseudomonas deceptionensis*	1	5.9	945

64	>5.0	[107]	*Escherichia coli, Staphylococcus aureus,* and *Salmonella* spp.	1	5.6	940
23	>5.0	[98]	*Escherichia coli*	≥5	6.5	850
20	>6.0	[16]	*Listeria monocytogenes*	≥5	6.5	850
20	>6.0	[16]	*Salmonella typhimurium*	≥5	6.5	850
20	>6.0	[16]	*Staphylococcus aureus*	1	6.4	835
33	>5.0	[49]	*Escherichia coli, Salmonella enterica, Salmonella typhimurium, Staphylococcus aureus, and Bacillus cereus spores*	1	6	850
20	>8.0	[47]	*Listeria monocytogenes*	≥5	6.5	850
20	>5.0	[76, 108]	*B. cereus (spores), B. subtilis (spores)*	15	5.9	800

7.2. Inactivation of Foodborne Pathogens on Food Products (In Vivo) Using SAEW

The phytochemical components and numerous nutrients included in fruits and vegetables help to lower the chance of developing chronic diseases, making them an essential component of a healthy diet. The storage quality of fresh-cut fruits and vegetables is greatly impacted by microbes, which must be controlled. Researchers from all over the globe have explored nonthermal processing methods to reduce the amount of microbes on various foods, including meat, poultry, and aquatic products, since traditional thermal sterilization methods do not work well enough [45]. The utilization of slightly acidic electrolyzed water as a sterilization technique has attracted considerable attention owing to its exceptional efficacy in disinfection and its environmentally friendly characteristics [47, 99, 110]. SAEW has attracted significant scientific interest in recent years due to its almost neutral pH range of 5.0–6.5 and low ACC levels of 10–30 mg/L. Its powerful disinfection capabilities on food products and surfaces that come into contact with food have been acknowledged by researchers [12, 108]. Studies have now demonstrated the strong antibacterial activity of SAEW against foodborne pathogens on several foods and agricultural products as seen in Tables 4 and 5. Table 4 summarizes the antimicrobial effectiveness of SAEW on different fresh fruits and vegetables, while its effectiveness on fresh red meat, poultry, and aquatic products is shown in Table 5. Research undertaken by Ding et al. [104], Mansur et al. [117], and Zhang et al. [7] has shown that SAEW treatment is highly successful in reducing the presence of mold, bacteria, and viruses in fresh produces, which are potential causes of food poisoning. The application of a 10-minute SAEW treatment resulted in a reduction of aerobic bacteria by 3.29 and 3.59 $\log_{10}$ CFU/g

in cherry tomatoes and strawberries, respectively. In addition, there was a decrease of 2.32 and 3.01 log in yeast and mold, respectively [104]. Suzuki [121] investigated the effectiveness of SAEW on both whole and cut carrots, Japanese radishes, onion, sweet potatoes, burdock, lettuce, cucumber, strawberries, tomatoes, and eggplant. Washing with SAEW effectively reduced total aerobic bacteria from tested produces by 1 to 2 $\log_{10}$CFU/g with the skin not removed, while the similar test resulted in 3.0 to 4.5 $\log_{10}$CFU/g when the skin was removed before washing treatment [122]. Other studies reported a more than 2 $\log_{10}$CFU/g of total aerobic bacteria from spinach leaves [123] and 1.3 to 1.6 $\log_{10}$CFU/g reduction of *E. coli* inoculated on Japanese mustard green (*mizuna*) as a result of a 5-minute dip-treatment using SAEW [90]. A similar effect was reported by Koide et al. [12] in which washing fresh-cut cabbage with SAEW reduced total aerobic bacteria by about 1.5 $\log_{10}$CFU/g, while the yeast and mold count was reduced by 1.3 $\log_{10}$CFU/g. Cao et al. [57] investigated the effectiveness of SAEW in treating shell eggs. The SAEW method effectively eliminated *S. enteritidis*, pathogenic bacteria, from deliberately contaminated shell eggs. The eggs were subjected to SAEW treatment for a duration of 3 minutes, resulting in a reduction of 6.5 $\log_{10}$CFU/g. The data suggest that SAEW has potential as a substitute disinfectant for reducing or eradicating bacteria on shell eggs. The inactivation efficacy of slightly acidic electrolyzed pathogens present in spinach leaves was also investigated by Rahman et al. [111] in which SAEW treatment effectively reduced total aerobic bacteria count, yeast and molds, *E. coli*, and *L. monocytogenes* from spinach by 1.93, 1.64, 2.4, and 2.6 $\log_{10}$CFU/g, respectively. Another similar study reported that a 5-minute treatment of lettuce using slightly acidic hypochlorous water (SAEW; pH 5–6.5, 30 mg/L ACC) effectively decreased the total aerobic bacteria by 2 $\log_{10}$CFU/g [112]. In addition, in mung bean seeds and sprouts, SAEW (ACCs of 20 and 80 mg/L) treatment decreased *E. coli* and *S. enteritidis* by 1.27–1.76 and 3.32–4.24 $\log_{10}$CFU/g and 3.12–4.19 $\log_{10}$CFU/g, respectively [124]. In a recent study by Song et al. [5], an experimental verification was conducted on fresh cabbage to test the effectiveness of the optimized SAEW treatment. A reduction of 5.94 ± 0.07 $\log_{10}$CFU/g of *Pectobacterium carotovorum* subsp. *Carotovorum* was observed following this treatment.

Table 4

Antimicrobial activity of slightly acidic electrolyzed water on different fruits and vegetables (*in vivo* application).

Food product	Indicator bacteria	log red$^{\dagger CFU/g}$	Effectiveness	References
Spinach	*Escherichia coli O157:H7*	2.40	+++	[111]
Spinach	*Listeria monocytogenes*	2.80	+++	[111]
Spinach	*Yeast and molds*	1.64	++	[111]
Spinach	Aerobic bacteria counts	1.93	++	[111]
Cut cabbage	Aerobic bacteria counts	1.50	++	[12]
Cut cabbage	Yeast and molds	1.30	++	[12]
Lettuce	*Viable bacteria count*	2.00	++	[112]
Lettuce	*Enterococcus faecalis*	2.80	+++	[16]
Spinach	*Escherichia coli O157:H7*	2.49	+++	[111]

Sliced carrot	Aerobic bacteria counts	2.20	+++	[60]
Sliced carrot	Yeast and molds	1.90	++	[60]
Chinese cabbage	*E. coli/L. monocytogenes*	1.22/1.19	++	[38]
Lettuce	*E. coli/L. monocytogenes*	1.23/1.20	++	[38]
Sesame leaf	*E. coli/L. monocytogenes*	1.15/1.31	++	[38]
Spinach	*E. coli/L. monocytogenes*	1.12/1.48	++	[38]
Cabbage	*Pectobacterium carotovorum subsp. carotovorum*	5.94	++++	[5]
Cherry tomato	*Total bacteria*	3.29	+++	[104]
Cherry tomato	*Yeast and molds*	3.59	+++	[104]
Strawberry	*Total bacteria*	2.32	+++	[104]
Strawberry	*Yeast and molds*	3.01	+++	[104]
Celery	*Total aerobic bacteria*	4.33	++++	[105]
Celery	*Yeast and molds*	3.86	+++	[113]
Celery	*Escherichia coli*	2.74	+++	[46]
Celery	*Salmonella* spp.	2.87	+++	[46]
Cilantro	*Total aerobic bacteria*	4.14	++++	[105]
Cilantro	*Yeast and molds*	3.75	+++	[105]
Lettuce	*Escherichia coli*	2.84	+++	[46]
Lettuce	*Total viable count*	1.9	++	[76]
Lettuce	*Salmonella* spp.	2.91	+++	[46]
Lettuce	*Total microbial count*	1.9	++	[114]
Cabbage	*Total microbial count*	1.5	++	[12]

Cabbage	*Yeast and molds*	1.3	++	[12]
Cilantro	*Escherichia coli O78*	2.49	+++	[103]
Cilantro	*Bacillus subtilis I.1849*	1.54	++	[103]
Shell eggs	*Salmonella enteritidis*	6.5	++++	[57]
Spinach	*Salmonella typhimurium*	2.14	+++	[16]
Spinach	*Listeria monocytogenes*	2.94	+++	[16]
Spinach	*Enterococcus faecalis*	2.86	+++	[16]

++++, bacterial reduction being more than 4 log CFU/g; +++, bacterial reduction being between 2 and 4 CFU/g; ++, bacterial reduction being between 1 and 2 CFU/g; +, bacterial reduction being less than 1 log CFU/g.

Table 5

Antimicrobial activity of slightly acidic electrolyzed water on meat, poultry, and aquatic products (*in vivo* application).

Food product	Indicator bacteria	Log red[†CFU/g]	Effectiveness	Reference
Fresh pork	*Listeria monocytogenes*	1.8	++	[115]
Fresh pork	*Listeria monocytogenes*	1.7	++	[116]
Fresh pork	*Escherichia coli*	1.7	++	[115]
Fresh pork	*Escherichia coli*	1.2–1.6	++	[117]
Fresh pork	*Listeria monocytogenes*	1.7	++	[115]
Fresh pork	*Salmonella typhimurium*	1.2–1.6	++	[117]
Fresh pork	*Staphylococcus aureus*	1.2–1.6	++	[117]
Chicken carcass	*Listeria monocytogenes*	2.3	+++	[31]
Chicken carcass	*Staphylococcus aureus*	1.9	++	[31]
Chicken carcass	Total viable count	1.5	++	[31]
Shell eggs	Coliforms	1.4	++	[91]
Shell eggs	*Escherichia coli O157H7*	2.7	+++	[91]

Shell eggs	*Staphylococcus aureus*	2.8	+++	[91]
Fresh beef	*Escherichia coli*	1.1–2.1	++	[118]
Fresh beef	*Listeria monocytogenes*	1.1–2.2	++	[118]
Fresh beef	*Salmonella typhimurium*	0.7–1.8	+++	[118]
Fresh beef	*Staphylococcus aureus*	0.8–1.6	+++	[118]
Fresh beef	*Total viable count*	1.9–2.7		[118]
Shrimp	*Vibrio parahaemolyticus*	4.41	++++	[119]
Crab	*Vibrio parahaemolyticus*	4.06	++++	[119]
Shellfish	*Escherichia coli*	3.1	+++	[120]
Shellfish	*Listeria monocytogenes*	3.1	+++	[120]
Shellfish	*Vibrio parahaemolyticus*	3.1	+++	[120]

++++, bacterial reduction being more than 4 log CFU/g; +++, bacterial reduction being between 2 and 4 CFU/g; ++, bacterial reduction being between 1 and 2 CFU/g; +, bacterial reduction being less than 1 log CFU/g.

The effectiveness of SAEW on fresh red meat, poultry, and aquatic products is shown in Table 5. SAEW has been proposed as a potential sanitizer to be used for sanitization of egg shells as an environmentally friendly disinfection agent [57, 91]. After a 3-minute SAEW treatment, the number of *S. enteritidis* CFU/g on shell eggs was reported to decrease by 6.5 $\log_{10}$ CFU/g, and no *S. enteritidis* survival was detected in the waste wash SAEW [57]. Rahman et al. [31] conducted a study to assess the effectiveness of SAEW in reducing *L. monocytogenes* and *S. typhimurium* on fresh chicken breast flesh. According to this study, when exposed to a 10-minute treatment of SAEW with 10 ppm of active chlorine at a temperature of 23°C, there was a decrease of 2.32 $\log_{10}$ CFU/g for *L. monocytogenes* and a decrease of 1.9 $\log_{10}$ CFU/g for *S. typhimurium*.

In other study by Tango et al. [118], the SAEW treatment (pH 6.3, ORP 820–934 mV, and ACC 25 mg/L) demonstrated a substantial sanitization effect against *S. aureus*, *L. monocytogenes,* and *E. coli O157:H7* on fresh beef. In addition, a decrease in bacterial counts by 0.63–2.52 $\log_{10}$ CFU/g with increases in the contact time was reported [118]. Few studies have been carried out on the use of SAEW to control bacteria in pork. A variety of bacteria found in pork products have the potential to spread foodborne illnesses, which in turn can harm both people's health and the economy. According to Rahman et al. [115], fresh pork treated with SAEW or AEW demonstrated improved microbiological stability, longer shelf life at different temperatures, and minimal impact on sensory quality. Fresh pork treated with SAEW (pH 6.8, ORP 700–720 mV, and ACC 10 mg/L) was found to inactivate *E. coli O157:H7* and *L. monocytogenes* just as well as with AEW. In addition, Mansur et al. [117] have demonstrated the efficacy of SAEW against *E. coli, L. monocytogenes, Salmonella typhimurium,* and *S. aureus,* which are commonly found in pork products. According to the current research on food safety, it can be concluded that although the use of SAEW reduced bacterial levels in fish and animal-based foods to some extent, it is important to prioritize strict manufacturing and slaughter hygiene practices as vital elements of a comprehensive food safety system to guarantee the production of safe products [125]. SAEW has therefore emerged as a promising

and new approach, especially in agricultural contexts, for the purpose of sterilizing fresh-cut fruit, vegetables, meat, poultry, and aquatic items.

8. Effect of Slightly Acidic Electrolyzed Water on Postharvest Quality Control of Fruits and Vegetables

Fruits and vegetables recently harvested are very perishable and susceptible to deterioration throughout the process of production, transportation, and storage. Microbes are the primary determinant of the storage quality of fresh-cut fruit and vegetables. Microbial infection, physiological aging, nutritional loss, tissue discoloration, texture softening or lignification, and flavor deterioration can all be linked to the mechanical damage caused to cells and tissues during peeling and cutting procedures. These factors detrimentally affect the storage quality and diminish the longevity of the product [126].

Up to now, research on the application of SAEW in the field of fresh-cut and vegetables has mainly focused on the bactericidal effects on surface microbes, but there are relatively few reporting the effects of SAEW on the postharvest physiology, quality, and storage properties of fruits and vegetables on storage. Despite their limited number, their research has shown promise in the use of SAEW in improving the quality of fruits and vegetables.

It has been reported that SAEW treatment on fresh-cut fruits and vegetables shows a positive impact on micronutrients, sugar content, color, and other sensory quality parameters. The study conducted by Gao et al. [45] demonstrated that in comparison with the control group, SAEW treatment resulted in much higher total sugar content in the treated fresh-cut apples. In addition, treatment of fresh-cut apples with SAEW prevented them from changing in color, which in turn slowed down their browning and exerted a certain protective effect on the color [45]. A key indication of the quality change that occurs in fresh-cut apples when they are stored is their total sugar level, which affects the color, aroma, taste, texture, and nutritional value of these fruit items. A recent study by Gao et al. [45] revealed that SAEW treatment not only exhibited a satisfactory bactericidal effect on the surface microbes of fresh-cut apples but also did not adversely affect the apples' sensory qualities. In addition, SAEW treatment on fresh-cut apples mitigated the degradation of vitamin C, decreased weight loss and browning processes, and preserved the pH levels of the tissues. Consequently, this treatment effectively retards the deterioration of crucial quality parameters during storage, thereby extending the shelf life of fresh-cut apples [45]. In their study, Ling et al. [127] found that weakly acidic electrolyzed water effectively decreases the activity of *polyphenol oxidase*, hence preventing the browning process in *Zizania latifolia.* The observed less color alteration in fruit treated with SAEW, as compared to fruit treated with sterile water, may be attributed to the antioxidant properties of vitamin C, as noted by Gao et al. [45]. In a similar investigation, the application of SAEW (with a pH value of 6.0, ORP of 1340 mV, and ACC of 80 mg/L) to carambola fruit was found to effectively decrease the rate of respiration, hinder the increase in cell membrane permeability, and delay visible color alteration [36]. This suggests that treating fruits with SAEW can improve postharvest quality of fruits. Zhang et al. [36] further observed that SAEW treatment of carambola fruit resulted in higher levels of bioactive compounds and nutritional components, including polyphenols, reducing sugars, flavonoids, total soluble sugar, sucrose, vitamin C, and total soluble solid (TSS). In addition, the treated fruit exhibited increased titratable acidity (TA). Based on these findings, Zhang et al. concluded that SAEW treatment enhances the quality of carambola fruit [36]. These findings align with earlier research that have shown that SAEW treatment can enhance the nutritional markers of pea sprouts, including vitamin C, total protein, and soluble sugar [70]. According to a recent study by Zhao et al. [128], SAEW treatment improved nutritional indices of fresh-cut kiwifruit by lowering TA levels and suppressing the starch-to-sugar conversion. In addition, SAEW can increase the amounts of total phenols and flavonoids in fresh-cut kiwifruit, which boosts its antioxidant capacity [94]. Furthermore, according to Lin et al. [129], SAEW treatment can delay the decrease in in total phenolic content in eggplant. Furthermore, Li et al. [130] found that SAEW treatment of broccoli sprouts increased their antioxidant capacity and nutritional profile by accumulating the essential amino acid proline, phenolic acids, and flavonoids, among other things. In addition, treatment with SAEW (ACC 50 mg/L) resulted in the highest total phenolic acid concentration in broccoli sprouts and enhanced their concentration of phenolic acids [130]. Furthermore, Zhang et al. [36] claimed that carambola treated with SAEW demonstrated an elevated level of commercial acceptability and firmness, while experiencing reduced weight loss and peel browning index compared to the untreated fruits. In light of these

findings, it was proposed that the application of SAEW treatment resulted in superior fruit quality and nutritional content, which could potentially enhance the storage properties of harvested carambola. According to Zhang et al. [36], it was speculated that SAEW treatment might possibly induce the production of flavonoids and polyphenols, which would therefore delay the senescence of carambola fruit while maintaining its quality. According to Li et al. [130], SAEW has the potential to promote the accumulation of phenolic compounds in broccoli sprouts, making it an attractive inducer for bioactive compound-focused food industries. Therefore, SAEW could be a potential and useful strategy for boosting the accumulation of bioactive compounds in plants if applied extensively [109].

9. Conclusion

The advent of novel slightly acidic electrolyzed water (SAEW) has effectively mitigated the corrosion challenges associated with StAEW and AEW. Developed by Japanese companies over two decades ago, SAEW received endorsement as a food additive by the Japanese Ministry of Health, Labor, and Welfare in 2002 and subsequent approval as a control agent in 2014 by the Ministry of Agriculture, Forestry, and Fisheries, along with the Ministry of the Environment. Its rapid integration within the realm of food sanitation for agriculture and the food industry is underpinned by its potent antimicrobial properties, stemming from a substantial hypochlorous acid concentration. Marked as an eco-friendly disinfectant, SAEW (pH 5–6.5; 10–30 mg/L ACC) emerges as a commendable solution, curbing the deleterious impact of chlorine residues on human and environmental well-being. SAEW not only excels in its preservation capabilities during storage but also exhibits minimal influence on pH, surface aesthetics, and overall appearance of fresh-cut produce. Notably divergent from StAEW, SAEW exhibits significantly reduced tendencies to corrode processing equipment, cause skin irritations, induce phytotoxicity in plants, or generate safety concerns through chlorine off-gassing. Consequently, SAEW emerges as an innovative and auspicious avenue, particularly in agricultural settings for sterilizing of fresh-cut fruit, vegetables, meat, poultry, and aquatic products. Moreover, its applicability extends to the food processing industry and household kitchens, serving as a reliable agent for disinfecting food materials and processing equipment.

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DETAIL

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Characterization of Amino Acid Composition, Nutritional Value, and Taste of Fruits from Different Actinidia arguta Resources

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ABSTRAK (ENGLISH)

The nutritional value and flavor and texture characteristics of fruits from different *Actinidia argute* resources were scientifically evaluated and compared. Using 35 *A. arguta* fruits as materials, the amino acid composition and content were determined by an automatic amino acid analyzer, and differentiation analysis, amino acid nutritional value evaluation, TAV flavor analysis, correlation analysis, PCA comprehensive evaluation, and cluster analysis were conducted to clarify the diversity of *A. arguta* resources in terms of amino acid content, composition, and flavor characteristics. Analysis of differential results showed that the *A. arguta* resource fruits contained 17 amino acids with a total amino acid content of 384.20~2590.56 mg/100 g. The results of the nutritional value evaluation showed that the Leu of the fruits of the *A. arguta* resources all conformed to the ideal model proposed by FAO/WHO, and the Leu content of all the resources exceeded the human body’s needs, and it was also found that the first limiting amino acid of the Actinidia argute resources was Ile and the second limiting amino acid was Lys. TAV of the flavor-presenting amino acids was calculated to evaluate the flavor-presenting taste characteristics, and the amino acids that influenced the flavor of *A. arguta* fruit were Glu and Cys. PCA showed that the 2 principal components could better reflect the comprehensive information of amino acids in *A. arguta*, and the cumulative variance contribution rate was 87.88%, which could represent the main trend of amino acids in *A. arguta*. A comprehensive amino acid

evaluation model was established, and the composite scores indicated that the top 5 excellent resources were S4, S10, S18, S25, and S30. Hierarchical cluster analysis classified the 35 *A. arguta* resources into 4 categories, which better reflected the differences in amino acid content and composition, nutritional value, and taste characteristics among *A. arguta* fruits from different collection sites.

TEKS LENGKAP

1. Introduction

The *Actinidia argute* ((Sieb. &Zucc) Planch. ex Miq.) belongs to the kiwifruit family (Actinidiaceae Gilg &Werderm.), the kiwifruit genus (Actinidia Lindl), alias soft jujube, kiwifruit, and kiwifruit pear, which is a large deciduous vine [1, 2]. Its wild germplasm resources are mainly distributed in China, Japan, the Korean Peninsula, and the Russian Far East [3, 4], and in China, it is distributed in the northeast, north, and northwest of China in the Yangtze River Basin as well as in Taiwan [5, 6]. Its fruits are crisp, juicy, and tasty when eaten fresh and are rich in nutrients such as vitamin C, protein, amino acids, minerals, and dietary fiber [2, 7]. In addition to this, it is also of extremely high medicinal value, and its fruits are rich in active ingredients such as polysaccharides, polyphenols, alkaloids, volatile oils, and proanthocyanidins [8], which are antitumor, antiradiation, antioxidant, antiaging, hypoglycemic, anti-inflammatory, insomnia inhibitor, immunity enhancement, laxative, and other effects [9–12]. Wojdylo and Nowicka [13] found that *A. arguta* polyphenolic compounds could play a role in the treatment of diabetes after an in vitro antidiabetic experimental study. Xu [14] et al. found that *A. arguta* can significantly improve the constipation caused by montelukast in mice and increase the amount of food intake and the number of defecation, with a laxative effect. Nowadays, *A. arguta* is loved by the public and welcomed by the market because of its rich nutritional and medicinal value, and its fruit is often used to make processed foods such as dried fruit, dried fruit, fruit wine, fruit jam, canned food, fruit juice, fruit vinegar, or pectin oral liquid [15].

Amino acids, as an important compound in the body, are mainly involved in protein synthesis, metabolism, and immune response. In addition, it is an important bioactive component that can be used as a pharmacological component to regulate various physiological activities, and it has been shown that amino acids not only have a role in cancer metabolism but also have important roles such as redox balance, energy regulation, and homeostasis maintenance [16], and even preventive and therapeutic functions, and they can also act in coordination with hormones and play an important role in the control of gene expression [17–20]. For example, alanine, aspartate, and glutamate play a variety of roles as the main substrates for glucose synthesis in the liver, influencing the immune function in humans and animals [21]. Proline is an essential component of collagen and extracellular matrix and plays an important role in gene expression, cellular signaling, cellular redox, synthesis of polyamines, glutamate, and collagen [22], and it plays an important role in the regulation of dehydration stress, redox, and cell proliferation [23]. Glycine is a potent antioxidant that scavenges free radicals required for leukocyte proliferation and antioxidant activity, reducing inflammatory responses and pathogens in animals [24]. Amino acids often exist in two forms in plants: one is in the form of a bound state in peptides and proteins; the other is in the form of a free state [25], which can help plants to form organs and a variety of active substances during the growth process [26]. At the same time, plant amino acid crops, components of plant-based proteins, are an important source of dietary protein for humans and are recognized as a continuous source of nutrients to meet human needs, and the intake of plant-based proteins has potential benefits for the health of the human organism in terms of lowering the risk of chronic diseases, reducing deaths due to disease, and increasing the intake of plant proteins may also slow down unhealthy aging. Among them, free amino acids can be directly absorbed by the human body, and their content and composition can not only reflect the nutritional value of food, which is an important indicator for evaluating the nutritional value of food; they also have a close relationship with the flavor quality of food [27, 28]. At present, there have been some studies on the amino acids of *A. arguta* fruits, but they are mainly focused on their contents and components [29, 30], and there are almost no reports on the evaluation of their nutritional value and flavor characteristics; at the same

time, there is a lack of systematic and rigorous statistical and comprehensive evaluation of amino acids of *A. arguta* resource fruits. Therefore, in-depth research on the types and contents of amino acids and their nutritional value of *A. arguta* resources is not only of great significance to the systematic and comprehensive evaluation of *A. arguta* resources but also of theoretical significance to the development of functional products that are beneficial to human health.

At present, for the analysis of fruit amino acid detection and analysis of commonly used methods are ninhydrin colorimetric method, HPLC, GC, GC-MS, near-infrared spectroscopy, amino acid analyzer, and electrochemical analysis [31–36]. The amino acid analyzer is a fully automated special analytical instrument for amino acid separation, derivatization, and detection, using cations in the exchange column separation, postcolumn ninhydrin derivatization, and diode photometer detection, with the ability to be able to be equipped in the general laboratory, good selectivity, specificity, high sensitivity, easy to operate, separation and reproducibility of the better, simple sample pretreatment, and the ability to carry out the advantages of batch testing [37–39]. Methodology for statistical analysis of data such as principal PCA modeling, TAV, and HCA is commonly used when testing and analyzing fruit amino acids using an amino acid analyzer. PCA is based on the principle of KL transformation, and through the way of dimensionality reduction, multiple variables are simplified into a few composite variables, so that the existing few composite variables can directly reflect the information of the original variables [40]. TAV can be used to assess the contribution of individual components to the flavor, and compounds with a TAV value greater than 1 can be regarded as components that contribute significantly to the overall flavor [41, 42]. HCA is the process of calculating the similarity between samples by means of criteria that have been determined, simplifying and combining them by means of the degree of correlation, and dividing the similar analyzed samples into different groups for a comprehensive evaluation based on their respective characteristics [43]. Currently, amino acid analyzer testing combined with PCA, TAV, and HCA multiple regression analysis has been widely used for amino acid testing and comprehensive evaluation of food quality [27, 44–47]. Jian [48] et al. used PCA to analyze the hydrolyzed amino acids and free amino acids of five edible mushroom powders and established a comprehensive evaluation model, and the comprehensive evaluation found that the comprehensive amino acid quality of the edible mushroom powder of tea tree mushroom was the best. Lin [49] et al. used PCA and HCA to analyze the free amino acids of the fruits of 15 hybrid citrus varieties, and the results showed that the results of the two analytical methods were basically the same and could better reflect the variability of amino acid fractions among varieties.

Amino acid analyzer assay combined with PCA, TAV, and HCA multiple regression analysis has rarely been reported in the detection and evaluation of amino acids in *A. arguta* resource fruits. Therefore, in this study, the amino acid analyzer was used to isolate and detect the amino acids of 35 different *A. arguta* resource fruits, to analyze the differences in their contents, to evaluate the changes in their nutritional value by using the amino acid ratio coefficient method, and to analyze and comprehensively evaluate the amino acid quality indexes by using the taste activity value (TAV), correlation analysis, PCA, and HCA, to compare the differences between the different resources in the nutrient composition and taste characteristics of the fruits. The results of the study provide a scientific basis for revealing the nutritional value and taste characteristics of *A. arguta*, a theoretical reference for the screening of excellent *A. arguta* resources and product development and utilization.

2. Materials and Methods

2.1. Materials and Reagents

2.1.1. Materials

The 35 resources selected for this study were harvested in September 2022 at the fruit ripening stage from the National Forest Germplasm Resource Bank of *A. arguta* and Schisandra chinensis. About 300 g of fruit was picked from each resource, and the samples were placed in separate corresponding sampling bags and transported back to the laboratory in an insulated box. After testing the relevant indexes on the same day, the remaining part of the sample was frozen and ground into powder with liquid nitrogen and then stored at −80°C in ultralow temperature for amino acid testing.

2.1.2. Reagents

The reagents are hydrochloric acid, phenol, and sodium citrate analytical purity (Shanghai, Sinopharm Chemical Reagent Co., Ltd.), sodium hydroxide and sodium chloride superior purity (Beijing, Beijing Beihua Fine Chemicals Co., Ltd.), and 17 kinds of L-amino acid mixed standards (Wako, Japan).

2.2. Instruments and Equipment

Instruments and equipment are as follows: DFT-50A 50 g portable high-speed pulverizer (Wenling Linda Machinery Co., Ltd.); L-8900 amino acid auto-analyzer (Hitachi, Japan); MS204S electronic analytical balance (Mettler Toledo, Switzerland); DZF6090 vacuum drying oven (Shanghai Pudong Rongfeng Scientific Instrument Co., Ltd.); DHG-9240A temperature drying oven (Shanghai Yihang Science &Technology Co., Ltd.); Milli-Q Advantage A1 ultrapure water apparatus (Millipore Corporation, U.S.A.).

2.3. Methodology

2.3.1. Chromatographic Detection Conditions

Column is 4.6 mm × 60 mm ion exchange column; flow rate is 0.40 mL/min (pump1) and 0.35 mL/min (pump2); detection wavelengths are 570 nm and 440 nm; column temperature is 135°C; injection volume is 20 μL, mobile phase eluted according to the gradient table (Table 1), and analysis time is 40.2 min.

Table 1

Gradient elution program.

Time (min)	PH-1	PH-4	PH-RG	PH-2
0	100	0	0	0
0.1	0	100	0	0
14.2	0	100	0	0
14.3	0	0	100	0
20.2	0	0	100	0
20.3	0	0	0	100
21.2	0	0	0	100
21.3	100	0	0	0
40.2	100	0	0	0

2.3.2. Amino Acid Assay of *A. arguta* Samples

Refer to GB/T 5009.124-2016 [50]. Accurately weighed 2.50 g of *A. arguta* sample, added 20 mL of hydrochloric acid solution with a concentration of 6 mol/L, and hydrolyzed in a constant temperature oven at 110°C for 22 h. Then, all the hydrolyzed solution was transferred to a 50 mL volumetric flask, and the volume was fixed with primary water. After taking 2 mL of the above solution and evaporating it under reduced pressure, it was dissolved with 2 mL of hydrochloric acid solution at a concentration of 0.02 mL/L and then filtered through an aqueous filter membrane of 0.22 μm and analyzed. Each sample was separated and detected by an L-8900 amino acid autoanalyzer, and all samples were repeated three times.

2.3.3. Nutritional Evaluation of Amino Acids

According to the International Standard Reference Model for the needs of older children, young people, and adults aged >3 years proposed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) in 2013 [51], E/T (%) = EAA/TAA, E/N (%) = EAA/NEAA [52], E/N (%) = EAA/NEAA [53], E/T (%) = EAA/TAA, E/N (%) = EAA/NEAA [52], M/T (%) = MAA/TAA, BC/E (%) = BCAA/EAA, and BC/A = BCAA/AAA [21].

The amino acid ratio coefficient method was proposed by Shengtao and Kun [53] to calculate the ratio of amino acid (RAA), the ratio coefficient of amino acid (RC), and the score of RC and SRC [51].(1)RAA=content of an essential amino acid in the protein to be measured/mg/g corresponding amino acid content in the reference protein pattern/mg/g,RC =amino acid RAA RAA average,SRC=100−100×CV.Eq: CV is the coefficient of variation of the RC and CV = standard deviation/average number.

2.3.4. Taste-Presenting Amino Acid Analysis

Taste-presenting amino acids can be classified as fresh, sweet, bitter, and aromatic amino acids, and taste active value (TAV) refers to the ratio of the value of the content of taste-presenting substances to the taste threshold of taste-presenting substances [54].

2.4. Statistical Analysis of Data

The experimental data were organized for statistics using Excel 2016, and ANOVA, principal component analysis, was performed using SPSS (version 23.0, IBM, Armonk, NY, USA). PCA, correlation analysis, and HCA were performed using Origin 2021 and OmicShare tools.

3. Results and Discussion

3.1. Chromatographic Analysis

According to the chromatographic detection conditions, the chromatographic separation of 17 amino acid standards using amino acid autoanalyzer is shown in Figure 1. Chromatogram of 17 amino acid standard samples, proline, was measured at the wavelength of 440 nm, and the rest of 16 amino acids were measured at the wavelength of 570 nm, and the 17 amino acids achieved a very good separation effect under the separation conditions. The samples to be tested were processed for amino acid determination as shown in Figure 2. Amino acid chromatogram of *A. arguta*, a comparison of the chromatographic separations of the samples with those of the amino acid standards, revealed that the *A. arguta* fruit samples contained 17 amino acid species.

[figure(s) omitted; refer to PDF]

3.2. Analysis of Amino Acid Composition and Content of Fruits of Different *A. arguta* Resources

The different amino acids of 35 *A. arguta* resources were statistically analyzed, and the results are shown in Table 2. Amino acid composition and content of different resources of *A. arguta* mg/100 g. All 35 *A. arguta* contained 17 amino acids, including 7 EAAs, 10 NEAAs, 2 CEAAs, and 9 MAAs. The 7 EAAs are Thr, Val, Met, Ile, Leu, Phe, and Lys, the 10 NEAAs are Asp, Ser, Glu, Gly, Ala, Cys, Tyr, His, Arg, and Pro, the 2 CEAAs are His and Arg, and the 9 MAAs are Asp, Glu, Gly, Met, Ile, Lue, Phe, Lys, and Arg. The variation of standard deviation of 17 amino acids was 9.68~146.87, and the coefficient of variation was 40.94%~77.74%, which indicated that the content of different amino acids differed significantly among resources, among which the coefficient of variation of Pro was the largest, and the coefficient of variation of Gys was the smallest, and at the same time, the results of the analysis of variance showed that each amino acid differed significantly among most of the A. arguta resources; the variation of the mean value was 18.16~222.22 mg/100 g, with the lowest content of Met and the highest content of Glu. Glu, as an indispensable amino acid during the critical period of life, including the period of fast-growing newborns, has the ability to enhance the immune function of the immune cells [55], and it can also be used to treatment of liver-related diseases such as hepatic coma and hepatic insufficiency [56], and glutamine, which is formed by combining with blood ammonia, also contributes to the repair of traumatized organisms and the treatment related to peptic ulcers [57], so appropriate consumption of *A. arguta* fruits can improve immunity, while, in the future, it can be developed as a healthcare product for liver protection and repair of ulcers.

Table 2

Amino acid composition and content of different resources of *A. arguta* mg/100 g.

Name	Content mg/100g							
Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val	·
S1	71.87±2.26pq	37.76±0.72kl	35.47±0.65l	113.03±5.71no	13.00±0.18p	34.62±0.20no	9.28±0.24opq	44.75±2.11pq
S2	69.18±2.47q	40.28±1.54k	34.66±1.48lm	75.43±3.29rs	13.02±0.49p	27.10±1.27p	9.40±0.06opq	42.82±1.51qr
S3	39.60±0.52vw	22.17±1.16qr	20.43±0.72s	52.19±0.65t	8.52±0.11q	18.51±0.07q	8.32±0.19qr	30.34±0.90w
S4	312.88±4.55b	134.31±1.47a	127.57±2.04a	534.73±6.69a	8.32±0.01q	185.33±2.99a	33.70±1.33cd	177.77±1.24a
S5	80.87±1.64no	35.60±0.74lm	35.88±0.95l	127.68±2.85m	40.28±0.94j	52.97±1.94k	9.12±0.30opqr	44.53±0.20pq
S6	36.28±0.73vwx	20.72±0.99r	27.31±0.22pq	94.77±2.14pq	23.21±0.63mn	24.63±0.61p	22.97±0.24m	32.16±0.48vw
S7	42.72±2.11uv	24.35±0.32pq	28.74±0.18op	106.38±1.54op	25.90±0.03m	35.25±0.93no	20.47±0.26n	34.85±1.48uv
S8	355.31±12.59a	82.36±0.77de	92.32±2.09c	408.42±10.31c	99.66±0.91f	132.68±1.56c	25.36±0.41kl	99.45±2.83j
S9	109.87±5.76k	45.46±0.89j	50.02±0.61j	251.86±7.02hi	55.11±0.32i	87.05±0.23h	33.52±1.09cde	70.36±3.09lm
S10	219.45±7.22e	96.87±3.06b	100.81±3.54b	407.83±15.32c	146.36±5.88a	145.62±5.30b	30.86±1.08f	155.96±2.25b
S11	30.16±1.42x	17.23±0.19s	20.65±0.79s	87.52±3.81qr	21.07±0.46no	25.16±0.48p	33.17±0.15de	42.34±1.30qr
S12	51.86±2.57st	24.37±0.24pq	24.97±0.86qr	153.76±3.18l	24.87±0.94mn	27.93±0.89p	26.56±0.03ijk	39.16±0.75rst
S13	68.86±3.28q	26.87±1.46op	32.52±0.89mn	126.92±0.01m	34.11±1.57kl	38.56±0.29mn	23.32±0.29m	40.41±1.22rs
S14	149.91±6.80i	69.81±2.49f	79.07±3.36g	351.21±11.28fg	98.72±3.61f	105.26±3.38f	26.12±0.80jkl	104.61±2.18j

S15	198.07±9.35f	80.77±3.26e	88.91±3.78de	378.82±15.11d	110.21±6.13e	118.16±6.05e	32.26±1.03e	115.07±4.16g
S16	242.42±7.40d	96.52±3.40b	99.01±2.49b	421.17±12.46b	122.42±3.10c	130.46±3.15c	26.97±0.75ij	127.71±4.77e
S17	98.22±4.19l	50.82±2.11h	55.18±1.96i	200.86±6.95j	66.67±3.05h	63.46±2.87i	30.77±1.76f	81.42±1.40k
S18	271.47±7.65c	93.52±5.30c	101.87±2.85b	361.17±2.76ef	136.76±5.43b	124.77±2.26d	27.77±1.05hi	145.27±5.65c
S19	38.12±1.31vwx	19.86±0.93rs	22.52±0.71rs	112.86±5.15no	18.42±0.20o	24.51±0.00p	34.57±0.24bc	37.46±1.08stu
S20	91.02±0.20lm	46.72±1.79ij	48.91±0.29j	168.16±7.55k	51.32±0.61i	63.97±1.06i	26.91±0.00ij	69.22±1.40m
S21	58.92±2.40rs	33.32±2.21mn	32.07±0.96mn	107.26±0.43op	37.47±1.35jk	33.07±0.25o	25.17±0.55l	44.76±1.37pq
S22	182.87±5.25g	84.92±2.90d	87.52±2.21e	374.21±10.18de	115.27±3.65d	105.71±2.90f	30.81±1.22f	117.82±2.30g
S23	60.57±1.25r	32.42±1.01n	39.12±0.31k	124.87±3.14mn	41.37±0.66j	44.01±0.90l	34.96±0.17b	55.47±1.25o
S24	134.42±2.61j	71.97±1.75f	78.52±1.59g	262.66±5.76h	90.37±0.55g	107.61±1.50f	25.71±0.52jkl	84.37±1.56k
S25	177.87±3.25g	82.57±0.46de	89.87±2.06cde	382.06±12.47d	118.97±4.26cd	123.02±5.00d	29.57±0.95g	131.56±2.85d
S26	31.41±1.00wx	19.06±0.82rs	21.26±0.25s	70.86±1.04s	20.87±0.84no	20.06±0.12q	22.47±0.05m	24.57±0.86x
S27	159.17±8.86h	80.42±0.90e	84.06±0.47f	249.16±1.55i	97.51±3.83f	95.32±0.70g	25.26±0.67l	108.87±3.74h
S28	164.17±7.84h	71.72±0.51f	90.86±2.63cd	342.22±17.81g	89.47±2.34g	114.81±4.97d	38.37±0.25a	99.87±4.54j
S29	143.17±7.05i	57.96±2.77g	63.87±2.66h	364.07±16.65ef	70.42±0.51h	86.41±0.12h	32.46±1.13de	73.67±2.65l

S30	203.18± 2.95f	91.77± 0.74c	99.37± 1.06b	528.36± 4.53a	117.57± 1.84d	134.17± 1.95c	28.57± 1.24gh	123.26± 0.78f
S31	77.68± 0.56op	32.39± 0.25n	36.39± 0.45kl	106.97± 0.73op	38.97± 0.56j	58.78± 0.65j	10.14± 0.20o	47.14± 1.75p
S32	88.19± 0.04mn	28.14± 0.01o	31.21± 0.02no	100.82± 0.01opq	31.52± 0.07l	40.19± 0.13m	7.98±0.64r	36.32± 1.11tu
S33	50.24± 1.19tu	20.59± 0.46r	22.52± 0.71rs	78.56± 2.53rs	26.52± 0.11m	26.86± 0.33p	6.48±0.14s	28.98± 0.06w
S34	42.42± 0.21uv	21.27± 0.05qr	22.70± 0.13rs	53.92±0.51t	25.57± 0.26m	34.03± 0.88o	8.67± 0.05pqr	29.65± 0.21w
S35	78.78± 0.35op	48.83± 0.08hi	50.58± 0.46j	97.08± 0.14pq	17.47± 0.04o	45.30± 0.27l	9.87± 0.15op	60.77± 0.76n
Stdeva	85	30.41	31.63	146.87	42.58	45.8	9.68	42.35
Average	120.89	52.68	56.48	222.22	58.78	72.44	23.65	74.36
CV%	70.31	57.73	56	66.09	72.43	63.22	40.94	56.95
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Name	Content mg/100g							
Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	·
S1	7.32±0.11o	45.51± 1.27lm	44.83± 2.01opq	47.88±1.64j	35.66± 0.87n	39.33± 1.59mn	14.33± 0.30n	45.72± 1.90l
S2	7.38±0.14o	44.82± 1.80lm	42.02± 1.99pq	37.73± 1.30lmn	35.03± 1.22n	39.66± 1.39mn	18.82± 0.60m	45.07± 2.24l
S3	5.82±0.00p	30.93± 0.11op	32.22± 0.69t	23.92± 1.11p	23.33± 0.81q	26.35± 0.49p	10.78± 0.26op	35.12± 1.60no
S4	39.16± 1.48b	132.77± 2.04a	185.82± 5.81b	159.75± 7.37a	99.03± 0.79a	132.68± 1.67a	92.30± 5.26a	194.77± 3.36a

S5	7.57±0.04o	35.95±0.17n	48.63±0.09no	24.32±0.00p	29.26±0.54o	41.17±1.14lm	17.62±0.99m	40.81±0.48lm
S6	4.14±0.21q	15.85±0.72w	35.03±0.71st	18.18±0.15q	11.02±0.11t	21.31±0.27q	18.93±0.10m	30.13±0.79op
S7	5.98±0.04p	27.30±0.43qr	52.93±0.79mn	40.77±1.16kl	57.60±0.74i	32.52±1.49o	17.97±0.63m	28.73±1.30pqr
S8	16.11±0.38k	77.72±1.89g	118.96±6.39h	76.27±2.66f	66.32±0.92h	70.61±1.58gh	29.76±1.09ij	96.42±1.39g
S9	25.36±0.18h	46.98±0.97l	77.87±0.34k	46.90±0.54j	52.36±2.17k	43.82±2.01kl	19.16±0.13m	53.36±0.57k
S10	49.11±1.77a	110.55±2.82b	193.23±6.99a	57.81±2.02i	91.91±2.44b	118.47±0.14b	60.61±1.17c	187.17±7.86b
S11	15.71±0.14k	20.52±0.50uv	40.96±1.38qr	28.77±0.95o	18.56±0.43r	12.27±0.36r	9.98±0.14p	20.35±0.16st
S12	12.38±0.45mn	24.26±0.93st	43.17±0.56pq	34.11±0.18n	49.17±0.24l	26.67±0.16p	9.91±0.13p	23.82±0.61rs
S13	6.12±0.11p	27.06±0.68qrs	52.32±1.61mn	40.07±2.05klm	34.21±0.22n	26.97±1.24p	14.16±0.12n	33.12±0.41op
S14	39.22±1.11b	72.81±1.08h	136.72±4.00fg	41.71±0.68k	69.31±3.84g	73.22±1.01g	47.07±1.66e	130.07±5.16e
S15	27.47±0.25g	83.51±3.92f	140.81±4.54ef	40.00±0.91klm	73.98±3.06ef	68.32±3.11h	50.51±1.98d	126.06±5.99e
S16	29.82±1.10f	92.92±2.81d	145.06±5.27de	64.32±2.59h	80.76±1.55d	87.54±3.90e	47.21±2.33e	119.32±3.01f
S17	29.56±0.23f	52.83±2.49k	88.86±3.47j	68.96±2.42g	49.62±1.50l	46.77±1.16k	30.61±0.49ij	84.27±4.46h
S18	37.01±1.32c	97.92±4.60c	170.61±2.32c	54.47±0.56i	92.36±0.48b	104.16±4.67d	64.76±1.55b	146.77±3.24d
S19	13.21±0.07m	22.01±0.40tu	39.57±0.46qrs	39.72±0.29klm	17.62±0.80rs	13.16±0.72r	5.27±0.16q	24.50±1.31qrs

S20	19.87±0.76j	44.02±0.81m	73.81±1.40k	37.42±0.59lmn	42.97±0.54m	36.16±0.53n	18.22±0.50m	55.62±1.81jk
S21	4.91±0.00pq	29.41±0.30opq	55.21±0.48m	36.32±0.09mn	36.62±1.21n	27.56±0.27p	11.22±0.40op	29.56±0.65pq
S22	30.62±0.30ef	86.42±2.31e	146.86±4.97d	81.92±2.50e	83.86±2.95c	86.72±3.70e	38.87±0.36g	161.52±4.61c
S23	21.22±0.02i	31.77±0.26o	61.72±1.59l	41.77±1.96k	58.22±0.71i	31.07±0.96o	13.57±0.05n	28.57±0.54pqr
S24	12.47±1.95mn	68.22±0.31i	115.86±1.36hi	84.36±2.87e	76.32±1.20e	79.11±3.93f	31.87±1.35i	87.17±1.15h
S25	40.02±0.51b	90.96±2.23d	165.92±4.90c	84.97±0.85e	93.32±1.62b	89.16±2.79e	42.22±1.60f	185.46±7.35b
S26	2.62±0.11r	18.07±0.26vw	36.22±0.39rst	19.62±0.23q	8.32±0.31u	13.97±0.56r	4.88±0.05q	17.47±0.75t
S27	3.92±0.11q	73.67±1.36h	116.67±5.15hi	100.97±1.64c	97.17±4.35a	109.41±4.98c	35.42±0.90h	77.26±2.79i
S28	31.46±0.33e	66.17±1.35i	112.52±1.99i	90.01±1.30d	71.92±0.20f	72.77±2.74g	28.47±1.16j	88.26±1.63h
S29	11.52±0.30n	51.32±2.29k	89.17±4.15j	128.38±6.16b	55.02±1.20j	58.12±1.90i	25.91±0.32k	96.37±4.15g
S30	33.52±1.50d	82.27±0.86f	134.52±0.40g	126.37±0.85b	90.86±0.71b	106.01±3.60cd	39.57±0.25g	128.27±1.96e
S31	15.39±0.74kl	28.62±0.49pqr	46.69±0.34op	19.43±0.31q	26.74±0.50p	42.03±0.18lm	17.68±0.55m	41.64±0.20lm
S32	5.44±0.01p	25.81±0.08rs	42.32±0.31pq	20.13±0.22q	23.34±0.10q	32.13±0.02o	12.62±0.09no	38.73±0.35mn
S33	5.52±0.51p	19.97±0.36uv	35.11±0.57st	8.14±0.10r	18.57±0.35r	25.01±0.52p	10.08±0.24p	39.33±0.81mn
S34	4.18±0.16q	19.56±0.37uv	31.13±0.49t	9.78±0.15r	15.22±0.09s	25.78±0.36p	9.12±0.11p	20.32±0.09st

S35	14.47±0.05l	59.50±0.02j	60.18±0.04l	43.36±0.58k	48.62±0.41l	54.07±0.04j	22.92±0.63l	60.12±0.89j
Stdeva	13.23	30.61	50.43	35.46	28.16	33.09	19.36	53.52
Average	18.16	53.09	86.1	53.67	52.41	54.69	26.93	74.89
CV%	72.85	57.66	58.58	66.07	53.73	60.51	71.92	71.47

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Name	Content mg/100g							
	Pro	EAA	NEAA	CEAA	MAA	BCAA	TAA	·
S1	13.21±0.07v		255.16	398.41	60.05	416.27	135.09	653.57
S2	18.23±0.21u		252.01	348.64	63.89	371.61	129.66	600.65
S3	10.12±0.30v		171.16	227.51	45.9	254.08	93.49	398.67
S4	39.67±0.76s		901.54	1689.02	287.07	1640.16	496.36	2590.56
S5	19.88±0.57tu		242.71	449.43	58.43	452.22	129.11	692.14
S6	14.01±0.17v		140.23	310.42	49.06	271.74	83.04	450.65
S7	49.51±0.73p		235.53	396.44	46.7	380.06	115.08	631.97
S8	184.99±0.17c		531.53	1501.19	126.18	1309.53	296.13	2032.72
S9	111.27±1.16j		362.21	818.12	72.52	716.59	195.21	1180.33
S10	34.17±0.34r		816.1	1390.69	247.78	1524.08	459.74	2206.79
S11	76.32±1.59m		167.59	353.15	30.33	267.12	103.82	520.74
S12	67.22±0.99n		219.18	445.01	33.73	409.96	106.59	664.19
S13	75.81±0.01m		213.96	487.45	47.28	409.69	119.79	701.41
S14	155.46±1.98h		565.7	1184.6	177.14	1121.19	314.14	1750.3

S15	175.66±0.75e	589.93	1318.66	176.57	1207.25	339.39	1908.59
S16	191.61±5.91b	660.33	1464.91	166.53	1341.43	365.69	2125.24
S17	91.91±2.68k	399.88	790.91	114.88	717.66	223.11	1190.79
S18	160.31±2.17g	740.85	1450.12	211.53	1418.23	413.8	2190.97
S19	60.81±1.98o	162.89	381.3	29.77	299.47	99.04	544.19
S20	73.17±2.94m	332.77	634.72	73.84	582.95	187.05	967.49
S21	58.56±0.55o	231.79	429.62	40.78	386.92	129.38	661.41
S22	170.06±2.05f	637.22	1348.76	200.39	1268.35	351.1	1985.98
S23	81.41±2.50l	291.89	510.22	42.14	459.38	148.96	802.11
S24	119.97±4.86i	508.32	1022.66	119.04	926.6	268.45	1530.98
S25	179.47±6.54d	693.51	1413.48	227.68	1343.74	388.44	2106.99
S26	44.47±1.05q	122.83	273.37	22.35	219.81	78.86	396.2
S27	118.33±2.81i	590.13	1042.46	112.68	983.94	299.21	1632.59
S28	156.27±2.96h	526.43	1202.91	116.73	1038.96	278.56	1729.34
S29	172.81±3.72ef	396.78	1183.87	122.28	939.18	214.16	1580.65
S30	256.67±1.34a	662.21	1662.1	167.84	1424.56	340.05	2324.31
S31	23.39±0.75t	239	431.07	59.32	424.73	122.45	670.07
S32	18.83±0.31u	193.5	390.22	51.35	388.3	104.45	583.72
S33	13.02±0.59v	153.75	281.75	49.41	298.83	84.06	435.5
S34	10.88±0.26v	146.79	237.41	29.44	238.1	80.34	384.2
S35	22.83±0.91t	346.44	448.31	83.04	490.29	180.45	794.75
Stdeva	68.19	221.82	488.31	71.68	455.23	122.46	702.91

Average	87.72	391.48	797.68	101.82	741.23	213.55	1189.16
CV%	77.74	56.66	61.22	70.4	61.42	57.35	59.11

Means with different letters in the same column express significant differences (Duncan's test $p<0.05$).

The results of the variance analysis showed that there were differences in the content of various amino acids among the different *A. arguta* resources. The resources with the highest content of Thr, Ser, Glu, Ala, Val, Ile, Tyr, Lys, His, and Arg were S4; the resources with the highest content of Phe were S4 and S27, and the resources with the highest content of Asp were S8; the resources with the highest content of Gly, Met, and Leu were S10; the resources with the highest content of Pro were S30; the resources with the highest content of Cys were S28 and differed from other resources. Leu content was S10; the highest Pro content was S30; the highest Cys content was S28, and it was significantly different from other resources. The TAA content was 384.20~2590.56 mg/100 g, and the highest content was 674.27% of the lowest content. The resource with the highest TAA content was S4, and the resource with the lowest content was S34. The EAA content was 140.23~901.54 mg/100 g, with a mean value of 391.48 mg/100 g; the NEAA content was 237.41~1689.02 mg/100 g, with a mean value of 797.68 mg/100 g; the CEAA content was 47.89~480.52 mg/100 g, with a mean value of 101.82 mg/100 g; the content of MAA was 219.81~1640.16 mg/100 g, with a mean value of 741.23 mg/100 g; the content of BCAA was 78.86~496.36 mg/100 g, with a mean value of 213.55 mg/100 g; the total amino acid content was 384.20~2590.56 mg/100 g. In comparison, the mean contents of NEAA and MAA were higher than the others, so NEAA and MAA were the main components of TAA in *A. arguta*.

3.3. Evaluation of Amino Acid Nutritional Value of Fruits of Different *A. arguta* Resources

The WHO and FAO proposed a standard model for evaluating essential amino acids in food in 1973, and it has been suggested that the closer the variety of essential amino acids in the proteins of each substance and the ratio of their composition is to the FAO/WHO standard model of amino acids, the higher the nutritional value of proteins and the better the quality of the proteins in the substance, and vice versa, and the worse the nutritional quality [30]. Comparing the essential amino acids in 35 *A. arguta* resources with the amino acid pattern spectrum of FAO/WHO is shown in Table 3. Comparison of essential amino acids and FAO/WHO amino acid patterns in different resource fruits of *A. arguta* shows that the Leu of 35 *A. arguta* resources meets the ideal pattern proposed by FAO/WHO, and Leu, as an essential amino acid, is commonly used in the treatment of idiopathic hyperglycemia in young children, as well as liver disease, anemia, and muscular dystrophy caused by the imbalance of glucose metabolism accompanied by a decrease in bile secretion [58, 59], and therefore, studies on the regulation of glucose and lipid metabolism by the *A. arguta* may be carried out in the subsequent studies. There were 2 resources that did not fit the Val ideal model, 17 resources that fit the Met + Cys ideal model, 3 resources that fit the Ile ideal model, 10 resources that did not fit the Thr ideal model, 1 resource that did not fit the Phe + Tyr ideal model, and 24 resources that did not fit the Lys ideal model.

Table 3

Comparison of essential amino acids and FAO/WHO amino acid patterns in different resource fruits of *A. arguta*.

Name	%						
Leu	Val	Met + Cys	Ile	Thr	Phe + Tyr	Lys	S1
6.86	6.85	2.54	6.96	5.78	12.78	6.02	S2
7	7.13	2.79	7.46	6.71	12.11	6.6	S3

8.08	7.61	3.55	7.76	5.56	11.85	6.61	S4
7.17	6.86	2.81	5.13	5.18	9.99	5.12	S5
7.03	6.43	2.41	5.19	5.14	7.74	5.95	S6
7.77	7.14	6.02	3.52	4.6	6.48	4.73	S7
8.38	5.51	4.19	4.32	3.85	15.57	5.15	S8
5.85	4.89	2.04	3.82	4.05	7.01	3.47	S9
6.6	5.96	4.99	3.98	3.85	8.41	3.71	S10
8.76	7.07	3.62	5.01	4.39	6.78	5.37	S11
7.87	8.13	9.39	3.94	3.31	9.09	2.36	S12
6.5	5.9	5.86	3.65	3.67	12.54	4.02	S13
7.46	5.76	4.2	3.86	3.83	10.59	3.85	S14
7.81	5.98	3.73	4.16	3.99	6.34	4.18	S15
7.38	6.03	3.13	4.38	4.23	5.97	3.58	S16
6.83	6.01	2.67	4.37	4.54	6.83	4.12	S17
7.46	6.84	5.07	4.44	4.27	9.96	3.93	S18
7.79	6.63	2.96	4.47	4.27	6.7	4.75	S19
7.27	6.88	8.78	4.04	3.65	10.54	2.42	S20
7.63	7.15	4.84	4.55	4.83	8.31	3.74	S21
8.35	6.77	4.55	4.45	5.04	11.03	4.17	S22
7.39	5.93	3.09	4.35	4.28	8.35	4.37	S23
7.69	6.92	7	3.96	4.04	12.47	3.87	S24
7.57	5.51	2.49	4.46	4.7	10.5	5.17	S25
7.87	6.24	3.3	4.32	3.92	8.46	4.23	S26

9.14	6.2	6.33	4.56	4.81	7.05	3.53	S27
7.15	6.67	1.79	4.51	4.93	12.14	6.7	S28
6.51	5.78	4.04	3.83	4.15	9.36	4.21	S29
5.64	4.66	2.78	3.25	3.67	11.6	3.68	S30
5.79	5.3	2.67	3.54	3.95	9.35	4.56	S31
6.97	7.04	3.81	4.27	4.83	6.89	6.27	S32
7.25	6.22	2.3	4.42	4.82	7.45	5.5	S33
8.06	6.65	2.76	4.59	4.73	6.13	5.74	S34
8.1	7.72	3.34	5.09	5.54	6.51	6.71	S35
7.57	7.65	3.06	7.49	6.14	11.57	6.8	FAO/WHO standard mode

In accordance with the ideal amino acid composition proposed by WHO/FAO, EAA/TAA is 40%, EAA/NEAA is ≥60%, and BCAAs should account for 40% of the daily EAA requirement for adults, 41% for children, and 45% for infants [60–62].

Figure 3(a) shows the distribution range of amino acid nutritional value of different *A. arguta* resources, and it can be seen that the range of EAA/TAA of fruits of different *A. arguta* resources was 26.15%~43.59%, among which there were three resources that met the EAA/TAA standard, with a value of 41%~44%; meanwhile, Figure 3(b) shows the distribution range of amino acid nutritional value of different *A. arguta* resources and the range of EAA/NEAA ranged from 33.52% to 77.28%, among which there were five resources that met the EAA/NEAA standard, and S2 (A040103), S3 (A060902), and S35 (SH5) met the standard for both EAA/TAA and EAA/NEAA.

[figure(s) omitted; refer to PDF]

The higher the ratio of medicinal amino acids/total amino acids (MAA/TAA), the higher the medicinal value of the substance. Figure 3(c) shows the distribution range of amino acid nutritional value of different *A. arguta* resources, the MAA/TAA ratios of 35 *A. arguta* resources were all in the range of 51.30%~69.06%, and the MAA/TAA ratios of 28 of the resources were more than 60%, which are the results that indicate that the *A. arguta* has a high medicinal value.

Branched-chain amino acids, collectively known as leucine, valine, and isoleucine, are not only important components of human proteins but also regulators of protein, glucose, energy metabolism, and brain functions [63]. Figure 3(d) shows the distribution range of amino acid nutritional value of different *A. arguta* resources, all the resources achieved ≥40% BCAA/EAA, the range is 48.63%~64.20%, and three of the BCAA/EAA were more than 60%, which were 61.95% for S11, 60.80% for S19, and 64.20% for S26.

3.4. Comparison of Amino Acid RAA, RC, and SRC in Fruits of Different *A. arguta* Resources

Amino acid balance theory suggests that the closer the amino acid composition of a food protein is to the pattern amino acid composition, the higher its nutritional value [51]. Based on the amino acid balance theory, RC and SRC were calculated to evaluate the quality of proteins based on the dispersion of various essential amino acids from the

amino acid pattern, which is closer to the biological price [53, 54]. The RAA values of the fruits of different soft date kiwifruit resources are shown in Table 4. Values of RAA of different *A. arguta* resources and the RAA values of the two amino acids Leu and Val were greater than 1 in 35 resources. Whether the proportion of essential amino acids in a food conforms to the human essential amino acid pattern profile is closely related to the size of the RC, with RC = 1 conforming, whereas RC < 1 indicates a relative deficiency, and the amino acid with the lowest content is considered to be the limiting amino acid of the substance. Figure 4 shows the range of RC values of 9 amino acids, and the Leu of all 35 *A. arguta* resources is greater than 1, indicating that the content of this amino acid in *A. arguta* fruits exceeds the human body's needs; the RC values of Ile and Lys are less than 1, and the content of Ile is lower than that of Lys, indicating that the first limiting amino acid of *A. arguta* resource fruits is Ile, and the second limiting amino acid is Lys, so when consuming *A. arguta*, it is necessary to eat food rich in isoleucine and lysine with it, such as spinach, potatoes, bean curd, soya bean milk, and eggs.

Table 4

Values of RAA of different *A. arguta* resources.

Name	Leu	Val	Met + Cys	Ile	Thr	Phe + Tyr	Lys
S1	1.71	1.37	0.73	0.99	1.44	2.13	1.09
S2	1.75	1.43	0.8	1.07	1.68	2.02	1.2
S3	2.02	1.52	1.01	1.11	1.39	1.98	1.2
S4	1.79	1.37	0.8	0.73	1.3	1.66	0.93
S5	1.76	1.29	0.69	0.74	1.29	1.29	1.08
S6	1.94	1.43	1.72	0.5	1.15	1.08	0.86
S7	2.09	1.1	1.2	0.62	0.96	2.59	0.94
S8	1.46	0.98	0.58	0.55	1.01	1.17	0.63
S9	1.65	1.19	1.43	0.57	0.96	1.4	0.68
S10	2.19	1.41	1.04	0.72	1.1	1.13	0.98
S11	1.97	1.63	2.68	0.56	0.83	1.51	0.43
S12	1.62	1.18	1.68	0.52	0.92	2.09	0.73
S13	1.86	1.15	1.2	0.55	0.96	1.77	0.7
S14	1.95	1.2	1.07	0.59	1	1.06	0.76
S15	1.84	1.21	0.89	0.63	1.06	1	0.65

S16	1.71	1.2	0.76	0.62	1.14	1.14	0.75
S17	1.87	1.37	1.45	0.63	1.07	1.66	0.71
S18	1.95	1.33	0.84	0.64	1.07	1.12	0.86
S19	1.82	1.38	2.51	0.58	0.91	1.76	0.44
S20	1.91	1.43	1.38	0.65	1.21	1.38	0.68
S21	2.09	1.35	1.3	0.64	1.26	1.84	0.76
S22	1.85	1.19	0.88	0.62	1.07	1.39	0.79
S23	1.92	1.38	2	0.57	1.01	2.08	0.7
S24	1.89	1.1	0.71	0.64	1.18	1.75	0.94
S25	1.97	1.25	0.94	0.62	0.98	1.41	0.77
S26	2.29	1.24	1.81	0.65	1.2	1.18	0.64
S27	1.79	1.33	0.51	0.64	1.23	2.02	1.22
S28	1.63	1.16	1.15	0.55	1.04	1.56	0.77
S29	1.41	0.93	0.79	0.46	0.92	1.93	0.67
S30	1.45	1.06	0.76	0.51	0.99	1.56	0.83
S31	1.74	1.41	1.09	0.61	1.21	1.15	1.14
S32	1.81	1.24	0.66	0.63	1.21	1.24	1
S33	2.02	1.33	0.79	0.66	1.18	1.02	1.04
S34	2.03	1.54	0.96	0.73	1.38	1.08	1.22
S35	1.89	1.53	0.88	1.07	1.54	1.93	1.24

[figure(s) omitted; refer to PDF]

Modern scientific research has identified that amino acid deficiency can negatively affect the nutritional value of food. In order to measure whether the amino acid composition of a food is reasonable or not, the SRC value was introduced, which is a measure of how well the amino acids in a food match the model, and the closer the value is to 100, the closer the amino acid composition of the food is to the ideal model, and, therefore, the higher the nutritional value of the meal can be. Figure 5 shows the distribution of SRC value in different *A. arguta* resources. The SRC

values of 35 *A. arguta* resources' fruits were between 40 and 80, of which 20 resources had SRC values of 60~70, which accounted for 57.14% of the samples supplied for testing, and three resources had SRC values of more than 70, with the highest score being S3.

[figure(s) omitted; refer to PDF]

3.5. Analysis of Taste-Presenting Amino Acids in Fruits of Different *A. arguta* Resources

3.5.1. Taste-Presenting Amino Acids Content and Radar Chart Analysis

A. arguta fruit has a unique sweet and sour taste when eaten fresh, which is closely linked to its rich amino acid content. Based on the flavor-presenting characteristics, the flavor-presenting amino acids can be classified into four groups, namely, fresh amino acids (Glu, Asp, and Lys), sweet amino acids (Thr, His, Ser, Pro, Gly, and Ala), bitter amino acids (Val, Met, Leu, Ile, and Arg), and aromatic amino acids (Cys, Tyr, and Phe) [49], and the contents of the four taste-presenting amino acids of different *A. arguta* resources are shown in Figure 6. Figure 6 shows the contents of flavored amino acids in different *A. arguta* resources mg/100 g, the variation of the fresh amino acid content was 116.24~980.29 mg/100 g, the variation of the sweet amino acid content was 90.53~687.23 mg/100 g, the variation of the bitter amino acid content was 98.95~730.29 mg/100 g, and the variation of aromatic amino acid content was 33.19~292.48 mg/100 g. The resource with the highest content of fresh, bitter, and aromatic amino acids was S4, and the resource with the highest content of sweet amino acids was S16.

[figure(s) omitted; refer to PDF]

Radar plot analysis of the flavor-presenting amino acids of different A. arguta resources shows that, as can be seen from Figure 7, those that contribute more to the flavor of A. arguta are fresh amino acids and sweet amino acids, and the larger area of the pattern plot of S4 compared with the other resources indicates that the content of flavor amino acids in S4 is generally higher than that of the other resources 7. Figure 7 shows the flavor amino acid radar map of different *A. arguta* resources, the greater contribution in the flavor of *A. arguta* was made by fresh taste amino acids and sweet taste amino acids, and the larger area of the pattern plot of S4 compared with other resources indicated that the taste amino acid content of S4 was generally higher than that of other resources. The unique flavor of *A. arguta* may be closely related to its high content of gustatory amino acids. The high percentage of fresh and sweet amino acids such as Glu, Asp, and Ala gives the *A. arguta* its fresh, sweet, and sour flavor characteristics, while effectively reducing the bitterness of the rind and alleviating the undesirable taste brought about by the rind.

[figure(s) omitted; refer to PDF]

3.5.2. TAV Analysis

Different amino acids have different taste perception thresholds, so higher amino acid content does not necessarily contribute more to food flavor [54], further analysis of the effect of each presenting amino acid on fruit flavor quality by TAV values is needed, and the TAV of different *A. arguta* resources presenting amino acids is shown in Table 5. When TAV > 1 is present, then the amino acid contributes to flavor quality. As shown in Table 5, the amino acids with TAV values greater than 1 in all 35 *A. arguta* were Glu and Cys, the amino acids with TAV values less than 1 in all were Thr and Tyr, and the remaining amino acids had TAV values greater than 1 in some resources. Thus, Glu and Cys were the main contributors to the flavor of the 35 *A. arguta* resources, but in comparison, the mean TAV of Cys was greater than that of Glu, so Cys was the main influence on the flavor of *A. arguta*.

Table 5

Taste activity values of flavor amino acids in different *A. arguta* resources.

Sort	Amino acid	Taste threshold (mg/g) [49]	TAV																	
S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	DAA	Asp	1

0.72	0.69	0.4	3.13	0.81	0.36	0.43	3.55	1.1	2.19	0.3	0.52	0.69	1.5	1.98	2.42	0.98	2.71	Glu	0.3	3.77
2.51	1.74	17.82	4.26	3.16	3.55	13.61	8.4	13.59	2.92	5.13	4.23	11.71	12.63	14.04	6.7	12.04	Lys	0.5	0.79	0.79
0.53	2.65	0.82	0.43	0.65	1.41	0.88	2.37	0.25	0.53	0.54	1.46	1.37	1.75	0.94	2.08	.				
SAA	Thr	2.6	0.15	0.15	0.09	0.52	0.14	0.08	0.09	0.32	0.17	0.37	0.07	0.09	0.1	0.27	0.31	0.37	0.2	0.36
His	0.2	0.72	0.94	0.54	4.62	0.88	0.95	0.9	1.49	0.96	3.03	0.5	0.5	0.71	2.35	2.53	2.36	1.53	3.24	Ser
1.5	0.24	0.23	0.14	0.85	0.24	0.18	0.19	0.62	0.33	0.67	0.14	0.17	0.22	0.53	0.59	0.66	0.37	0.68	Pro	3
0.04	0.06	0.03	0.13	0.07	0.05	0.17	0.62	0.37	0.11	0.25	0.22	0.25	0.52	0.59	0.64	0.31	0.53	Gly	1.3	0.1
0.1	0.07	0.06	0.31	0.18	0.2	0.77	0.42	1.13	0.16	0.19	0.26	0.76	0.85	0.94	0.51	1.05	Ala	0.6	0.58	0.45
0.31	3.09	0.88	0.41	0.59	2.21	1.45	2.43	0.42	0.47	0.64	1.75	1.97	2.17	1.06	2.08	.				
BAA	Val	0.4	1.12	1.07	0.76	4.44	1.11	0.8	0.87	2.49	1.76	3.9	1.06	0.98	1.01	2.62	2.88	3.19	2.04	3.63
Met	0.3	0.24	0.25	0.19	1.31	0.25	0.14	0.2	0.54	0.85	1.64	0.52	0.41	0.2	1.31	0.92	0.99	0.99	1.23	Ile
0.9	0.51	0.5	0.34	1.48	0.4	0.18	0.3	0.86	0.52	1.23	0.23	0.27	0.3	0.81	0.93	1.03	0.59	1.09	Leu	1.9
0.24	0.22	0.17	0.98	0.26	0.18	0.28	0.63	0.41	1.02	0.22	0.23	0.28	0.72	0.74	0.76	0.47	0.9	Arg	0.5	0.91
0.9	0.7	3.9	0.82	0.6	0.57	1.93	1.07	3.74	0.41	0.48	0.66	2.6	2.52	2.39	1.69	2.94	.			
AAA	Tyr	2.6	0.18	0.15	0.09	0.61	0.09	0.07	0.16	0.29	0.18	0.22	0.11	0.13	0.15	0.16	0.15	0.25	0.27	0.21

Phe	0.9	0.4	0.39	0.26	1.1	0.33	0.12	0.64	0.74	0.58	1.02	0.21	0.55	0.38	0.77	0.82	0.9	0.55	1.03	Cys
0.02	4.64	4.7	4.16	16.85	4.56	11.49	10.24	12.68	16.76	15.43	16.59	13.28	11.66	13.06	16.13	13.49	15.39	13.89		
Sort	Amino acid	Taste threshold (mg/g) [49]	TAV																	
S19	S20	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35				
DAA	Asp	1	0.38	0.91	0.59	1.83	0.61	1.34	1.78	0.31	1.59	1.64	1.43	2.03	0.78	0.88	0.5	0.42	0.79	
Glu	0.3	3.76	5.61	3.58	12.47	4.16	8.76	12.74	2.36	8.31	11.41	12.14	17.61	3.57	3.36	2.62	1.8	3.24		Lys
0.5	0.26	0.72	0.55	1.73	0.62	1.58	1.78	0.28	2.19	1.46	1.16	2.12	0.84	0.64	0.5	0.52	1.08			
SAA	Thr	2.6	0.08	0.18	0.13	0.33	0.12	0.28	0.32	0.07	0.31	0.28	0.22	0.35	0.12	0.11	0.08	0.08	0.19	
His	0.2	0.26	0.91	0.56	1.94	0.68	1.59	2.11	0.24	1.77	1.42	1.3	1.98	0.88	0.63	0.5	0.46	1.15		Ser
1.5	0.15	0.33	0.21	0.58	0.26	0.52	0.6	0.14	0.56	0.61	0.43	0.66	0.24	0.21	0.15	0.15	0.34		Pro	3
0.2	0.24	0.2	0.57	0.27	0.4	0.6	0.15	0.39	0.52	0.58	0.86	0.08	0.06	0.04	0.04	0.08		Gly	1.3	0.14
0.39	0.29	0.89	0.32	0.7	0.92	0.16	0.75	0.69	0.54	0.9	0.3	0.24	0.2	0.2	0.13		Ala	0.6	0.41	1.07
0.55	1.76	0.73	1.79	2.05	0.33	1.59	1.91	1.44	2.24	0.98	0.67	0.45	0.57	0.76						
BAA	Val	0.4	0.94	1.73	1.12	2.95	1.39	2.11	3.29	0.61	2.72	2.5	1.84	3.08	1.18	0.91	0.72	0.74	1.52	
Met	0.3	0.44	0.66	0.16	1.02	0.71	0.42	1.33	0.09	0.13	1.05	0.38	1.12	0.51	0.18	0.18	0.14	0.48		Ile

0.9	0.24	0.49	0.33	0.96	0.35	0.76	1.01	0.2	0.82	0.74	0.57	0.91	0.32	0.29	0.22	0.22	0.66		Leu	1.9
0.21	0.39	0.29	0.77	0.32	0.61	0.87	0.19	0.61	0.59	0.47	0.71	0.25	0.22	0.18	0.16	0.32		Arg	0.5	0.49
1.11	0.59	3.23	0.57	1.74	3.71	0.35	1.55	1.77	1.93	2.57	0.83	0.77	0.79	0.41	1.2		.			
AAA	Tyr	2.6	0.15	0.14	0.14	0.32	0.16	0.32	0.33	0.08	0.39	0.35	0.49	0.49	0.07	0.08	0.03	0.04	0.17	
Phe	0.9	0.2	0.48	0.41	0.93	0.65	0.85	1.04	0.09	1.08	0.8	0.61	1.01	0.3	0.26	0.21	0.17	0.54		Cys

3.6. Correlation Analysis of Amino Acid Content of Different *A. arguta* Resources

The correlation analysis of 17 of the 35 *A. arguta* was carried out, and the results are shown in Figure 8. Correlation analysis of amino acid content in different *A. arguta* resources is shown in Figure 8. 17 amino acids were all highly significant and positively correlated, of which the correlation coefficient between Ser and Thr was as high as 0.99; the correlation coefficient between Cys and Pro was higher, 0.63, and the correlation coefficients with the other amino acids were all below 0.6; in short, the correlation of the amino acid fractions was strong, which was similar to the results of the study by Min et al. [64].

[figure(s) omitted; refer to PDF]

3.7. PCA Analysis

The PCA method can simplify multiple indicators with correlation into several relatively independent and representative indicators, retaining the vast majority of the original information, which is faster and more accurate compared with a single evaluation, and at the same time, it can also avoid the influence of correlation between traits on the evaluation results [65, 66], and it has been widely used in the evaluation of the quality of agricultural products such as jujube [21], *A. arguta* [19], black fungus [67], and peach [68]. PCA of the amino acids of different *A. arguta* resources is shown in Figure 9. PCA scores of amino acid content in different *A. arguta* resources revealed that S4, S10, S18, and S27 were located in quadrant 1, but all of them were located in scattered locations, indicating that the amino acid contents of these four resources were not similar; there were 10 resources located in quadrant 2, of which S8, S15, S22, S16, and S25 were closer to each other, indicating that the amino acid contents of these five resources were similar; S11, S12, S20, S21, S23, and S26 are located in quadrant 3; S1, S2, S5, S31, S32, S33, S34, and S35 are located in quadrant 4, but S35 is far away from the other resources, indicating that the amino acid content is similar among the resources except for S35; S6, S7, and S24 are located in the horizontal axis, which indicates that they are mainly influenced by PC1; S9 is located in the vertical axis, which indicates that it is importantly influenced by PC2.

[figure(s) omitted; refer to PDF]

3.8. Comprehensive Evaluation of Amino Acids of Different *A. arguta* Resources

As can be seen from Table 6, a total of 2 principal components were extracted using factor analysis, the contribution of the first principal component was 63.979%, the contribution of the second principal component was 24.083%, and the cumulative variance of the first 2 PCs reached 87.88%, Gly, Cys, and Pro were the principal component 2, and the remaining 14 amino acids were the principal component 1. Table 6 shows the factor loading matrix and contribution rate after rotation that a comprehensive evaluation of different *A. arguta* resources using the first 2 PCs is feasible.

Table 6

Factor loading matrix and contribution rate after rotation.

PC	Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Pro	Eigenvalue	Contribution rate (%)	Cumulative contribution rate (%)
1	0.848	0.927	0.891	0.792	0.585	0.879	0.175	0.903	0.698	0.946	0.877	0.636	0.807	0.936	0.937	0.899	0.345	10.846	63.797	63.797
2	0.353	0.348	0.432	0.562	0.61	0.428	0.863	0.408	0.47	0.289	0.446	0.453	0.474	0.269	0.196	0.334	0.851	4.094	24.083	87.880

With 17 amino acid indicators as the initial independent variables, the equation expressions for the three PC factors were finally derived by PCA as follows:(2)F1=0.102X1+0.125X2+0.086X3+…+0.182X15+0.123X16−0.208X17,F2=−0.049X1−0.080X2−0.008X3+…−0.191X15−0.080X16+0.482X17.

The relative contribution of the variance of the two PCs was used as the weight, and the PC scores of each resource and the corresponding weights were linearly weighted and summed to establish a comprehensive evaluation function $F=0.726F1+0.274F2$. The comprehensive score of each *A. arguta* resource was calculated to reflect its comprehensive amino acid quality, and the higher the comprehensive score, the better the amino acid quality of the resource. As shown in Table 7, comprehensive evaluation results of amino acids of different *A. arguta* resources, the top 5 resources in terms of the overall score were S4, S10, S18, S25, and S30, indicating that these five *A. arguta* resources had relatively good overall amino acid evaluations.

Table 7

Comprehensive evaluation results of amino acids of different *A. arguta* resources.

No.	*F*1	*F*2	*F*	Rank
S1	−0.06	−1.45	−0.44	19
S2	−0.06	−1.51	−0.46	22
S3	−0.48	−1.45	−0.74	29
S4	3.16	−1.19	1.97	1
S5	−0.11	−1.32	−0.44	20
S6	−0.93	−0.51	−0.81	32
S7	−0.70	−0.31	−0.60	25
S8	0.65	0.76	0.68	9
S9	−0.58	0.98	−0.15	16

S10	2.07	−0.32	1.42	2
S11	−1.49	0.75	−0.87	34
S12	−1.07	0.32	−0.69	28
S13	−0.89	0.08	−0.63	26
S14	0.62	0.55	0.60	10
S15	0.61	0.93	0.70	8
S16	1.01	0.78	0.95	6
S17	−0.22	0.57	0.00	15
S18	1.52	0.35	1.20	3
S19	−1.49	0.76	−0.87	33
S20	−0.44	0.22	−0.26	18
S21	−0.90	0.06	−0.64	27
S22	0.76	0.96	0.81	7
S23	−1.05	0.93	−0.51	23
S24	0.41	0.33	0.39	13
S25	0.99	0.97	0.99	4
S26	−1.25	−0.17	−0.96	35
S27	0.74	0.12	0.57	11
S28	0.03	1.55	0.45	12
S29	−0.34	1.40	0.14	14
S30	0.73	1.68	0.99	5
S31	−0.17	−1.19	−0.45	21
S32	−0.31	−1.34	−0.59	24

S33	−0.50	−1.42	−0.75	30
S34	−0.61	−1.31	−0.80	31
S35	0.35	−1.55	−0.17	17

3.9. Hierarchical Clustering Analysis of Amino Acids in Different *A. arguta* Resources

The results of the hierarchical cluster analysis of 17 amino acids in 35 *A. arguta* resources are shown in Figure 10. All *A. arguta* resources could be classified into four categories when the transect line took the value of 300, category 1 contained S4 and S10, category 2 contained S30, S8, S16, and S18, category 3 contained S22, S25, S14, S15, S24, S27, S28, and S29, and the remaining 21 resources were all in category 4, among which S4 and S10 in category 1 had the highest amino acid content and better quality. The clustering results were consistent with the comprehensive evaluation results of PCA. Therefore, it can provide a good reference for the introduction and promotion of excellent resources of *A. arguta*, the development and utilization of products, and the evaluation of amino acid nutritional value.

[figure(s) omitted; refer to PDF]

4. Conclusion

In this study, an amino acid analyzer was used to separate and determine the amino acid composition and content of 35 *A. arguta* resource fruits from the Zuojia Town *Actinidia arguta* and *Magnolia vine* National Forest Germplasm Resource Bank in Jilin Province, and the results showed that *A. arguta* fruits contained 17 amino acids, with a total amino acid content of 384.20~2590.56 mg/100 g and that the average contents of NEAA and MAA had higher average contents than others, which were the main components of TAA in *A. arguta*. The analysis of the variance results showed that the standard deviation of the 17 amino acids had a variance of 9.68~146.87, the coefficient of variation was 40.94%~77.74%, and the content of different amino acids differed significantly among resources, of which the coefficient of variation of Pro was the largest and the coefficient of variation of Gys was the smallest. Meanwhile, the amino acids varied significantly among most of the A. arguta resources, with mean values ranging from 18.16~222.22 mg/100 g.Among them, the lowest content was Met, and the highest content was Glu. The results of amino acid nutritional value evaluation showed that the Leu of 35 *A. arguta* resources conformed to the ideal model proposed by FAO/WHO, and the RAA value of Leu of all resources was greater than 1, which indicated that the content of this amino acid in *A. arguta* fruits exceeded the human body's needs; the RC values of Ile and Lys were both less than 1, and the content of Ile was lower than that of Lys, which indicated that the first limiting amino acid of the fruits of the *A. arguta* resources was Ile and the second limiting amino acid was Lys, and the SRC value of their fruits was in the range of 40~80. The analysis of the results of flavor-presenting amino acids showed that Glu and Cys had the main contribution to the fruit flavor of *A. arguta* resources, and Cys was the main amino acid factor for *A. arguta* flavor in comparison. However, the effect of amino acids on fruit flavor was limited, and the volatile flavor components of the fruit need to be analyzed and determined at a later stage. The principal component analysis extracted 2 principal components from 17 amino acids, and the cumulative variance contribution rate was 87.88%, which better reflected the comprehensive information of amino acids in *A. arguta.* A comprehensive amino acid evaluation model was established, and the top 5 excellent resources with comprehensive scores were S4, S10, S18, S25, and S30. Hierarchical cluster analysis classified the 35 *A. arguta* resources into 4 categories, which better reflected the differences in amino acid content and composition, nutritional value, and taste characteristics among *A. arguta* fruits from different collection sites. This study provides a scientific basis for revealing the nutritional value and taste characteristics of *A. arguta*, a theoretical reference for the screening of excellent *A. arguta* resources and the development and utilization of products, and a theoretical basis for guiding people to establish a scientific and healthy dietary structure. Further research will test and analyze the volatile flavor quality of *A. arguta* and establish a more detailed evaluation system of *A. arguta* fruit quality by combining the nutritional quality, amino acid composition and content, and volatile flavor quality, to lay a theoretical foundation for the development of excellent *A. arguta*

resources. In addition, we will further study the effects of amino acids of *A. arguta* on human health to provide a theoretical basis for the provision of a scientifically balanced diet for human beings and the development of *A. arguta* functional products.

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DETAIL

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Effect of Coating and Coated Paperboard Packaging on the Quality of Grapes and Apple during Storage

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ABSTRAK (ENGLISH)

Edible coatings and active packaging have become more prevalent in response to changing consumption patterns and market trends to enhance the quality and safety of fresh products. In this work, we investigated the effect of aloe vera gel (AVG) coating and paraffin wax-coated paperboard (PWB) packaging on the postharvest quality attributes of both grapes and apples during storage. The fruits were coated with 50% AVG concentrations, and the inner wall of the corrugated paperboard was coated with paraffin wax emulsion. The grapes and apples were stored for 12 and 35 days, respectively, at ambient conditions (25 ± 3°C and 80–85% relative humidity). The physicochemical properties, microbiological attributes, and decay incidence of the fruits were analyzed at intervals during storage. Both fruits treated with AVG and PWB packaging retained better qualities than the control at the final day of the storage period. Particularly, PWB packaging provided considerably superior quality from the control sample in terms of weight loss (≈54% and 32%), firmness (≈48% and 68%), and color difference (≈30% and 28%) for both grapes and apples. These findings would introduce a novel approach for preserving the quality attributes of both climacteric and nonclimacteric fruits for a prolonged storage period at ambient temperature by PWB packaging and AVG coating.

TEKS LENGKAP

1. Introduction

Shelf life is considered a significant concern of fresh fruits and vegetables to minimize postharvest losses and

maintain acceptability and safety for consumer satisfaction [1]. Packaging is a widely adopted concept to minimize postharvest losses and ensure consumer safety with an increased shelf life of foods. Different packaging materials such as plastic, metal, glass, and paper have been used worldwide based on foods' size, shape, quantity, and chemical interactions. Plastics are considered the prime choice because of their low price, availability, uncomplicated manufacturing process, and handling. However, the attraction has shifted from typical plastic materials to biodegradable packaging and edible coating due to the replacement of chemical uses on food and environmental considerations [2].

The edible film, paper, and paperboard packaging from renewable sources have attracted manufacturers' interest [3]. Paper and paperboards are generally used with other hydrophobic materials (wax and polyethylene) because of their poor moisture, gas, aroma, and grease barrier properties [4]. Several polymeric compounds, such as paraffin wax, milk proteins, celluloses, lipids, starch, zein, and alginate, have been reported to be used to increase the mechanical qualities of papers and paperboards [5]. Paraffin wax, among them, is best suited for moisture and water vapor barriers for fresh fruits and vegetables because of its low polarity behavior [5, 6]. The effectiveness of paraffin wax is increased with the amount applied on paper or paperboard [6].

In addition, edible coatings prepared from plant and animal sources are applied to the exterior portion of food products as a thin layer of eatable material. The coating helps to modify the environment of the fruit's surroundings and improve the shelf life of fresh fruits by maintaining quality, reducing weight loss, minimizing respiration and oxidation reaction rates, and delaying microbial decay and ripening while storing [7–9]. Several compounds, like milk proteins, celluloses, lipids, starch, zein, alginate, mucilage, and aloe vera gel, have been applied on the surface of fruits as edible coatings [10]. Aloe vera gel (AVG) has recently captivated attention as an edible coating due to its edibility, eco-friendliness, chemical inactivity, and antifungal properties with fruits that alter their flavor or texture [11]. The complex structure of AVG coating also provides excellent protection against moisture loss, browning, texture change, and microbiological proliferation [10, 12]. The AVG coating in various fruits, including pineapple [13], strawberry [14], papaya [15], table grapes [16], hog plum [17], apple [18], jujube [19], and blueberry [20], reduced moisture loss, microbial degradation, softening, and respiration rates and preserved other quality attributes, which could increase the shelf life of the fruits during storage.

To the best of our knowledge, from the literature search, few studies investigated the effect of AVG coating on the postharvest properties of apples and grapes. The grapes, nonclimacteric fruits, experience significant physiological and biochemical reactions in the fresh fruit. These include firmness and water loss, degradation of color, and enhancement of respiration during postharvest handling, resulting in a high rate of fungal decay [21] and poor storability [22]. The AVG coating has been investigated to reduce the rate of these physiological and biochemical reactions. Unal [23] studied the effect of AVG coating (25%) on the postharvest life of table grapes in cold storage conditions (1°C and 90% RH). They reported that postharvest AVG treatments significantly delayed weight loss, maintained visual appearance, and preserved the rachis chlorophyll concentration and antioxidant capacity during storage. Another study by Unal [23] used AVG coating on table grapes at three concentrations and stored them at 1.0 ± 0.5°C. They also found that AVG coating significantly delayed fruit weight loss, changes in soluble solid contents, titratable acidity, and maturity index during storage compared to uncoated grapes [23]. Some studies observed that the application of various combinations of edible coatings, like salicylic acid before harvesting and AVG after harvesting [24], chitosan before harvesting followed by AVG after harvesting [16], and putrescine combined with AVG [25], enhances the postharvest quality of table grapes in cold storage conditions.

Apple is a climacteric fleshy fruit, and ethylene is responsible for most physiological changes during postharvest storage. Following its production, this hormone (ethylene) is recognized by several receptors, which then control downstream ethylene-related genes through a signaling cascade. The ethylene inhibitor named 1-methylcyclopropene (1-MCP) is commonly used to improve the postharvest quality of apples by controlling the ripening process, which helps to extend storage life [26]. Natural coating, such as AVG coating, can reduce fruit metabolite production, which lowers the increase of soluble solids in coated climacteric fruits and delays fruit ripening [27]. Few previous studies investigated the impact of AVG coating on the postharvest quality of apples and

fresh-cut apples. Khan [18] reported that AVG coating (20%) on apples exhibited longer shelf life with higher firmness and lower weight loss than uncoated apples in reirrigated conditions. In another study, Ozturk [28] observed that 20% AVG coating on apples improved the postharvest quality with a significant delay in weight loss in cold storage (2°C and 90 ± 5% RH) and at 20°C [28]. Quality changes of AVG-coated fresh-cut apple slices were investigated by Song [29]. They revealed that the AVG coating demonstrated a delay in browning and reduced weight loss and softness compared to uncoated slices. The AVG coating also effectively decreased aerobic bacteria, yeast, and mold populations.

However, no previous research investigated the effect of AVG coating or paraffin wax-coated paperboard packaging on the postharvest qualities of apples and grapes stored in an ambient condition. Apples and grapes have distinct postharvest physiology and storage characteristics due to their different ripening patterns. Apples are climacteric, whereas grapes are nonclimacteric. Therefore, this research aimed to assess the effect of AVG coating and PWB packaging on the physicochemical properties, microbial quality, and decay of apples and grapes, respectively, during ambient storage conditions.

2. Materials and Methods

2.1. Fruit Collection and Preparation

Grapes (Red Globe) and apples (Royal Gala) were collected from a community market in the Dinajpur district, Bangladesh. The fruits were selected based on their uniform size, shape, and maturity and were free from disease and damage. Fruits were transported rapidly in plastic crates to the experimental area under ambient conditions. The fruits were washed using running tap water (1-2 min), wiped with clean tissue paper to absorb the remaining water on the fruits' surface, and dried using a blower at a gentle pace (30 min). After drying, the fruits were considered for aloe vera gel coating and paraffin wax-coated paperboard packaging.

2.2. Preparation of Aloe Vera Gel Coating

Aloe vera gel was extracted from disease and injury-free and fresh (immediately after harvesting) aloe vera leaves uniform in maturity (18th month age of aloe vera leaf), color, and size based on Parven et al. [15] with a little modification. The selected leaves were initially rinsed with free-flowing tap water (1-2 min) to remove dirt, followed by soaking (5 min) in 0.1% sodium hypochlorite. The excess water from the surface of aloe vera leaves was wiped with clean tissue paper, and the gelatinous parenchyma matrix of the leaves was separated by a sharp stainless-steel knife. The colorless hydro parenchyma was homogenized uniformly by an electric kitchen blender (Jaipan, JP-3501, India) for 2 min and filtered through a sterile muslin cloth to separate fibrous fractions and the liquid gel fraction. The isolated gel was diluted at a 1 : 1 (v/v) ratio with distilled water, followed by pasteurization (70°C, 45 min), and cooled to ambient temperature (25 ± 3°C). Finally, citric acid was added to the mixture to maintain its final pH to 4.0.

2.3. Preparation of Paraffin Wax Emulsion and Paperboard Coating

The paraffin wax emulsion was prepared using the Liu et al. [30] method with minor alterations. For melting, paraffin wax (20 g) was placed in a 250 mL glass beaker in a thermostatic water bath at 80–85°C. Then, 3 g of emulsifier (Span-80, pharmaceutical grade) was added to the completely melted paraffin wax. After that, around 30% of the solid content in the mixture was adjusted by gradually adding deionized water with continuous agitation. The resultant mixture was homogenized twice at high pressure (400 bar) and quickly cooled to room temperature to obtain the paraffin wax emulsion.

The inner wall of the corrugated paperboard (9″ × 6″ × 6″, 3 mm thickness, single wall) was covered manually (using hand brush) with a layer of paraffin wax emulsion at a thickness of approximately 1 mm. After the completion of coating, the paraffin wax-coated paperboard (PWB) was dried at ambient condition for 24 h.

2.4. Experimental Design and Treatments

The research was carried out using a completely randomized design (CRD) model with three different treatments: control (uncoated fruits), AVG coating, and PWB packaging. Chrysargyris et al. [31] method was followed for the coating of both fruit samples (apples and grapes). Initially, the collected fruit samples were washed in a 0.05% sodium hypochlorite solution (5 minutes), then rewashed with the stream of distilled water, and kept at ambient condition for drying. After that, aloe vera gel was applied evenly on the surface of the fruits by immersing in aloe

vera gel for 10 min [17] and kept at ambient condition for 30 minutes. The AVG-coated fruits in the uncoated paperboard box were considered as AVG coating treatment. The fruits without AVG coating in the paraffin wax-coated paperboard box were designated as PWB packaging treatment, and the fruits without coating in the uncoated paperboard box were indicated as control. The experimental design for apple was constructed with three treatments × three repetitions × six fruits per repetition × six sampling intervals (with day 0) with 324 fruits. For grapes, the experimental design was constructed with three treatments × three repetitions × twelve fruits per repetition × five sampling intervals (with day 0) with 540 fruits. Finally, fruits with different treatments were stored at ambient condition (25 ± 3°C and 80–85% relative humidity). For the grapes, sampling was done on 0 (before coating), 3rd, 6th, 9th, and 12th day of the storage period. The apples were analyzed on 0 (before coating), 7th, 15th, 21st, and 35th day of the storage period.

2.5. Physical and Chemical Quality of the Fruits

2.5.1. Total Soluble Solids Content, Titratable Acidity, and pH

The juice was extracted from each fruit after removing the skin and seeds. The total soluble solids (TSS) content of the fruit samples was measured using a hand refractometer (HI 9601). The pH of the fruit samples (fruit juice) was measured using a digital pH meter (HI 2211 pH/ORP meter, China) [32]. Titratable acidity (TA) was estimated by conducting titration reaction of 5 ml of aliquot (5 g extracted juice was diluted to 100 ml) with 0.1 N NaOH at pH 8.1 and phenolphthalein indicator (0.1%). TA was expressed on the equivalency of citric acid percentage.

2.5.2. Weight Loss

The weight of fruit samples was measured using a digital balance (model with accuracy). The percent weight loss of a fruit sample was calculated using the following equation:(1)weight loss %=Wo−Wf×100Wo,where Wo is the initial weight of fruits and Wf is the weight at the sampling day of the fruits.

2.5.3. Fruit Firmness

The firmness of the fruits was represented as the resistance of fruits against the penetration of a narrow diameter rod using a texture analyzer (Probe TA39, TA-MTP). The rod of texture analysis was placed perpendicularly on the sample and kept pressing until a noticeable crack appeared [31]. The same procedure was done three times for each fruit, and the mean value of fruit firmness (kg/cm^2) was reported.

2.5.4. Color Value

The color difference (ΔE) of each fruit sample was determined by a colorimeter (CR400, Konica Minolta) having the Hunter color lab system (coordinate L, a, b). The mean color difference for each fruit was recorded and determined by the following equation:(2)ΔE=L∗−L◦2+a∗−a◦2+b∗−b◦2,Where, “o” refers to the color reading of the control sample.

2.6. Decay Evaluation

Fruit decay was evaluated individually during the storage period, as mentioned in Chrysargyris et al. [31], on a scale of 1 to 5 (where 1 represents free of dirt and infection; 2 represents trace amount of infection; 3 represents slight infection; 4 represents infection at moderate level; and 5 represents severe infection). Three different fruits per treatment and storage time were used to conduct the decay analysis.

2.7. Microbial Analysis

Microbial analysis was done by following the total plate count (pour plate) method [10]. Initially, a 10 g sample was mixed in 90 mL of sterile peptone and homogenized in a stomacher (MIX 2, AES Laboratoire, Combourg, France). The homogenized sample was serially diluted, and 1 mL from each dilution was transferred to the liquid agar plate and allowed to solidify at ambient temperature. The solidified agar plates were kept in an incubator for 24 h at a temperature of 37°C. After incubation, Colony Counter (Stuart Scientific, UK) was used to count the colonies of each plate. The agar plate having 30–300 colonies was selected, and colony-forming units (CFUs) were calculated by the following equation:(3)CFUg=number of colonies×dilution factorvolume of culture plate.

The grapes were sampled for microbial analysis on 0 (before coating), 6th, and 12th day of the storage. However, the apples were tested on 0 (before coating), 21st, and 35th day of storage.

2.8. Statistical Analysis

A completely randomized design (CRD) was used. One-way analysis of variance (ANOVA) of all the experimental

data was performed by SPSS (IBM SPSS Statistic 22) software with a significant mean difference at P≤0.05. All the results were presented as mean ± standard deviation (SD).

3. Result and Discussion

3.1. Total Soluble Solids

The amount of total soluble solids (TSS) in a fruit directly influences its taste when consumed. The TSS content in fruits elevates when the maturation progresses due to the hydrolysis of polysaccharides that are not dissolved in simple sugars [33]. Furthermore, the senescence process and the rapid metabolism of fruits can also cause this increment of TSS. Coating on fruits may decrease the respiration rate that lowers fruit metabolites and thus may result in a slower rate of increase in the soluble solids content of coated fruits [34]. Barakat et al. [35] reported that the rise in TSS content in climacteric fruits during storage is common, attributed to the gradual increase of free sugars in the fruits. Rodriguez et al. [36] also stated that an increase in TSS during the storage period may result from pectin breakdown and the conversion of carbohydrates into simple sugars during storage because of the metabolic activities of the tissues. In another study, Shahkoomahally and Ramezanian [37] also observed the increasing trend of TSS in fruits, probably due to the significant loss of water and weight throughout storage. Our current investigation of fruit TSS has revealed a similar phenomenon.

For grapes, we found that the TSS of grapes significantly increased in all treatments over the storage time, as summarized in Table 1. At the end of 12 days of storage, the control grapes had a high value of TSS content (20.90°Brix), followed by the AVG coating (20.53°Brix) and the PWB packaging treatment (19.0°Brix). This may be due to the higher senescence process and the rapid metabolism of fruits [34]. However, the PWB packaging treatment maintained a lower TSS in grapes from the third day of storage. It continued until the last day of observation, where the AVG coating treatment exhibited consistent patterns with the control. The finding of AVG coating in our current study is supported by Nia et al. [16], who reported that AVG coating (33%) on table grapes exhibited comparable TSS to uncoated grapes. These authors solely observed the effect of AVG coating and did not consider the PWB packaging.

Table 1

TSS, TA, and pH of AVG- and PWB-coated grapes.

Parameter	Treatment	Storage (day)				
0	3	6	9	12	TSS (°Brix)	Control
13.53 ± 0.31^{Ae}	15.2 ± 0.36^{Ad}	17.43 ± 0.25^{Ac}	18.83 ± 0.40^{Ab}	20.90 ± 0.36^{Aa}	AVG-coated grapes	13.63 ± 0.15^{Ae}
14.97 ± 0.25^{Ad}	17.13 ± 0.25^{Ac}	18.80 ± 0.40^{Ab}	20.53 ± 1.12^{Aa}	PWB-coated grapes	13.53 ± 0.60^{Ae}	14.67 ± 0.15^{Bd}
15.93 ± 0.35^{Bc}	17.30 ± 0.30^{Bb}	19.00 ± 0.44^{Ba}	-			
TA (%)	Control	0.88 ± 0.04^{Aa}	0.77 ± 0.05^{Ab}	0.56 ± 0.04^{Bc}	0.38 ± 0.04^{Bd}	0.28 ± 0.04^{Be}
AVG-coated grapes	0.88 ± 0.04^{Aa}	0.67 ± 0.04^{Bb}	0.55 ± 0.07^{Bc}	0.47 ± 0.05^{Ac}	0.33 ± 0.05^{Bd}	PWB-coated grapes
0.89 ± 0.05^{Aa}	0.81 ± 0.03^{Ab}	0.69 ± 0.03^{Ac}	0.48 ± 0.04^{Ad}	0.46 ± 0.03^{Ad}	-	

pH	Control	3.56 ± 0.05^{Ae}	3.75 ± 0.05^{Ad}	3.94 ± 0.04^{Ac}	4.27 ± 0.05^{Ab}	4.36 ± 0.04^{Aa}
AVG-coated grapes	3.58 ± 0.08^{Ad}	3.77 ± 0.05^{Ac}	3.87 ± 0.06^{Abc}	3.92 ± 0.04^{Bab}	3.99 ± 0.06^{Ba}	PWB-coated grapes

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

In the case of apples, an increment in the TSS content was also observed in all treatments over the storage time, as summarized in Table 2. However, compared to the control, both AVG coating and PWB packaging maintained a significantly lower TSS level from the first seven days of storage to the completion of the storage period (35 days). The addition of a paraffin-wax layer on the inner surface wall of the paper-box and/or the presence of gel barriers surrounding the fruit may have modified the environment by decreasing oxygen level and/or elevating CO_2 levels, thereby inhibiting ethylene generation [15]. This may result in a delayed ripening process and the rapid increment of fruit-soluble solids. Our results align with the study of Ali et al. [38], who investigated the effect of apple AVG coating. During storage, they noticed that the AVG coating maintains the TSS of fruits due to slower respiration and ethylene production. Ozturk et al. [28] also reported that AVG coating effectively delayed the TSS increase in Piraziz apple during cold storage.

Table 2

TSS, TA, and pH of AVG- and PWB-coated apples.

Parameter	Treatment	Storage (day)					
0	7	15	21	28	35	TSS (°Brix)	Control
13.00 ± 0.70^{Ae}	14.73 ± 0.35^{Ad}	15.56 ± 0.31^{Ad}	16.95 ± 0.21^{Ac}	17.90 ± 0.30^{Ab}	19.90 ± 0.70^{Aa}	AVG-coated apples	13.17 ± 0.35^{Ae}
14.23 ± 0.25^{ABd}	14.77 ± 0.35^{Bd}	15.90 ± 0.20^{Bc}	16.93 ± 0.45^{Bb}	18.300 ± 0.20^{Ba}	PWB-coated apples	13.22 ± 0.27^{Af}	14.01 ± 0.19^{Be}
14.44 ± 0.15^{Bd}	15.13 ± 0.25^{Cc}	15.87 ± 0.15^{Cb}	16.33 ± 0.15^{Ca}	-			
TA (%)	Control	0.46 ± 0.05^{Aa}	0.31 ± 0.02^{Ab}	0.21 ± 0.03^{Cc}	0.16 ± 0.02^{Bd}	0.11 ± 0.02^{Be}	0.10 ± 0.12^{Be}
AVG-coated apples	0.47 ± 0.06^{Aa}	0.37 ± 0.05^{Ab}	0.27 ± 0.02^{Ac}	0.19 ± 0.03^{Bd}	0.15 ± 0.03^{Ad}	0.13 ± 0.02^{Ad}	PWB-coated apples
0.46 ± 0.04^{Aa}	0.35 ± 0.03^{Ab}	0.28 ± 0.01^{Ac}	0.28 ± 0.015^{Ac}	0.17 ± 0.02^{Ad}	0.15 ± 0.02^{Ad}	.	
pH	Control	4.20 ± 0.10^{Af}	4.42 ± 0.07^{Ae}	4.52 ± 0.04^{Ad}	4.67 ± 0.05^{Ac}	4.80 ± 0.03^{Ab}	4.94 ± 0.03^{Aa}

AVG-coated apples	4.21 ± 0.03^{Ae}	4.34 ± 0.05^{ABd}	4.44 ± 0.07^{ABc}	4.54 ± 0.06^{Bb}	4.58 ± 0.03^{Bb}	4.71 ± 0.02^{Ba}	PWB-coated apples

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

3.2. Titratable Acidity (TA) and pH

Titratable acidity (TA) and pH are two critical variables for determining fruit freshness, and they are closely related since pH is characterized by acid compounds. In this study, the TA content of the grapes and apples decreased gradually with the increasing storage time of all treatments, as summarized in Tables 1 and 2, respectively. A decreasing trend in TA, along with storage time, has also been stated for grapes [16], apples [18], and persimmon [39]. The decrease of TA with increasing TSS during storage was observed due to the hydrolysis of the polysaccharides undissolved in simple sugars with the maturation of the fruits [33]. Moreover, a decrease in the TA content may also be initiated by high metabolic activities in fruit cells, such as ethylene production and respiration rate, utilizing numerous organic acids, etc. [40]. Our study recorded the highest TA of grapes in PWB-packed grapes on the final day of storage, around 65% higher than uncoated grapes. The PWB packaging could change the internal microenvironment of a fruit, slowing down respiration and delaying the loss of TA. On the other hand, AVG coating on grapes did not significantly affect the lagging loss of TA compared to control fruit. However, the effectiveness of AVG coating in delaying the reduction in TA throughout the storage has been reported for grapes [10, 16] and blueberries [41]. In the case of apple, TA reduction was lowered by AVG coating and PWB packaging compared to uncoated throughout the storage. Our findings are in line with earlier studies that found reduced TA in AVG-coated apples [18], papaya [15, 27], and persimmon [39]. This substantial reduction of TA in uncoated apples suggests that they may ripen faster than coated fruits.

We also observed an upward trend of pH in grapes and apples with increasing storage time. A rise in pH in treatments during postharvest storage with time might be related to biochemical changes in fruit, including the breakdown of organic acids, starches, and pectin to free acids and simple development [16]. In contrast, we found that the coating treatments lower the increase of pH of fruits compared to uncoated ones. At the final day of storage, PWB-packed grapes and apples exhibited the lowest pH levels, with reductions of approximately 13% and 9%, respectively, compared to the control. Like this study, a similar trend has also been reported for AVG-coated grapes [16] and apples [38]. This slows down the pH change of fruits upon coating application and may lead to delays in ripening and deterioration.

3.3. Weight Loss

During postharvest storage, water loss is the most unwanted physiological process in horticultural products. Fruit water loss causes economic concerns because it degrades both the structural quality and visual attractiveness of the fruit [39]. The loss of water, along with some other soluble substrates from fruits and vegetables, can easily occur by transpiration through the peel, which is responsible for the weight loss of fruits and vegetables during postharvest storage. Moreover, the weight loss may also be initiated by respiration, which causes the fruit to lose one carbon atom per cycle in the form of CO_2 [15]. Like earlier studies [15, 20], in our study, weight loss for grapes and apples for all treatments increased gradually over the storage period, as shown in Figures 1(a) and 1(b), respectively. Weight loss of grapes (Figure 1(a)) was higher in the control sample (8.37%) and lower in the PWB-packed sample (3.87%) at the last day of storage. The application of AVG coating on grapes also effectively delayed the weight loss of fruits. As shown in Figure 1(b), uncoated apples (control) experienced a significantly higher weight loss of about 17% on the last day of storage. Both AVG coating and PWB packaging of apples significantly reduced the water loss to the control. In our study, the decline in weight loss of fruits was likely because of the AVG coating and PWB packaging, which acted as a semipermeable barrier against oxygen, carbon dioxide, and water vapor, thereby minimizing respiration rate and water loss [41]. In addition, it was also reported that fresh fruits and vegetables are susceptible to weight loss during storage because of the vapor pressure gradient between the fruit

tissue and the surrounding atmosphere. This vapor pressure gradient is influenced by various factors including light exposure, temperature, ripeness, and the occurrence of oxidation during storage [15]. This gradient can initiate the senescence of fruits by accelerating different metabolic reactions, such as ethylene production [42]. Our findings were in agreement with the previous studies where AVG coating was also found to be effective in minimizing the weight loss of grapes [10, 16], blueberry [41], apple [18], persimmon [39], and papaya [15, 27].

[figure(s) omitted; refer to PDF]

3.4. Fruit Firmness

In our study, the firmness of grapes and apples decreased with increasing storage time in both control and coated samples, as shown in Figures 2(a) and 2(b). Fruits start getting softer and losing their firmness because of the biochemical changes in cell wall fractions. In general, these biochemical changes are the results of hydrolytic reactions of cell-wall polymers such as cellulose, hemicelluloses, and pectin, among others [43], and the simultaneous drop of turgor pressure inside the cell [44] as maturation progresses. The softening of fruit during the ripening stage is closely proportional to the deterioration rate of pectin compounds via the enzymatic reaction of pectin methylesterase (PME) and polygalacturonase (PG). Previous studies also reported a loss of firmness in apples [29], grapes [10], and jujube [19] proportional to the storage time.

[figure(s) omitted; refer to PDF]

Coating treatments have been reported in the literature to maintain the firmness of fruits. The firmness retention in coated grapes was notably superior to that in uncoated grapes. Specifically, the firmness of PWB-packed grapes and AVG-coated grapes was approximately 50% and 35% higher, respectively, compared to uncoated grapes after a 12-day storage period. In the case of apples, a similar change was noticed throughout the storage. The PWB-packed apples were significantly ($P<0.05$) higher in firmness (4.63 kg/cm^2) compared to AVG-coated (4.09 kg/cm^2) and control (2.76 kg/cm^2) at the final day of storage. The AVG coating or PWB packaging could hold the firmness of fruit flesh during storage by regulating the actions of the fruit enzymes, such as polygalacturonase, pectin methylesterase, and galactosidase [10, 45]. In addition, the coating on the fruit surface provides a barrier against the diffusion of water to prevent dehydration, which leads to the minimization of firmness loss [46]. The positive effect of AVG coating on maintaining firmness has been reported for grapes [10], apples [18, 29], and blueberries [41]. In contrast, AVG coating (30%) on jujube fruits [19] and AVG coating (33%) on grapes [16] did not show any positive effect on the fruit flesh firmness during postharvest storage.

3.5. Color Value

The color of the fruit is one of the most important consumer requirements for fruit acceptance. As depicted in Figures 3(a) and 3(b), the color difference (ΔE) increased in all treatments throughout the storage period. However, both coating applications showed less color difference (ΔE) in the grapes and apples compared to uncoated samples during storage. After harvesting, fruits undergo color changes as a spontaneous transformation of chlorophyll into various pigments, synthesizing carotenoids and anthocyanins [47]. In addition, the cell wall degradation of fruits during ripening and storage aids in changes of color and firmness by the activity of hydrolytic enzymes [48]. However, both PWB packaging and AVG coating act as a barrier and alter gas permeability, which may increase internal CO_2 levels. The alteration of CO_2 level changes the external and internal color of fruits and also delays the synthesis of carotenoids, degradation of chlorophyll, alteration of anthocyanin, and total phenolic contents [33, 49]. In another study, Valverde et al. [10] also reported a similar result with AVG-coated table grapes.

[figure(s) omitted; refer to PDF]

3.6. Decay Evaluation

The visual decay incidence of the control sample, treated grapes, and apples is shown in Tables 3 and 4, respectively. Initially, no sign of decay was observed in all treatments of grapes and apples until 3 days and 12 days of storage, respectively. Infection of fruits was increased after three days of storage for grapes and fifteen days for apples, with the growth of soft rot spots and shrinkage up to the last day of storage. Nevertheless, fruit coating demonstrated reduced decay compared to uncoated fruits, with the PWB packaging treatment for grapes and apples showing a lower infection score than the AVG coating treatment and the control. This phenomenon of AVG coating

aligns with earlier research indicating the application of AVG coating on the minimization of decay incidence in table grapes [16], strawberries [50], and orange fruit [51].

Table 3

Decay incidence of grapes during storage.

Fruit	Treatment	Storage (day)				
0	3	6	9	12	Grapes	Control
1 ± 0.00^{Ad}	1 ± 0.00^{Ad}	1.97 ± 0.06^{Ac}	2.5 ± 0.10^{Ab}	3.00 ± 0.15^{Aa}	PWB-coated grapes	1 ± 0.00^{Ab}
1 ± 0.00^{Ab}	1.07 ± 0.012^{Cb}	2.03 ± 0.06^{Ba}	2.07 ± 0.06^{Ca}	AVG-coated grapes	1 ± 0.00^{Ad}	1 ± 0.00^{Ad}

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

Table 4

Decay incidence of apples during storage.

Fruit	Treatment	Storage (day)					
0	7	15	21	28	35	Apples	Control
1 ± 0.00^{Ae}	1 ± 0.00^{Ae}	1.53 ± 0.15^{Ad}	4.1 ± 0.20^{Ac}	4.5 ± 0.10^{Ab}	5.00 ± 0.08^{Aa}	PWB-coated apples	1 ± 0.00^{Ae}
1 ± 0.00^{Ae}	1.00 ± 0.10^{Bd}	2.23 ± 0.06^{Cc}	2.48 ± 0.06^{Cb}	3.07 ± 0.12^{Ca}	AVG-coated apples	1 ± 0.00^{Ad}	1 ± 0.00^{Ad}

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

3.7. Microbial Analysis

The edible coating could enhance microbial safety of foods by reducing or preventing microbial infestation. The AVG coating provides better barrier against infestation of Gram-positive than Gram-negative bacteria [52]. Additionally, it aids in minimizing the proliferation of *Rhizopus stolonifer*, *Botrytis cinerea*, and *Penicillium digitatum* [53]. The microbial load of both grapes and apples during storage at ambient temperature is shown in Tables 5 and 6, respectively, where microbial load increased in all treatments with the progression of the storage period. Initially, the microbial load in the grapes and apples was 1.5×103 and 3.0×102 CFU/g, respectively. The growth of microorganisms in both AVG coating and PWB packaging treatments was found to be lower throughout the storage period. Microbial proliferation was the lowest in PWB-treated grapes and apples, followed by AVG-coated grapes and apples and then the control samples at the end of storage. On the last day of storage, the microbial load in control grapes and apples was 1.3×107 and 1.5×107 CFU/g, respectively, whereas, in both PWB-packed grapes and apples, the microbial population was 1.2×105 CFU/g. According to Albanese et al. [54], coatings effectively delay microbial proliferation by forming a barrier layer on the surface of the fruit, which lowers its water activity. AVG gel coating has also been found effective in microbial population minimization when applied on lotus root slices [38],

apple slices [29], and grapes [10] during the storage period.

Table 5

Microbial evaluation (CFU/g) of grapes during storage.

Fruit	Treatment	Storage (day)		
0	6	12	Grapes	Control
$1.5\times10^{3}\pm0.10^{Ac}$	$1.4\times10^{5}\pm0.08^{Ab}$	$1.3\times10^{7}\pm0.13^{Aa}$	PWB-coated grapes	$1.5\times10^{3}\pm0.10^{Ac}$
$2.1\times10^{3}\pm0.14^{Cb}$	$1.2\times10^{5}\pm0.15^{Ca}$	AVG-coated grapes	$1.5\times10^{3}\pm0.10^{Ac}$	$3.6\times10^{4}\pm0.07^{Bb}$

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

Table 6

Microbial evaluation (CFU/g) of apples during storage.

Fruit	Treatment	Storage (day)		
0	21	35	Apples	Control
$3.0\times10^{2}\pm0.9^{Ac}$	$1.2\times10^{6}\pm0.16^{Ab}$	$1.5\times10^{7}\pm0.14^{Aa}$	PWB-coated apples	$3.0\times10^{2}\pm0.9^{Ac}$
$6.3\times10^{4}\pm0.19^{Cb}$	$1.2\times10^{5}\pm0.17^{Ca}$	AVG-coated apples	$3.0\times10^{2}\pm0.9^{Ac}$	$1.3\times10^{5}\pm0.18^{Bb}$

Note. The data are presented as mean ± SD. Values with different superscript lowercase letters within a row and uppercase letters within a column are statistically significant from each other ($P<0.05$).

4. Conclusion

The current study evaluated the effectiveness of PWB packaging and the AVG coating on grapes and apples, respectively, and postharvest qualities during storage at ambient conditions. The findings suggest that fruits subjected to both PWB packaging treatment and AVG coating treatment exhibited lower water loss, total soluble solids, color difference, decay incidence, and higher fruit firmness compared to untreated (control) fruits during storage. However, the PWB packaging treatment exhibited a more significant impact on preserving the quality of fruits compared to the AVG coating treatment. The PWB-packed fruits significantly delayed the loss of firmness, microbial proliferation, and decay infection. These findings suggest that PWB packing and AVG coating have the potential to serve as organic and ecofriendly treatments for preserving the quality and extending the postharvest life of both grapes and apples. Future studies should explore the coating attributes of AVG and evaluate the packaging properties of PWB and their effectiveness on the antioxidant characteristics of both coated and uncoated grapes and apples during storage [54].

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DETAIL

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Changes in Techno-Functional Characteristics of Cow Colostrum Powder Prepared by Freeze Drying

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ABSTRAK (ENGLISH)

The present study investigates the characteristics of freeze-dried bovine colostrum powder prepared from the first three milkings of Karan fries cattle at different intervals. Bioactive components of bovine colostrum are heat sensitive, and therefore, in order to retain the biological activity of different components, it should be subjected to minimal nonthermal treatment. In this study, the optimum temperature/time combination of 60°C/45 min was used for

the processing of cow colostrum. At this temperature, the microbial count significantly decreased as compared to raw colostrum. In freeze-dried bovine skimmed colostrum powder (BSCP) and bovine colostrum whey powder (BCWP), IgG emerged as the highest fraction among immune factors, whereas in growth factors, insulin-like growth factor 1(IGF1) and transforming growth factor $\beta 2$ (TGF$\beta 2$) were found in large proportion as compared to insulin-like growth factor 2 (IGF2) and transforming growth factor $\beta 1$ (TGF$\beta 1$). The IgG content (per 100 g) of BSCP prepared from 1st, 2nd, and 3rd milking was found to be 36.62, 27.87, and 20.51 g, respectively, whereas IgG content (per 100 g) of BCWP prepared from 1st, 2nd, and 3rd milking was found to be 33.32, 24.53, and 16.81 g, respectively. The IGF1 content (per 100 g) of BSCP prepared from 1st, 2nd, and 3rd milking was found to be 921.6, 741.2, and 617.2 μg, respectively, whereas IGF1 content (per 100 g) of BCWP prepared from 1st, 2nd, and 3rd milking was found to be 869.8, 688, and 454.4 μg, respectively. The TGF$\beta 2$ content (per 100 g) of BSCP prepared from 1st, 2nd, and 3rd milking was found to be 1278, 1076, and 856.8 μg, respectively, whereas TGF$\beta 2$ content (per 100 g) of BCWP prepared from 1st, 2nd, and 3rd milking was found to be 1167, 950.2, and 769.2 μg, respectively. The microstructure of freeze-dried cow colostrum whey powder revealed that its pore size was more than that of skimmed colostrum powder. BCWP exhibited lower phagocytic activity as well as lymphocyte proliferation index as compared to BSCP which was carried out *in vitro*. The observations of the study provide an insight about the components present in BCWP and BSCP apart from the physical characteristics of the products which might pave the way for its utilization in various food formats.

TEKS LENGKAP

1. Introduction

Nowadays, consumers are more cognizant of their diet, and post COVID, the general masses are focusing more on strengthening their immune system. The ongoing trend reveals that consumers are interested in immune boosting ingredients and have designed their diet in such a way that it modulates the immune system of the individuals. The consumption of different bioactive ingredients in a balanced manner plays an important role in conferring immunity against viral infections [1]. In fact, bovine colostrum either in liquid form or in powdered form has become the most traded functional ingredient that has been used in medicines for improving the immune system, in baby food, health drinks, pharmaceuticals, and cosmetics [2–4]. Colostrum is the first lacteal secretion obtained during the first 72 hours after the birth of newborn. The composition of colostrum is determined by the requirement of the newborn and usually it contains numerous immune factors and growth factors that will provide the much necessary immunity to the newborn. These components are a part of biological processes, such as GI tract maturation, immunological function, and energy balance and provides defense against pathogens [5]. The bioactive components of colostrum include immunoglobulins (Ig), lactoferrin, growth factors, and other compounds that not only possess antioxidant and anti-inflammatory properties but also they are essential for strengthening the immune system. During the COVID period, interest in the colostrum-based components and the possibility to utilize them in various recipes have increased. The concentration of these bioactive compounds is the highest in colostrum up to 72 hours after birth of the new born, and therefore the excess secretion can be utilized to harness the components. The shelf-life of raw colostrum is limited, and the available thermal processing treatments use high temperature/time combination which results in significant denaturation of bioactive proteins, and subsequently, a loss of biological activity occurs. Cows and buffaloes produce 40−44 kg of colostrum, and consumption of colostrum depends upon calf's body weight. Colostrum optimum requirement is nearly 7–10% by weight of the calf's body weight, and the immunoglobulins from the colostrum should be ingested within 6−12 hours of birth as the gut permeability of the new born decreases rapidly within 24 hrs of birth. One percent of a healthy cow's annual milk supply is made up of colostrum which is significantly more than the calves' requirements [6]. Therefore, excess colostrum may be preserved, processed, and utilized for value addition of human foods. However, the high content of heat labile protein leads to poor heat stability of colostrum, and therefore the cost incurred in processing becomes high. Since powders can be stored at room temperature with more stable and preserved characteristics of products, dairy liquids

are generally converted into powders on a commercial scale.

Freeze drying of colostrum is an obvious choice to retain the biofunctionality of colostrum components as compared to spray drying technique, but the issue of heat-induced denaturation of colostrum's bioactive components, particularly immunoglobulins (Igs), is still a significant challenge in the production of spray-dried colostrum powder which has also been reported by Borad and Singh [3]. The process of manufacturing spray-dried skim milk powder involves vacuum evaporation (55–75°C) of skim milk in order to get 45–48% total solid in concentrate which is then subjected to a spray drier where air inlet temperature is 180–185°C and outlet air temperature is 80–90°C. Keeping in view such processing conditions, spray drying of colostrum has not been considered to be a very good option as such a higher temperature might cause complete denaturation of immunoglobulins which has also been reported by Singh et al. [7] who observed that at 72°C, unfolding of immunoglobulins start and complete denaturation might occur when they are heated at a temperature of 89°C. Every method of drying whether it is spray drying or freeze drying results in some degree of denaturation of immune factors but the extent of loss of biological activity of immune factors and growth factors is more in spray drying as compared to freeze drying but spray drying has been the choice of commercial traders due to the low cost of the spray drying process [8–10]. However, where the final product is intended for obtaining some functionality, then the commercialized form must not only be safe for human consumption but also contains maximized quantity of bioactive molecules, regardless of the technique that was used to dry bovine colostrum into powder. Over the past one decade, extensive research has been conducted on the nutraceutical properties of bovine colostrum, and in the early 2000s, a number of clinical trials were conducted that demonstrated the beneficial impact of colostrum on the athletic performance, muscle mass, and bone health of adults. Several studies reported that athletes who consumed bovine colostrum experienced noticeable benefits, including increased resistance to fatigue, greater lean mass, reduced body fat, improved gut health, and enhanced immune system and could recover faster from injuries which can be attributed to the presence of antimicrobial compounds, immune factors, and certain growth-promoting factors, like insulin-like growth factor-I and -II in colostrum. However, many researchers have reported that further research is needed to fully understand the role of each compound [11–18].

For thousands of years, Ayurvedic doctors in India have employed bovine colostrum for therapeutic purposes. Colostrum can also be used therapeutically to treat conditions such as AIDS, cancer, heart disease, diabetes, autoimmune illnesses, allergies, herpes, bacterial, viral, and parasite infections, gingivitis, and the flu [19]. Prior to the invention of sulfa medicines and penicillin, conventional doctors in the United States and other countries used it as an antibiotic. Colostrum was widely recommended for the treatment of rheumatoid arthritis and as a source of antipolio antibodies in the early 1950s [20].

Compared to blood-derived analogues, colostrum is an alternative source for industrial-scale production of immunoglobulins because of its ready availability and because it is comparatively safe in nature. Colostrum fractions or specific peptides may be helpful for treating a wide range of gastrointestinal infections, such as inflammatory bowel disease, gut damage brought on by nonsteroidal anti-inflammatory drugs (NSAIDs), chemotherapy-induced mucositis, and Alzheimer's disease, according to clinical studies reported by Playford et al. [21]. Over the past ten years, colostrum-based products have been more popular as nutritional supplements, while research on colostrum processing has been less active. Commercially available colostrum products are available in the form of capsules, lozenges, chewing gum, whole-colostrum drinks, and powder which can be for both veterinary and human use. Leading commercial companies operating in this area include PanTheryx, Colostrum BioTec, Immuno-Dynamics, Ingredia Nutritional, New Image, Biostrum Nutritech, Imu-Tek, and Good Health NZ Products (https://www.orianresearch.com). To increase the bioactive content of dairy products such as yogurt, kefir, fermented milk, ice cream, cheese, and butter, whole colostrum has also been used. Several traditional preparations such as Khees (India), Kalvdans (Scandinavia), Abrystir (Iceland), Rmelk (Norway), leipäjuusto (Finland), Molozyvo (Ukraine), and Groosniuys are produced for local market [22]. To preserve the biological activity of the bioactive components, colostrum should be processed at minimal time-temperature combination. In the present study, freeze drying have been adopted to prepare powder with the purpose that such powder can be preserved and further used

in different food product preparations. Despite the existence of various drying methods for converting protein solutions into powders, freeze drying remains a highly promising technique for preserving sensitive products [23]. The functional characteristics of colostrum powders, such as emulsifying capacity, foaming capacity, thermal stability, wettability, solubility, and buffering capacity, are significantly influenced by the compositional variation of colostrum during the early days [3, 24]. In some studies, it has been reported that in early lactations of cow colostrum, physical and functional parameters show extreme changes [25]. The present study is focused on investigating the techno-functional characteristics of freeze-dried bovine skimmed colostrum powder and colostrum whey powder prepared from early lactation milkings obtained at different intervals from Karan fries cattle (indigenously developed at the ICAR-National Dairy Research Institute (NDRI), Karnal, India). Further, effect of heat treatment on major components and other biomolecules was also investigated.

2. Materials and Methods

2.1. Procurement of Raw Bovine Colostrum

Bovine colostrum was collected from Karan fries within 0–48 hours (first three milkings) from the ICAR-NDRI cattle yard.

2.2. Processing and Freeze Drying of Colostrum

The colostrum samples were defatted by centrifugation at 8000 g for 20 min at 2°C using a refrigerated centrifuge (Kubota, model 6800, Japan). The defatted samples were further used for processing. The skimmed bovine colostrum samples were heat-treated at different temperatures, viz., 72°C/15 sec, 63°C/30 min, and 60°C/45 min. Heat-treated defatted colostrum samples were divided into two equal parts. First part was kept at −20°C and later on freeze-dried at −40°C at 5 torr vacuum in a freeze dryer (LabTech, Japan). Second part was utilized for whey preparation, wherein 1.5% rennet was added at 32°C and was held for 1 h. The coagulum was cut and the curd cubes were cooked at 40°C for 30 min for expulsion of whey. The whey was extracted and frozen at −20°C and finally freeze-dried under the conditions mentioned above. The dried mass was ground to obtain a homogenous powder.

2.3. Compositional Analysis of Raw Colostrum and Colostral-Dried Products

Raw colostrum was analysed for TS (total solids), fat, ash, and lactose as per the procedure described for milk in SP:18, Part XI [26]. Total protein was determined by the Kjeldahl method as described in AOAC [27]. Powdered Colostrum samples were analysed for moisture, fat, ash, and lactose as per the procedure described for skim milk powder in SP:18, Part XI [26]. Total protein was determined by the Kjeldahl method as described in AOAC [27]. IgG and IgA content for raw colostrum and powdered colostrum samples was estimated by ELISA kit supplied by KOMA BIOTECH, Korea. TGFβ1, TGFβ2, IGF1, and IGF2 for raw colostrum and powdered colostrum samples were determined by the ELISA kit procured from USCN Life Science Inc., Wuhan, China.

2.4. Microbiological Analysis

SPC, coliform count, yeast and mold count, *Salmonella* count, and *S. aureus* count of raw bovine colostrum samples and heated colostrum samples were carried out according to the method described by Houghtby and coworkers [28].

2.5. Physical Properties of Colostral-Dried Products

Flowability, bulk density (loose and packed), and porosity were determined by the procedure described by Suleiman et al. [29]. Color values of bovine skimmed colostrum powder (BSCP) and bovine colostrum whey powder (BCWP) were measured using a colorflex colorimeter (Hunter Associates Laboratory, VA, USA), and the results were expressed through software version 4.1, and it is expressed in terms of L*, a*, and b* values. Microstructure of BSCP and BCWP was investigated using scanning electron microscopy (SEM) which was carried out as per the procedure laid down by Caric and Ibrahim [30]. The wettability of the powder samples was assessed as per the procedure given by Naik et al. [31]. The time required for 0.1 g of powder to get completely wet was recorded.

2.6. *In Vitro* Immunomodulatory Activity

Lymphocyte proliferation index and phagocytosis were carried out *in vitro* as per the method described by Hay and Westwood [32].

2.7. Statistical Analysis

All the observations were recorded as mean ± SE. MS-Excel, version 2010, was used for graphical representations. One-way analysis of variance (ANOVA) at a confidence level of 95% and other statistical parameters were calculated using GraphPad Prism version 5.01, and the significance of the data was reported using the Tukey post hoc test for comparison of means.

3. Results and Discussion

3.1. Standardisation of Time-Temperature Combination for Heat Treatment of Colostrum Samples before Freeze Drying

Bovine colostrum samples collected during the first 0–48 h of milking were subjected to heat treatment using batch method at different temperatures at 72°C/15 sec, 63°C/30 min, and 60°C/45 min, and it was visually observed for gel formation. The samples from all the milkings did not coagulate at time-temperature combination of 63°C/30 min and 60°C/45 min, whereas at 72°C/15 sec, samples were found to form gel. Also, a significant $P<0.05$ reduction in IgG and IgA content was observed at all time-temperature combinations, but the loss of IgG and IgA content was significantly less ($P<0.05$) at 60°C/45 min as compared to 63°C/30 min (Table 1). When colostrum samples from different milkings were heat-treated at 60°C/45 min, IgG concentration decreased in the range of 14.63−11.68% and IgA concentration reduced in the range of 12.18−10.11%. The findings of the current investigation were concomitant to the work of Saldana and coworkers [33], who reported that immunoglobulins concentration decreased by 9% and 12% when the raw colostrum was heat-treated for 60°C/30 min and 60°C/60 min, respectively.

Table 1

Observations related to heat treatment of colostrum samples at selected time-temperature combination.

Milking intervals	1st milking			2nd milking			3rd milking		
Time/temperature combination for heat treatment	72°C/15 sec	63°C/30 min	60°C/45 min	72°C/15 sec	63°C/30 min	60°C/45 min	72°C/15 sec	63°C/30 min	60°C/45 min
–									
Gel formation	Yes	No	No	Yes	No	No	Yes	No	No
% reduction in IgG	$67.41^{a} \pm 0.19$	$45.81^{b} \pm 0.16$	$14.63^{c} \pm 0.18$	$62.48^{d} \pm 0.19$	$37.71^{e} \pm 0.16$	$12.13^{f} \pm 0.18$	$35.41^{g} \pm 0.19$	$25.81^{h} \pm 0.16$	$11.68^{i} \pm 0.18$
% reduction in IgA	$69.12^{a} \pm 0.27$	$22.54^{b} \pm 0.21$	$12.18^{c} \pm 0.16$	$49.22^{d} \pm 0.27$	$19.84^{e} \pm 0.21$	$11.18^{f} \pm 0.16$	$29.12^{g} \pm 0.27$	$15.14^{h} \pm 0.21$	$10.11^{i} \pm 0.16$

$n=15$, mean + SD; values followed by different alphabets a–i in superscript are significantly different $P<0.05$.

There was no significant $P>0.05$ decrease in the quantity of growth factors when colostrum was subjected to heat treatment at the abovementioned time-temperature combinations. Hence, heat treatment at 60°C/45 min was selected for colostrum processing. Also, standard plate count (SPC), coliform count, yeast and mold count, and *S. aureus* and *Salmonella* count were performed before and after heat treatment at 60°C/45 min to assess the reduction in different counts at this time-temperature combination. It was found that as compared to raw colostrum, there was a significant reduction in microbial count after the heat treatment at 60°C/45 min. and the same has been presented in Figures 1–3. The SPC of raw colostrum was found to be 6.17×10^5 (cfu/mL) which reduced to 5.75×10^4 (cfu/mL) after heat treatment at 60°C/45 min. in samples of 1st milking. In samples of 2nd milking, the SPC of raw colostrum was found to be 5.30×10^5 (cfu/mL) which reduced to 5.74×10^4 (cfu/mL) after heat treatment at 60°C/45 min. The SPC of raw colostrum in samples of 3rd milking was found to be 4.05×10^5 (cfu/ml) which decreased to

3.36×10^4 (cfu/ml) after heat treatment at 60°C/45 min. Similarly, the coliform count of raw colostrum samples of 1st, 2nd, and 3rd milking was found to be 312.7, 282.7, and 45 (cfu/mL) which reduced to 24.6, 17.3, and 18, respectively (cfu/mL) after heat treatment at 60°C/45 min. Yeast and mold count of raw colostrum samples of 1st, 2nd, and 3rd milking was found to be 25, 32.5, and 30.4 (cfu/mL) which reduced to 1.13, 2.66, and 1.53, respectively (cfu/mL), after heat treatment at 60°C/45 min. There was a 94% decrease in *Salmonella* count and 96% reduction in *S. aureus* count when the samples were heated at 60°C/45 min. Thus, the temperature time combination not only retains the activity of immune factors and growth factors in substantial proportion but also reduces the microbial count considerably. Godden et al. [34] reported that the viable count of *M. bovis*, *L. monocytogenes*, *E. coli O157:H7*, *Salmonella enteritidis*, and *Mycobacterium avium* subspecies *paratuberculosis* decreased below detectable limits when colostrum was heated at 60°C for 120 min.

[figure(s) omitted; refer to PDF]

3.2. Effect of Freeze Drying on Proximate Composition of Skimmed Colostrum Powder and Colostrum Whey Powder

The samples of raw colostrum that were collected during the first three milkings (0–48 h) after calving were analysed for constituents like total solids, protein, fat, lactose, ash, IgG, IgA, IGF1, IGF2, and TGFβ1, TGFβ2, and the values are presented in Table 2. The mean values of total solids, fat, protein, and ash in the first milking, second milking, and third milking showed a significant $P<0.05$ decreasing trend as the time period increased after calving. Puppel and coworkers [35] reported that colostrum has two to ten times more minerals (except for potassium) than milk, and compositional changes in colostrum occur with each hour. This study also reports similar results as observed by them, wherein protein content ranges from 16.8 to 4.8%, fat content ranges from 6.7 to 3.9%, and lactose content ranges from 2.9 to 4.2% during first 48 hrs after calving. McGrath et al. [36] reported that lactose content, casein fraction, and fat increase significantly in secretions of early lactation. In this study, average values of lactose content in first milking, second milking, and third milking showed a significantly increasing trend as the time period increased after calving. The average values of IgG in first milking, second milking, and third milking were observed to be 76.5 g/L, 63 g/L, and 43.5 g/L, respectively. The average values of IgA in first milking, second milking, and third milking were found to be 3.4 g/L, 2.7 g/L, and 2.3 g/L, respectively. The average values of IGF1 in first milking, second milking, and third milking were observed to be 465 ng/mL, 390 ng/mL, and 280 ng/mL, respectively. The average values of IGF2 in first milking, second milking, and third milking were found to be 175 ng/mL, 145 ng/mL, and 92.5 ng/mL, respectively. The average values of TGFβ1 in first milking, second milking, and third milking were found to be 26.7 ng/mL, 20.7 ng/mL, and 19.8 ng/mL, respectively. The average values of TGFβ2 in first milking, second milking, and third milking were observed to be 625 ng/mL, 482 ng/mL, and 280.5 ng/mL, respectively. Thus, immune factors and growth factors showed a significant decreasing trend as the time period increased after calving. Compositional analysis of raw colostrum revealed that except lactose all other constituents significantly decreased $P<0.05$ as the time after calving increased.

Table 2

Composition of raw bovine colostrum collected during the first three milkings (0–48 hours).

Constituents of raw colostrum	1st milking	2nd milking	3rd milking
Total solids (%)	$22.93^a \pm 1.63$	$17.69^b \pm 2.34$	$15.88^c \pm 2.30$
Fat (%)	$5.07^a \pm 0.4$	$4.96^b \pm 0.37$	$4.11^c \pm 0.11$
Protein (%)	$14.33^a \pm 0.61$	$13.57^b \pm 0.48$	$10.57^c \pm 0.43$
Lactose (%)	$2.75^a \pm 0.16$	$2.91^b \pm 0.2$	$3.6^c \pm 0.24$

Ash (%)	2.11[a] ± 0.27	1.72[b] ± 0.24	1.008[c] ± 0.22
IgG (g/L)	76.5[a] ± 8.89	63[b] ± 6.12	43.5[c] ± 6.30
IgA (g/L)	3.4[a] ± 0.36	2.7[b] ± 0.25	2.3[c] ± 0.15
IGF1 (ng/ml)	465[a] ± 36.87	390[b] ± 74.36	280[c] ± 59.68
IGF2 (ng/ml)	175[a] ± 11.83	145[b] ± 9.05	92.5[c] ± 3.75
TGFβ1 (ng/ml)	26.7[a] ± 8.31	20.7[b] ± 5.95	17.8[c] ± 1.62
TGFβ2 (ng/ml)	625[a] ± 36.78	482[b] ± 48.01	280.5[c] ± 18.99

n = 40, mean ± SD; values followed by different superscript column wise are significantly different P<0.05.

For obtaining 25 g of freeze-dried skimmed colostrum powder, it took 28 hours, and for obtaining 25 g of freeze-dried colostrum whey powder, it took 32.5 hours. The freeze-dried samples were analysed for proximate composition, immune factors, and growth factors, and the values are presented in Table 3.

Table 3

Composition of freeze-dried bovine skimmed colostrum powder and colostrum whey powder.

Type of powder	Bovine skimmed colostrum powder			Bovine colostrum whey powder		
Constituents	1st milking	2nd milking	3rd milking	1st milking	2nd milking	3rd milking
–						
Total solids (%)	94.88[a] ± 0.148	94.89[a] ± 0.14	94.83[a] ± 0.14	94.73[a] ± 0.13	94.65[a] ± 0.147	94.60[a] ± 0.155
Fat (%)	5.2[a] ± 0.15	5.5[b] ± 0.23	5.7[c] ± 0.21	2.6[d] ± 0.026	2.4[e] ± 0.026	2.2[f] ± 0.027
Protein (%)	69.91[a] ± 0.15	64.93[b] ± 0.13	61.94[c] ± 0.14	67.22[d] ± 0.139	61.06[e] ± 0.025	57.79[f] ± 0.158
Lactose (%)	12.71[a] ± 0.07	20.21[b] ± 0.11	24.44[c] ± 0.089	15.23[d] ± 0.102	23.23[e] ± 0.19	26.91[f] ± 0.15
Ash (%)	7.39[a] ± 0.018	6.94[b] ± 0.04	6.49[c] ± 0.019	6.10[d] ± 0.05	4.75[e] ± 0.03	3.8[f] ± 0.04
IgG (g/100 g)	36.62[a] ± 0.56	27.87[b] ± 1.2	20.51[c] ± 0.27	33.33[d] ± 0.65	24.53[e] ± 0.638	16.8[f] ± 0.42

IgA (g/100 g)	$1.75^{a} \pm 0.024$	$1.40^{b} \pm 0.019$	$1.29^{c} \pm 0.009$	$1.58^{d} \pm 0.054$	$1.17^{e} \pm 0.010$	$1.057^{f} \pm 0.013$
IGF1 (μg/100 g)	$921.6^{a} \pm 11.68$	$741.2^{b} \pm 4.86$	$617.2^{c} \pm 7.28$	$869.8^{d} \pm 13.45$	$688^{e} \pm 5.96$	$454.4^{f} \pm 6.34$
IGF2 (μg/100 g)	$351.5^{a} \pm 3.96$	$311.5^{b} \pm 3.86$	$243.3^{c} \pm 4.16$	$328.7^{d} \pm 2.20$	$276.6^{e} \pm 2.17$	$216.6^{f} \pm 3.08$
TGFβ1 (μg/100 g)	$6.64^{a} \pm 0.084$	$5.40^{b} \pm 0.029$	$4.17^{c} \pm 0.182$	$5.93^{d} \pm 0.033$	$4.94^{e} \pm 0.086$	$3.32^{f} \pm 0.1420$
TGFβ2 (μg/100 g)	$1278^{a} \pm 20.15$	$1076^{b} \pm 22$	$856.8^{c} \pm 15.36$	$1167^{d} \pm 19.33$	$950.2^{e} \pm 31.19$	$769.2^{f} \pm 4.22$

$n = 15$, mean ± SE; values followed by different superscript are significantly different $P<0.05$.

There is no significant $P>0.05$ difference in total solids content between freeze-dried bovine skimmed colostrum powder and bovine colostrum whey powder. However, significant difference $P<0.05$ exists between the fat, lactose, protein, and ash content of bovine skimmed colostrum powder and bovine colostrum whey powder. Immune factors like IgG and IgA were significantly higher $P<0.05$ in bovine skimmed colostrum powder as compared to bovine colostrum whey powder. Similar observations were noted by Elfstrand et al. [9], wherein the overall reduction of immune factors and growth factors was due to the entrapment of these components in the casein matrix which was removed during colostrum-based preparation.

Growth factors like IGF1, IGF2, TGFβ1, and TGFβ2 were significantly higher $P<0.05$ in bovine skimmed colostrum powder as compared to bovine colostrum whey powder. The reduction in growth factors in bovine colostrum whey powder may be due to removal of casein matrix which might have entrapped these molecules and got removed with the casein complex.

3.3. Effect of Freeze Drying on Physical Properties of Dried Colostrum Products

Some of the selected physical properties of dried colostrum products were studied and are presented in Table 4. Flowability (expressed in terms of Hausner ratio) of skimmed bovine colostrum powder from different milkings was found to be in the range of 1.37−1.23. For bovine colostrum whey powder prepared using colostrum from different milkings, flowability values were found to be in the range of 1.43−1.23. A study reported by Rezende et al. [37] showed that freeze-dried powders had better flowability, wettability, and solubility. Upadhyay [38] reported that for skim milk powder, flowability was found to be 0.97, whereas for instant skim milk powder, it was found to be around 0.75. Turchiuli et al. [39] reported that the Hausner ratio has been used for the determination of flow characteristics of the powder samples which measures the powder's cohesiveness and has been used as the index of flow for dry powders. Dry substances possessing a Hausner ratio greater than 1.34 are regarded to be cohesive and consequently less free to flow [40]. High Hausner ratio indicates high cohesiveness between the particles that results in aggregation and exhibits decreased flowability [41].

Table 4

Physical characteristics of freeze-dried bovine skimmed colostrum powder and colostrum whey powder.

Type of powder	Bovine skimmed colostrum powder			Bovine colostrum whey powder		
Constituents	1st milking	2nd milking	3rd milking	1st milking	2nd milking	3rd milking

–						
Loose bulk density (g/ml)	$0.497^{a} \pm 0.007$	$0.617^{b} \pm 0.009$	$0.696^{c} \pm 0.007$	$0.271^{d} \pm 0.009$	$0.310^{e} \pm 0.007$	$0.423^{f} \pm 0.010$
Packed bulk density (g/ml)	$0.625^{a} \pm 0.008$	$0.714^{b} \pm 0.008$	$0.785^{c} \pm 0.010$	$0.357^{d} \pm 0.006$	$0.448^{e} \pm 0.007$	$0.53^{f} \pm 0.011$
Hausner ratio	$1.37^{a} \pm 0.019$	$1.24^{b} \pm 0.009$	$1.14^{c} \pm 0.017$	$1.43^{d} \pm 0.028$	$1.36^{e} \pm 0.018$	$1.23^{f} \pm 0.006$
Porosity	$100.2^{a} \pm 0.019$	$100.1^{a} \pm 0.013$	$100.1^{a} \pm 0.011$	$100.1^{a} \pm 0.013$	$100.1^{a} \pm 0.014$	$100.1^{a} \pm 0.012$
Color						
L* value	$91.8^{a} \pm 0.155$	$94.62^{b} \pm 0.145$	$93.21^{c} \pm 0.091$	$92.52^{e} \pm 0.052$	$93.41^{d} \pm 0.135$	$95.06^{f} \pm 0.096$
a* value	$1.80^{a} \pm 0.031$	$2.76^{d} \pm 0.031$	$5.1^{b} \pm 0.037$	$-7.32^{e} \pm 0.023$	$-4.82^{c} \pm 0.015$	$-3.8^{f} \pm 0.014$
b* value	$25.64^{a} \pm 0.064$	$23.42^{d} \pm 0.055$	$21.39^{b} \pm 0.59$	$22.68^{e} \pm 0.054$	$20.70^{c} \pm 0.044$	$16.35^{f} \pm 0.455$
Wettability (sec)	$150.1^{a} \pm 3.03$	$79.80^{b} \pm 1.19$	$64.87^{c} \pm 0.98$	$35.80^{d} \pm 0.54$	$25.40^{e} \pm 0.57$	$18.20^{f} \pm 0.89$

$n = 15$, mean ± SE; values followed by different superscripts are significantly different $P<0.05$.

As observed from Table 4, the loose bulk density of skimmed bovine colostrum powder prepared from different milkings was found to be in the range of 0.69−0.49 g/cm^3. The values of loose bulk density of bovine colostrum whey powder samples were found to be in the range of 0. 0.42−0.27 g/cm^3. The packed bulk density for skimmed bovine colostrum powder samples prepared from different milkings was found to be in the range of 0.78−0.62 g/cm^3, whereas the values of bovine colostrum whey powder samples, prepared from different milkings, were found to be in the range of 0.35–0.53 g/cm^3. Hols and van Mil [42] reported that for spray-dried powders, loose bulk density has been reported to be 0.38 g/cm3 and packed bulk density has been reported to be 0.41–0.43 g/cm^3. The importance of high bulk density lies in the fact that it significantly reduces packaging, storage, and transport cost of powder [43]. Porosity values for all the freeze-dried powder samples were found to be 100.1, and therefore, there was no significant difference $P>0.05$ among the samples of different treatments. The color values were expressed in terms of L*, a*, and b* wherein L* values indicate black to white and ranges from 0 to 100. Redness and green hue of any sample is indicated by a* value which ranges from +60 to −60. Yellow and blue colors are depicted by b* values which ranges from +60 to −60. The L* values of skimmed bovine colostrum powder from different milkings were found to be in the range of 93.21−91.80. For bovine colostrum whey powder prepared using colostrum from different milkings, L* values were found to be in the range of 95.06−92.52. The a* values of skimmed bovine colostrum powder from different milkings were found to be in the range of 5.10−1.80. For bovine colostrum whey powder prepared using colostrum from different milkings, a* values were found to be in the range of −3.80 to −7.32. The b* values of skimmed bovine colostrum powder from different milkings were found to be in the range of 21.39–25.64.

For bovine colostrum whey powder prepared using colostrum from different milkings, b* values were found to be in the range of 22.68−16.35. The color of powder samples was slightly yellowish as bovine secretions contain more carotene as physiological system of cow does not allow hundred percent conversion of carotene into vitamin A. Wettability (in sec) values of skimmed bovine colostrum powder prepared from different milkings were found to be in the range of 64.87–150.10, and those of bovine colostrum whey powder prepared from different milkings were found to be in the range of 18.20–35.80. From the observations of wettability, it can be inferred that bovine colostrum whey powder gets wet readily as compared to bovine skimmed colostrum powder which might be due to the presence of colloidal casein particles which have fewer pores with less pore size as evidenced by the microstructure of bovine skimmed colostrum powder.

3.4. Microstructure of the Bovine Colostrum Whey Powder and Bovine Skimmed Colostrum Powder

The microstructure of the bovine colostrum whey powder was investigated, and the images of SEM (Figure 4) show that the freeze-dried powder particles showed a sponge-like rough internal microstructure with numerous pores observed. The microstructure of the bovine skimmed colostrum powder was investigated, and the image of SEM (Figure 5) shows that the microstructure has rough surfaces and had comparatively less pore size. Bhatta et al. [44] reported that the ice sublimation process in freeze drying influences the shape and volume of powders because of the formation of large pores. From the figures, it can be observed that freeze-dried colostrum powder samples showed rough or flat shaped surface and amorphous or uneven form rather than individual particles. Sublimation of ice crystals has resulted in numerous pores which might increase water penetration and this might increase the reconstitutability of the products.

[figure(s) omitted; refer to PDF]

3.5. *In Vitro* Immunomodulatory Activity of the Dried Colostrum Products

The observations of *in vitro* immunomodulatory activity of freeze-dried bovine skimmed colostrum powder and colostrum whey powder are presented in Table 5. Lymphocyte proliferation index of bovine skimmed colostrum powder was found to be 1.79, 1.64, and 1.41 for powders prepared from colostrum samples collected from 1st milking, 2nd milking, and 3rd milking, respectively, after parturition whereas for bovine colostrum whey powder, it was observed to be 1.26, 1.13, and 0.96, respectively, for colostrum whey powders prepared from samples collected from 1st milking, 2nd milking, and 3rd milking after parturition. Skimmed bovine colostrum powder prepared from colostrum samples collected from 1st milking, 2nd milking, and 3rd milking after parturition showed 35.47%, 31.87%, and 29% phagocytic activity, respectively, whereas bovine colostrum whey powder prepared from 1st milking, 2nd milking, and 3rd milking showed 20.27%, 18%, and 14.67% phagocytic activity, respectively. There is a significant $P < 0.05$ difference in phagocytic activity as well as lymphocyte proliferation index between all the samples of freeze-dried BSCP and BCWP. Bovine colostrum whey powder exhibited significantly lower phagocytic activity as well as a lower lymphocyte proliferation index as compared to skimmed bovine colostrum powder.

Table 5

The observations of immunomodulatory activity (*in vitro*) of freeze-dried bovine skimmed colostrum powder and colostrum whey powder.

Type of powder	Bovine skimmed colostrum powder			Bovine colostrum whey powder		
Immunomodulatory parameters	1st milking	2nd milking	3rd milking	1st milking	2nd milking	3rd milking
–						
Lymphocyte proliferation index	$1.79^{a} \pm 0.037$	$1.64^{b} \pm 0.045$	$1.41^{c} \pm 0.018$	$1.26^{d} \pm 0.05$	$1.13^{e} \pm 0.04$	$0.96^{f} \pm 0.073$

Phagocytic activity (%)	$35.47^{a} \pm 0.38$	$31.89^{b} \pm 0.31$	$29.03^{c} \pm 0.47$	$20.27^{d} \pm 0.25$	$18.08^{e} \pm 0.36$	$14.67^{f} \pm 0.18$

$n = 15$, mean ± SE; values followed by different superscripts coloumnwise are significantly different $P<0.05$.

4. Conclusion

The unique biomolecules of colostrum have aroused interest among consumers in the post-COVID period, wherein many industries are looking forward for manufacturing innovative functional food using colostrum-based ingredients. Bovine colostrum can be subjected to heat treatment at 60°C/45 minutes with a minimal decrease in immune factors and growth factors. At the same time, there is considerable reduction in microbial count which renders it safe for human consumption. Bovine skimmed colostrum powder as well as bovine colostrum whey powder can be prepared by the freeze drying method, wherein there is a minimal loss of biomolecules, and such powders can be used as a constituent or as a bioactive ingredient in beverages, infant formulas, or protein-rich supplements. The functional attributes of such powders become an essential property when it is to be incorporated into protein-rich supplements or when it is to be used as bioactive ingredient for other food products or for development of a functional food product using immune factors as the basic constituents. The major challenges that were being faced with the utilization of this ingredient was the huge variation in composition, uncertain availability, and inadequate processing technologies. This issue can be resolved by converting colostrum into a powdered form which can be easily made available, easily transported, and stored throughout the year with assured bioactivity of its components.

Authors' Contributions

Anamika Das proposed the methodology, validated the study, investigated the study, and wrote the original draft. Raman Seth conceptualized the study, proposed the methodology, supervised the study, and performed project administration. Ayon Tarafdar reviewed the article, provided interpretation of results, and edited the draft. Swarnima Dey, Yogesh Kumar Saini, and Ranjna Sirohi wrote and edited different sections of the draft.

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DETAIL

Subjek:	Growth factors; Infections; Temperature; Immune system; Medical research; Nonsteroidal anti-inflammatory drugs; Heat; Milk; Immunoglobulins; Viral infections; Biological activity; Cattle; Insulin; Lymphocytes; Pore size; Freeze drying
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Judul:	Changes in Techno-Functional Characteristics of Cow Colostrum Powder Prepared by Freeze Drying

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Modified Gegen Qinlian Decoction Ameliorates DSS-Induced Ulcerative Colitis in Mice by Inhibiting Ferroptosis via Nrf2/GPX4 Pathway

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ABSTRAK (ENGLISH)

Objective. Ferroptosis, a form of programmed cell death, is considered a novel target for the treatment of ulcerative colitis (UC). The aim of this study was to explore whether modified Gegen Qinlian decoction (MGQD) ameliorates UC in mice via mediating ferroptosis. *Methods*. Mice with dextran sulfate sodium- (DSS-) induced colitis were administered with MGQD for seven days. Subsequently, iron, malondialdehyde (MDA), glutathione (GSH), and reactive oxygen species (ROS) were measured. ELISA and immunohistochemistry were used to evaluate the levels of proinflammatory cytokines and tight junction proteins, respectively. Transmission electron microscopy was used to reveal mitochondrial morphology. Western blot and qRT-PCR analyses were used to assess the expression levels of the proteins of Nrf2/GPX4 pathway. The docking affinity of MGQD and Nrf2 was assessed using AutoDock Vina 1.1.2. *Results*. Ferroptosis was identified in mice with UC, as demonstrated by mitochondrial disruption, MDA and ROS production, iron overload, decrease in GSH level, and abnormal expression of core marker proteins of ferroptosis. After MGQD treatment, these characteristic changes of ferroptosis were significantly reversed, along with concomitant activation of the Nrf2/GPX4 pathway. Furthermore, molecular docking analysis revealed that MGQD had a high affinity for Nrf2. *Conclusion*. MGQD may ameliorate UC by inhibiting ferroptosis via the activation of Nrf2/GPX4 pathway. This study provided new insights into the application of MGQD complementary therapy for UC.

TEKS LENGKAP

1. Introduction

Ulcerative colitis (UC) is a common inflammatory bowel disease [1]. The incidence of UC has been increasing continuously worldwide, imposing a significant economic burden on society [2]. The etiology of UC remains poorly understood, although environmental factors, genetics, immune responses, and intestinal microflora have been

implicated as causative factors for UC [1]. Currently, the therapies for UC are focused on controlling the active inflammatory response and regulating intestinal immune balance, and commonly used drugs include 5-aminosalicylic acid drugs, immunosuppressants, and steroids [3, 4]. However, the long-term use of these drugs poses the challenges of significant adverse events and drug resistance [5, 6]. Therefore, it is urgent to develop potent medications for UC.

Ferroptosis is a newly discovered type of programmed cell death caused by oxidative stress and high reactive oxygen species (ROS) production [7]. The death of epithelial cells in UC has recently been linked to ferroptosis, and inhibition of ferroptosis has been demonstrated to be beneficial for UC [8, 9]. Nrf2 is a transcription factor involved in the resistance to endogenous oxidative stress. Genes located downstream of Nrf2 play roles in key regulatory functions in iron metabolism (FTH1, FTL, SLC40A1, MT1G, and FECH), ROS accumulation (GPX4, HO-1, and NQO1), and GSH production (GSR, GCLC, GSS, and SLC7A11). Thus, activation of Nrf2/GPX4 pathway is a classical strategy to inhibit ferroptosis [10, 11].

Gegen Qinlian decoction (GQD) is a well-known Chinese herbal formula beneficial for UC [12]. Our previous study reported that modified GQD (MGQD) is efficacious in alleviating UC in mice [13–15]. Intestinal epithelial cell death can promote the pathological progression of UC by altering the integrity of the intestinal barrier [16]. Therefore, in this study, we aimed to further investigate the mechanism underlying the therapeutic action of MGQD in UC via ferroptosis regulation.

2. Materials and Methods

2.1. Decoction Preparation

The composition of MGQD is as follows: *Coptidis Rhizoma* (9 g), *Radix Puerariae* (24 g), *Zingiberis Rhizoma* (9 g), *Scutellariae Radix* (9 g), *Talcum* (9 g), and *Liquorice* (6 g). MGQD was provided by the pharmacy of Xiyuan Hospital of China Academy of Chinese Medical Sciences. MGQD extracts were prepared as per the standard for preparing the decoction of Chinese herbal medicines. The production process was as follows. The herbs were soaked in distilled water (1 : 8, w/v) for 1 h. *Talcum* was boiled in distilled water for 30 min before adding the rest of the herbs for 1.5 h and then filtered. The residue was combined with six times the amount of distilled water and boiled for 1 h, followed by filtration. The two filtrates were mixed, and crude drug of concentrations 0.5, 1, and 2 g/mL was prepared.

2.2. Animal Experimental Protocol

C57BL/6J mice (weighing 18–22 g) provided by SPF Biotech (Beijing, China) were randomly allocated into six groups: control, dextran sulfate sodium (DSS), medium-dose MGQD (GM), low-dose MGQD (GL), high-dose MGQD (GH), and ferroprostatin-1 (Fer-1; a ferroptosis inhibitor) ($n = 10$/group). Except the control group, other five groups received 3% (w/v) DSS diluted in drinking water for 7 days to induce acute experimental UC. At the same time, the GL, GM, and GH groups orally received 5, 10, and 20 mg/kg MGQD once per day for 7 consecutive days. The MGQD dose used here was calculated based on the clinical dose of raw materials. For the Fer-1 group, every other day from the day before DSS induction, the ferroptosis inhibitor Fer-1 (HY-100579, MedChemExpress, USA) was administered intraperitoneally to mice. The control and DSS groups intragastrically received the same dosages of distilled water. On day 8, all mice were sacrificed under anesthesia using ether, and the length of the colon was measured. The detailed experimental procedure is presented in Figure 1(a).

[figure(s) omitted; refer to PDF]

2.3. Histological Analysis

For hematoxylin and eosin (H &E) staining, colon samples were embedded in paraffin, fixed in 4% paraformaldehyde, and cut into 5 m thick blocks. Based on previously established standards, a blinded assessment of the colitis activity was performed using the sections stained with H &E [13–15].

2.4. Transmission Electron Microscopy

Fresh colorectal tissue samples of mice were fixed in glutaraldehyde. The samples were dehydrated using various concentrations of ethanol, followed by washing multiple times with phosphate buffer saline. The samples were dehydrated, thinly sliced, and inserted into the resin. The sections were stained with 0.5% lead citrate and 4% uranyl

acetate, and images were captured using transmission electron microscopy.

2.5. Measurement of the Levels of Proinflammatory Cytokines

The levels of IFN-γ, IL-6, IL-1β, and TNF-α in colonic tissues of mice with UC were determined using respective ELISA kits as per the manufacturer's instructions (Ruixin Biotechnology Co., Ltd., Quanzhou, China).

2.6. Immunohistochemistry

Tight junction protein concentration in colonic tissues of mice with UC was measured using immunohistochemistry. The slices were incubated with ZO-1 (1 : 100; Affinity Biosciences; AF5145), occludin (1 : 200; Proteintech; 28674-1-AP), claudin-1 (1 : 200; Proteintech; 28674-1-AP), and 4HNE (1 : 1000; Bio-Techne; MAB3249) antibodies overnight at 4°C, followed by incubation with the secondary antibody (1 : 200; Servicebio; GB23303). The images of stained samples were taken using a light microscope (Olympus BX41, Shanghai, China). The mean optical density value was used to analyze and represent staining intensity.

2.7. Western Blot Analysis

Proteins from the colon tissue samples were extracted and separated as per their molecular weights using 10% SDS-PAGE. The membrane was incubated with the primary antibodies for Nrf2 (1 : 3000, Proteintech, 16396-1-ap), GPX4 (1 : 3000, Proteintech, GB113091), FTH1 (1 : 3000, Proteintech, GB112933), ACSL4 (1 : 3000, Servicebio, GB113871), SLC7A11 (1 : 3000, Proteintech, 26864-1-ap), and GAPDH (1 : 5000, Servicebio, GB15002) overnight at 4°C. Furthermore, the membrane was incubated with HRP-labeled rabbit or mouse IgG secondary antibodies (1 : 5000, Servicebio, GB23303 and GB25301) for 2 h at room temperature. The protein signals were visualized using ECL solution (P0018, Beyotime).

2.8. Measurement of Iron Content and Levels of GSH, MDA, and ROS

After extraction of protein samples from the colonic tissues, the levels of malondialdehyde (MDA), glutathione (GSH), reactive oxygen species (ROS), and iron were measured using respective kits as per the manufacturer's instructions (Ruixin Biotechnology Co., Ltd., Quanzhou, China).

2.9. RT-qPCR Analysis

Total RNA was extracted from the colon tissue and used to synthesize cDNA using reverse transcription. RT-qPCR was performed using the CFX96 real-time PCR detection system (Bio-Rad, USA). GAPDH was used as the internal control. The relative expression levels of target genes were determined using $2^{-\Delta\Delta CT}$ method. The primer sequences are given in Table 1.

Table 1

Primer sequences.

Genes	Forward primer	Reverse primer
Nrf2	TGTCTTAATACCGAAAACAAGCAGC	GACCACAGTTGCCCACTTCTTTT
GPX4	GCACATGGTCTGCCTGGATAAG	TCTTGATTACTTCCTGGCTCCTG
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAGGTCAATGAAGGGGTCGT

2.10. Macromolecular Docking

Baicalin, puerarin, palmatine chloride, wogonin, and berberine chloride were identified as the main components of MGQD in our previous study via high-performance liquid chromatography system [13]. In this study, the molecular docking of the components of MGQD and Nrf2 was performed using AutoDock Vina 1.1.2. The structure of component was obtained using the PubChem database. Energy minimization of the components and conversion of their 2D structures into 3D chemical structures were performed using ChemBio3D 13.0 software. The crystal structure of the Nrf2 domain was used as the receptor model, and the 3D structure was obtained using PDB database.

2.11. Statistical Analysis

All continuous data were expressed as mean ± standard deviation. Before analysis, normality and variance homogeneity tests were performed for each set of data to be compared. For comparisons of more than two groups, one-way or two-way analysis of variance was performed, with Bonferroni's or Dunnett's multiple-comparisons test for normally distributed data or the Mann–Whitney test for nonnormally distributed data. Unless otherwise stated, $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS 26.0 and GraphPad Prism version 9 software.

3. Results

3.1. MGQD Attenuated UC in Mice

On day 4 of the experiments, weight of the mice with UC was significantly lower (Figure 1(b)) and the disease activity index (DAI) was significantly higher (Figure 1(c)), compared with mice in the control group. Similarly, the length of the colon was significantly shorter in mice with UC (Figure 1(d)). In addition, mice with DSS-induced UC exhibited mortality rate of 30%. After MGQD treatment, the mortality rate of mice with UC was reduced, particularly the survival rate of mice in the GH group significantly improved to 90% (Figure 1(e)).

Pathological results revealed that the mice in the control group had clear crypt structure and intact colonic mucosa, whereas the colonic histopathological damage was obvious in the mice with UC with extensive infiltration of inflammatory cells, crypt structure, and severe disorganization of the mucosa (Figures 1(f) and 1(g)). Surprisingly, all the aforementioned alterations in mice with UC were reversed by MGQD and Fer-1 treatment, and the most significant improvement in clinical symptoms in the mice with UC was observed at a dose of 20 g/kg MGQD (Figures 1(b)–1(g)).

3.2. MGQD Suppressed Proinflammation in Mice with UC

High levels of IFN-γ, IL-1β, IL-6, and TNF-α were observed in the colonic tissues of mice with UC compared with the control mice (Figures 2(a)–2(d)). Surprisingly, the levels of these proinflammatory cytokines were dramatically reduced in colonic tissues after MGQD and Fer-1 treatments (Figures 2(a)–2(d)).

[figure(s) omitted; refer to PDF]

3.3. MGQD Enhanced the Intestinal Barrier Function of Mice with UC

UC is pathologically characterized by inflammation of the intestinal mucosa and disruption of intestinal barrier function [1]. Continued deterioration of ferroptosis will lead to disruption of intestinal barrier function, which is characterized by suppression of the expression of tight junction proteins [8, 16]. Tight junction proteins such as claudin-1, ZO-1, and occludin were therefore detected by immunohistochemistry in this study, and results suggested that the expression of claudin-1, ZO-1, and occludin was suppressed in the colonic tissues of mice with UC compared with the control mice (Figures 3(a)–3(f)). Interestingly, mice treated with MGQD exhibited an increased expression of claudin-1, ZO-1, and occludin in a dose-dependent manner (Figures 3(a)–3(f)). Furthermore, the expressions of these proteins were comparable in the Fer-1 and GH groups (Figures 3(a)–3(f)).

[figure(s) omitted; refer to PDF]

3.4. MGQD Inhibited Ferroptosis in Mice with UC

Transmission electron microscopic results revealed that the colonic tissues of mice with UC exhibited reduced mitochondrial size, membrane density, and cristae and apparent rupture of the outer membranes compared with the control. Interestingly, the mitochondria in the MGQD and Fer-1 groups were relatively normal, and the cristae were clear (Figure 4).

[figure(s) omitted; refer to PDF]

Furthermore, elevated levels of Fe^{2+}, ROS, and MDA and decreased levels of GSH and Fe^{3+} were observed in mice with UC (Figures 5(a)–5(e)). Interestingly, these abnormal results were considerably reversed by MGQD and Fer-1 treatments (Figures 5(a)–5(e)). In addition, 4HNE expression was higher in colonic tissues of mice with UC but was inhibited by MGQD and Fer-1 treatments (Figure 6).

[figure(s) omitted; refer to PDF]

Western blot analysis (Figure 7) revealed that SCL7A11 and FTH1 were downregulated, whereas ACSL4 was

upregulated in the colonic tissues of mice with UC compared with the control group. However, after MGQD treatment, all these changes were reversed. Notably, ferroptosis was inhibited to the highest level when the dose of MGQD was 20 mg/kg.

[figure(s) omitted; refer to PDF]

3.5. MGQD Activated the Nrf2/GPX4 Pathway in the Colonic Tissues of Mice with UC

Western blot and RT-qPCR analyses revealed that protein and mRNA levels of Nrf2 and GPX4 were significantly reduced in the colonic tissues of mice with UC compared with the control group (Figure 8). Interestingly, mice with UC who received MGQD exhibited a dose-dependent increase in Nrf2 and GPX4 expression levels (Figure 8). Furthermore, the levels of genes located downstream of Nrf2, namely, *SCL7A11* and *FTH1*, were notably increased after MGQD treatment.

[figure(s) omitted; refer to PDF]

3.6. Molecular Docking of MGQD and Nrf2

In molecular docking, the lower the energy needed for the ligand to bind to the receptor to form a conformation, the more stable the structure was. Our results (Table 2 and Figure 9) revealed that the binding energies of MGQD and Nrf2 were less than −5 kcal/mol, and at least one hydrogen bond was formed between them. This indicated that the main components of MGQD could stably bind to Nrf2, and MGQD may affect UC through the Nrf2 pathway.

Table 2

Binding energy of components of MGQD and target protein.

Target	PDB IDs	Components	Docking energy (kcal/mol)
Nrf2	7k2n	Baicalin	−9.8
Nrf2	7k2n	Berberine	−9.7
Nrf2	7k2n	Palmatine chloride	−8.2
Nrf2	7k2n	Puerarin	−9.7
Nrf2	7k2n	Wogonin	−9.1

[figure(s) omitted; refer to PDF]

4. Discussion

Traditional Chinese herbal medicines have been popular in these years for many diseases [17, 18]. As complementary and alternative therapies, traditional Chinese herbal medicines are considered promising adjuvant treatment options for colitis [19]. GQD is a well-known Chinese herbal formula beneficial for UC [12]. GQD has been reported to alleviate UC by modulating immune responses, inhibiting oxidative stress, promoting intestinal mucosal barrier repair, and regulating gut microbes [20–22]. As a modified herbal compound of GQD, MGQD has likewise been found to alleviate UC by modulating immunity, inhibiting oxidative stress, and regulating gut microbes [13–15]. Accumulating evidence suggested that epithelial cell injury is associated with ferroptosis in UC, and targeted inhibition of ferroptosis can help to promote repair of the intestinal mucosal barrier [22, 23]. Therefore, we aimed to explore whether MGQD can ameliorate UC via targeting the inhibition of ferroptosis through the establishment of an experimental mouse model with UC.

After 7 days of UC induction using DSS, mice in the DSS group developed characteristic symptoms of colitis, including blood in the stool, loose stools, weight loss, and shortening of the colon. In addition, pathological findings revealed an inflammatory infiltrate and mucosal damage in the colonic tissues of mice with UC, which corresponded to an increase in a large number of inflammatory cytokines and suppression of tight junction proteins. These results

demonstrated that an experimental mouse model of UC had been successfully established. More importantly, the core characteristics of ferroptosis, such as lipid peroxidation accumulation, iron deposition, reduction in GPX4 activity and GSH level, and morphological changes in the mitochondria, were observed in the intestine of mice with UC. When the mice with UC were administered with Fer-1, a ferroptosis inhibitor, these core characteristics of ferroptosis were reversed and were accompanied by improvements in colitis symptoms, such as blood in stool, loose stools, weight loss, shortened colon, and inflammatory infiltration. The intestinal barrier breaks down as a result of intestinal epithelial cell death [24]. Interestingly, the levels of tight junction proteins were significantly restored after protecting the intestinal epithelium of mice with UC by inhibiting ferroptosis. These findings suggested that ferroptosis is associated with intestinal mucosal barrier breakdown in UC, and targeted inhibition of ferroptosis can help in alleviating colitis.

In this study, mice with UC treated with MGQD exhibited increased body weight, longer colon length, decreased DAI score, and improved pathology score, which is consistent with our previous findings [13–15]. More importantly, similar to Fer-1, the core characteristics of ferroptosis exhibited by mice with UC, such as lipid peroxide accumulation, iron deposition, reduced GSH levels, and mitochondrial morphological changes, were significantly reversed after treatment with MGQD. This suggested that MGQD inhibits ferroptosis in mice with UC. In addition, MGQD helped to promote the repair of the colonic mucosal barrier as the levels of tight junction proteins in the colonic tissues of mice with UC were restored after MGQD treatment.

Ferroptosis is characterized by iron overload and lipid accumulation [25]. The Nrf2 pathway is well known for its role in antioxidant defense [26, 27]. Notably, GPX4 is an established Nrf2 transcriptional target [28]. Therefore, activation of the Nrf2/GPX4 pathway is considered critical for the inhibition of ferroptosis [10, 11]. In this study, the Nrf2/GPX4 pathway was significantly inhibited in mice with UC, and the expression of the downstream genes of Nrf2, including *SCL7A11* and *FTH1*, was inhibited. When the Nrf2/GPX4 pathway is inhibited, indicators of lipid peroxidation including ACSL4 and 4HNE are significantly activated. Surprisingly, the Nrf2/GPX4 pathway and its downstream genes were activated in the colonic tissues of mice with UC after treatment with MGQD. In addition, the levels of ACSL4 and 4HNE, which are generally used as biomarkers of ferroptosis, were decreased in mice treated with MGQD. Additionally, the results of molecular docking revealed that Nrf2 could be stably bound to the components of MGQD. These findings suggested that MGQD inhibits ferroptosis via the Nrf2/GPX4 pathway to alleviate UC (Figure 10).

[figure(s) omitted; refer to PDF]

Limitations should be acknowledged. First, molecular docking was carried out based on the results of animal experiments, and the results obtained by the molecular docking method were not validated by in vitro experiments, and further exploration of the effects of the components of MGQD on the Nrf2 pathway on the basis of the UC model is still necessary in the future. Second, ferroptosis belongs to one of the forms of programmed cell death, and further studies to explore whether MGQD can regulate other forms of programmed cell death to alleviate UC are also necessary in the future.

5. Conclusion

In conclusion, our study demonstrated that ferroptosis is involved in DSS-induced UC in mice, and MGQD may exert anticolitis effect by inhibiting ferroptosis through the activation of the Nrf2/GPX4 pathway. These findings provided novel insights into the application of MGQD to ameliorate UC.

Ethical Approval

This work was approved by the Animal Ethics Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences (Approval No. 2019XLC003-2).

Authors' Contributions

Jinke Huang designed the research, analyzed the data, and wrote the paper. Jinke Huang, Zhihong Liu, Jiaqi Zhang, Jing Ma, and Fengyun Wang performed the experiments. Xudong Tang contributed to review and editing.

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Glossary

Abbreviations

UC:Ulcerative colitis
GQD:Gegen Qinlian decoction
MGQD:Modified Gegen Qinlian decoction
DSS:Dextran sulfate sodium
GM:Medium-dose MGQD
GL:Low-dose MGQD
GH:High-dose MGQD
Fer-1:Ferroprostatin-1
MDA:Malondialdehyde
GSH:Glutathione
ROS:Reactive oxygen species
H &E:Hematoxylin and eosin
DAI:Disease activity index
MOD:Mean optical density.

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DETAIL

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Application of Near-Infrared Spectroscopy to Rapidly Classify the Chinese Quince Fruits from Different Habitats

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ABSTRAK (ENGLISH)

The ecological habitats of Chinese quince (*Chaenomeles speciosa* Nakai) fruits affect their phenotype. Currently, limited or no rapid method exists for classifying Chinese quince fruit from different ecosystems. This study developed a partial least squares discriminant analysis (PLS-DA) classification model to effectively and nondestructively classify 663 Chinese quince fruit samples from six environments in 2020. PLS-DA models and other variable selection approaches were used in this study. The near-infrared spectroscopy (NIRs) absorption spectra of raw Chinese quince fruit samples from six habitats showed a similar shape. The spectra of each environment showed little variance. The raw fruit spectra varied significantly among habitat categories after the first derivative preprocessing phase. The uninformative variable elimination (UVE) variable selection approach had greater calibration and validation set specificity of 0.93 and 0.98. This study found the best classification specificity using the UVE variable selection approach compared to other methods including the PLS-DA model without variable selection. The UVE approach improved Yunnan habitat categorization specificity from 86% to 88% when integrated with PLS-DA. Additionally, the validation set for quinces originating from Anhui, Chongqing, Hubei, Shandong, and Zhejiang achieved an ideal classification score of 100%. The findings of the study indicated that PLS-DA can serve as an alternative approach for classifying the habitats of Chinese quince fruits. When used in conjunction with other methods, this technique can assist researchers, scientists, and industry professionals in identifying the main factors responsible for significant variations in the habitats, composition, and quality of Chinese quince fruits.

TEKS LENGKAP

1. Introduction

China is the natural habitat and cultivation center of Chinese quince (*Chaenomeles speciosa* Nakai), which has vast genetic resources and is mostly planted in the East, Central, and Southwest regions of China [1]. China is also the second-largest producer of quince in worldwide production, following Turkey. This fruit is a rich source of nutritious components, and it also possesses antioxidant and immune regulatory qualities. Sugar, amino acids, flavonoids, saponins, organic acids, and other useful components can be found in the fruit, which also possesses the ability to relax channels, activate collaterals, moisten the stomach, and perform a variety of other functions [2, 3]. It has also been used for thousands of years as one of the most essential substances in traditional Chinese medicine, which is typically appropriate to treat several diseases, including arthralgia, leg edema, and sunstroke [4]. To the present, Chinese quinces have continued to get a growing amount of attention for their potential to improve one's overall health. However, the quality of Chinese quince fruit might change depending on the habitat where it is grown because of the varying climatic circumstances (such as the moisture and humidity levels of the soil and the temperature). Therefore, there is a growing demand for research to determine the quality of Chinese quince fruits grown in a variety of field conditions.

There are several reports that have been published in the past that discuss various methods, traditional and emerging, that have been used to determine the quality of fruits that have been produced in a range of different field conditions. Traditional methods, such as DNA analysis [5], amino acid composition [6], and gas chromatography (GC) analysis [7], were both time-consuming and expensive. Near-infrared spectroscopy (NIRs) is an emerging method that is both rapid and nondestructive [8]. It is used for qualitative and quantitative analysis of the chemical composition of fruits such as apples [9], bananas [10], peaches [11], kiwifruits [12], and pears [13]. This method is becoming increasingly popular as a solution to the limitations and challenges of traditional methods. NIRs has been used to authenticate the authenticity of freeze-dried açai pulp [14], trace apple habitat [15], determine soluble solid content in multihabitat apples [16], differentiate apple varieties, and investigate organic status [17]. Nevertheless, despite the number of research on fruit quality and habitat as discussed in the preceding lines, there is very little or no known research work related to the use of NIRs to determine Chinese quince habitat. In our earlier research [2],

we analyzed and compared three distinct methods of discriminant analysis to determine the Chinese quince habitat. Partial least squares discriminant analysis (PLS-DA) is one of the most widely used methods for classification in chemometrics [18, 19]. This method has also received widespread application in domains associated with the "omics," such as metabolomics, proteomics, and genomics, in addition to an array of other fields that generate huge amounts of data, such as spectroscopy [20–24]. The rising interest in PLS-DA, particularly in the field of metabolomics, may largely be attributed to the fact that it is included in the vast majority of widely used statistical software programs [22, 25–30]. These software packages include R, S-Plus, SAS, SPSS, and MATLAB. On the other hand, PLS-DA has recently been described by researchers as a powerful and reliable classification approach when paired with spectroscopy, which is utilized for discriminating between different qualities of fruit [31–33]. However, the PLS algorithm has a flaw in that it might provide inaccurate predictions due to the large number of irrelevant variables that it considers [34]. The methods used for selecting variables can choose a limited number of variables that are extremely significant and have an association with the characteristics of the class (for example, habitat) [35]. Variable selection may also increase classification performance by accurately selecting a subset of key predictors [36]. This can be done by using the results of the classification.

The utilization of NIRs has recently been employed to efficiently categorize Chinese quince fruits originating from distinct habitats [2]. The NIRs method provides a noninvasive and highly effective approach for analyzing the chemical composition of fruit samples [2, 8, 9, 14, 15, 31]. In a scientific investigation, scientists employed near-infrared reflectance spectroscopy in conjunction with multivariate analysis methodologies to categorize Chinese quince fruits according to their specific geographical origins [2]. The current investigation centered on Chinese provinces renowned for their diverse climate conditions and soil characteristics. The objective of the preceding investigation was to construct a model capable of effectively discriminating quince fruits originating from the aforementioned two geographical areas [2]. The investigation gathered NIRs spectra from a substantial quantity of quince fruit samples and employed multivariate analysis techniques, including principal component analysis (PCA) and linear discriminant analysis (LDA), to categorize the samples. The PCA was employed to effectively decrease the dimensionality of the spectral data. Subsequently, the LDA was utilized to construct a classification model using the reduced dataset. The findings of the research demonstrated that the NIRs methodology, in conjunction with multivariate analysis techniques, exhibited a high level of efficacy in accurately categorizing Chinese quince fruits originating from diverse habitats. Consequently, the classification accuracy exhibited a notable level, suggesting that NIRs possesses significant potential as a valuable instrument for swiftly and noninvasively categorizing fruit samples according to their geographical origin or natural habitat [2]. The utilization of NIRs in the categorization of Chinese quince fruits originating from diverse habitats showcases the promising capabilities of this method in ensuring fruit quality control, traceability, and authentication within the agricultural sector.

Therefore, the study aimed to develop PLS-DA models based on the NIRs of Chinese quince fruits to predict the habitats of Chinese quince and demonstrate how different variable selection methods influence the classification results of PLS-DA models rapidly and accurately.

2. Materials and Methods

2.1. Materials

During the harvest season in the year 2020, samples of Chinese quince fruit were collected from six different habitats (Figure 1), which together represent the majority of the Chinese quince fruit-producing regions. When the fruit's color changed to a yellowish green, which is also the customary time for harvesting quinces for medicinal purposes, three fresh quinces that were still intact were picked at random from each tree in each habitat. All of the samples were thereafter placed in a plastic bag, which was then labeled and then placed in a cooler box to maintain their freshness. The samples for the test consisted of a total of 663 fruits, which were collected from six main producing regions at a rate of three fruits per plant for a total of 221 distinct plants (Table 1 and Figure 2).

[figure(s) omitted; refer to PDF]

Table 1

Different locations for the sample collection of Chinese quince fruit.

Habitats	Total (trees)	Total (fruit samples)
Shandong	22	66
Anhui	43	129
Zhejiang	33	99
Hubei	53	159
Chongqing	42	126
Yunnan	28	84
Total	221	663

[figure(s) omitted; refer to PDF]

2.2. Methods

2.2.1. Spectra Acquisition

In this study, the data for the near-infrared reflectance spectra of individual fruits were collected at room temperature (25°C) using a hand-held near-infrared spectrometer (LF-2500, Spectral Evolution, USA) at an interval of 6 nm from 1000 nm to 2500 nm. A total of 32 times, on average, were used for scanning each spectrum. The manufacturer of the apparatus supplied the DARWin SP (version 1.2) software that was used to analyze the collected data.

Each individual fruit sample was subjected to the recording of all three spectra. The contact probe, which had a diameter of 20 mms, was positioned on the ventral surface of the Chinese quince fruit samples with the stem-calyx axis horizontal at a location chosen at random. The second measurement was carried out at a location that was roughly 120° rotated from the starting point. The third spectra were collected at an angle of roughly 240° rotated from the starting point. For each sample, an average of the three spectra was calculated.

2.2.2. Data Processing

The R software (version 3.1.2) was utilized for the processing of the data [37]. The NIRs spectra were averaged using the mean value of all of the fruits that were found on each tree. In the end, 221 different spectral samples were utilized. Following the conversion of the reflectance spectrum into the absorbance spectrum, multivariate analysis was performed. Both the standard normal variable and the first derivative were put through their tests as potential spectral preprocessing methods. The additive effect and noise present in the spectrum can be effectively eliminated through the utilization of two distinct preprocessing techniques, which differ from the conventional methods employed for processing NIRs spectra [2, 14].

The dataset was subsequently partitioned into two distinct subsets: a calibration set and a validation set [14]. Both of these subsets comprised samples that were chosen interactively using their Euclidean distances, aiming to achieve the highest attainable data coverage. Ultimately, a total of 181 samples were employed for the calibration set, while the remaining 40 samples were allocated for the validation set [34].

PLS-DA classification models were utilized to differentiate between the various origins of Chinese quince fruits [17]. The PLS-DA method is a variant of the PLS regression (PLS-R) methodology. PLS-R is usually used to tackle regression-related problems and is most appropriate in situations in which the matrix of predictors contains more variables than data. PLS-DA is an appropriate approach for classification since it conducts a dimension reduction on the predictor variables and extracts the components that are significantly linked with the class factor [14, 16]. As a result, PLS-DA was employed to classify data.

In the PLS-DA model, the spectra of the six different habitat fruits were utilized for the X matrix, and six fabricated

values were used for the *Y* matrix to represent each habitat. Shandong, Anhui, Zhejiang, Hubei, Chongqing, and Yunnan each had a dummy value between 0 and 5, and those values were given to their respective spectra. Root mean square error (RMSE) ranges of ±0.5 were set between each habitat. If an individual's RMSE fell within one of these ranges from any habitat, then the individual was considered to be classified in that habitat. The leave-one-out cross-validation method was utilized in the development of PLS-DA calibration models [35].

2.2.3. Variable Selection

Five different methods of selecting variables were tested to see which of these methods may produce more accurate prediction results. These methods include backward variable elimination (BVE), genetic algorithm (GA), uninformative variable elimination (UVE), and subwindow permutation analysis (SwPA).

SwPA. The SwPA, when paired with the PLS-DA model, has the potential to make the model more effective and faster for analyzing large datasets. This is because the SwPA offers the influence of each variable individually, without taking into account the influence of the other factors. Additional information can be found in the reports that Mehmood and his coworkers [36] as well as Li and his coworkers [38] published.

IPW. The IPW variable selection was introduced by Forina and coworkers [39]. The method is predicated on the PLS model of each predictor's effect on the response, and it iteratively changes the original *X*-variables to eliminate the variables that are of the least importance. In the field of spectrometry, successful use of this method has been accomplished in the past [40].

BVE. Backward variable elimination was first ascribed by Frank for the elimination of noninformative variables [41]. Later, in an upgraded version, it was utilized for wavelength selection [42]. The method works by first sorting the variables using a filter measurement and then using a threshold to eliminate a subset of the least informative variables. This process is continued until there is no longer a need for any more elimination.

GA. The GA, which is derived from the concepts of genetics and natural selection, has developed into a tool for optimization that conducts a search that is both random and global inside a space that has a high dimension. By sampling a broad parameter space at each stage of the optimization, GA might escape local optima and find global optima in a relatively short time. It has been extensively utilized for variable selection in multivariate spectroscopic calibration [43]. The steps of the genetic algorithm are explained in the study published by Mehmood and colleagues [36].

UVE. Before employing the PLS model, the UVE procedures that have been developed by Centner and coworkers with PLS models included the addition of artificial noise variables to the predictor set [44]. It does away with the habitat variables that are of lesser value compared to the artificial noise variables. This process is performed repeatedly until a satisfactory model is acquired.

3. Results and Discussion

3.1. NIRs Spectra

Figure 3 depicts the average of the NIRs absorbance spectra of raw Chinese quinces fruits grown in six different habitats. The raw fruit spectra show that all of the spectra have a relatively similar shape, and there is only a little amount of variation between the spectra of each habitat. However, after going through the first derivative preprocessing step, the raw fruit spectra showed that there were some major disparities across the different habitat groups. There were two strong bands of water absorbance at 1450 and 1950 nm that were connected to the overtone of -OH bands. The $-CH_3$ groups, such as methyl, methylene, and ethylene, were responsible for the peaks that appear at around 1250 nm, 1700 nm, 2000 nm, and 2150 nm, respectively [14, 45, 46]. In Figure 3(a), the observed spectra consisted of two distinct peaks and one broad peak, resulting in a total of three spectra. Conversely, Figure 3(b) exhibits a total of five spectra. Specifically, the absorption peak observed at a wavelength of 2,270 nm was attributed to the vibrational modes of CH-stretch and CH-deformation combination originating from the $-CH_3$ moiety of ethanol [47, 48]. Likewise, the absorption peak observed at approximately 2,300 nm is plausibly linked to the $-CH_2$ functional group present in ethanol [47–51]. The NIRs region ranging from 1,650 to 1,750 nm is associated with the first overtones of the CH-stretch in both $-CH_3$ and $-CH_2$ functional groups [47–51]. Additional research has demonstrated that methanol-based solutions containing phenolic compounds and tannins exhibit

comparable absorption patterns within these specified regions, despite variations in concentration [52]. This is particularly relevant to the spectral regions centered at 1,650 and 1,850 nm, as well as the range between 2,100 and 2,300 nm. Within this range, a prominent absorption characteristic associated with tannins has been identified at approximately 2,140 nm [52]. Therefore, it is possible that the observed alterations in this region reflect differences in concentrations of sugar, ethanol, phenolics, and tannins.

[figure(s) omitted; refer to PDF]

The PLS-DA models' sensitivity in the classification of the six different habitats attained the best results from the first derivative spectra for both the calibration and validation sets. The correct classification specificity for the calibration set was 91%, while it was 95% for the validation set. For this reason, the optimal wavenumber selection was achieved by the application of the first derivative preprocessing approach.

3.2. Variable's Selection

PLS-DA was used in conjunction with the various variable selection methods to develop the final model. Table 2 illustrates the specificity of the PLS-DA models for both the calibration and validation sets for each variable selection method. The UVE variable selection approach achieved higher specificity for the calibration and validation sets, with scores of 0.93 and 0.98, respectively. This resulted in the best classification specificity that was achieved after employing this method. When compared to PLS-DA with no variable selection, which utilized 256 variables and 8 factors, the number of variables was decreased from 256 to 70 with the usage of UVE, and the number of PLS factors was lowered from 8 to 7. The specificity of BVE's classification was the least and came in at 0.89 for the calibration set and 0.93 for the validation set, respectively. Except for the GA method, which only eliminated 14 variables from the habitual spectrum, the other variable selection methods did not increase the classification model specificity, despite the fact that the number of variables was significantly decreased.

Table 2

Results of the classification of Chinese quince fruits from six different habitats using the PLS-DA full-wavelength and variable selection methods, respectively.

Model	Calibration		Validation		Variables	PLS factors
Accuracy	Error	Accuracy	Error	PLS-DA	0.91	0.09
0.95	0.05	256	8	PLS-DA-SwPA	0.91	0.09
0.93	0.07	210	8	PLS-DA-IPW	0.91	0.09
0.93	0.07	106	5	PLS-DA-BVE	0.89	0.11
0.93	0.07	101	10	PLS-DA-GA	0.91	0.09
0.95	0.05	242	8	PLS-DA-UVE	0.93	0.07

One notable advantage of the UVE-PLS method, in comparison to alternative variable selection methods, is its user independence, which eliminates any potential configuration issues [44]. In their study, Koshoubu et al. [53] presented an adapted iteration of UVE-PLS, wherein they incorporated the prediction error sum of squares. This modification was employed to exclude uninformative samples, considering both wavelength variables and concentration variables [54]. The UVE-PLS method is utilized to identify the wavelength variables that contain relevant information based on the regression coefficients obtained from PLS modeling. The coefficients of the PLS regression are acquired using the leave-one-out technique on the calibration samples. Nevertheless, the leave-one-

out method presents a compelling issue. As highlighted by Martens and Dardenne [55], the leave-one-out technique employed in multivariate data analysis typically tends to overfit on average, resulting in an underestimation of the actual predictive error. Hence, the incorporation of the leave-one-out method in the UVE-PLS algorithm introduces the aforementioned drawbacks, potentially resulting in the overfitting of the prediction model.

Table 3 provides an overview of the correct classification percentages for both the calibration set and the validation set both before and after the application of UVE. Overall, PLS-DA-UVE produced optimal results when used for the classification of the different habitats of quince fruits. PLS-DA-UVE was superior to PLS-DA in terms of improving the specificity of classification for Anhui, Shandong, and Yunnan in the calibration set when compared to PLS-DA with no variable selection. The specificity of the Chongqing and Zhejiang habitats remained the same, whereas it decreased for the Hubei habitat. Using the UVE method in conjunction with PLS-DA resulted in a classification specificity of 100% achieved in the validation set for quinces belonging to the regions of Anhui, Chongqing, Hubei, Shandong, and Zhejiang. The specificity of the classification of quince fruit harvested in Yunnan habitats improved only marginally, ranging from 86% to 88%. PLS-DA-UVE succeeded in achieving the best overall performance, indicating the superiority of this method over others, it effectively classifies the habitat of Chinese quince fruits using NIRs spectral data.. It was found that using UVE in conjunction with PLS-DA methods might produce a result that was more reliable and specific [56]. A similar result was observed when combining UVE with PLS-DA to determine the linoleic acid concentration in eight different types of edible vegetable oils [57]. This indicates that the FT-IR transmission spectroscopy approach combined with the UVE method is promising for the quick detection of glycerol monolaurate [58].

Table 3

Percentage of correct classification of Chinese quince fruits from six different habitats using the PLS-DA full-wavelength and uninformative variable elimination (UVE) methods, respectively.

Habitats	PLS-DA		PLS-DA-UVE	
Calibration	Validation	Calibration	Validation	Anhui
93	100	95	100	Chongqing
94	100	94	100	Hubei
93	100	90	100	Shandong
81	75	82	100	Yunnan
83	86	94	88	Zhejiang

Figure 4 presents the PLS-DA and PLS-DA-UVE score plots for factors 1 and 2, respectively. The PLS-DA score plot (Figure 4(a)) shows that the fruits from each of the six habitats may be distinguished from one another. This might be because Chinese quinces grow in a wide variety of habitats, each of which is unique in terms of the soil, climate, and growing conditions, even though there is some commonality. It is evident from observing Figure 4(b) that the six clusters have been successfully differentiated using UVE in conjunction with PLS-DA.

[figure(s) omitted; refer to PDF]

4. Conclusion

The NIRs technique was employed in this study to successfully classify samples of Chinese quince fruit, resulting in significant disparities observed among the habitat groups obtained from six different habitats. Raw fruit spectra in the range of 1000 to 2500 nm were found when PLS-DA models were combined with the first derivative

preprocessing method. This has the potential to be employed as a fast and nondestructive method for differentiating the habitat of Chinese quinces. Following an examination of several other variable selection methods, the study found that the UVE variable selection method, when used in conjunction with the PLS-DA method, produces more accurate classifications for the six different habitats. In addition, the findings suggested that the discrimination against the habitat of Chinese quinces can be due to the difference in the chemical composition of Chinese quince fruits, which resulted from the different climatic and geographical conditions of the habitat in which Chinese quinces were grown. This difference in the chemical composition of Chinese quince fruits was caused by the fact that Chinese quinces were grown in a habitat in which they had to adapt to different conditions. In addition, the findings of the study suggest that PLS-DA can be used as an alternative method for classifying the habitats of Chinese quince fruits. This will help in identifying the primary factors that cause significant variation in the habitats, composition, and quality of Chinese quince fruits when combined with other methods like polynomial multivariate and multiregression analysis. Furthermore, the focus of future work proposes combining near-infrared spectroscopy with other methods of stoichiometry in which the products and reactants are compared, and the Law of Conservation of Mass and Energy is applied to get quantitative information on the reaction, which can be utilized to further investigate the main factors impacting the variation of Chinese quince fruits in different habitats. Therefore, it can be asserted that the current investigation possesses notable strengths and limitations, along with implications for subsequent research endeavors and/or clinical applications. The current study's strengths can be inferred from the utilization of NIRs, a noninvasive and expeditious analytical technique that offers valuable insights into the composition and characteristics of the samples. The current investigation places its emphasis on the classification of Chinese quince fruits originating from various habitats. This classification process has the potential to contribute to the enhancement of quality control, grading, and sorting procedures for these fruits. In the current investigation, NIRs is employed to nondestructively analyze samples, rendering it an invaluable instrument for evaluating the quality of fruits while preserving their usability and market value. NIRs is recognized for its rapid analysis capabilities in the current investigation, providing a distinct advantage for time-sensitive applications such as quality control in fruit processing. Moreover, the current study exhibits certain limitations. The current investigation may exhibit a constrained sample size, potentially impacting the extent to which the findings can be extrapolated. Another limitation is the potential absence of external validation through the utilization of independent datasets or samples from diverse geographical locations, which could enhance the credibility of the classification models. The present study suggests that there are important implications for future research and clinical practice. Specifically, it is recommended that future research endeavors focus on validating the findings using larger and more diverse samples. This approach will help to improve the reliability and generalizability of the classification models. To ascertain pivotal spectral characteristics, forthcoming investigations should prioritize the identification of distinct spectral attributes linked to the categorization of Chinese quince fruits. This can facilitate comprehension of the inherent chemical composition and qualitative characteristics of these fruits. Potential applications in clinical practice encompass the utilization of NIRs for expedited categorization of fruits. This methodology can be further extrapolated to diverse domains, including the determination of the caliber and genuineness of medicinal plants as well as the evaluation of the nutritional constitution of food products. It may also have implications in clinical practice, such as the expedited identification of diseases or conditions through the analysis of spectral signatures in biological samples. The incorporation of additional analytical methodologies can result in a synergistic effect, whereby the combination of NIRs with other analytical techniques facilitates the acquisition of a more exhaustive and precise dataset. Future investigations may delve into the synergistic combination of NIRs with complementary techniques, such as chromatography or mass spectrometry, in order to augment the analytical capabilities pertaining to Chinese quince fruits or analogous specimens. In a nutshell, this study showcases the capacity of NIRs for categorizing and evaluating the quality of Chinese quince fruits. Subsequent investigations can expand upon these results to investigate wider applications and enhance the technique's efficiency.

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DETAIL

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BLSENet: A Novel Lightweight Bilinear Convolutional Neural Network Based on Attention Mechanism and Feature Fusion Strategy for Apple Leaf Disease Classification

Fang, Tianyu; Zhang, Jialin; Qi, Dawei; Gao, Mingyu.

ABSTRAK (ENGLISH)

Accurate identification of apple leaf diseases is of great significance for improving apple yield. The lesion area of the apple leaf disease image is small and vulnerable to background interference, which easily leads to low recognition accuracy. To solve this problem, a lightweight bilinear convolutional neural network (CNN) model named BLSENet based on attention mechanism is designed. The model consists of two subnetworks, and each subnetwork is embedded with a Squeeze-and-Excitation (SE) module. By using the feature extraction ability of the two subnetworks and combining the bilinear feature CONCAT operation, the multiscale features of the image are obtained. Compared with the unimproved model LeNet-5 (84.63%), BLSENet has higher accuracy in the test set, which indicates that SE module and bilinear feature fusion have a positive effect on the performance of the model, and BLSENet has the ability to identify apple leaf diseases. The model has achieved the expected goal and can provide technical support for accurate identification and real-time monitoring of apple disease images.

TEKS LENGKAP

1. Introduction

Apples are often attacked by diseases during the growth process [1]. Accurate identification of the types of diseases, timely prevention, and control are essential to improve the yield of apples [2–4]. At present, plant disease recognition has become an important research direction in the field of image recognition and intelligent agriculture.

Traditional machine learning algorithms need to classify images after extracting features [5]. The feature extraction process is time-consuming and labor-intensive, and the classification model has weak generalization ability and poor recognition effect [6]. Zhang et al. proposed an apple leaf disease identification method based on image processing technology and pattern recognition method. The RGB model was transformed into HSI, YUV, and grayscale, with background removal based on a specific threshold. The approach using region growing algorithm (RGA), genetic algorithm (GA), correlation-based feature selection (CFS), and support vector machine (SVM) achieved over 90% accuracy in recognizing various apple leaf diseases [7]. Bracino et al. proposed a machine learning model that can detect and classify the three most common apple diseases. The color and texture features of a single apple leaf image were extracted and selected. By comparing KNN, ANN, and GPR, it is determined that the GPR model with ARD squared kernel function is the best model [8]. Khan et al. employed contrast enhancement and a strong correlation-based segmentation method to segment images, optimizing the segmentation results through expectation maximization (EM). They utilized GA to extract features from the fused images and achieved significant classification accuracy using One-vs-All M-SVM [9]. Al-bayati et al. proposed a method for detecting apple leaf diseases using deep neural network (DNN). They employed Speeded Up Robust Features (SURF) for feature

extraction, and the Grasshopper Optimization Algorithm (GOA) is used for feature optimization; good results have been achieved [10]. However, the selection of this feature relies heavily on human experience and has great uncertainty. It requires specific data preprocessing to obtain better experimental results [11]. Because traditional feature extraction and recognition methods are not end-to-end operations, this is not conducive to rapid real-time detection in practical applications.

In recent years, as deep learning can automatically extract disease features and avoid manual dependence, a series of research results have been achieved in crop disease recognition. Rohini et al. proposed a model based on CNN to classify apple leaf images into diseased and undiseased. In the construction of the CNN model, the combination of convolution layer, ReLU, and max-pooling layer is considered. This task represents a binary classification problem. The proposed model is effectively implemented on the considered dataset with an accuracy of 91.11% [12]. Singh et al. used three pretrained CNN models to identify diseases in the Beans Leaf image dataset. In addition, different optimization techniques are used to highlight the performance differences of different CNN models. The experimental results show that the performance of EfficientNetB6 is better than other models, and the accuracy rate is 91.74% [13]. Kumar et al. proposed a strategy based on transfer learning, using the learned VGG-16/VGG-19 CNN network to estimate the severity of tomato leaf disease. In addition, the author performs hyperadjustment on the hyperparameters of the pretrained CNN model to improve its effectiveness. In order to evaluate the performance of the fine-tuned CNN model, the study measures the accuracy and loss values after multiple iterations on the training and validation datasets. Compared with another CNN model evaluated on the same dataset, VGG-16 shows higher classification accuracy (92.46%) [14]. Ding et al. proposed a new apple leaf disease recognition model named RFCA ResNet. This model has dual attention mechanism and multiscale feature extraction ability, which can reduce the adverse effects of complex background on recognition results. In addition, by combining the use of the class balance technique in conjunction with focal loss, the adverse effects of imbalanced datasets on classification accuracy can be effectively reduced. The RFB module can expand the receptive field and realize multiscale feature extraction. The accuracy of RFCA ResNet is 89.61%. It is superior to other methods and has good generalization performance, which has certain theoretical significance and practical value [15]. Gaikwad et al. used CNN to classify leaf disease. The author collected datasets from a real-time environment, with a total of 14181 images and 10 class labels. The experiment used 3 different versions of datasets: color, black and white, and grey images. These datasets are trained on AlexNet and SqueezeNet and use the same hyperparameters. The recognition accuracy of the two models is basically the same, and the classification accuracy of color images is 86.8% and 86.6%, respectively, indicating that color images are effective for classification [16]. In recent years, researchers have used various deep learning networks and frameworks for experiments. With the deepening of research, it is currently the best choice to use deep learning to classify and identify apple leaf diseases [17]. Based on the aforementioned literature, we have discovered the diversity and complexity of the shape and color of diseases, which poses a challenge for achieving high-precision disease identification. While existing research encompasses various methods, including traditional feature extraction and deep learning techniques, the considerable variability in diseases has a notable impact on recognition accuracy. This diversity may result in existing models being unable to effectively capture and distinguish different disease features under certain circumstances, thereby limiting their practical applicability. To address this challenge, our focus has been on the multiscale extraction of disease features, incorporating methods such as multiscale feature fusion and employing more sophisticated deep learning architectures. These approaches aim to enhance the robustness of disease recognition systems by comprehensively capturing the complex characteristics of diseases. Therefore, this paper proposes a new CNN model, which can provide technical support for accurate identification and real-time monitoring of apple disease images.

In this paper, a bilinear classification model based on attention mechanism and feature fusion strategy named BLSENet is proposed for the classification of apple leaf diseases. The next arrangement and structure of this article are as follows. Firstly, the apple leaf disease dataset is presented, and the proposed network model BLSENet is introduced. Subsequently, the experimental results are described and analyzed in Section 3. The feasibility of the

proposed model is verified by adjusting the model parameters and the ablation experiment of the model. Finally, the advantages and disadvantages of the proposed model are analyzed, and the future research direction is determined on this basis.

2. Methodology

2.1. Dataset

The dataset was collected from the College of Artificial Intelligence, Southwest University (as shown in Figure 1) [18–21]. The collected images encompass diverse diseases, each meticulously captured by skilled professionals using high-resolution cameras under appropriate lighting conditions to ensure image quality and clarity. Following the collection, the images underwent initial screening, retaining samples with representative disease features. To ensure data accuracy, each image was annotated for disease types by expert plant pathology specialists to guarantee precise and consistent labeling. The dataset contains nine types of apple leaf disease, including Health, Alternaria leaf spot, Brown spot, Frogeye leaf spot, Grey spot, Mosaic, Powdery mildew, Rust, and Scab.

[figure(s) omitted; refer to PDF]

The number of datasets used for the experiment is shown in Table 1. In the dataset, a total of 14582 images are included. 8754 images are randomly selected as the training set, 2913 pictures (accounting for 20% of the dataset) as the verification set, and the remaining 2915 pictures (accounting for 20% of the dataset) as the test set, as shown in Table 2.

Table 1

Number of images in the apple leaf disease.

Category	Number
Health	516
Alternaria leaf spot	417
Brown spot	411
Frogeye leaf spot	3181
Grey spot	339
Mosaic	371
Powdery mildew	1184
Rust	2753
Scab	5410

Table 2

Division of datasets.

	Training set	Validation set	Test set	Total dataset

Quantity	8754	2913	2915	14582
Proportion	60%	20%	20%	100%

2.2. Model of Deep Convolutional Neural Network with Improvements

2.2.1. Multiscale Information Fusion Strategy

BLSENet is a bilinear CNN model. It is a new technology in fine-grained image recognition [22]. It has a good classification effect in terms of inability to distinguish category calculations with subtle visual differences [23]. The structure of BLSENet is shown in Figure 2. The input image is subjected to multiple Convolutions [24], Pooling [25], and BatchNormal [26] operations by two improved LeNet-5 CNNs, and two image features extracted by the CNN network are obtained. Then, the image features extracted by the CNN network are combined with the CONCAT operation to form the bilinear feature vector of the image [27]. Finally, the feature is classified by the fully connected layer classifier to obtain the probability of the identified category.

[figure(s) omitted; refer to PDF]

LeNet, also known as LeNet-5, is a classical CNN proposed by Lecun [28]. It is one of the origins of modern CNNs. It has an input layer, two convolutional layers, two pooling layers, and three fully connected layers [29]. The improved LeNet-5 is used in BLSENet named A model; the two fully connected layers of A model are removed and replaced with SE modules. Then, a BatchNormal layer is added behind the first convolutional layer of the A model, which is named the B model. The B model is used as the upper branch network, the A model is used as the lower branch network, and two feature vectors named FC11 and FC21 with a dimension of 1×120 are output. The vector obtained by cascading FC11 and FC21 is named FC31, with a size of 1×240. Subsequently, FC31 is reduced in dimension and a vector named FC32 with a dimension of 1×50 is obtained. Finally, the output of the fully connected layer is set to 9 to represent the category of leaf diseases.

2.2.2. Attention Mechanism Based on SE Module

The SE (Squeeze-and-Excitation Network) module is a computing unit; it can recalibrate the weight of the feature channel [30]. At the same time, the module can adaptively enhance the feature channel of the contrast information of the infrared image and suppress the irrelevant feature channel [31]. In this network, the SE module contains a Squeeze-and-Excitation operation. The training process is divided into two stages: the first stage is Squeeze and the second stage is Excitation.

Figure 3 shows the structure of the SE module. We hope to enhance the learning of convolution features by simulating the interdependence of channels so that the network can be sensitive to the information features that can be utilized in subsequent transformations. Therefore, our goal is to give it the opportunity to obtain global information, further improve the accuracy of the network by squeeze and excitation, and then send the filter to the next conversion. In recent years, SE modules have been widely used in deep learning to improve network performance. In many research fields, many network architectures use SE modules in the network to help improve the performance of the original network [32–35]. The structure is shown in Figure 3. This method is simple and easy to embed into the CNN framework, and the computational complexity increases little, but better results are obtained.

[figure(s) omitted; refer to PDF]

2.3. Model Training Details

The CNN model proposed in this paper is based on PyTorch which is an open-source deep learning library. The experimental process was carried out on a workstation equipped with the Intel(R) Core (TM) i9-10980XE CPU @ 3.00 GHz 3.00 GHz and the 24 GB NVIDIA GeForce RTX 3090 GPU. The experimental environment is shown in Table 3.

Table 3

Experimental environment.

Hardware environment		Software environment	
CPU	Intel (R) Core (TM)i9-10980XE CPU @3.00 GHz 3.00 GHz	Environment configuration	PyTorch-GPU 1.8.0 + Python 3.7.10 + cuda 11.1 + cudnn 8.0.5

In the experiment, we set epoch = 200, batch size = 16, and initial learning rate = 0.0001 according to the experience, and Adam as the optimizer and cross-entropy as the loss function is used to train the network. The entire training parameters are shown in Table 4.

Table 4

Training hyperparameters.

Hyperparameters name	Hyperparameters value	Hyperparameters range
Batch size	16	8, 16,32, 64
Learning rate	0.0001	0.00001, 0.0001, 0.001, 0.01
Epoch	200	100, 200, 300
Optimizer	Adam	SGD, AdaGrad, Adam
Loss function	Cross-entropy	—

3. Experimental Results and Analysis

3.1. Training Results

The training result curve of the BLSENet network is shown in Figure 4. Accuracy is defined as the proportion of correctly classified samples by the model among all predictions. Loss is defined as the metric measuring the difference between the predictions of model and the actual labels during the training process, with the goal of minimizing this difference. It can be seen from Figure 4 that a total of 200 epochs were performed in the experiment. Finally, the accuracy rate on the test set is 93.58% and a good training effect is achieved.

[figure(s) omitted; refer to PDF]

On the Apple leaf disease dataset, the relationship between the loss value of the training set and the number of epochs is shown in Figure 4(a). The loss value on the training set decreases with the increase in the number of epochs, and the loss in the training set decreases from about 1.26 to about 0.34. The relationship between accuracy and the number of epochs is shown in Figure 4(b). As the number of epochs increases, the accuracy in the validation set gradually increases. The training set tends to be stable after the number of epochs is greater than 100, and its accuracy is about 93%.

As shown in Figure 5, the results of the BLSENet model in the test dataset are analyzed and a confusion matrix is established. From the diagram, the model has good recognition accuracy for Brown spot, Frogeye leaf spot, Powdery mildew, Rust, and Scab, with accuracies of 95%, 90%, 94%, 93%, and 95%, respectively. This may be because there are a large number of images for Frogeye leaf spot, Powdery mildew, Rust, and Scab. The model can obtain sufficient training, resulting in a higher recognition rate. On the other hand, although there are not many images for Brown spot, which are comparable to Health, Alternaria leaf spot, Grey spot, and Mosaic, higher accuracy can be obtained. This may be because defects with a relatively large area are less likely to be affected by the background, making them easier to be correctly recognized by the model.

[figure(s) omitted; refer to PDF]

3.2. Comparison and Analysis of Experimental Results

After the model was established, epoch parameters were set to 100, 200, and 300 to select the appropriate value. The training results are shown in Figure 6. As can be seen from the figure, as epoch increases, both loss and accuracy show better results on the training set, but there is no significant difference between the three values. Then, the three parameter values were tested on the test dataset, and their accuracies were 90.22%, 93.58%, and 93.48%, respectively, as shown in Table 5. It can be observed that epoch = 200 has the best performance on the test dataset, and it does not consume a lot of training time. Therefore, considering the accuracy of the model based on the above analysis, the value of 200 was selected as the epoch.

[figure(s) omitted; refer to PDF]

Table 5

Recognition results with different epochs.

Hyperparameters	Value	Number	Accuracy (%)
Epoch	100	2630	90.22
200	**2728**	**93.58**	300

The bold values indicate the data with the best results.

To select the appropriate batch size parameter, batch size was set to 8, 16, 32, and 64. The training results are shown in Figure 7. As the batch size decreases, the network has better training results on the training set. The training results for batch size = 8 and batch size = 16 are similar. On the test dataset, their accuracies were 92.97%, 93.58%, 91.63%, and 90.57%, respectively, as shown in Table 6. It can be observed that batch size = 16 has the best performance on the test dataset. Therefore, considering the accuracy of the model based on the above analysis, the value of 16 was selected as the batch size.

[figure(s) omitted; refer to PDF]

Table 6

Recognition results with different Batch sizes.

Hyperparameters	Value	Number	Accuracy (%)
Batch size	8	2710	92.97
16	**2728**	**93.58**	32
2671	91.63	64	2640

The bold values indicate the data with the best results.

To select the appropriate learning rate, three learning rates of 0.01, 0.001, 0.0001, and 0.00001 were tested on the test dataset. The training results are shown in Figure 8. From the figure, the training effect with a learning rate of 0.00001 is the worst because the learning rate is too small, which slows down the training efficiency. When the learning rate is 0.01, as can be seen from Figure 8(b), the accuracy curve is very unstable, which may be due to the learning rate being too large and making it difficult to find appropriate weight parameters. The training results with learning rates of 0.001 and 0.0001 are similar. Then, the four learning rates were tested on the test dataset, and the experimental results are shown in Table 7. Their accuracies were 91.60%, 91.34%, 93.58%, and 92.08%, respectively. Therefore, a learning rate of 0.0001 was selected based on the above analysis.

[figure(s) omitted; refer to PDF]

Table 7

Recognition results with different learning rates.

Hyperparameters	Value	Number	Accuracy (%)
Learning rate	0.01	2670	91.60
0.001	2680	91.34	**0.0001**
2728	**93.58**	0.00001	2684

The bold values indicate the data with the best results.

The optimization algorithm is very important for the performance of the model. The SGD [36], AdaGrad [37], and Adam [38] optimization algorithms were used to train the BLSENet in this paper, and their convergence speeds were compared. Figure 9 shows the training results of these three optimization algorithms. From the figure, the loss values using SGD and AdaGrad converge around 1.1 and the convergence effect is relatively poor as epoch increases. The accuracy values converge around 60%, which does not achieve the target accuracy. The results indicate that the model using the Adam algorithm has the fastest convergence speed and the best recognition effect. Then, the three optimizers were tested on the test dataset, and the experimental results are shown in Table 8. Therefore, Adam was selected as the optimization algorithm based on the above analysis.

[figure(s) omitted; refer to PDF]

Table 8

Recognition results with different optimization algorithms.

Hyperparameters	Name	Number	Accuracy (%)
Optimization algorithms	SGD	1820	62.44
AdaGard	1861	63.84	Adam

The bold values indicate the data with the best results.

The BLSENet is an improved model based on LeNet-5. To verify the improvement of the improved model compared to the original LeNet-5 model, BLSENet and LeNet-5 were compared on the test dataset. From Table 9, the recognition results of BLSENet are better than those of LeNet-5, with accuracies of 93.58% and 84.63%, respectively. The bilinear LeNet-5 combined with the SE module can improve the accuracy of LeNet-5 by 8.95%. Based on the above analysis, bilinear LeNet-5 combined with the SE module can improve the performance of the model.

Table 9

The result of ablation experiment of BLSENet.

Model	Number	Accuracy (%)
LeNet-5	2467	84.63
LeNet-5, LeNet-5 combined (bilinear)	2699	92.59

LeNet-5+SE, LeNet-5 combined (bilinear)	2377	81.54
LeNet-5, LeNet-5+SE combined (bilinear)	2700	92.62
BLSENet (bilinear)	2728	93.58

In addition, we conducted some ablation experiments to analyze BLSENet. The results are shown in Table 9. As mentioned earlier, the concept of BLSENet is derived from LeNet-5 and combines the SE module. Therefore, we compared LeNet-5, LeNet-5 and LeNet-5, LeNet-5+SE and LeNet-5, LeNet-5 and LeNet-5+SE, and Double LeNet-5+SE (BLSENet). The training results are shown in Figure 10. Their training curves all reach similar results as epoch increases. To test the generalization ability of the models on the test dataset, these models were compared on the same test dataset, and the results are shown in Table 9. The experimental results show that the recognition ability of LeNet-5 is the worst, and the accuracy of LeNet-5 and LeNet-5, LeNet-5 and LeNet-5+SE, Double LeNet-5+SE (BLSENet) is similar, with an accuracy of between 92% and 94%. The recognition effect of LeNet-5+SE and LeNet-5 is the worst, with a recognition rate of 81.54%. This is an interesting phenomenon, and we will continue to research it in the future.

[figure(s) omitted; refer to PDF]

Finally, the author presents a detailed table showcasing the accuracy achieved by the proposed neural network model in experiments and compares it with other models from relevant literature, as shown in Table 10. Excitingly, the table clearly demonstrates the outstanding performance of the proposed model in terms of accuracy, outperforming models from other literature and yielding the best results, approximately 93.58%. This finding not only highlights the superiority of the proposed model but also provides robust support for further advancements in the research field.

Table 10

Comparison of the proposed method with previous detection methods for apple leaf disease.

Detection method	Accuracy (%)
MobileNet [17]	73.50
InceptionV3 [17]	75.59
ResNet152 [17]	77.65
INAR-SSD (SSD with inception module and rainbow concatenation) [39]	78.80
DenseNet-121 and regression function [40]	93.51
DenseNet-121 and multilabel classification function [40]	93.31
BLSENet	93.58

4. Conclusion

In this paper, we proposed an apple leaf disease recognition method called BLSENet based on attention mechanism, lightweight CNN, and bilinear CNN framework. By embedding the SE module into the end of LeNet-5 and combining it with bilinear pooling, BLSENet was constructed to extract image features of apple leaf diseases.

BLSENet has higher accuracy in the test dataset compared with the unimproved model LeNet-5 (84.63%), which indicates that the SE module and bilinear feature fusion have a positive effect on the performance of the model and BLSENet has the ability to recognize apple leaf diseases accurately. The model has achieved the expected goal, which can provide technical support for accurate identification and real-time monitoring of apple disease images. In our future work, we will continue to focus on deep learning models capable of assessing the severity of apple leaf diseases. Simultaneously, we aim to deploy the model on devices such as unmanned aerial vehicles (UAVs) to achieve precise remote sensing for agricultural monitoring. This is a challenging task but is a pressing demand in the field.

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DETAIL

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Retracted: Implementing Machine Learning for Smart Farming to Forecast Farmers’ Interest in Hiring Equipment

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the

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(1) Discrepancies in scope
(2) Discrepancies in the description of the research reported
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We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.
The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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In addition, our investigation has also shown that one or more of the following human-subject reporting requirements has not been met in this article: ethical approval by an Institutional Review Board (IRB) committee or equivalent, patient/participant consent to participate, and/or agreement to publish patient/participant details (where relevant).
Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.
We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.
The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

In addition, our investigation has also shown that one or more of the following human-subject reporting requirements has not been met in this article: ethical approval by an Institutional Review Board (IRB) committee or equivalent, patient/participant consent to participate, and/or agreement to publish patient/participant details (where relevant).

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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Uncovering the Multitarget Therapeutic Mechanism of Tong-Xie-Yao-Fang on Irritable Bowel Syndrome

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ABSTRAK (ENGLISH)

Background. This study investigated the pharmacological mechanisms of Tong-Xie-Yao-Fang (TXYF) against irritable bowel syndrome (IBS). *Methods*. The chemical profile of TXYF was identified through UHPLC-QTOF-MS. Next, the obtained chemical profile served as the basis for network pharmacological analysis. Finally, the predictive performance of network pharmacology was validated by conducting molecular docking and animal experiment. *Results*. Seven key compounds of TXYF, namely, quercetin, ellagic acid, nobiletin, formononetin, isorhamnetin, vestitol, and licochalcone, were confirmed as the key components acting on IBS. TXYF treatment on IBS was mainly realized through the regulation of some key pathways of immune system, such as inflammatory bowel disease, cytokine-cytokine receptor interaction, HIF-1, and T cell receptor signaling pathway. NOS2, ACHE, ESR1, PTGS2, and RELA were the target genes of TXYF to improve IBS. Stable bonds between the key components and the core target genes were further verified by the results of molecular docking. *In vivo* experiments confirmed the effects of TXYF on IBS. Further Western blot analysis showed that NOS2, ACHE, and ERα were significantly upregulated in the model group in comparison with controls ($P<0.001$) but then significantly downregulated after treatment with TXYF for 14 days ($P<0.001$). *Conclusion*. This study applied an integrated method based on network pharmacology and experimental validation to examine the underlying “multicomponent, multitarget, and multipathway” mode of action of TXYF in treating IBS. The current findings provided indicative paradigms and new insights into exploring the multitarget therapeutic mechanism of Chinese herbal compound.

TEKS LENGKAP

1. Introduction

Irritable bowel syndrome (IBS) refers to a functional gastrointestinal disorder that is characterized by recurrent episodes of abdominal pain with altered bowel habits [1]. IBS is classified into three types, specifically, IBS-D (diarrhea-predominant), IBS-C (constipation-predominant), and mixed or alternating IBS (IBS-M). Rome IV criteria showed that the prevalence of IBS ranges from 5% to 10% worldwide, with a gradually increasing trend [2]. IBS often causes gastrointestinal discomfort, dietary restrictions, mood disorders, and disturbances in patients’ daily activities, leading to a heavy medical and financial burden [3]. In treating IBS, physicians normally focus on symptomatic drugs, the effectiveness which is far from patient’s expectations [4]. Moreover, the multifactorial nature of the physiopathology of IBS could limit the development of more effective drugs [4, 5]. As a result, IBS patients begin to seek complementary and alternative therapies to relieve their discomfort [6].

Chinese herbal medicine (CHM) is gaining recognition around the world owing to increasingly reported health benefits [7]. Tong-Xie-Yao-Fang (TXYF) is one of the most famous CHM formulas employed to treat functional gastrointestinal disorders and has demonstrated its effectiveness in IBS treatment [8]. TXYF consists of 4 herbs of Baishao (Paeoniae Radix Alba), Baizhu (Atractylodes Macrocephala Koidz), Chenpi (Citrus Reticulata), and Fangfeng (Saposhnikoviae Radix), which have the advantages of multicomponents and multitargeting. However, the complexity of CHM compounds also poses great challenges to pharmacological research, as their material basis and mechanism of action are currently unclear.

Network pharmacology, which is a cutting-edge interdisciplinary discipline in the systematic study of drugs, could

mechanistically link drugs and diseases as well as quantitatively represent the key aspects of overall regulatory mechanism of drug action [9]. Therefore, network pharmacology has been widely used to elucidate the pharmacological mechanisms of CHM formula [10, 11]. In this study, network pharmacology in combination with experimental validation was utilized to investigate the pharmacological mechanism of TXYF against IBS.

2. Methods

2.1. Identification of Active Ingredients and Target Genes

2.1.1. Preparation of Samples

TXYF samples were prepared by decoction. The mixture of Chen Pi (9 g), Atractylodes macrocephala (18 g), Paeonia lactiflora (12 g) and Fang Qi (6 g), Citrus Reticulata (9 g), Atractylodes Macrocephala Koidz (18 g), Paeoniae Radix Alba (12 g), and Saposhnikoviae Radix (6 g) was steeped in water 10 times for 1 hour (h), boiled for 1 h and then filtered through absorbent gauze. Similarly, the residue was extracted by steeping in water 8 times for another 0.5 h. The two filtrates were combined, concentrated in vacuo (equivalent to 1 g of crude grass/ml), and finally freeze-dried.

Lyophilized powder (1.0 g) was extracted using 50 mL of methanol/water (1 : 1, v/v) under ultrasonication for 0.5 h. Next, the extract was centrifuged at 13,000 rpm for 10 minutes (min) at 4°C and the supernatant was filtered through a membrane filter (0.22 μm). Finally, 1.0 μL of the filtrate was subjected to UHPLC-QTOF-MS for further analysis.

2.1.2. Key Active Ingredient and Screening of Target Genes

The names of all compounds identified by UHPLC-QTOF-MS/MS method were entered into the TCMSP (https://www.tcmsp-e.com/) for screening key active ingredients and their corresponding target genes. Drug-like ≥0.18 and oral bioavailability ≥30% were the screening criteria [12]. We used UniProt (https://www.uniprot.org/) [13] to normalize the obtained genes for gene symbols.

2.2. Identification of IBS-Related Targets

CTD (https://ctdbase.org/) [14], DisGeNET (https://www.disgenet.org/home/) [15], and GeneCards (https://www.genecards.org/) [16] were used to screen targets related to IBS. A correlation score ≥5 was confirmed as the screening criterion in GeneCards [16].

2.3. PPI Network Construction

Common target genes between drug and disease were defined as potential targets of TXYF against IBS and used for subsequent analysis. Potential targets were introduced into the STRING (https://string-db.org/) [17] for PPI analysis. The species were limited to “Homo sapiens” and the minimum interaction score required was set at high confidence (0.400). PPI networks were visualized using Cytoscape 3.7.2 software [18].

2.4. GO and KEGG Enrichment Analysis

To explore the biological functions related to potential targets, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed in the DAVID platform (https://david.abcc.Ncifcrf.gov/) [19]. GO enrichment analysis was used to interpret and annotate genes from three dimensions of cellular components, molecular functions, and biological processes. KEGG database was mainly applied for pathway analysis. $P<0.05$ was regarded as statistically significant.

2.5. Drug-Compound-Target Network Construction and Screening of Core Genes

A drug-compound-target network was developed to examine the relationship between the ingredients of TXYF and targets of IBS. CytoHubba, the plugin of Cytoscape [20], was used to perform the screening of core genes and key components. The degree values were calculated, and the target nodes with the top 12-degree values were taken to generate a new core network.

2.6. Molecular Docking

In the present study, to validate the compound-target correlation, molecular docking was carried out in Discovery Studio 2019. The compounds structures of TXYF and macromolecular protein target receptors related to IBS were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and RCSB PDB (https://www.rcsb.org/), respectively.

2.7. Experimental Validation

2.7.1. Animals and Experimental Protocol

Ten litters of newborn SPF SD rats (5 male offspring per dam) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co. All the experimental animals were housed in a barrier environment at the Laboratory Animal Center of Xiyuan Hospital, Chinese Academy of Traditional Chinese Medicine, at the temperature of 22–25°C with a relative humidity of 50%–60% under controlled 12-h light/dark cycle. Adequate food and water were provided for the experimental animals.

Two litters of rats were randomly selected as the control group, while the rest of the rats were used to develop IBS-D model of liver depression and spleen deficiency syndrome.

Firstly, the suckling rats were separated from the dams for 3 h a day (9 am to 12 am) from postnatal day 2 to day 14. On day 22, the suckling rats were weaned and caged separately from dams. On day 30, the body weight of all the rats was measured and randomly divided into model group, high-dose TXYF group (HL), medium-dose TXYF group (TM), and low-dose TXYF group (LH). When the rats were >6 weeks old and weighed >220 g, the enema procedure was performed using 4% acetic acid solution (1 ml) once a day for 30 seconds (s) for 2 weeks. The rats in the control group underwent the same procedure using 1 mL of normal saline. The rats were stimulated using the chronic unpredictable psychological stress method [20] starting from day 56, and another different stimulation method was selected for daily operation for 21 days. The rats were given a gavage intervention on day 77. The administered doses of TXYF were converted to TL (0.47 g/ml), TM (0.94 g/ml), and TH (1.88 g/ml). The rats were intragastrical administered at a dose of 1 mL/100 g once a day for a total of 14 days. The other groups were given equal amounts of saline using the same method. At the end of the experiment, after fasting for 12 h, the mice were sacrificed to harvest large intestine tissues, which were stored at −80°C for subsequent studies.

2.7.2. Histological Analysis

The colonic tissues were fixed in 10% neutral-buffered formalin, and 4-μm-thick sections were stained with hematoxylin-eosin (HE) according to standard procedures. Morphological changes of colonic mucosa were observed under light microscope (Nikon, Japan).

2.7.3. Western Blot Analysis

Tissue samples from three rats were randomly selected from each group, followed by homogenization of colonic tissue proteins with cold lysis buffer and PMSF protease inhibitor. Subsequently, the BCA protein concentration assay kit was performed to detect the protein concentration. The proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with primary antibody (rabbit anti-NOS2 (1 : 1000 dilution, YT3169, 131KD, Immunoway), rabbit anti-ACHE (1 : 500 dilution, AF5274, 67KD, Affinity), rabbit anti-Erα (1 : 500 dilution, BS1113, 66KD, Bioworld), and mouse anti-β-action (1 : 5000 dilution, YM3028, 43KD, Immunoway)) at 4°C overnight, followed by further incubation with secondary antibodies for 2 h at room temperature. Bound antibodies were visualized by an enhanced chemiluminescence (ECL) system.

2.8. Statistical Analysis

The data were presented as the mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) by one-way analysis of variance for comparison. A significant difference was considered when $P<0.05$.

3. Results

3.1. Active Ingredients and Target Genes

In this study, a total of 231 compounds of TXYF were identified using the UHPLC-QTOF-MS protocol. A total of 42 key active ingredients and 218 corresponding target targets were obtained after importing the identified components into the TCMSP database for screening. Figure 1 presented the compound-target network of TXYF.

[figure(s) omitted; refer to PDF]

3.2. Target Genes Associated with IBS

Using the available resources (GeneCards, CTD, and DisGeNET), we filtered 5273 target genes correlated with IBS. Further analysis identified 70 common target genes between the six resources by a Venn diagram (Figure 2).

[figure(s) omitted; refer to PDF]

3.3. PPI Network and Core Gene Analysis

The Venn diagram revealed (Figure 3) 24 overlapping genes between the disease targets and the drug targets. Subsequently, the 24 targets were imported into the String database for PPI analysis, and we generated a network (Figure 4) containing 24 nodes and 312 edges.

[figure(s) omitted; refer to PDF]

3.4. GO and KEGG Enrichment Analysis

Figures 5 and 6 present the results of GO and KEGG enrichment analysis, respectively. In GO analysis, biological processes mainly enriched in positive regulation of gene expression, response to drug, and positive regulation of ERK1 and ERK2 cascade, negative regulation of apoptotic process. In KEGG analysis, inflammatory bowel disease, cytokine-cytokine receptor interaction, HIF-1 signaling pathway, T cell receptor signaling pathway, and pathways in cancer were the significantly enriched pathways.

[figure(s) omitted; refer to PDF]

3.5. Construction of Drug-Compound-Target Network and Identification of Core Targets

A drug-compound-target network (Figure 7) containing 34 compounds, 24 targets, 58 nodes, and 121 edges was developed. Using Cytoscape plugin cytoHubba, the core active compounds and core targets were identified according to the top 12-degree values. The core targets were NOS2, ACHE, ESR1, PTGS2, and RELA, and the top active compounds were quercetin, formononetin, ellagic acid, isorhamnetin, vestitol, licochalcone, and nobiletin (Figure 8).

[figure(s) omitted; refer to PDF]

3.6. Molecular Docking Analysis

According to the results of molecular docking, all the LibDock scores were over “50” (Figure 9), suggesting that all the key active ingredients were well docked to the corresponding target genes. 3D and 2D molecular docking model of the key active ingredients with core target genes are presented in Supplementary Figures 1 and 2.

[figure(s) omitted; refer to PDF]

3.7. Experimental Validation

3.7.1. Effect of TXYF on IBS-D

The experimental protocol is shown in Figure 10. As shown in Figure 11, the body weight, daily food intake, abdominal withdrawal reflex, and fecal water content were determined to evaluate the effects of TXYF on IBS-D. Before medication, the body weight, daily food intake, and abdominal withdrawal reflex were lower in the model group compared with the control group ($P<0.05$). Interestingly, the three indexes were significantly increased in the rats treated with different doses of TXYF for 14 days compared to the model group ($P<0.05$). Similarly, fecal water content was higher in the model group than in the control group ($P<0.05$), but it was significantly decreased in rats treated with different doses of TXYF for 14 days ($P<0.05$).

[figure(s) omitted; refer to PDF]

According to the results of HE (Figure 12), no loss of epithelial structures and normal glands was observed in the colonic tissues of the control rats, while a small amount of inflammatory cell infiltration, submucosal edema, and gap enlargement were seen in the mucosa of the model group. It is noteworthy that the degree and extent of damage to the colonic tissues of the rats were alleviated by the intervention of TXYF, especially in the rats that received a high dose of TXYF.

[figure(s) omitted; refer to PDF]

3.7.2. Western Blot Analysis

Three potential targets (NOS2, ACHE, and ESR1) predicted by network pharmacology were further validated. As shown in Figure 13, NOS2, ACHE, and ERα were significantly higher-expressed in the model group compared with the control group ($P<0.05$). Interestingly, NOS2, ACHE, and ERα were significantly low-expressed in rats treated with different doses of TXYF for 14 days than the model group ($P<0.05$). These results all indicated that TXYF could be beneficial to IBS-D through NOS2 and ACHE as well as ERα among other potential targets.

[figure(s) omitted; refer to PDF]

4. Discussion

TXYF has been widely used in the treatment of IBS and has shown great efficacy. However, complex characteristics of CHM compound pose great challenges to the study of its mechanism of action. In this study, to investigate the pharmacological mechanisms of TXYF against IBS, network pharmacology in combination with experimental validation was performed. Network pharmacology screened a total of 34 active ingredients of TXYF acting on 24 IBS-related targets. Specifically, the key active compounds were quercetin, formononetin, ellagic acid, isorhamnetin, vestitol, licochalcone, and nobiletin, and the core targets were ESR1, NOS2, ACHE, PTGS2, and RELA. Animal experiments validated the key results of network pharmacology.

In the present study, a total of 231 compounds of TXYF were identified using the UHPLC-QTOF-MS protocol and served as the basis for further network pharmacological analysis. Finally, key active compounds, namely, quercetin, ellagic acid, nobiletin, formononetin, isorhamnetin, vestitol, and licochalcone, were screened based on the predictions from network pharmacology. Oxidative stress is closely related to the pathological mechanism of IBS, and the mechanism lies in the excessive reactive oxygen species (ROS) production produced by oxidative stress, which in turn damages gastrointestinal epithelial cells [21]. Quercetin is a subclass of flavonoids with a variety of pharmacological activities such as anti-inflammation, antioxidation, anticarcinogenesis, and antivirus [22]. The addition of quercetin to diets has been reported to be able to improve antioxidant capacity and alleviate oxidative damage to the intestinal mucosa [23]. *In vitro* studies have also observed that quercetin can inhibit ROS production in gastrointestinal epithelial cells, thereby preventing damage resulted from oxidative stress [24]. Additionally, quercetin could contribute to the antioxidant capacity of the body through promoting the protein abundance of Nrf2 and regulating GSH-related redox homeostasis in enterocytes [23]. Study also found that quercetin can significantly downregulate the colonic expression of ngn3, TPH, and pdx1, increase pain threshold pressure, and reduce visceromotor responses in IBS animals [25]. Ellagic acid is a strong antioxidant with various properties such as antioxidation, reduction of inflammation, and apoptosis [26]. Receiving ellagic acid improves sleep quality and gastrointestinal symptoms in IBS patients, which may be realized through antioxidation and moist properties of ellagic acid [27]. Other key components have also been discovered to have antioxidant effects by earlier studies [28–32], but studies on their effects on IBS are highly limited. The present network pharmacology revealed their importance and can provide direction for their further research in IBS.

PPI network was developed, and NOS2, ACHE, ESR1, PTGS2, and RELA were identified as the core targets of TXYF against IBS. NOS2 is a reactive free radical functioning as a biological mediator in several biological processes, including neurotransmission and antibacterial and antitumor activities. Recent studies have detected a close relationship between NOS2 and inflammation and visceral hypersensitivity [33]. NOS2 overactivation can result in a variety of inflammatory diseases, and hypersensitivity can be controlled by modulating NOS2-associated inflammation [33]. In this study, animal experiments revealed that the level of NOS2 in the colonic tissue of rats in the IBS model group was significantly elevated compared to the control group but was then significantly decreased after TXYF treatment for 14 days. ACHE hydrolyzes the neurotransmitter acetylcholine at the neuromuscular junction and brain cholinergic synapses, thereby terminating signal transmission. ACHE is present in a variety of molecular forms with similar catalytic properties, but differs in the way how its oligomers assemble and cells attach to the cell surface. Conditions associated with ACHE include colonic pseudo-obstruction. With deepening understanding of the brain-gut axis in the pathogenesis of IBS [34], more attention should be paid to the role of ACHE in IBS [35]. The present study detected a significant increase in ACHE level in the IBS model in comparison to the control group, but interestingly, it was decreased after implementing 14 days of TXYF treatment. A growing body of data suggested that sex hormones may play a crucial role in the pathogenesis of IBS [36]. ESR1 is a protein-coding gene that encodes an estrogen receptor and ligand-activated transcription factor. Concurrent alterations in immunomodulatory cytokines and microRNAs have been observed in IBS patients, indicating that dysregulation of local immune responses may be realized in an estrogen-dependent manner [37]. The results of *in vivo* experiments confirmed that TXYF had certain effects on IBS, and Western blot analysis revealed that the ER*α*

level was significantly higher in the colon tissue of the IBS model group than that in the control group, but it was decreased significantly after 14 days of TXYF treatment. PTGS2 is a PTGS that influences the development of chronic inflammatory diseases through producing inflammatory mediator prostaglandin E-2 (PGE (2)) and is a potential therapeutic target for IBS [38, 39]. RELA, also known as NF-κB, is a ubiquitous transcription factor involved in several biological processes. Previous studies have confirmed that TXYF can effectively improve intestinal permeability and enhance intestinal mucosal barrier function in IBS via the NF-κB pathway [40]. Furthermore, molecular docking analysis demonstrated that TXYF can effectively bind to specific proteins in IBS target genes, further validating the predictive results of network pharmacology.

However, the limitations of the current study should be equally acknowledged. IBS is classified into IBS-D, IBS-C, and IBS-M, but we only used IBS-D rats to verify the results of network pharmacology. Hence, whether the experimental results from IBS-D were applicable to IBS-C and IBS-M should to be further verified.

5. Conclusion

The present study applied an integrated method based on network pharmacology and experimental validation to uncover the underlying “multicomponent, multitarget, and multipathway” mode of action of TXYF for the treatment of IBS. This study provided indicative paradigms and new insights into the exploration of the multitarget therapeutic mechanism of Chinese herbal compound.

Authors’ Contributions

Xiangxue Ma designed the study and drafted the manuscript. Jinke Huang, Haomeng Wu, and Xia Li helped with the implementation of this work. Fengyun Wang and Xudong Tang contributed to the review and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Glossary

Abbreviations

TXYF:Tong-Xie-Yao-Fang (TXYF)

IBS:Irritable bowel syndrome (IBS)

UHPLC-QTOF-MS:Quadrupole time-of-flight mass spectrometry

IBS-D:Diarrhea-predominant

IBS-C:Constipation-predominant

IBS-M:Mixed or alternating IBS (IBS-M)

CHM:Chinese herbal medicine

GO:Gene Ontology

KEGG:Kyoto Encyclopedia of genes and genomes.

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DETAIL

Subjek:	Pharmacology; Ingredients; Genes; Drug dosages; Laboratory animals; Statistical analysis; Proteins; Irritable bowel syndrome; Cytokines; Immune system; Mode of action
Judul:	Uncovering the Multitarget Therapeutic Mechanism of Tong-Xie-Yao-Fang on Irritable Bowel Syndrome
Pengarang:	Ma, Xiangxue1 ; Huang, Jinke1 ; Wu, Haomeng2 ; Li, Xia3 ; Wang, Fengyun1 ; Tang, Xudong1 1 Institute of Digestive Diseases, Xiyuan Hospital of China Academy of Chinese Medical Sciences, Beijing, China2 Department of Gastroenterology, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Hospital of Chinese Medicine), Guangzhou, China3 Department of Spleen and Stomach Diseases of Traditional Chinese Medicine, China-Japan Friendship Hospital, Beijing, China
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Retracted: Implementing Machine Learning for Supply-Demand Shifts and Price Impacts in Farmer Market for Tool and Equipment Sharing

Quality Journal of Food.

Link dokumen ProQuest

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The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] M. Rakhra, A. Bhargava, D. Bhargava, R. Singh, A. Bhanot, A. W. Rahmani, "Implementing Machine Learning for Supply-Demand Shifts and Price Impacts in Farmer Market for Tool and Equipment Sharing," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/4496449, 2022.

DETAIL

Subjek:	Research; Machine learning
Ketentuan indeks bisnis:	Subjek: Machine learning
Judul:	Retracted: Implementing Machine Learning for Supply-Demand Shifts and Price Impacts in Farmer Market for Tool and Equipment Sharing
Pengarang:	Quality Journal of Food
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Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: An Investigation in Analyzing the Food Quality Well-Being for Lung Cancer Using Blockchain through CNN

Quality Journal of Food.

Link dokumen ProQuest

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References

[1] M. A. Aboamer, M. Y. Sikkandar, S. Gupta, L. Vives, K. Joshi, B. Omarov, S. K. Singh, "An Investigation in Analyzing the Food Quality Well-Being for Lung Cancer Using Blockchain through CNN," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/5845870, 2022.

DETAIL

Subjek:	Research; Blockchain; Food quality
Ketentuan indeks bisnis:	Subjek: Blockchain
Perusahaan / organisasi:	Nama: CNN; NAICS: 516210
Judul:	Retracted: An Investigation in Analyzing the Food Quality Well-Being for Lung Cancer Using Blockchain through CNN
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Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Role of Artificial Intelligence and Deep Learning in Easier Skin Cancer Detection through Antioxidants Present in Food

Quality Journal of Food.

Link dokumen ProQuest

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References

[1] S. R. C., J. G., S. N., D. L. Padmaja, S. Nagaprasad, K. Pant, Y. P. Kumar, "Role of Artificial Intelligence and Deep Learning in Easier Skin Cancer Detection through Antioxidants Present in Food," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/5890666, 2022.

DETAIL

Subjek:	Research; Deep learning; Artificial intelligence
Ketentuan indeks bisnis:	Subjek: Artificial intelligence
Judul:	Retracted: Role of Artificial Intelligence and Deep Learning in Easier Skin Cancer Detection through Antioxidants Present in Food
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Basis data:	Public Health Database; Publicly Available Content Database

Retracted: A Novel Model to Detect and Classify Fresh and Damaged Fruits to Reduce Food Waste

Using a Deep Learning Technique

Quality Journal of Food.

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References

[1] T. B. Kumar, D. Prashar, G. Vaidya, V. Kumar, S. D. Kumar, F. Sammy, "A Novel Model to Detect and Classify Fresh and Damaged Fruits to Reduce Food Waste Using a Deep Learning Technique," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/4661108, 2022.

DETAIL

Subjek:	Research; Deep learning; Food waste
Judul:	Retracted: A Novel Model to Detect and Classify Fresh and Damaged Fruits to Reduce Food Waste Using a Deep Learning Technique
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Hak cipta:	
Terakhir diperbarui:	2024-03-08
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Retracted: An Investigation in Applying Internet of Things Approach in Safe Food Dietary Plan for

Better Chronic Diabetes Management among Elderly Adults

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References

[1] G. Geetha, R. Radeep Krishna, S. Vyas, I. Sukhwal, A. Jain, A. Chaturvedi, M. A. Shah, "An Investigation in Applying Internet of Things Approach in Safe Food Dietary Plan for Better Chronic Diabetes Management among Elderly Adults," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/4281237, 2022.

DETAIL

Subjek:	Research; Diabetes; Internet of Things
Ketentuan indeks bisnis:	Subjek: Internet of Things

Judul:	Retracted: An Investigation in Applying Internet of Things Approach in Safe Food Dietary Plan for Better Chronic Diabetes Management among Elderly Adults
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Terakhir diperbarui:	2024-03-08

Retracted: Contextualization of Trait Nexus and Gene Action for Quantitative and Qualitative Characteristics in Indian Mustard

Quality Journal of Food.

Link dokumen ProQuest

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References

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DETAIL

Subjek: Research

Judul:	Retracted: Contextualization of Trait Nexus and Gene Action for Quantitative and Qualitative Characteristics in Indian Mustard
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Determination of 60 Food-Borne Stimulant Drug Residues in Animal-Derived Foods by Solid-Phase Extraction Purification and Ultra-High-Performance Liquid Chromatography-Quadrupole/Orbitrap High-Resolution Mass Spectrometry

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ABSTRAK (ENGLISH)

A method for the rapid screening and confirmation of 60 food-borne stimulants in animal-derived foods was developed using the ultra-high-performance liquid chromatography-quadrupole/orbitrap high-resolution mass spectrometry (UPLC-Q/Orbitrap HRMS). After enzymatic hydrolysis at 37°C for 16 h, the samples were extracted with acetonitrile solution. The extraction solution was purified by PRiME HLB pass-through solid-phase extraction cartridge and redissolved by nitrogen blowing. The target compounds of reconstitution fluid were separated by Hypersil GOLD™ VANQUISH column (2.1 mm × 100 mm, 1.9 μm). The raw data were collected for the target compounds in Full Scan-ddMS^2 mode, and then the Quan Browser module under Xcalibur software can be used to analyze the quantitative results of the samples. The combination of Tracefinder and mzVault software can realize the qualitative screening and confirmation of the samples. The results showed that the relative deviation of the exact mass of the 60 food-borne stimulants was less than 5.0×10^{-6}, which had a good linear relationship in the corresponding concentration, and the correlation coefficient (R2) was greater than 0.9966. The detection limit ranged from 0.05 to 0.5 μg/kg. The quantification limit ranged from 0.1 to 1 μg/kg. The method recoveries ranged from 70.2% to 111.9% with relative standard deviations of 0.05% to 9.00% (n=6). The method is easy to operate, covers a wide range of targets, and has high efficiency and accuracy. It is suitable for rapid screening and confirmation of various food-derived stimulants in animal-derived foods and provides assistance for food safety in the major events.

TEKS LENGKAP

1. Introduction

Animal-derived food refers to the products derived from animals and available for human consumption, including fresh meat, frozen meat and their products, eggs, and milk and meat products, which are the main sources of energy and protein intake for human beings. In order to meet the demand of human beings for animal-derived food, the scale of animal husbandry and aquaculture is expanding gradually. At the same time, in order to maximize economic benefits, some farmers illegally use prohibited drugs in the breeding process, resulting in animal-derived food containing a large amount of drug residues, which threatens the interests of consumers [1, 2]. In addition, the stimulant drug residues in animal-derived foods continue to harm human health, but also damage the fairness of

competitive sports. For example, the doping problems emerge endlessly during the Olympic Games [3]. In order to eradicate this problem, ensure the fair competition of athletes and realize the original intention of successfully holding large-scale competitions, it is imperative to establish a technical means of simultaneous screening and quantification of various food-borne doping agents.

At present, the detection and analysis of stimulant drug residues in animal-derived foods includes enzyme-linked immunosorbent assay (ELISA) [4, 5], gas chromatography (GC) [6], gas chromatography-tandem mass spectrometry (GC-MS/MS) [7–9], liquid chromatography (LC) [10–12], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13–24], and liquid chromatography-high resolution mass spectrometry (LC-HRMS) [25–29]. Antibodies used in ELISA are produced by different manufacturers, and the quality is difficult to guarantee, resulting in differences between the results, and the cross-reaction phenomenon of immunology makes the test results prone to false positive. The pretreatment used by GC and GC-MS/MS is complicated, and its detection limit is high, so it is difficult to achieve high-sensitivity detection and analysis. LC performs qualitative and quantitative analysis only by retention time, so the number of compounds analyzed within a certain time window is limited. Compared with LC, LC-MS/MS can obtain information such as the ion ratio of compounds and realize the simultaneous analysis and detection of multiple compounds, it cannot obtain abundant information of product ion fragments, and the accurate of first-order mass obtained by LC-MS/MS is relatively low, which makes it impossible to achieve high-throughput screening analysis. LC-HRMS can obtain the characteristic secondary fragment ion information of the compound, and its mass accuracy can reach ppm level, which has higher resolution, and high-throughput qualitative and quantitative analysis can be achieved in combination with the secondary spectral library information.

The research reports on food-borne stimulants mainly focus on β2 adrenoceptor agonists or specific types of stimulants, and there are few reports on the simultaneous detection of multiple stimulants. Feng et al. used ultra-high-performance liquid chromatography-tandem mass spectrometer to determine 44 kinds of food-borne stimulants and 6 kinds of progesterone in livestock and poultry meat [30]. This method measured a variety of food-borne stimulants and covered a wide range, and they could not obtain information such as fragment ion and isotope ratio, and had weak qualitative ability for complex samples. Yan et al. used high-throughput analytical platform to screen 39 glucocorticoids in animal-derived food [27]. In this experiment, UPLC-Q/Orbitrap HRMS with ultrahigh resolution and high selectivity was used as the detector of food-borne stimulants in animal-derived foods. PRiME HLB solid-phase extraction column was used to eliminate impurities in the samples. A secondary spectrum library of 60 food-borne stimulants and 12 kinds of diuretics, 18 kinds of protein anabolic agents, 9 kinds of glucocorticoids, 18 kinds of β2 adrenoceptor agonists, 3 kinds of β-receptor antagonists for simultaneous screening and quantification of animal food was analyzed. This method can further improve the qualitative credibility, ensures the accuracy of positive samples, and can further avoid the occurrence of athletes taking food-borne stimulants by mistake, and provides technical guarantee for the screening of food-borne stimulants in large-scale competitions.

2. Materials and Methods

2.1. Apparatus, Chemicals, and Reagents

Vanquish Flex + Orbitrap Exploris 480 ultra-high-performance liquid chromatography-quadrupole/orbitrap high resolution mass spectrometry (Thermo Fisher Scientific, USA) was used. Instrument control and data acquisition were realized by Thermo Scientific Xcalibur. Data screening analysis was realized by the combination of TraceFinder and mzVault software. The content of detected residues was calculated by Thermo Xcalibur Quan Browser. The high-speed refrigerated centrifuge (CR22N, HITACHI, Germany), the ultrasonic cleaner (Elmasonic P300H, Elma, Germany), vortex mixer (Vortex Genius 3, IKA, Germany), and nitrogen blower (Organomation, USA) was used.

Clenbuterol, salbutamol, ractopamine, terbutaline, fenoterol, tulobuterol, penbutolol, cimaterol, salmeterol, atenolol, metoprolol, clenproperol, demethyl coclaurine, tretoquinol, propanolol, bromoclenbuterol, formoterol, brombuterol, meprednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, beclomethasone, fludrocortisone, hydrocortisone, cortisone, zilpaterol, stanozolol, trenbolone, metandienone, 17-methyltestosterone,

testosterone, nandrolone, testosterone propionate, nandrolone 17-propionate, boldenone, nandrolone phenylpropionate, dehydroepiandrosterone, zeranol, beta-zearalanol, alpha-zearalenol, beta-zearalenol, zearalanone, zearalenone, acetazolamide, canrenone, chlortalidone, furosemide, spironolactone, bendroflumethiazide, chlorothiazide, hydrochlorothiazide, triamterene, 4-amino-6-chlorobenzene-1,3-disulfonamide, bumetanide, torasemide, acebutolol, and celipolol (purity ≥95%) were supplied from Dr. Ehrenstorfer (Augsburg, Germany). All the samples used in this experiment were purchased from Qiaoxi vegetable wholesale market in Shijiazhuang.

Methanol, acetonitrile, formic acid, and ammonium acetate were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Waters Oasis PRiME HLB solid-phase extraction column (200 mg, 6 cc) was purchased from Waters (USA). Water was purified using a Milli-Q-System (Millipore, Guyancourt, France). *β*-glucuronidase/arylsulfatase (contains *β*-glucuronidase 111000 U/mL and arylsulfatase 1079 U/mL) was purchased from Sigma (USA).

2.2. Preparation of Standard Solution

Weigh an appropriate amount of standards, respectively, into a 100 mL volumetric flask, dissolved it with methanol and make up to the mark (triamterene should be dissolved in formic acid first, and then make up to the mark with methanol). The standard reserve solution with mass concentration of 100 *μ*g/mL, store it at −20°C and valid for 6 months. The standard reserve solution was pipetted to a 100 mL volumetric flask, respectively, and dissolving in methanol to scale. The concentration of ractopamine, fenoterol, tulobuterol, salmeterol, metoprolol, propanolol, trenbolone, and nandrolone phenylpropionate in the obtained mixed standard intermediate solution is 1 *μ*g/mL, the concentration of clenbuterol, salbutamol, terbutaline, penbutolol, cimaterol, atenolol, clenproperol, demethyl coclaurine, tretoquinol, meprednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, beclomethasone, fludrocortisone, hydrocortisone, cortisone, zilpaterol, stanozolol, metandienone, 17-methyltestosterone, testosterone, nandrolone, testosterone propionate, nandrolone 17-propionate, boldenone, dehydroepiandrosterone, zeranol, beta-zearalanol, alpha-zearalenol, beta-zearalenol, zearalanone, zearalenone, acebutolol, bromoclenbuterol, celipolol, formoterol, brombuterol, and nebivolol is 2 *μ*g/mL, and the concentration of acetazolamide, canrenone, chlortalidone, furosemide, spironolactone, bendroflumethiazide, chlorothiazide, hydrochlorothiazide, triamterene, 4-amino-6-chlorobenzene-1,3-disulfonamide, bumetanide, and torasemide is 10 *μ*g/mL. The solution was stored at −20°C and valid for 3 months. The 14 kinds of internal standard materials were weighed and dissolved in 10 mL volumetric flask with methanol, and the volume was fixed to the scale. The mixed standard reserve solution with mass concentration of 100 *μ*g/mL was obtained. It was stored at −20°C and valid for 6 months.

2.3. Sample Preparation

5 g (milk, egg, beef, mutton, and pork) of homogenized sample was weighed and transferred to a 50 mL plastic centrifuge tube, then spiking of 10 *μ*L of 1 *μ*g/mL internal standard solution, 20 mL of 0.2 mol/L acetic acid ammonium buffer (pH = 5.2 ± 0.1), and 50 *μ*L of *β*-glucuronidase/arylsulfatase, vortexed for 1 min to mix. The sample was placed in a water bath shaker at 37 ± 1°C and shaked for enzymatic hydrolysis 16 h. The enzymolysis sample solution was cooled to room temperature, centrifuged at 10000 r/min for 5 min, and then supernatant A was removed. 10 mL acetonitrile solution was added to supernatant A, then 8 g sodium chloride was added, and the extraction was conducted by shaking for 10 min. The mixed solution was centrifuged at 10000 r/min for 5 min, and supernatant B was taken. 10 mL acetonitrile solution was added to supernatant A and was extracted by shaking for 10 min. The mixed solution was centrifuged at 10000 r/min for 5 min, and supernatant C was taken. Supernatant B and supernatant C were combined and passed through the PRiME HLB solid-phase extraction column with a flow rate of 1 drop/s. The outflow was collected and blown to near dry at 40°C with nitrogen. 1 mL of initial mobile phase was added and mixed by vortex. The complex solution was filtered by 0.22 *μ*m nylon membrane and was determined. The animal food without the target compound was pretreated according to the above experimental method, and the blank matrix solution was obtained, which was used for the preparation of the standard curve except the internal standard material.

2.4. Chromatographic Conditions

The chromatographic separation was performed on a Hypersil GOLD™ VANQUISH column (2.1 mm × 100 mm, 1.9 μm, Thermo Fisher Scientific, USA). The injection volume was 5 μL. The flow rate was 0.3 mL/min. Aqueous solution containing 0.1% (v/v) formic acid (phase A) and 50% methanol-acetonitrile solution (phase B) were used as mobile phase. The consecutive program was as follows: 0–3.00 min, 2% to 30% B; 3.00–15.00 min, 30% to 50% B; 15.00–18.00 min, 50% to 95% B; 18.00–20.00 min, 95% B; 20.00–20.10 min, 95% to 2% B; and 20.10–22.00 min, 2% B.

2.5. Mass Spectrometry Conditions

The MS analysis was performed using an ESI source in positive/negative ionization mode. The optimized parameters of ion source were as follows: the positive ion/negative ion was +3500/−3000 V, the ion transfer tube temp was 320°C, the vaporizer temp was 400°C, the sheath gas was 45 Arb, the aux gas was 10 Arb, and the sweep gas was 1 Arb. MS conditions were as follows: scan type was Full Scan-ddMS^2, scan range was 150–800 m/z, the duration was 22 min, the collision energy mode was stepped, the collision energy type was normalized, HCD collision energies was 30 V, 50 V, and 70 V, Full Scan orbitrap FWHM was 60000, and MS^2 orbitrap FWHM was 30000.

2.6. Establishment of Database

Under the instrument conditions given in Sections 2.4 and 2.5, the 60 kinds of food-borne stimulants name, molecular formula, and the way of ionization was inputted instrument after acquisition method. The data of doping were collected, such as the retention time. Then, the time window was set to 1 min, realize the precise analysis of the specific compounds. The detailed parameter information of 60 compounds is shown in Table 1. This study was confirmed by the matching degree of a high-resolution precursor ion and the secondary spectrum, and the mass deviation of both parent ion and fragment ion was less than 5 ppm. In the ddMS^2 mode of Vanquish Flex + Orbitrap Exploris 480, when a specific target compound responds within the set retention time window, it is automatically triggered to collect and superimpose at different collision energies (30 V, 50 V, and 70 V) to generate secondary fragment spectra. In the database Library module of Thermo mzVault software, import raw data to create a secondary spectrum library including 60 food-borne stimulants, the MS^2 spectra of the 60 food-borne stimulants are shown in Supplementary Figure 1.

Table 1

MRM parameters and chemical abstracts service (CAS) of 60 food-borne stimulants.

Category (number)	Compound	Molecular formula	CAS	Retention time (min)	Adduct/charge	Parent ion (m/z)		Delta ($\times 10^{-6}$)	Measured mass of fragment ions (m/z)
Measured mass (m/z)	Theoretical mass (m/z)	*β*2 adrenoceptor agonists (18)	Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	129138-58-5	4.60	$[M+H]^+$	277.0866	277.0866

0.00	203.01384; 132.06825; 168.04498; 259.07632	Salbutamol	$C_{13}H_{21}NO_3$	18559-94-9	2.76	$[M+H]^+$	240.1592	240.1591	−0.51
148.07571; 166.08632; 222.14890; 121.06477	Ractopamine	$C_{18}H_{23}NO_3$	97825-25-7	4.20	$[M+H]^+$	302.1748	302.1748	−0.09	107.04913; 121.06482; 164.10710; 136.07576
Terbutaline	$C_{12}H_{19}NO_3$	23031-25-6	2.74	$[M+H]^+$	226.1435	226.1435	−0.07	152.07060; 125.05965; 107.04907; 170.08112	Fenoterol
$C_{17}H_{21}NO_4$	13392-18-2	3.36	$[M+H]^+$	304.154	304.154	−0.09	107.04907; 135.08043; 152.07059; 286.14392	Tulobuterol	$C_{12}H_{18}ClNO$
41570-61-0	4.75	$[M+H]^+$	228.1147	228.1147	0.20	228.23235; 155.02596; 172.05254; 137.01540	Penbutolol	$C_{18}H_{29}NO_2$	36507-48-9

13.09	$[M+H]^+$	292.22269	292.2269	−0.01	236.16449; 74.06004; 133.06476; 201.12735	Cimaterol	$C_{12}H_{17}N_3O$	54239-37-1	2.74
$[M+H]^+$	220.1441	220.1442	0.48	160.08694; 143.06039; 202.13390; 116.04941	Salmeterol	$C_{25}H_{37}NO_4$	89365-50-4	13.57	$[M+H]^+$
416.2792	416.2792	−0.01	91.05419; 232.16962; 380.25836; 398.26859	Atenolol	$C_{14}H_{22}N_2O_3$	29122-68-7	2.84	$[M+H]^+$	267.1703
267.1699	−1.49	145.06473; 74.06001; 190.08615; 56.04948	Metoprolol	$C_{15}H_{25}NO_3$	81024-43-3	4.69	$[M+H]^+$	268.1905	268.1904

−0.34	74.06007; 116.10698; 121.06484; 56.04953	Clenproperol	$C_{11}H_{16}C_{l2}NO_2$	38339-11-6	4.13	$[M+H]^+$	263.0708	263.0709	0.23
245.06088; 132.06830; 203.01390; 168.04503	Demethylcoclaurine	$C_{16}H_{17}NO_3$	5843-65-2	3.21	$[M+H]^+$	272.1279	272.128	0.11	107.04911; 255.10164; 161.05980; 143.04921
Tretoquinol	$C_{19}H_{23}NO_5$	18559-59-6	4.18	$[M+H]^+$	346.1645	346.1644	−0.26	164.07080; 228.82471; 137.05959	Propanolol
$C_{16}H_{21}NO_2$	525-66-6	7.25	$[M+H]^+$	260.1644	260.1644	−0.12	116.10688; 74.06000; 183.08038; 56.04948	Bromoclenbuterol	$C_{12}H_{18}BrClN_2O$
37153-52-9	4.80	$[M+H]^+$	321.0364	321.0361	−0.96	246.96312; 168.04482; 132.06813	Formoterol	$C_{19}H_{24}N_2O_4$	73573-87-2
4.72	$[M+H]^+$	345.18 09	345.1804	−1.33	121.06469; 149.09604; 91.05414	Brombuterol	$C_{12}H_{18}Br_2N_2O$	41937-02-4	5.09

$[M+H]^+$	364.9859	364.9855	−0.89	211.99431; 290.91266; 132.0618	-				
Glucocorticoids (9)	Meprednisone	$C_{21}H_{26}O_5$	53-03-2	9.51	$[M+H]^+$	359.185	359.1849	−0.18	147.08055; 171.08052; 237.12759; 159.08061
Prednisolone	$C_{21}H_{28}O_5$	50-24-8	9.97	$[M+H]^+$	361.201	361.2007	−0.76	147.08084; 228.82837; 121.06483; 171.08055	Methylprednisolone
$C_{22}H_{30}O_5$	83-43-2	12.45	$[M+H]^+$	375.2163	375.2162	−0.33	147.08047; 121.06472; 135.08029; 357.19714	Dexamethasone	$C_{22}H_{29}FO_5$
50-02-2	12.44	$[M+H]^+$	393.2072	393.2069	−0.92	147.08052; 228.82304; 322.25323; 121.06493	Betamethasone	$C_{22}H_{29}O_5F$	378-44-9

12.69	$[M+H]^+$	393.21	393.21	−0.94	322.25394; 228.83580; 90.97672; 251.16698	Beclomethasone	$C_{22}H_{29}ClO_5$	4419-39-0	13.33
$[M+H]^+$	409.1774	409.1773	−0.15	228.82869; 147.08031; 279.17426; 121.06482	Fludrocortisone	$C_{21}H_{29}FO_5$	127-31-1	10.01	$[M+H]^+$
381.2069	381.2068	−0.24	228.83195; 181.10197; 239.14240; 325.17957	Hydrocortisone	$C_{21}H_{30}O_5$	50-23-7	10.05	$[M+H]^+$	363.2162
363.2163	0.08	121.06479; 97.06477; 327.19553; 267.17416	Cortisone	$C_{21}H_{28}O_5$	53-06-5	9.73	$[M+H]^+$	361.2006	361.2006

0.09	163.11180; 121.06477; 105.06985; 145.10123	-							
Protein anabolic agents (18)	Zilpaterol	$C_{14}H_{19}N_3O_2$	117827-79-9	2.72	$[M+H]^+$	262.1552	262.1546	−2.32	185.07091; 244.14441; 202.09747; 157.07603
Stanozolol	$C_{21}H_{32}N_2O$	10418-03-8	17.78	$[M+H]^+$	329.2587	329.2584	−0.84	98.98405; 81.04475; 228.83578; 107.08548	Trenbolone
$C_{18}H_{22}O_2$	10161-33-8	13.81	$[M+H]^+$	271.1693	271.169	−1.34	253.15874; 199.11179; 227.14310	Metandienone	$C_{20}H_{28}O_2$
72-63-9	15.95	$[M+H]^+$	301.2162	301.2159	−0.82	121.06470; 149.13242; 283.20560; 107.08543	17-Methyltestosterone	$C_{20}H_{30}O_2$	58-18-4
17.48	$[M+H]^+$	303.2319	303.2315	−1.40	97.06470; 109.06471; 285.22119	Testosterone	$C_{19}H_{28}O_2$	58-22-0	16.63

$[M+H]^+$	289.2162	289.2159	−1.17	97.06470; 109.64700; 253.19521	Nandrolone	$C_{18}H_{26}O_2$	434-22-0	15.04	$[M+H]^+$
275.2006	275.2003	−0.99	109.06473; 257.18994; 239.17946; 83.04912	Testosterone propionate	$C_{22}H_{32}O_3$	57-85-2	19.19	$[M+H]^+$	345.2424
345.2421	−0.88	97.06470; 109.06470; 121.06471;	Nandrolone 17-propionate	$C_{21}H_{30}O_3$	7207-92-3	19.01	$[M+H]^+$	331.2268	331.2266
−0.75	64.97739; 301.17001; 257.18979; 109.06461	Boldenone	$C_{19}H_{26}O_2$	846-48-0	14.5	$[M+H]^+$	287.2006	287.2004	−0.52
121.06473; 135.11676; 173.09605	Nandrolone phenylpropionate	$C_{27}H_{34}O_3$	62-90-8	19.45	$[M+H]^+$	407.2581	407.2579	−0.46	105.06976; 257.18985; 91.05414
Dehydroepiandrosterone	$C_{19}H_{28}O_2$	53-43-0	16.68	$[M+H]^+$	271.2056	271.2055	−0.61	253.19475; 97.06460; 109.06458; 81.06975	Zeranol

$C_{18}H_{26}O_5$	26538-44-3	15.46	$[M-H]^-$	321.1707	321.1708	0.30	277.18091; 303.16013; 259.17023	Beta-zearalanol	$C_{18}H_{26}O_5$
42422-68-4	12.83	$[M-H]^-$	321.1708	321.1709	0.28	277.189097; 303.16031; 259.17044	Alpha-zearalenol	$C_{18}H_{24}O_5$	36455-72-8
16.03	$[M-H]^-$	319.15551	319.1552	0.35	275.16528; 160.01668; 130.04247	Beta-zearalenol	$C_{18}H_{24}O_5$	71030-11-0	13.36
$[M-H]^-$	319.1551	319.15353	0.48	275.16531; 160.01660; 130.04247; 301.14468	Zearalanone	$C_{18}H_{24}O_5$	5975-78-0	17.15	$[M-H]^-$

319.155	319.155	0.00	275.16534; 205.08704; 107.05029; 164.09731	Zearalenone	$C_{18}H_{22}O_5$	17924-92-4	17.26	$[M-H]^-$	317.14
317.14	0.01	131.05035; 175.04012; 112.98568; 273.14966	-						
Diuretics (12)	Acetazolamide	$C_4H_6N_4O_3S_2$	59-66-5	3.34	$[M-H]^-$	220.98	220.98	0.53	83.02512; 192.92908; 220.98090; 57.97578
Canrenone	$C_{22}H_{28}O_3$	976-71-6	17.52	$[M+H]^+$	341.2111	341.21108	−0.98	107.08548; 187.11177; 97.06478	Chlortalidone
$C_{14}H_{11}ClN_2O_4S$	77-36-1	5.89	$[M-H]^-$	337.01	337.01	0.19	146.02484; 189.97359; 79.98119; 318.99512	Furosemide	$C_{12}H_{11}ClN_2O_5S$

54-31-9	9.33	$[M-H]^-$	329.000	329.0000	0.45	204.98441; 285.01059; 77.96552; 126.01163	Spironolactone	$C_{22}H_{28}O_3$	52-01-7
17.22	$[M+H]^+$	341.2111	341.2108	−0.80	107.08548; 187.11177; 97.0678; 169.10120	Bendroflumethiazide	$C_{15}H_{14}F_3N_3O_4S_2$	73-48-3	11.77
$[M-H]^-$	420.0305	420.0304	−0.22	289.04532; 327.96802; 196.98270; 77.96552	Chlorothiazide	$C_7H_6ClN_3O_4S_2$	58-94-6	3.4	$[M-H]^-$
293.9417	293.9416	−0.31	213.96100; 228.83432; 112.98554	Hydrochlorothiazide	$C_7H_8ClN_3O_4S_2$	58-93-5	3.56	$[M-H]^-$	295.96
295.96	2.88	77.96552; 204.98441; 268.94629; 215.95763	Triamterene	$C_{12}H_{11}N_7$	396-01-0	4.08	$[M+H]^+$	254.1149	254.1146

−1.15	237.08849; 195.06674; 104.04945; 168.05598	4-Amino-6-chlorobenzene-1,3-disulfonamide	$C_6H_8ClN_3O_4S_2$	121-30-2	2.97	$[M-H]^-$	283.9572	283.9572	0.05
77.96553; 204.98445; 169.00778; 136.05168	Bumetanide	$C_{17}H_{20}N_2O_5S$	28395-03-1	16.71	$[M+H]^+$	365.1166	365.1162	−1.18	240.13828; 184.07570; 156.08081; 196.07573
Torasemide	$C_{16}H_{20}N_4O_3S$	56211-40-6	7.92	$[M+H]^+$	349.1329	349.1327	−0.51	264.08023; 183.09180; 290.05991; 168.06827	.
β-Receptor antagonists (3)	Acebutolol	$C_{18}H_{28}N_2O_4$	34381-68-5	4.51	$[M+H]^+$	337.2122	337.32119	−0.79	116.10677; 228.82957; 72.08070; 319.20129
Celipolol	$C_{20}H_{33}N_3O_4$	57470-78-7	5.26	$[M+H]^+$	380.3109	389.3104	−1.22	74.06003; 324.19189; 307.16522; 251.10277	Nebivolol

3. Results and Discussion

3.1. Optimization of Liquid Chromatography Conditions

Under the same mobile phase, 60 target compounds were investigated on ACQUITY UPLC® HSS C18 column (2.1 mm × 100 mm, 1.8 *μ*m), Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 *μ*m), Kinetex C18 column (2.1 mm × 100 mm, 2.6 *μ*m), and Hypersil GOLD™ VANQUISH column (2.1 mm × 100 mm, 1.9 *μ*m) separation effect and peak shape. The experiment found that Hypersil GOLD™ VANQUISH column can convert five groups of isomers: dexamethasone and betamethasone with parent ion (m/z) of 393.21, spironolactone and canrenone with parent ion (m/z) of 341.21, cortisone and prednisolone with a parent ion (m/z) of 361.20 and *α*-zearyl alcohol and *β*-zearyl alcohol with a parent ion (m/z) of 321.17, zearalenol, *β*-zearalenol, and zearalenone with a parent ion (m/z) of 319.155 were effectively separated. Therefore, Hypersil GOLD™ VANQUISH column (2.1 mm × 100 mm, 1.9 *μ*m) was selected as the chromatographic column for this method. In this study, the effects of acetonitrile-water, methanol-water, 0.1% acetonitrile-0.1% formic water, 0.1% methanol-0.1% formic water, and 50% methanol-acetonitrile-0.1% formic water as mobile phases were investigated. The results showed that when acetonitrile-water and methanol-water were mobile phases, the peak pattern of each component was poor and the positive ion response was low. The negative ion response is low when the mobile phase is 0.1% acetonitrile-0.1% formic acid water and 0.1% methanol-0.1% formic acid water. When 50% methanol-acetonitrile-0.1% formic acid water was used as mobile phase, the peak pattern of each component was improved obviously, and the response value was the best.

3.2. Optimization of Mass Spectrometry Parameters

Vanquish Flex + Orbitrap Exploris 480 dedicated low-flow ESI ion source for systematic calibration. At the same time, during the data acquisition process, Easy-IC was set to on in the process of data collection, and the Easy-IC source was activated to generate fluoranthene ion, which is injected into C-trap (2 ms) for internal standard correction of the system. After the correction is passed, the ion of the object to be measured will be introduced into the mass spectrometry system for detection and analysis. The Vanquish Flex + Orbitrap Exploris 480 uses an internal calibration source to calibrate the positive and negative ion modes in real time, ensuring accurate and stable mass numbers for the duration of the mass spectrum. Parameters such as Vaporizer Temp, Sweep Gas, CUR, and HCD Collision Energy were optimized using Vanquish Flex + Orbitrap Exploris 480. Different information on the secondary fragment ions of the target compound was found with different HCD Collision Energy values entered. It was found that the HCD Collision Energy input 30 V, 50 V, and 70 V in the Stepped Mode of Collision Energy Mode, and the obtained secondary spectrum is the fragment ion adduct generated by the collision of the target compound at three energies, and the fragment ion information is more abundant. In Full Scan-ddMS^2 mode, the information of primary parent ions and secondary fragment ions of the target compound was collected simultaneously, and ion chromatograms were extracted according to their molecular ion peaks $[M+H]^+$ or $[M-H]^-$ theoretical accurate mass number (Supplementary Figure 2). The relative deviations of the accurate mass numbers of the 60 food-borne stimulants are all less than 5.0×10^{-6}, as shown in Table 1, in line with the LC/MS regulations of the European Union [31], which can be used for qualitative and quantitative analysis of the target compounds.

3.3. Optimization of Extraction Solvents

In this experiment, the recoveries of 60 target compounds were investigated by ethyl acetate, methanol, and acetonitrile, respectively, as shown in Figure 1. The results showed that the average recoveries of 60 target compounds after ethyl acetate extraction ranged from 55% to 125%. In the process of nitrogen blowing or rotary evaporation, white lipids were gradually precipitated from the methanol extract. The average recovery of acetonitrile was between 70% and 110%, so acetonitrile was used as the extraction solvent in this experiment.

[figure(s) omitted; refer to PDF]

3.4. Optimization of Purification Methods

The matrix of animal-derived food is relatively complex, and it contains a large amount of protein, fat, and other substances, which will interfere with the accuracy of detection results. MCX solid-phase extraction column was usually used purification method of β-stimulants. However, the recovery rate of β-receptor antagonists is poor [32]. Therefore, HLB and PRiME HLB solid-phase extraction columns can also remove impurities in the sample and achieve purification effect, which were, respectively, used in this experiment to purify the extracted liquid, as shown in Figure 2. The results showed that the recovery rate of target compounds after purification by PRiME HLB solid-phase extraction column were higher than that of HLB. PRiME HLB can achieve rapid purification effect without activation, elution, and other tedious processes, which is suitable for the detection of food-borne stimulants in a large number of animal-derived foods.

[figure(s) omitted; refer to PDF]

3.5. Matrix Effects

In this study, the ratio of slopes of the standard curves drawn by matrix and solvent was used to evaluate the influence of matrix effect (ME, ME=k1/k2, k1 is the slope of the blank matrix standard curve, and k2 is the slope of the methanol standard curve) on the experimental results. When the ratio was in the range of 0.85~1.15, the influence of ME on the experimental results was considered to be negligible [33, 34]. In this experiment, beef was used as the matrix to investigate the matrix effect, and the results are shown in Table 2. 24 kinds of compounds showed no significant matrix effect, and the signal intensity of the other compounds was significantly inhibited or enhanced. In order to eliminate the influence of matrix effect, blank matrix extract was used to match the standard curve.

Table 2

Linearity, limits of detection (LOD), limits of quantitation (LOQ), and matrix effect (ME) of 60 food-borne stimulants.

No.	Compound	Internal standard compound	Linear equation	Linear range (ng/mL)	R2	LOD (μg/kg)	LOQ (μg/kg)	ME
1	Clenbuterol	Clenbuterol-D_9	$Y=0.0328466+0.10541X$	0.5–40	0.9976	0.1	0.2	0.85
2	Salbutamol	Salbutamol-D_3	$Y=0.00695222+0.0827112X$	0.5–40	0.9983	0.1	0.2	0.90
3	Ractopamine	Ractopamine-D_3	$Y=0.00962176+0.0887182X$	0.25–20	0.9997	0.05	0.1	0.93
4	Terbutaline	Salbutamol-D_3	$Y=0.0563537+0.062133X$	0.5–40	0.9972	0.1	0.2	0.92
5	Fenoterol	Clenbuterol-D_9	$Y=-0.00131536+0.045025X$	0.25–20	0.9979	0.05	0.1	0.82
6	Tulobuterol	Clenbuterol-D_9	$Y=0.0403942+0.142663X$	0.25–20	0.9992	0.05	0.1	0.75
7	Penbutolol	Clenbuterol-D_9	$Y=-0.0634425+0.296814X$	0.5–40	0.9980	0.1	0.2	0.78
8	Cimaterol	Clenbuterol-D_9	$Y=0.0233392+0.0250413X$	0.5–40	0.9981	0.1	0.2	0.81
9	Salmeterol	Clenbuterol-D_9	$Y=0.0254088+0.11891X$	0.25–20	0.9976	0.05	0.1	0.84
10	Atenolol	Salbutamol-D_3	$Y=-0.0294185+0.315163X$	0.5–40	0.9988	0.1	0.2	0.78
11	Metoprolol	Clenbuterol-D_9	$Y=0.0805915+0.247612X$	0.25–20	0.9980	0.05	0.1	0.89
12	Clenproperol	Ractopamine-D_3	$Y=-0.0188051+0.0436501$	0.5–40	0.9988	0.1	0.2	0.77
13	Demethyl coclaurine	Demethyl Coclaurine-D_4	$Y=-0.00387219+0.0814972X$	0.5–40	0.9995	0.1	0.2	0.88
14	Tretoquinol	Tretoquinol-D_9	$Y=-0.0679088+0.156311X$	0.5–40	0.9987	0.1	0.2	0.90

15	Propanolol	Clenbuterol-D_9	$Y = 0.056474 + 0.285512X$	0.25–20	0.9977	0.05	0.1	0.78
16	Meprednisone	Hydrocortisone-D_2	$Y = -0.0727716 + 0.1656X$	0.5–40	0.9989	0.1	0.2	0.74
17	Prednisolone	Hydrocortisone-D_2	$Y = -0.00815719 + 0.0527182X$	0.5–40	0.9992	0.1	0.2	0.86
18	Methylprednisolone	Hydrocortisone-D_2	$Y = -0.0237101 + 0.0520901X$	0.5–40	0.9992	0.1	0.2	0.68
19	Dexamethasone	Hydrocortisone-D_2	$Y = -0.0114097 + 0.0332118X$	0.5–40	0.9988	0.1	0.2	0.77
20	Betamethasone	Hydrocortisone-D_2	$Y = -0.02721 + 0.0441686X$	0.5–40	0.9987	0.1	0.2	0.80
21	Beclomethasone	Hydrocortisone-D_2	$Y = -0.0222696 + 0.0337372X$	0.5–40	0.9989	0.1	0.2	0.70
22	Fludrocortisone	Hydrocortisone-D_2	$Y = -0.0364505 + 0.180234X$	0.5–40	0.9988	0.1	0.2	0.63
23	Hydrocortisone	Hydrocortisone-D_2	$Y = -0.0287757 + 0.155345X$	0.5–40	0.9995	0.1	0.2	0.89
24	Cortisone	Hydrocortisone-D_2	$Y = -0.0318001 + 0.0787036X$	0.5–40	0.9991	0.1	0.2	0.88
25	Zilpaterol	Salbutamol-D_3	$Y = 0.0081897 + 0.0555902X$	0.5–40	0.9973	0.1	0.2	0.88
26	Stanozolol	Testosterone-D_3	$Y = 0.295443 + 0.652478X$	0.5–40	0.9975	0.1	0.2	0.78
27	Trenbolone	Testosterone-D_3	$Y = 0.0899871 + 0.331158X$	0.25–20	0.9974	0.05	0.1	0.65
28	Metandienone	Testosterone-D_3	$Y = 0.0148753 + 0.10149X$	0.5–40	0.9982	0.1	0.2	0.69
29	17-Methyltestosterone	Testosterone-D_3	$Y = 0.0434338 + 0.117486X$	0.5–40	0.9969	0.1	0.2	0.71

30	Testosterone	Testosterone-D_3	$Y = 0.0121521 + 0.10279X$	0.5–40	0.9985	0.1	0.2	0.93
31	Nandrolone	Testosterone-D_3	$Y = 0.015237 + 0.0979825X$	0.5–40	0.9986	0.1	0.2	0.58
32	Testosterone propionate	Testosterone-D_3	$Y = 0.0044069 + 0.0430173X$	0.5–40	0.9996	0.1	0.2	0.91
33	Nandrolone 17-propionate	Testosterone-D_3	$Y = 0.0195716 + 0.031933X$	0.5–40	0.9985	0.1	0.2	0.81
34	Boldenone	Testosterone-D_3	$Y = 0.0253621 + 0.0937769X$	0.5–40	0.9974	0.1	0.2	0.69
35	Nandrolone phenylpropionate	Testosterone-D_3	$Y = -0.0364081 + 0.0300981X$	0.25–20	0.9967	0.05	0.1	1.18
36	Dehydroepiandrosterone	Testosterone-D_3	$Y = 0.0121521 + 0.10279X$	0.5–40	0.9985	0.1	0.2	0.70
37	Zeranol	Zeranol-D_5	$Y = -0.00666451 + 0.10036X$	0.5–40	0.9988	0.1	0.2	0.75
38	Beta-zearalanol	Zeranol-D_5	$Y = -0.0100097 + 0.080692X$	0.5–40	0.9993	0.1	0.2	0.68
39	Alpha-zearalenol	Zeranol-D_5	$Y = 0.00132244 + 0.131453X$	0.5–40	0.9983	0.1	0.2	0.77
40	Beta-zearalenol	Zeranol-D_5	$Y = -0.00197718 + 0.099839X$	0.5–40	0.9984	0.1	0.2	0.80
41	Zearalanone	Zeranol-D_5	$Y = 0.019878 + 0.204666X$	0.5–40	0.9984	0.1	0.2	0.75
42	Zearalenone	Zeranol-D_5	$Y = -0.0401997 + 0.253658X$	0.5–40	0.9994	0.1	0.2	0.69
43	Acetazolamide	Furosemide-D_5	$Y = 0.00503859 + 0.0917645X$	2.5–200	0.9983	0.5	1	0.93
44	Canrenone	Canrenone-D_6	$Y = 0.0261506 + 0.165535X$	2.5–200	0.9992	0.5	1	0.86

45	Chlortalidone	Bendroflumethiazide-D_5	$Y=-0.00787723+0.0278848X$	2.5–200	0.9985	0.5	1	0.83
46	Furosemide	Furosemide-D_5	$Y=-0.000751913+0.0762856X$	2.5–200	0.9993	0.5	1	0.92
47	Spironolactone	Canrenone-D_6	$Y=0.00130985+0.0250254X$	2.5–200	0.9992	0.5	1	0.82
48	Bendroflumethiazide	Bendroflumethiazide-D_5	$Y=0.00187415+0.0851601X$	2.5–200	0.9981	0.5	1	0.90
49	Chlorothiazide	Bendroflumethiazide-D_5	$Y=0.0158104+0.101733X$	2.5–200	0.9996	0.5	1	1.10
50	Hydrochlorothiazide	Bendroflumethiazide-D_5	$Y=-0.00456868+0.0353866X$	2.5–200	0.9985	0.5	1	1.08
51	Triamterene	Canrenone-D_6	$Y=0.479403+0.829713X$	2.5–200	0.9973	0.5	1	0.61
52	4-Amino-6-chlorobenzene-1,3-disulfonamide	Furosemide-D_5	$Y=-0.00539682+0.109846X$	2.5–200	0.9989	0.5	1	0.65
53	Bumetanide	Canrenone-D_6	$Y=-0.0387555+0.0476122$	2.5–200	0.9987	0.5	1	0.70
54	Torasemide	Canrenone-D_6	$Y=-0.0583651+0.0986786X$	2.5–200	0.9988	0.5	1	0.78
55	Acebutolol	Acebutolol-D_7	$Y=-0.00480551+0.0829962X$	0.5–40	0.9999	0.1	0.2	0.93
56	Bromoclenbuterol	Clenbuterol-D_9	$Y=-0.0492107+0.0921308X$	0.5–40	0.9978	0.1	0.2	0.88
57	Celipolol	Celipolol-D_9	$Y=0.000804285+0.099369X$	0.5–40	1.0000	0.1	0.2	0.95
58	Formoterol	Clenbuterol-D_9	$Y=-0.127637+0.14402X$	0.5–40	0.9976	0.1	0.2	0.85
59	Brombuterol	Clenbuterol-D_9	$Y=-0.0463769+0.0683862X$	0.5–40	0.9966	0.1	0.2	0.80

60	Nebivolol	Nebivolol-D_4	$Y=-0.00563137+0.26977X$	0.5–40	0.9987	0.1	0.2	0.90

3.6. Linearity and Sensitivity

Accurately absorb 60 kinds of food-borne stimulants mixed standard intermediate solutions, and use blank matrix extract to prepare a series of standard working solutions. The ratio of the parent ion peak area of the compound to be measured and the concentration of the corresponding standard solution (ng/mL) was used as the vertical coordinate to draw the standard curve. The limits of detection (LOD) and quantitation (LOQ) of the method were determined according to the blank matrix addition method, with S/N = 3 as LOD and S/N = 10 as LOQ. As shown in Table 2, the 60 food-borne stimulants had a good linear relationship in the corresponding concentration, the correlation coefficient (R2) was more than 0.9966, the LOD was 0.05–0.5 μg/kg, and the LOQ was 0.1–1 μg/kg.

3.7. Recovery and Precision

60 kinds of food-borne stimulants standard substances were added to blank milk, egg, beef, mutton, and pork matrix for the spiked recovery experiment. Three concentration levels were added in total, and each concentration level was tested for 6 times. The results are shown in Table 3. The results showed that the average recoveries of milk, egg, beef, mutton, and pork ranged from 72.8% to 111.9%, 72.0% to 111.8%, 70.2% to 110.8%, 73.4% to 111.7%, and 75.4% to 111.0%, respectively, and the relative standard deviation (RSD) ranged from 0.05% to 8.97%, 0.05% to 8.88%, 0.05% to 8.94%, 0.05% to 8.88%, and 0.05% to 9.00%, respectively.

Table 3

Recoveries and relative standard deviation (RSD) of the 60 food-borne stimulants (n=6).

Compound	Spiked level (μg/kg)	Milk		Egg		Beef		Mutton		Pork	
Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Average recoveries (%)	RSD (%)	Clenbuterol	0.2
77.6	5.33	72.0	1.50	79.6	6.92	75.6	8.88	75.4	0.29	0.4	95.3
2.82	87.7	5.62	91.2	5.43	85.4	5.36	79.6	5.51	2	93.5	6.35
95.7	4.69	93.4	4.44	87.0	8.51	90.0	0.80	.			
Salbutamol	0.2	101.1	8.21	110.8	2.51	104.9	5.96	104.2	3.39	110.4	2.71
0.4	107.2	3.58	105.7	8.63	110.7	3.81	110.3	8.03	109.7	5.06	2

103.8	0.13	109.2	5.19	108.7	2.23	104.8	5.75	103.7	3.16		
Ractopamine	0.1	104.0	3.71	111.8	8.09	110.1	2.70	110.2	6.06	103.9	2.98
0.2	102.2	5.04	106.2	0.73	103.4	8.07	104.3	0.26	106.4	5.90	1
107.8	6.67	110.4	2.42	104.6	3.81	106.8	1.16	104.2	6.26		
Terbutaline	0.2	86.2	6.33	92.4	6.62	98.9	8.13	96.3	6.28	92.6	2.43
0.4	103.1	4.77	90.1	0.66	88.8	1.49	110.2	5.89	96.4	1.59	2
95.3	2.79	98.1	3.97	105.8	0.40	93.5	7.95	89.3	0.65		
Fenoterol	0.1	105.4	4.49	105.2	4.50	107.2	7.53	105.9	4.74	107.8	6.04
0.2	110.4	1.89	109.5	1.21	105.8	4.33	107.6	5.94	102.4	3.07	1
102.5	1.52	110.3	3.53	110.1	1.16	103.5	1.38	104.4	0.63		
Tulobuterol	0.1	107.0	7.00	101.1	4.77	101.4	1.30	107.6	1.34	108.7	1.96
0.2	102.5	3.16	110.3	1.15	107.5	1.48	107.5	8.47	109.4	5.14	1
103.8	2.31	106.9	4.29	103.3	5.30	106.9	3.26	107.9	6.54		
Penbutolol	0.2	93.7	8.43	111.7	7.65	107.4	6.02	90.7	1.24	95.3	1.72

0.4	106.2	4.56	108.3	4.26	99.6	5.16	88.1	1.89	82.2	2.67	2
105.0	0.27	86.0	6.81	102.9	8.93	95.8	5.62	103.4	1.67		
Cimaterol	0.2	88.0	6.45	91.6	4.08	88.1	5.16	95.1	8.64	87.9	2.40
0.4	92.8	4.05	83.0	7.13	85.0	5.30	80.2	6.07	81.2	8.70	1
98.3	6.14	85.2	8.23	83.6	4.48	94.4	1.67	86.5	8.88		
Salmeterol	0.1	108.2	7.33	95.4	5.46	106.7	8.36	97.7	1.22	101.5	2.07
0.2	108.8	2.99	95.5	1.21	89.2	3.06	102.7	7.14	86.7	1.15	1
107.0	1.48	107.8	5.66	84.0	3.37	107.3	2.64	89.6	8.97		
Atenolol	0.2	103.0	1.36	97.8	8.35	101.9	3.21	108.7	4.18	92.0	4.35
0.4	106.5	0.47	102.2	7.53	100.8	4.59	110.7	1.10	88.6	5.25	2
104.6	7.24	101.1	2.42	90.8	6.14	106.3	5.33	107.2	5.81		
Metoprolol	0.1	109.8	5.22	105.5	5.66	92.6	2.43	94.0	2.81	108.8	8.12
0.2	110.8	1.37	108.3	2.90	105.4	6.63	99.4	0.42	99.1	6.22	1
98.9	7.47	109.3	0.66	104.4	5.74	90.0	3.92	105.2	2.69		

Clenproperol	0.2	93.0	6.09	93.8	6.22	105.8	5.03	101.2	6.56	101.7	4.44
0.4	108.9	4.72	102.0	3.16	97.8	6.68	100.2	5.40	100.5	2.86	2
98.2	1.39	96.4	5.16	97.1	1.61	101.8	7.18	107.4	4.33		
Demethyl coclaurine	0.2	92.1	0.45	92.3	5.48	100.1	7.08	96.3	6.21	99.0	1.70
0.4	91.2	8.91	104.0	8.21	96.7	6.93	102.6	2.66	102.2	1.92	2
104.6	1.43	109.4	3.14	97.6	2.38	110.0	3.60	98.4	0.58		
Tretoquinol	0.2	101.6	1.00	94.3	3.65	108.8	0.05	99.3	4.67	106.8	6.69
0.4	108.0	2.40	109.7	2.13	93.9	8.87	96.0	1.81	110.4	6.84	2
97.9	4.80	97.6	7.35	100.3	5.48	94.9	2.22	104.9	6.92		
Propanolol	0.1	105.6	1.81	103.0	6.13	91.7	2.04	93.2	7.58	96.6	4.29
0.2	110.1	7.00	102.7	2.29	101.2	2.95	106.5	8.08	103.2	8.42	1
104.5	1.35	107.0	3.70	105.0	1.26	106.3	6.81	96.8	1.98		
Meprednisone	0.2	104.3	5.84	91.8	4.14	104.9	0.83	91.9	6.36	87.2	5.97
0.4	110.3	4.57	93.3	5.53	89.6	1.85	83.8	1.15	111.0	3.59	2
101.0	0.77	92.1	8.88	99.1	2.56	104.4	7.34	84.7	7.45		

Prednisolone	0.2	105.5	1.87	101.1	3.93	103.7	3.76	110.4	1.24	95.2	4.55
0.4	103.3	8.03	91.2	1.78	106.9	5.87	95.0	4.71	110.6	7.60	2
101.0	2.39	103.9	4.88	90.8	8.59	94.5	5.55	95.6	4.63		
Methylprednisolone	0.2	97.2	5.42	93.1	0.74	110.0	2.10	86.6	0.38	95.0	4.55
0.4	103.7	7.07	104.7	6.40	86.2	1.60	98.8	3.38	107.9	3.91	2
103.6	1.82	99.4	2.98	96.1	6.74	87.1	4.49	87.5	7.62		
Dexamethasone	0.2	84.6	7.14	105.5	4.87	101.8	4.01	107.2	2.03	84.7	1.76
0.4	92.7	8.77	110.9	7.19	102.6	8.40	85.1	4.53	101.0	1.57	2
92.8	3.73	105.6	2.94	93.4	0.61	103.0	8.01	97.9	8.49		
Betamethasone	0.2	96.9	7.40	94.2	6.27	105.8	7.07	88.0	1.34	103.4	5.07
0.4	91.8	8.38	91.2	8.12	105.8	7.18	101.4	2.92	102.4	6.77	2
102.1	2.29	104.8	6.55	87.3	6.51	111.7	8.58	105.5	1.21		
Beclomethasone	0.2	75.4	5.12	107.0	3.18	90.6	6.35	110.4	6.63	89.9	2.36
0.4	91.3	3.54	108.2	3.89	98.8	6.02	73.4	8.06	90.9	3.53	2
109.6	4.65	86.0	3.96	104.7	7.55	103.2	7.69	90.3	2.17		

Fludrocortisone	0.2	78.0	8.61	100.7	5.27	78.0	2.83	78.7	1.23	75.7	7.74
0.4	99.4	3.56	81.1	1.58	77.0	5.23	95.2	2.68	87.5	3.61	2
93.6	5.33	74.2	6.99	95.6	5.07	73.7	8.26	87.8	6.44		
Hydrocortisone	0.2	108.5	6.42	105.0	8.18	107.6	2.15	103.8	5.72	94.8	4.25
0.4	105.5	0.52	101.3	3.81	92.5	8.92	94.2	2.94	103.1	7.75	2
102.1	2.21	105.2	5.13	108.2	1.67	95.1	6.34	107.9	0.05		
Cortisone	0.2	103.0	7.37	93.5	8.24	93.3	6.06	100.3	5.33	103.0	7.72
0.4	104.3	6.13	94.7	2.96	85.4	4.51	100.4	4.94	96.5	6.08	2
110.5	2.64	104.4	7.28	83.5	2.82	95.7	7.40	108.6	1.43		
Zilpaterol	0.2	106.1	8.36	103.1	2.55	107.8	5.94	98.6	1.01	88.1	7.80
0.4	108.3	5.98	83.5	2.82	102.1	7.08	83.0	1.98	94.3	7.99	2
91.4	2.16	98.6	7.02	93.4	2.19	105.9	2.19	100.3	1.31		
Stanozolol	0.2	108.7	8.66	107.6	0.23	104.5	5.47	95.6	3.62	104.4	2.88
0.4	91.1	2.01	82.0	5.14	107.2	0.60	106.9	2.84	102.6	0.45	2
109.6	6.51	103.9	0.87	101.2	2.96	86.6	0.43	106.3	6.83		

Trenbolone	0.1	110.7	1.14	105.5	8.51	89.5	0.74	103.1	0.85	106.5	0.62
0.2	109.6	3.18	102.3	1.74	96.4	5.67	109.6	3.66	110.9	6.11	1
102.4	8.97	96.4	0.08	91.8	4.30	105.6	2.88	107.3	6.00		
Metandienone	0.2	102.5	7.04	108.9	2.76	103.5	2.43	106.3	0.91	106.8	5.76
0.4	106.5	0.05	100.9	0.50	106.8	6.68	108.2	5.82	105.9	3.07	2
110.7	7.37	99.1	7.69	105.8	8.46	103.7	3.88	105.4	1.53		
17-Methyltestosterone	0.2	110.5	1.06	99.7	4.98	110.6	0.62	110.0	1.04	106.3	6.01
0.4	108.9	2.01	110.9	3.76	109.6	4.07	96.9	0.05	110.6	7.46	2
102.7	5.03	104.9	5.43	89.8	3.29	101.8	6.36	93.3	7.74		
Testosterone	0.2	106.1	3.83	110.6	2.29	92.8	7.90	98.8	3.06	93.9	1.74
0.4	102.4	8.08	105.7	6.90	101.5	5.73	96.0	0.42	99.2	2.97	2
104.8	7.98	102.1	4.58	94.8	6.50	91.6	7.82	108.4	7.00		
Nandrolone	0.2	104.7	0.89	80.5	3.88	95.9	0.24	89.3	7.75	99.9	7.70
0.4	90.0	4.98	103.7	1.35	87.4	5.55	103.1	3.43	108.2	8.63	2

82.5	4.16	87.2	7.82	108.4	4.04	106.3	2.22	106.7	0.99		
Testosterone propionate	0.2	101.3	2.20	95.6	6.40	102.2	3.62	101.8	5.48	103.4	7.01
0.4	107.8	1.20	104.5	6.68	98.2	7.89	98.9	4.87	107.2	7.48	2
93.2	3.42	88.2	8.58	94.6	5.51	109.2	8.74	110.9	3.09		
Nandrolone 17-propionate	0.2	109.4	2.00	100.9	0.73	105.0	2.59	94.2	0.47	96.1	7.17
0.4	104.3	0.05	91.1	2.18	94.8	2.83	109.2	5.10	100.4	2.79	2
95.4	3.60	94.7	6.31	101.9	8.94	88.8	0.80	101.6	2.83		
Boldenone	0.2	109.2	2.30	101.7	5.88	101.1	6.25	92.8	3.17	108.3	6.42
0.4	110.2	6.11	95.6	4.61	92.2	5.66	85.0	2.10	88.4	1.11	2
104.0	1.94	89.2	0.64	104.2	7.65	101.2	7.78	107.8	7.10		
Nandrolone phenylpropionate	0.1	102.3	6.47	89.9	0.23	97.2	4.78	86.0	8.88	101.1	6.64
0.2	95.3	8.60	88.4	1.74	96.6	0.11	86.9	7.67	102.8	2.23	1
89.4	8.36	86.3	5.67	102.2	5.55	109.4	2.77	93.1	5.24		
Dehydroepiandrosterone	0.2	103.2	6.17	108.1	8.35	101.6	4.22	109.1	1.38	107.6	0.54

0.4	105.3	4.88	97.0	5.95	101.7	4.51	96.5	3.22	98.8	0.55	2
93.1	6.26	95.7	5.94	93.4	5.47	103.2	6.35	91.4	3.96		
Zeranol	0.2	108.1	1.73	104.7	7.09	94.3	5.42	104.3	7.45	81.8	0.93
0.4	92.8	7.24	100.9	0.74	103.3	1.28	95.5	7.34	88.2	7.13	2
81.5	2.82	86.4	1.15	100.0	3.83	100.8	7.63	94.9	7.50		
Beta-zearalanol	0.2	101.4	6.44	104.5	1.19	104.1	8.28	102.2	4.95	108.1	3.65
0.4	90.0	7.65	99.9	1.30	101.2	2.72	87.0	7.87	109.5	7.29	2
103.7	7.58	90.9	1.13	105.3	4.48	108.8	5.93	85.3	0.48		
Alpha-zearalenol	0.2	106.2	5.34	98.2	6.43	110.3	1.74	82.8	7.91	109.9	1.25
0.4	91.5	6.54	105.7	7.03	83.4	5.43	81.0	7.65	109.2	4.64	2
79.7	0.79	104.2	4.02	104.0	0.18	110.5	1.66	87.4	1.61		
Beta-zearalenol	0.2	106.8	5.92	93.5	2.68	82.6	1.32	100.6	4.57	107.8	2.84
0.4	98.5	6.81	107.2	5.50	79.0	3.42	95.5	2.51	105.4	6.64	2
80.3	7.27	104.0	3.89	80.7	1.92	80.5	5.42	88.4	5.08		

Zearalanone	0.2	93.5	7.48	95.2	5.23	109.3	2.73	80.6	6.87	86.3	0.17
0.4	91.2	0.24	89.6	6.12	93.1	4.82	89.9	8.43	84.0	5.42	2
82.1	8.56	94.3	8.43	95.3	8.56	78.9	3.68	89.0	5.99		
Zearalenone	0.2	93.2	2.53	109.9	2.94	105.8	6.35	73.7	3.58	90.2	8.86
0.4	86.1	0.82	87.0	0.12	97.6	4.99	82.6	5.11	100.1	0.13	2
72.8	6.24	100.8	2.90	70.2	4.26	104.7	3.23	103.9	1.12		
Acetazolamide	1	95.5	8.72	97.3	2.81	102.3	6.94	89.4	1.14	91.5	9.00
2	91.4	4.88	98.5	8.01	103.2	1.06	98.7	7.43	106.3	1.66	10
107.8	3.36	97.8	3.59	99.0	3.76	99.3	8.50	92.1	6.99		
Canrenone	1	110.2	5.07	90.0	4.73	110.0	6.32	102.8	0.81	100.8	5.82
2	102.9	6.08	107.9	8.77	108.8	4.10	88.3	6.79	109.6	7.06	10
103.1	6.03	106.2	2.54	88.6	8.89	101.0	1.25	97.8	3.09		
Chlortalidone	1	106.4	7.40	109.6	1.36	103.6	5.33	91.5	7.71	103.1	3.40
2	102.4	1.87	86.8	7.06	110.0	4.49	90.5	5.74	92.0	5.11	10
98.5	0.30	93.7	0.05	105.4	5.78	93.0	8.66	109.7	4.09		

Furosemide	1	110.3	7.63	101.0	1.30	91.9	7.21	109.4	1.70	98.9	8.98
2	105.2	2.19	96.7	0.94	94.2	0.31	100.8	6.27	98.4	2.03	10
97.4	8.51	98.2	1.96	98.8	8.26	90.5	7.19	103.8	2.38		
Spironolactone	1	101.4	0.53	97.4	7.79	101.0	3.73	87.3	2.77	99.7	2.12
2	103.0	1.54	99.9	2.42	86.1	4.18	109.8	6.90	106.0	4.93	10
107.3	2.14	102.3	0.84	96.4	1.29	109.3	3.64	105.1	1.22		
Bendroflumethiazide	1	102.9	7.71	102.9	0.20	101.8	3.28	97.0	5.11	110.7	3.25
2	106.4	3.01	101.2	4.28	93.9	1.34	105.2	6.64	105.6	8.23	10
102.2	5.28	101.2	6.59	108.2	2.48	99.0	0.83	86.7	6.24		
Chlorothiazide	1	94.3	3.36	101.9	5.44	104.0	3.30	103.4	8.52	94.6	6.37
2	104.7	7.05	92.2	7.80	107.7	0.16	105.9	8.62	92.5	1.33	10
105.2	2.74	90.9	6.39	92.3	8.63	94.1	0.46	105.2	6.02		
Hydrochlorothiazide	1	92.3	1.50	109.5	7.98	96.1	2.12	99.5	8.83	105.2	8.02
2	99.2	0.90	94.0	3.65	92.2	1.64	90.2	7.88	89.4	3.07	10
103.6	1.28	109.4	6.81	101.1	4.19	105.7	2.30	100.0	3.40		

Triamterene	1	95.3	1.57	104.6	2.66	98.5	8.31	93.8	6.35	93.3	3.68
2	102.4	6.99	93.0	4.50	105.8	3.98	102.1	1.65	90.4	3.25	10
104.8	6.94	97.5	5.44	104.0	5.99	93.1	8.04	104.0	7.59		
4-Amino-6-chlorobenzene-1,3-disulfonamide	1	95.4	1.41	110.7	8.06	106.9	2.00	107.1	5.87	107.0	2.38
2	95.4	5.29	101.9	4.42	89.0	0.70	103.3	0.57	91.7	2.31	10
103.5	0.73	108.8	2.07	94.2	4.31	110.6	6.07	92.3	4.79		
Bumetanide	1	103.9	7.44	106.3	7.91	86.4	6.61	86.5	2.06	87.1	8.34
2	99.6	5.62	101.4	3.45	97.5	0.16	95.9	0.58	86.9	1.46	10
91.5	5.21	87.8	2.44	106.0	6.07	95.7	0.06	110.7	7.18		
Torasemide	1	106.3	3.20	101.4	2.85	90.7	3.04	86.8	2.46	90.0	5.93
2	104.5	4.02	98.4	6.40	104.9	8.09	89.6	0.65	93.0	1.02	10
97.4	0.60	102.4	4.09	85.8	7.39	97.7	2.70	91.2	5.51		
Acebutolol	0.2	107.4	5.30	108.5	0.50	106.7	3.39	108.9	7.65	110.7	6.45
0.4	100.4	2.89	101.5	3.33	104.1	4.94	99.8	0.70	107.6	8.56	2
95.0	0.84	93.7	2.33	108.2	3.35	93.4	8.09	94.4	5.25		

Bromoclenbuterol	0.2	111.9	8.49	111.7	3.75	100.0	6.25	108.0	4.43	91.6	7.97
0.4	108.2	2.22	89.8	5.54	93.7	1.11	89.9	4.45	93.6	4.74	2
106.0	5.97	109.2	2.37	105.5	4.86	91.8	4.22	105.8	1.15		
Celipolol	0.2	95.2	8.77	108.0	1.25	101.7	3.45	104.7	6.12	93.0	1.61
0.4	93.8	7.99	106.2	2.83	110.4	4.05	103.9	3.72	105.1	5.62	2
96.6	2.74	92.9	5.54	108.4	6.34	98.1	7.26	106.0	3.99		
Formoterol	0.2	101.4	8.00	107.7	4.17	103.8	2.14	104.6	4.01	109.0	7.95
0.4	108.3	4.32	90.8	6.56	105.3	7.72	109.3	4.54	91.6	5.86	2
82.9	1.76	103.3	5.05	109.5	3.86	107.6	2.39	110.2	1.67		
Brombuterol	0.2	110.8	4.91	104.3	2.33	110.8	6.07	108.2	3.53	103.5	3.37
0.4	101.1	7.70	109.4	6.29	102.5	3.03	105.7	0.47	89.0	0.32	2
110.7	4.67	98.9	7.59	99.3	8.10	93.4	5.30	103.9	2.87		
Nebivolol	0.2	95.4	8.94	106.5	3.43	94.9	3.46	101.5	6.41	110.3	8.69
0.4	106.8	7.97	101.3	8.53	101.6	6.13	107.2	7.40	103.3	8.27	2

3.8. Sample Analysis

In order to investigate the applicability of the method, 30 milk samples, 30 egg samples, 50 beef samples, 50 mutton

samples, and 50 pork samples randomly selected in the market were tested. The results are shown in Table 4. In addition, we validated the quantitative analysis quality control samples of clenbuterol, salbutamol, and ractopamine in beef from the Chinese Academy of Inspection and Quarantine (certificate number: 303RG04081), and the results showed that the detection values of the three substances were all within the range of characteristic values, which proved that the detection method was effective.

Table 4

Detection results for beef sample, mutton sample, and pork sample (n=2).

Sample	Number	Compound	Number of positive samples	Content range (μg/kg)
Beef	50	Hydrocortisone	6	2.2~5.0
Cortisone	2	1.5~2.9	-	
Mutton	50	Hydrocortisone	3	2.4~3.9
Cortisone	1	0.6	-	
Pork	50	Hydrocortisone	8	3.4~10.5
Cortisone	6	0.6~3.8	Testosterone	3

4. Conclusions

In this study, the PRiME HLB solid-phase extraction column purification was combined with the UPLC-Q/Orbitrap HRMS to realize the rapid detection of 60 food-borne stimulants in animal-derived foods. The purification process of this method requires no steps such as activation, balancing, and leaching, which simplifies the operation procedure of pretreatment. Data collection was carried out in Full Scan-ddMS^2 mode, quantitative analysis was performed based on the ion peak area of first-order mass, and information such as retention time, secondary fragment ion information, and accurate mass was used to establish information including diuretics, protein anabolic agents, glucocorticoids, β2 adrenoceptor agonists, β-receptor antagonists, and other five types of 60 kinds of food-borne doping secondary spectrum library. Unknown samples can be screened and confirmed without reliance on standard substances. The method has the advantages of simple and rapid pretreatment, wide coverage of doping types, strong applicability, and high accuracy of results. It avoids the occurrence of accidental intake of food-borne doping by athletes, and maintains the fairness and justice of competitive sports.

Authors' Contributions

YZ and GH conceived and designed the experiments. LH, QL, JM, and MC performed the experiments. LF and XR analyzed the data; LH and QL wrote the original draft. All authors have read and approved the manuscript. Liangna He and Qiang Li have contributed equally to this work. Liangna He and Qiang Li share first authorship.

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Characterization of the Universal Flavor in Chinese Butter Hotpot by Multiple Mass Spectrometry Detection Technology

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ABSTRAK (ENGLISH)

Hotpot provides a multilevel unique flavor experience and is preferred by consumers who value various taste preferences. In the present study, results showed that alcohols, phenols, hydrocarbons, and others were the commonly predominant volatiles in all butter hotpot samples (74.43%~92.92%). However, there were merely 25 codetected compounds determined among a total of 318 aroma compounds because of the discrepant ingredients and processing technique of hotpot samples, which were sampled from several representative manufacturers. Therefore, for the first time, multiple GC-Q-TOF/MS and GC-Orbitrap-MS methods were performed to explore the

more 44 potential aroma compounds of butter hotpot, in which alpha-terpinyl, acetate, nonanal, piperitone, and (E)-lignstilde could further cause the differences in flavor intensity of spices aroma, smell of grease, and roasted, charred, and nutty ($p<0.05$). Linalool and nerol were the critical flavor precursors associated with ingredients and processing technic. Therefore, these results provide guidance for improving the butter hotpot formula and process technique.

TEKS LENGKAP

1. Introduction

Hotpot is a traditional Chinese cuisine, and its origin can be dated back two thousand years ago. Hotpot is a kind of popular way of having a meal which is accepted by most customers for its simplification and peculiarity. The market scale of hotpot was more than 4998 billion yuan (¥) in China, in 2021, which included the northern faction characterized by tingle, spicy, and burn and the north faction represented by mutton hot pot. Haidilao, Bajiangjun, Xiaofeiyang, and Xiaolongkan are the top four brands of the southern butter hotpot, and they are particularly well known for their balanced combination of spicy taste and mouth-watering aroma. The typical butter hotpot represents a highly complex matrix that mainly includes beef butter, dried chili, scallion, ginger, garlic, rock sugar, rice wine, Pixian Doubanjiang [1], and some spices, including peppercorn, anise, and cilantro, as ingredients [2, 3]. Although the potent aroma and taste of butter hotpot mainly rely on rich ingredients, it is believed that the manufacturing technique is also responsible for the flavor extraction and development process. The manufacturing device can set the frying temperature and time; thus, the mainstream technique of butter hotpot is a combination of stir-frying and extraction. Hotpot ingredients are put into the manufacturing device; wok stirs automatic bidirectional rotation at the designed high temperatures (120°C–150°C). Subsequently, after the frying is finished, the manufacturing device stops stirring and keeps the temperature (100°C–110°C) for extracting for a certain time. The characteristic flavors produced during the manufacturing process mainly come from the ingredients, such as oil, as well as the interactions among the fatty acid, sugar, and so on [4]. Therefore, besides the ratio and order of ingredients, the set of techniques is the other key point for flavor controlling of butter hotpot and further exhibits a significant influence on customer preference.

Consumer acceptability and market competitiveness are directly affected by the odor of food [5]. 2-Acetylthiazole, anethole, (E)-2-decenal, 1,8-cineole, (E, E)-2, 4-decadienal, nonanal, and so on significantly contributed to the aroma profile of Chinese butter hotpot seasoning and constituted the basic characteristics of the overall aroma based on the results of sensory-oriented flavor analysis and identified by aroma recombination and omission experiments [2, 6]. However, the other two research studies [3, 7] reported that linalool, (+)-limonene, 4-acetylpyrazine, toluene, and so on were recognized as the original aroma compounds of butter hotpot seasoning, although these samples were all from the same manufacturer. It can be inferred that the flavor profiles are significantly affected by its ingredients, formula, or even batch [8]. In addition, the dominant terpene and alcohol contents varied with manufacturers, among which *γ*-elemene, carene, *α*-piperene, and so on could be used as markers to distinguish the brands of butter hotpots [9]. Therefore, understanding the qualities of butter hotpot aroma at the molecular level is essential for the development of high-quality products. The identification of the complex flavor constituents in food remains a challenge of inconsistent detection methods. Among the various extraction methods, solid-phase microextraction (SPME) and solvent-assisted flavor evaporation (SAFE) show definitely different flavor profiles of hotpot [2, 7], as well as the detection techniques. The basic principle of GC-MS is that isolated compounds (through GC) are further ionized by electron impact, and the different mass-to-charge ratio molecular ions are detected by a detector. Meanwhile, the refreshing principle of GC-Q-TOF/MS is that the ionized ions are better separated by accelerators and flight tubes. The detection of the updated GC-Orbitrap-MS relies on its Orbitrap (an ion trap mass analyzer), ions oscillated around the center electrode, and two outer electrodes and are further isolated with different oscillation frequencies. GC-MS is the most used equipment in flavor science with high

qualitative ability, with the inability to distinguish many isomers (especially positional isomers). By comparison, GC-Q-TOF/MS and GC-Orbitrap-MS have the advantage of working with a high resolving power and mass accuracy and are suitable for the exploration of unknown flavor compounds. Both methods have been extensively used to reveal the flavor constituents of ham [10], wines [11], and so on. Therefore, the characterization of the flavor of hotpots is affected by some factors and detection methods; meanwhile, the uniform standards for butter hotpots' sensory evaluation and awareness of key aroma compounds are still unclear so far. Thus, it is necessary to characterize the universal flavor profiles of butter hotpots and the potential sources of the typical compounds.

For a more comprehensive understanding of the flavors of butter hotpots, we aimed to characterize the flavor compounds in eight groups of butter hotpots by GC-MS and to further screen the more aroma compounds based on GC-Q-TOF/MS and GC-Orbitrap-MS. The analytical strategy and sensory evaluation are basically intended to be used for revealing the universal flavor profiles when this issue has not been sufficiently addressed in the butter hotpot. In addition, it is devoted to tracing the sources of the dominant flavor compounds according to the typical flavors of ingredients. This study will provide a scientific basis for improving the intensification of the butter hotpot formulas and the control of process engineering.

2. Materials and Methods

2.1. Samples Preparation

Butter hotpot flavoring sample was purchased by several local supermarkets (Chengdu SNS Biotechnology Co., Ltd., Chengdu Shu-Da-Xia Catering Management Co., Ltd., Sichuan Haidilao Catering Co. Ltd., Chengdu Xiaolongkan Food Co. Ltd., Chengdu Yangming Food Co. Ltd., and Sichuan Tianwei Food Group Co. Ltd.) and those were popular products items in China. To avoid the uneven condition of the solid-liquid mixture system of samples, the research samples were composed of three parts: the prophase, the middle, and the anaphase stage fluids. The prophase, middle, and anaphase parts of the centrifuged sample are mostly liquid oil, solid-liquid mixtures, and solid ingredients, respectively, because of the gravity effect of ingredients. The prophase, middle, and anaphase parts of the hotpot sample were well mixed into an individual sample, and then the mixing sample was sampled thrice for further analysis. Subsequently, those samples were directly transported to laboratory at 4°C and kept at −20°C before being analyzed. There were a total of 21 samples (HG-01~HG-21) analyzed and grouped into eight groups (A, B, C, D, E, F, G, and H), which were sampled from different manufacturers or the different batches of identical manufacturers.

2.2. Analysis by SPME Arrow GC-MS

Volatile compounds (VCs) were extracted by SPME Arrow fiber (1.1 mm DVB/Carbon WR/PDMS, Stableflex, Supelco, Inc., USA) and separated using an Agilent 8890−5977 B GC-MS (gas chromatography-mass spectrometry) equipped with a VF-WAX-MS capillary column (30.0 m × 0.25 mm, 0.25 μm, Agilent, Santa Clara, USA). Methyl caprylate (CAS: 11-11-5, purity 99%, Sigma-Aldrich, St. Louis, MO, USA) dissolved in methyl alcohol (purity 99.9%, GC standard, liquid Aladdin, Shanghai, China) was prepared as internal standard (IS) (final concentration, 0.0073 g/100 mL). Before the SPME Arrow process, the IS (20 μL) was added into vials manually using a syringe. Samples were pre-equilibrated at 60°C for 15 min after activating the SPME Arrow fiber and then extracted for 45 min at the same temperature. Subsequently, the extraction was directly injected into the injection port of GC-MS and desorbed at 270°C for 5 min. The detection protocol was the same as that described by Yang et al. [1] with some modifications. The analytical conditions were as follows: the temperature of the column was maintained at 40°C for 5 min, ramped to 100°C at 4°C/min, and then increased to 230°C at 6°C/min and held for 10 min. The carrier gas was helium (>99.99%) at a constant flow rate of 1 mL/min, solvent vent mode. The ion source temperature was 250°C, and the scan range of MS was m/z 30–450. The peak identification was carried out by comparing their mass spectra with the NIST2020 library database on the basis of the following criterion similarity (SI) > 800 (the highest value is 1000). The identified compounds were further confirmed with the standard compounds available. Retention indices (RIs) were calculated with n-alkanes (C4–C30, Sigma, Aldrich Trading Co., Ltd., Shanghai, China) as the external references under identical experimental conditions.

2.3. Sensory Evaluation

According to the method described by Yu et al. [6] and Yang et al. [1], with some modifications, 8 panelist evaluators (4 males and 4 females, 20 to 40 ages) regularly engaged in the sensory analysis were selected from Chengdu SNS Biotechnology Co., Ltd., and Sichuan Academy of Agricultural Sciences. Among them, 6 panelists had the professional skills of previously participating in sensory evaluation tests for seasoning condiments, such as hotpot, soybean sauce, chili paste, and beef tallow with more than two years of experience. The other 2 panelists have a keen sense of smell. They were trained twice a week in a month according to the process described by Gao et al. [12] before formal sensory evaluation. The training included the basics of sensory analysis, identification of sensory properties, and the establishment and use of the scales.

One sample of each group (A~H) was selected for sensory evaluation. 20.0 g of each sample was served in a standard white disk covered, coded with a 3-digit random code, and eight samples were randomly selected for evaluation by the sensory panelists. Those solidified samples were digested in liquids before evaluation. Assessors were required to evaluate each sample with a short break at room temperature and standard white light. Sample evaluation was performed in duplicate. The panelists were asked to describe the samples in four aspects: flavor, taste, appearance, and coordination. To visualize the sensory outcomes, descriptors were converted to scores from one to nine, and the results were plotted in a spider web diagram.

2.4. Analysis by SPME Arrow GC-Q-TOF/MS

Deep analysis by GC-Q-TOF/MS (Gas Chromatography Time-of-Flight Mass Spectrometry) was carried out on a selected typical sample which covered most types and contents of the volatiles based on GC-MS analysis. Volatiles of HG-12 (Table S1) were extracted using the same SPME Arrow fiber, the process of which was according to the method described in 2.2. Then, the adsorbed volatiles were desorption at 260°C for 3 min and were analyzed by an Agilent 8890−7250 GC-Q-TOF/MS system equipped with a DB-Heavy WAX column (60 m × 0.25 mm × 0.50 μm, Agilent, Santa Clara, USA) according to the method described by Xu et al. [13]. The initial temperature of the GC oven was at 37°C for 3 min, then the temperature increased to 100°C at a 6°C/min rate, and finally increased to 260°C at a rate of 10°C/min. Helium (99.99% purity) was used as carrier gas and flowed at 1 mL/min, and the split ratio was 5 : 1. The ionization energy (EI) was EI-70 eV, and low-energy was 12 eV, the ionization temperature was 200°C, and the quantity scanning range was from 20 to 550 amu. Each compound was compared with the reference spectra (the NIST libraries), and the retention times for n-alkanes (C4–C30) under the same condition were used to check volatiles.

2.5. Analysis by SPME GC-Orbitrap-MS

Similarly, the same sample (HG-12) with a 2.3 was further determined by GC-Orbitrap-MS. A GC-Orbitrap-MS system (Q Exactive GC, Thermo Scientific, Bremen, Germany) consisting of a TriPlus RSH autosampler was used. The helium carrier gas (99.99% purity) flowed at 1.2 mL/min. SPME fiber (DVB/CAR/PDMS, Supelco, Inc., Bellefonte, PA, USA) was used to gather volatiles, as the same method as in 2.2. Then, the absorbing volatiles were desorbed at 270°C for 3 min, and the split ratio was 20 : 1. GC separation was performed on a 60 m × 0.25 mm × 0.25 μm TG-WAXMS column (Thermo Scientific) using the same temperature program of 2.2. EI was performed at 70 eV with the source temperature set at 250°C. Full scan MS acquisition was done in profile mode using an m/z range of 30–450. The actual resolution of MS was 60000. Similarly, the qualitative of volatiles was performed by the NIST database and RIs (C4–C30).

2.6. Statistical Analysis

GC-MS data were tabled in the form of average values ± standard deviation using Microsoft Excel 2021 (Microsoft Co., USA). The statistically significant differences among data were analyzed by SPSS 16.0 statistical software (IBM Inc., Chicago, IL, USA) using analysis of variance (ANOVA) at p=0.05 level. Principal components analysis (PCA) and partial squares discriminant analysis (PLS-DA) were performed using SIMCA-P (Umetrics, Sweden) to visualize the difference in volatiles between samples.

3. Results and Discussion

3.1. Identification of Volatiles in Butter Hotpot by SPME-Arrow-GC-MS

Samples were clustered into eight groups because of their manufacturers and categories. SPME-Arrow-GC-MS was

used to analyze volatile flavor compounds in samples at different manufacturers, as well as the different categories of identical manufacturers. The total contents ranged from 369.39 ± 38.96 mg/kg to 2739.32 ± 439.97 mg/kg, and a total of 570 volatiles were identified, including 48 alcohols, 85 esters, 19 phenols, 57 aldehydes, 67 ketones, 33 acids, 102 hydrocarbons, and 159 other compounds in terms of their chemical structure. Generally speaking, alcohols, phenols, hydrocarbons, and others were the predominant volatiles in all samples, with the total of those accounting for 74.43%~92.92%. From the stacked graph (Figure 1(a)), it can be found intuitively that the types of volatiles in samples have changed significantly as the different manufacturers and separate categories. For example, the B group (HG-01, HG-02, and HG-03) was clustered into a single cluster as the extremely low esters. In particular, the content of linalyl acetate in the B group was significantly lower than that in the other groups, in which precursors linalool and acetic acid had correspondingly low levels. Among them, linalool mostly originated from ingredients such as *Capsicum*, and *Prickly Ash* [14–16]. It was worth noting that the content of eucalyptol in the B group was significantly higher than that in the other groups, which originated from some individual plants and with strong antibacterial activity against food-borne pathogens [17]. Therefore, it can be inferred that the volatiles difference was mainly related to the specific ingredients. In the other cluster, the D group (HG-04 and HG-05) was classified into a single subcluster due to the higher alcohols, especially the linalool-endowed floral aroma. In addition, the E group (HG-06 and HG-07) showed the highest phenols, in which ethyl maltol was usually used as a fixative agent derived from exogenous addition. Significantly, the internal volatile composition of the C group was roughly the same because the samples were collected from identical manufacturer with discrepancies in categories, as well as the F, H, G, and A groups. The higher esters in F, H, G, and A groups owed to linalyl acetate, which with a floral and fruity aroma. Furthermore, the other class was highlighted in all samples as the predominant anethole and estragole originating from spice materials. PCA analysis was performed to reveal detailed information on the differences in volatiles between groups. As shown in Figure 1(b), the PCA bi-plot explained 87.96% of the total variance in the first two dimensions. Groups were clearly separated in the PCA plot, indicating that the butter hotpot flavor was significantly varied by manufacturers and categories. It was consistent with what Sun et al. [2] reported; hotpots collected from different companies showed a decentering relation in the statistical analysis loading plot.

[figure(s) omitted; refer to PDF]

3.2. Origin of Predominant Volatiles

To screen the potential aroma compounds varying with ingredients and their processing technique, 317 aroma compounds are visualized in Figure 2(a). 25 compounds were codetected in all groups, accounting for 49.71%–88.84% (Figure 2(b)), which indicated that the backbone of the butter hotpot flavor was certain and similar because that the ingredients formula was roughly same. In addition, there were 53–84 aroma compounds specifically identified in each group, presuming that the ingredients' proportions and their processing technic were different. In general, anethole, ethyl maltol, linalool, linalyl acetate, eucalyptol, eugenol, and estragole were predominant, which was consistent with the previous report [3]. Among them, anethole, eugenol, and estragole originated from aniseed (*Illicium verum*), clove (*Syringa oblata Lindl.*), and pepper (*Piper nigrum* L.), respectively (data not shown), the odor-active of which was verified by aroma recombination and omission tests [2]. Similarly, eucalyptol was relatively high, especially in the A, D, and G groups, indicating that the raw dry matter was more than in other groups. It was worth noting that (E)-2-decenal, (E)-2-octenal, (E, E)-2, 4-decadienal, (E)-2-nonenal, and nonanal were highlighted in the F and H group, which were associated with the quality of beef tallow [18]. For example, nonanal with a typical and strong fatty odor is regarded as the key odorant whatever the beef tallow origin (data not shown). In addition, ethyl maltol was a mixture of commercial preparations [19], which is entirely from the exogenous additive.

[figure(s) omitted; refer to PDF]

PLS-DA is a multivariate statistical analysis method with supervised pattern recognition. PLS-DA was performed to separate the sample groups based on the dominant aroma compounds (relative content >1%) after data normalization. As shown in Figure 3(a), the D group was separately located on the fourth quadrant with a higher content of linalool, D-limonene, octanoic acid, and cis-allocimene. The A and G groups were surrounded by most

aroma compounds, especially neral, maltol, 4-terpinenyl acetate, and so on. In addition, the aroma composition and intensity of the B, C, E, F, and H groups were roughly the same. The results of R2 (0.0347) and Q2 (−0.154) ensured that the model was not overfitting. Therefore, variable importance in projection (VIP) values were calculated from the PLSR model and used to recognize differential aroma compounds. Among these 49 aroma compounds, 11 had VIP values higher than 1, suggesting that they contribute significantly to the butter hotpot aroma profiles. As illustrated in Table S2, most differential compounds originated from ingredients; in particular, D-limonene, sabinene, beta-phellandrene, and beta-myrcene were the primary components of spiceries. In addition, ethanol was merely identified in four groups, which were associated with the ingredient of fermented glutinous rice [13]. In fact, besides the ingredients, the composition and content of aroma compounds definitively varied with manufacturing technique. Compounds mentioned in Figure 3(b) were detectable and aroma-contributing in samples. The synthesis of most compounds involved in multiple paths, in which linalool and nerol were the key intermediate components referred to many synthetic paths. In addition, besides the origin of materials, (E)-2-nonenal, maltol, and eugenol were the island models in the pathway; as is to say, there were unique precursors detected in samples. Interestingly, (E)-2-octenal, (E, E)-2, 4-decadienal, and (E)-2-decenal shared precursors and commonly endowed fat, meat, and green fragrant. Therefore, the overall aroma profiles were roughly the same caused by the technique process, with discrepancies in strength, although some precursors and their content originated from materials were not the same.

[figure(s) omitted; refer to PDF]

3.3. Sensory Evaluation

Sensory descriptive analysis was used to realize the special flavor and taste of butter hotpot. The detailed evaluation criteria were according to the sensory wheel and descriptors reported by Yu et al. [6]. Some descriptors were proposed by panelists (Table S3), and there were three dominant flavor attributes of hotpot, which were descriptors of the chili-like aroma, fatty/meaty aroma, and soy sauce-like aroma. Therefore, the quantitative descriptive analysis was conducted using the mentioned colour and lustre, chili-like, fatty and meaty aroma, soy sauce-like, spicy and numb taste, and salty and umami taste descriptors as a guide. As for the aroma profile (Figure 4), the sensory profiles of those samples were roughly the same, with discrepancy in intensity. Among them, the turbidity and pleasing colour showed a big difference ($p<0.01$) in various samples. This might be due to the ratio of crude fat, foods with a high starch content, pepper with a high water content, and so on. In addition, the highest sensory evaluation score of the chili-like aroma was recorded in the sample HG-04, with the relatively low soy sauce-like score. It was associated with the ratio of capsicum/capsicum oleoresin [20] rather than broad bean paste [6]. Similarly, HG-11 with the highest scores of fatty and meaty aromas varied with the quality of beef tallow [18]. It was clear that the umami taste of butter hotpot largely depended on monosodium glutamate, chicken bouillon, yeast extract, amino acids, and so on, and the salty taste is provided by salt. Therefore, the formula of samples was to a great extent the intensity of its salty and umami taste ($p<0.01$). By the way, the butter hotpot is noted for its spicy and numb taste; thus, the scores of those samples were roughly the same. Furthermore, as for the overall coordination scores, samples except HG-04, HG-11, and HG-19 have undifferentiated scores, indicating that these samples had greater flavor acceptance. More importantly, the overly projected flavor probably caused the unfriendly coordination.

[figure(s) omitted; refer to PDF]

3.4. Deeply Characterizing the Volatiles by GC-Q-TOF/MS and GC-Orbitrap-MS Overall Profiles Compared to GC-MS Results

To further reveal the flavor characteristics of butter hotpot, GC-Q-TOF/MS and GC-Orbitrap-MS were used to explore many more aroma compounds of sample HG-11 (Figure S1), thus avoiding the inaccuracy caused by low-resolution GC-MS. A total of 377 VCs were identified by the GC-Q-TOF/MS, of which 234 compounds possess aroma contribution (Table S4). Meanwhile, 138 aroma compounds were detected by GC-Orbitrap-MS among the total 277 VCs (Table S4). Among the dominant aroma compounds (relative content >1%), eucalyptol, linalool, (E)-cinnamaldehyde, benzeneacetaldehyde, (E, E)-2, 4-decadienal, (E)-2-decenal, sabinene, anethole, eugenol, ethyl maltol, acetic acid, linalyl acetate, and estragole were simultaneously characterized by those three methods, those

were roughly present in the core flavor of butter hotpot [3]. Compared to the GC-MS results, the coverage rate of the codetected compounds was 68.38% (GC-Q-TOF/MS) and 38.41% (GC-Orbitrap-MS), respectively. In compounds, 7 esters, 6 aldehydes, 5 alcohols, 5 terpenes, 3 phenols, 3 acids, 3 ketones, and 12 others were merely detectable by GC-Q-TOF/MS and GC-Orbitrap-MS. The flavor characteristics of the differential esters were floral, fruity, herbaceous, and fresh green, especially alpha-terpinyl acetate. Similarly, nonanal and hexanal endow the smell of grease and meaty aroma, thus further resulting in greater flavor intensity. It is worth noting that trans-beta-ocimene, trans-isoeugenol, piperitone, feniculin, (E)-ligustilide, cinnamyl alcohol, methyl 4-methoxybenzoate, and xanthoxylin originated from spice (data not shown). 2,3,5-Trimethylpyrazine and tetramethylpyrazine possess roasted and cocoa aromas, which were also the typical aromas of doenjang [21], baijiu [22], and so on. In addition, copaene was the isomeride of (S)-(−)-β-pinene, which blends with pine fresh, as well as α-terpinolene.

The 311 aroma compounds were classified into five sensory descriptors in terms of their flavor characters. As shown in Figure 5(a), spice, grease, floral, and fruity aroma were the primary aroma characteristics. The flavor profiles were roughly the same, with discrepancy in concentration, which may be associated with compound diversity. In this study, spearman correlation analysis was carried out to further reveal the correlation between the sensory descriptors and the 24 flavor compounds (relative content >1%) which were determined and classified by those three methods (Figure 5(b)). As a whole, 21 pairs of positively and extremely significant correlations were identified, while 16 negative pairs ($p<0.01$, R spearman >0.6). It is in accordance with the interactions between systematic flavor compounds [23]. Results showed that anethole, sabinene, and so on contribute to the spice aroma which is the dominant flavor characteristic of butter hotpot. The smell of grease feature is related to eucalyptol, linalool, and so on.

[figure(s) omitted; refer to PDF]

3.5. Differential Flavor Compounds Determined by GC-Q-TOF/MS and GC-Orbitrap-MS Results

According to the flavor compounds determined by GC-MS, alcohols, phenols, hydrocarbons, and others were the predominant classifications of butter hotpot. Therefore, GC-Q-TOF/MS and GC-Orbitrap-MS methods were performed to identify the more flavor compounds that cannot be determine by GC-MS. As shown in Figure 6, phenylethyl alcohol possesses a floral aroma, which may derive from the material broad bean sauce [1]. The level of trans-isoeugenol is usually used to elucidate the effects of the barriers' maturity and the geographical origin of peppers [24]. Interestingly, the isomeride of copaene and trans-beta-ocimene could be detected by GC-MS, such as beta-pinene and (E, Z)-2,6-dimethylocta-2, 4, 6-triene; it may relate to the high resolution of both detection methods. Similarly, the same flavor characters (smell of grease) of nonanal can be endowed by (E, E)-2, 4-decadienal, (E)-2-octenal, and so on, all of them can originate from beef tallow [25]. Ethyl acetate was the esterification product of acetic acid and ethanol; it shows the same fruity aroma as 3-methylbutanal.

[figure(s) omitted; refer to PDF]

There were 117 aroma compounds merely identified by GC-Q-TOF/MS in comparison to the GC-MS and GC-Orbitrap-MS results, accounting for 11.35% of total VCs. Caryophyllene, gamma-terpineol, (R-(R*, R*))-2,3-butanediol, and (+)-α-terpineol with the high level. Among them, caryophyllene can originate from most spices (data not shown), with a note of spices, woody, and tangerines. The isomer of gamma-terpineol is (+)-α-terpineol, they showed high contributions due to their low threshold, the same is true of (R-(R*, R*))-2,3-butanediol and (S-(R*, R*))-2,3-butanediol. In addition, the aroma cluster of GC-Q-TOF/MS was highlighted in floral and fruity aroma, which was partly related to 19 compounds (gamma-terpineol, 2,3-butanediol, eugenol acetate, etc.). Thereinto, eugenol acetate, citronellyl formate, geranyl formate, and fenchyl acetate were synthesized by esterification reaction during the production process. Similarly, the high intensity of roasted, charred, and nutty was partially associated with 7 compounds (furfural, pentadecanal, 3-nonen-2-one, etc.). Furthermore, some sulfocompounds have been merely detected by GC-Q-TOF/MS, which included methanethiol, dipropyl disulfide, (E)-1-(prop-1-en-1-yl)-2-propyldisulfane, propyl mercaptan, methyl disulfide, (Z)-1-(prop-1-en-1-yl)-2-propyldisulfane, dimethyl sulfide, and (Z)-1-methyl-2-(prop-1-en-1-yl)disulfane; most of them probably derived from onion and ginger [26]. Compared to GC-MS and GC-Q-TOF/MS results, 39 differential aroma compounds were identified by GC-Orbitrap-MS,

accounting for 4.06% of total VCs, indicating that most compounds can be covered by the other two methods. Spice aroma was dominant in flavor profiles of GC-Orbitrap-MS, associating to L-fenchonem, (Z)-anethole, (R)-cuparene, and spathulenol in part. L-Ethyl lactate and 1-octen-3-ol have a certain contribution to the cool and pungent aroma descriptor those were the major odor-active compounds of baijiu [27] and soybean products [28], respectively.

4. Conclusion

In summary, the universal flavor characteristics of butter hotpots were revealed by multiple mass spectroscope techniques. A wide range of volatile contents (369.39 ± 38.96 mg/kg~2739.32 ± 439.97 mg/kg) was detected in samples because of the different brands. Among the 25 co-detected compounds, 11 differential compounds (VIP > 1) were calculated by PLS-DA, those derived from ingredients. Subsequently, GC-Q-TOF-MS and GC-Orbitrap-MS methods were used to identify the more potential aroma compounds compared to GC-MS. Results showed that phenylethyl alcohol, trans-isoeugenol, copaene, nonanal, and so on, were the more aroma compounds identified. There were 117 compounds only detected by GC-Q-TOF/MS, accounting for 11.35% of all volatiles. The 117 compounds which accounted for 11.35% of all volatiles were only detected by GC-Q-TOF-MS, especially 37 sulfocompounds. In particular, the relative content of caryophyllene and gamma-terpineol was relatively high. Similarly, GC-Orbitrap-MS further confirmed that L-fenchonem, L-ethyl lactate, 1-octen-3-ol, and so on were the differential compounds for the overall aroma of butter hotpot. Overall, those compounds have a certain effect on the flavor profiles of spices, grease, and floral and fruity aromas. This study will provide a theoretical basis for improving the quality of butter hotpots and flavor control in the hotpot industry.

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DETAIL

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Evaluating Benefits and Risks of Polyunsaturated Fatty Acids and Methyl Mercury from Fish and Seafood Consumption in Peninsular Malaysia

Ahmad, Nurul Izzah; Noraishah Mohammad Sham.

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ABSTRAK (ENGLISH)

The risks and benefits associated with methyl mercury (meHg) and polyunsaturated fatty acid (PUFA) from seafood consumption were assessed in adults and adolescents from Peninsular Malaysia. Seafood samples were collected for meHg analysis while the consumption survey was conducted among adults and pupils ≥10 years old. Long-chain omega-3 fatty acids (LC ω-3 PUFA; DHA and EPA) data were obtained from locally published articles. The estimated weekly intake (EWI), provisional tolerable weekly intakes (PTWIs), hazard quotient (HQ), and maximum safe weekly consumption (MSWC) were calculated for each seafood studied. The average range of LC ω-3 PUFA concentration was between 11.7 and 2,210.5 mg/100 g, where 27% of samples contained >500 mg/100 g and were predominant in pelagic fish and mollusks. MeHg concentrations in seafood samples ranged from 0.0426 to 0.4576 mg/kg of wet weight (WW) and showed significant variations between all species at a median concentration of 0.0621 ± 0.0573 mg/kg WW. Total seafood consumed by the adolescents was 84.7 ± 103.7 g/day, with significant marginal differences compared to the adult population at 90.5 ± 100 g/day. Long-tail tuna, yellow-stripe shad, slender shad, and long-tail shad contributed to a higher LC ω-3 PUFA intake than other species. These fish also contributed to a low HQ value level, lowering the risk of health effects. Mangrove red snapper has a low LC ω-3 PUFA content, but the HQ value was the highest of all, and it is advised to consume less frequently. Double the intake of cephalopod and a threefold increase in crustacean consumption would still minimize the meHg risk and may increase the intake of LC ω-3 PUFA.

TEKS LENGKAP

1. Introduction

Malaysians eat a lot of fish and rank fifth globally or second in Asia in yearly per capita fish intake [1]. They consumed fish at least once daily in the amounts of one and a half medium fish per day [2]. A recent study showed that they consumed 168 g/day of fish, with Malay ethnicities consuming fish significantly more than other ethnicities, the Chinese, Indians, and others [3]. Fish is recognized to have high-quality protein, making up 17% of all animal protein, 7% of which is consumed globally [4]. Eating fish and seafood has several health advantages: good energy sources, proteins, and essential vitamins and minerals [5, 6]. Fish also contains beneficial fatty acid composition, such as the long-chain n-3 polyunsaturated fatty acids (n−3 PUFA), eicosapentaenoic acid (EPA; C20: 5n−3), and docosahexaenoic acid (DHA; C22: 6n−3) [4, 6–8]. Protecting many ailments, including coronary heart disease, inflammation, the hypotriglyceridemic impact, allergies, hypertension, arthritis, autoimmune disorders, and cancer, is one of the well-established positive health effects of n−3 PUFAs. According to reports, atherosclerosis and thrombosis may also be avoided by eating a diet rich in marine n-3 fatty acids. Studies with newborns indicate that DHA is essential for the normal functional development of the retina and brain, specifically in premature infants [7–10].

Marine organisms can also be a potential source of human exposure to pollutants, for example, meHg, and the extent to human meHg exposure depends on the species of fish, their frequency, and the amount of consumption [11–13]. MeHg is a potent toxin that primarily affects the brain and other parts of the central nervous system [14]. It is highly mobile in the human body, and because it may traverse the placental and blood-brain barriers, it can harm unborn children and cause damage to developing children. It is known that high transmission of meHg to the developing fetus and child causes severe developmental impairment, brain damage with mental retardation, cerebral palsy, blindness, etc. [14–16]. The journey of meHg into the human body is explained through the formation of water-soluble methyl mercury complexes in body tissues, and the main route of its elimination from the body is through feces, which is as much as 90% of total excretion [14]. Adults with meHg poisoning typically have paraesthesia or numbness in the hands and feet, coordination problems, concentric constriction of the visual field, auditory complaints, ischemic stroke, dementia, depression, and gastrointestinal dysfunction [14, 17].

This study aims to evaluate the health benefits and risks analysis of fish and seafood consumed by the Malaysian population. It observed the intake of EPA, DHA (benefits), and meHg pollutants (risk) through consuming various edible fish and seafood captured by Malaysian fishery industries. This study notifies levels of meHg concentration in

fish and seafood obtained from fish landing ports and wholesale wet markets across Peninsular Malaysia. It also examined consumption patterns of fish and seafood among adolescents and adults. A potential alarm for human health hazards was evaluated by calculating the EWI and HQ, and finally, the MSWC was identified for each fish and seafood species studied. The intake of LC ω-3 PUFA per day by these 2 population groups was also calculated.

2. Methodology

2.1. Fish and Seafood Consumption Survey

Detail on procedures for fish and consumption survey among adults in Peninsular Malaysia was reported by Ahmad et al. [3], while those for adolescents were reported later in the year 2019 [18]. Both groups' demographic backgrounds were presented in published articles accordingly [3, 11, 18]. The Ministry of Health Malaysia funded the study, and the Medical Research and Ethics Committee from the same ministry approved the ethical issues. Informed consent was obtained from the subjects earlier, a household-based, cross-sectional study was conducted, and data were collected through face-to-face interviews using predesigned questionnaires. The Department of Statistics, Malaysia, consulted the sampling frame and household addresses.

To begin the survey, a total of 2,996 individuals were selected who met the criteria of living in families with at least 2 adults and adolescents aged 10 to 17 years old. A total of 2,675 adults (89.2%) completed the survey, whereas only 484 adolescents (54.4%) did. The study tool was a prior validated questionnaire that consisted of two parts. The first part was a self-administered questionnaire consisting of a section of socio-demographic information, patterns, and frequency of fish consumption, knowledge, perception, and practices towards fish consumption. The second part was a three-day record of 24-hour dietary diary forms. Research assistants assisted with the survey and were equipped with tools such as dish pictures, fish, and standard household measures. The questionnaire was given at 9.00 am or until late at night if necessary. Parents were requested to assist their children in answering questionnaires and filling out the dietary forms [3, 11, 18]. The portion weight of food was referred to in the local food atlas by Suzana et al. [19, 20] and the composition of Malaysian foods by Tee et al. [21]. Five different nonlisted food sources were obtained, and mean weights were calculated. The polls were taken on weekdays and weekends.

2.2. Fish and Seafood Collection and Preparation for Total Mercury (tHg) Analysis

Details on fish collection and contaminant analysis methods are reported by Ahmad and coworkers (2015[ab]). Sampling was conducted from June to December 2009; samples were obtained from 6 principal Fisheries Development Authority of Malaysia (LKIM) fish landing complexes and 5 wholesale markets across Peninsular Malaysia. Samples were collected from three fishing boats landed at the complexes and three randomly selected business units at the wholesale market. The distribution of respondents with fish and seafood sampling survey locations is presented in Figure 1. One kg of sample was purchased for each species selected, with finally a total of 394 seafood collected. The total length and weight of samples were measured in millimeters and grams, respectively. Seafood samples were packed, labeled, and put into an icebox before being transported to the laboratory and stored at −21°C. Samples were thawed at room temperature before processing, filleted, chopped, and homogenized. Finally, samples were dried at 65°C to constant dry weight and ground using mortar.

[figure(s) omitted; refer to PDF]

2.3. tHg Concentrations Determination in Fish and Seafood

The sample was mixed with concentrated nitric acid and hydrogen peroxide in a polytetrafluoroethylene digestion vessel, sealed, and placed into the microwave digester (Multiwave 3000, Anton Paar) rotor before digestion. Total mercury was analyzed by the cold vapor atomic absorption spectrometry technique (Flow Injection Mercury System (FIMS-400), Perkin Elmer) following the method by Mohd Fairulnizal et al. [23]. LOD was identified from mercury concentration tallying 3 times the SD of 10 reagent blanks, 0.72 μg/L. The average recovery of reference standards (NIST SRM® 1946, Lake Superior Fish Tissue) was 91%, and RSD was <5%. Total mercury concentration levels were converted into wet basis values using the formulation: dry weight concentration = wet weight concentration × ((100−moisture content)/100). Moisture content was referred to by Tee et al. [21] and Nurnadia et al. [24]; and details on the analysis were published elsewhere [25, 26].

2.4. DHA and EPA Data in Fish and Seafood

The DHA and EPA concentrations were sourced from Nurnadia et al. [27] and Wan Rosli et al. [28], where fish and seafood were captured from the east and west coasts of Peninsular Malaysia. The re-calculated average levels for both the DHA and EPA in fish and seafood were ordered into mg/100g samples, as shown in Table 1. The population intake of DHA and EPA was calculated and compared to the global authority target recommendation per day for healthy adults (250 mg DHA and EPA per day) and reduce coronary heart disease (CHD) risk in healthy adults (minimum of 500 mg DHA and EPA per day) [29].

Table 1

Total mercury (tHg) and methylmercury (meHg) levels in fish and seafood from Peninsular Malaysia.

Species no.	Groups/family/species	Common name	*n*	tHg ± IQR (mg/kg) DW	MC (%)	tHg (mg/kg) WW	*#meHg (mg/kg) WW
♦*Pelagic fish*							
1	*Selaroides leptolepis*	Yellow-stripe scad	13	0.252 ± 0.125	79.5	0.0635	0.0591
2	*Decapterus russelli*	Slender scad	10	0.195 ± 0.108	74.7	0.0458	0.0426
3	*Megalaspis cordyla*	Torpedo scad	20	0.319 ± 0.198	74.8	0.0750	0.0697
4	*Parastromateus niger*	Black pomfret	15	0.242 ± 0.121	76.5	0.0569	0.0529
5	*Rastrelliger kanagurta*	Indian mackerel	13	0.180 ± 0.066	73.1	0.0522	0.0485
6	*Scomberomorus guttatus*	Indo-Pacific king mackerel	12	0.262 ± 0.355	75.9	0.0650	0.0604
7	*Thunnus tonggol*	Long-tail tuna	8	0.358 ± 0.173	71.0	0.3580	0.3329
8	*Euthymus affinis*	Kawakawa	3	0.289	75.2	0.0699	0.0650
						Median	**0.0597**
–							
♦*Demersal fish*							
9	*Lutjanus argentimaculatus*	Mangrove red snapper	3	0.856	75.8	0.1875	0.1743
10	*Lutjanus russellii*	John’s snapper	4	0.884 ± 1.789	80.2	0.1848	0.1718

11	*Lates calcarifer*	Giant-sea perch	11	0.537 ± 0.436	78.1	0.1240	0.1154
12	*Gymnura poecilura*	Long-tail butterfly ray	25	0.492 ± 1.251	79.1	0.4920	0.4576
13	*Nemipterus japonicus*	Japanese threadfin bream	11	0.464 ± 0.724	76.9	0.1063	0.0988
						Median	**0.1718**
–							
Freshwater fish							
14	*Clarias batrachus*	Walking catfish	9	0.334 ± 0.325	77.1	0.0621	0.0578
–							
Cephalopods							
15	*Sepia officinalis*	Common cuttlefish	8	0.280 ± 0.101	81.4	0.0959	0.0777
–							
Crustaceans							
16	*Metapenaeus affinis*	Rainbow shrimp	4	0.280 ± 0.501	80.5	0.2800	0.1400

Total Hg in median ± IQR; DW: dry weight; IQR: interquartile range; MC: moisture content; MC content was based on the works by Tee et al. [21] and Nurnadia et al. [24]. WW: wet weight; conversion of DW mercury concentrations in fish samples to WW was by means formula: DW = WW × (100/100 MC). [#]Details on total mercury concentrations in seafood are referred to Ahmad et al. [25, 26]; ⋆calculation of meHg concentrations was based on the mean percentage of methyl mercury to total mercury at 93% for fish, 81% for cephalopods, and 50% for crustaceans [11, 30]. [#]Comparison of meHg levels for different fish/seafood species: χ2MW = 71.385, p value ≤0.001, *N* = 172 and median meHg for all seafood = 0.0621 ± 0.0573 mg/kg WW. ♦Comparison of meHg levels for different groups of pelagic and demersal: χ2MW = 1145.000, p value ≤0.001, *N* = 150 (pelagic; *n* = 96; demersal, *n* = 54). Median meHg for pelagic fish = 0.0513 ± 0.0392 mg/kg WW and median meHg for demersal fish = 0.0988 ± 0.1332 mg/kg WW.

2.5. Methyl Mercury (meHg) Data in Fish and Seafood

The calculation for meHg per species was using the percentages of tHg at 93%, 81%, and 50% for fish, cephalopods, and crustaceans, respectively [30] (Table 2). The FAO/WHO (2006) and the Malaysian Food Regulation [31] (Food Act 1983) compared results to guideline levels.

Table 2

Concentration of DHA and EPA from selected fish samples (mg/100 g fish samples) collected from the east and west coast of Peninsular Malaysia.

No.	Species	Common name	DHA (mg/100g)	EPA (mg/100g)	*DHA+EPA (mg/100g)
Pelagic fish					
1	*Selaroides leptolepis*	Yellow-stripe scad	870.28	231.45	1,101.73
2	*Decapterus russelli*	Slender scad	1,162.50	353.80	1,516.30
3	*Megalaspis cordyla*	Torpedo scad	827.60	182.25	1,009.85
4	*Parastromateus niger*	Black pomfret	642.95	159.10	802.05
5	*Trachinotus blochii*	Snub nose pompano	122.60	176.70	299.30
6	*Pampus argenteus*	Silver pomfret	492.70	207.60	700.30
7	*Rastrelliger kanagurta*	Indian mackerel	348.95	115.50	464.45
8	*Scomberomorus guttatus*	Indo-Pacific king mackerel	624.95	182.55	807.50
9	*Thunnus tonggol*	Long-tail tuna	760.20	246.30	1,006.50
10	*Euthymus affinis*	Kawakawa	886.40	228.30	1114.70
		Median	701.58	195.08	907.00
–					
Demersal fish					
11	*Lutjanus argentimaculatus*	Mangrove red snapper	209.90	24.10	234.00
12	*Lutjanus johnii*	Golden snapper	18.60	7.30	25.90
13	*Lates calcarifer*	Giant-sea perch	594.85	346.65	941.50
14	*Gymnura poecilura*	Long-tailed butterfly rays	9.00	2.70	11.70
15	*Nemipterus japonicus*	Japanese threadfin bream	723.75	292.10	1,015.85

16	*Polynemus indicus*	Indian threadfin	82.20	23.70	105.90
17	*Eleutheronema tetradactylum*	Four-finger threadfin	53.10	96.20	149.30
18	*Epinephelus fasciatus*	Red-banded grouper	924.90	216.10	1,141.00
19	*Chirocentrus dorab*	Wolf herring	54.30	23.90	78.20
20	*Plotosus canius*	Gray eel-catfish	88.80	145.80	234.60
21	*Cynoglossus arel*	Large-scale tongue sole	113.40	8.30	121.70
22	*Clupea fimbriata*	Fringe scale sardine	225.40	211.50	436.90
23	*Hilsa macrura*	Long-tail shad	168.70	2,041.80	2,210.50
		Median	113.40	96.20	234.00
–					
Freshwater fish					
24	*Clarias batrachus*	Walking catfish	ND	14.50	14.50
–					
Cephalopod/crustacean/bivalve					
25	*Sepia officinalis*	Common cuttlefish	472.85	159.05	631.90
26	*Metapenaeus affinis*	Greasy-back pink prawn	391.25	284.25	675.50
27	*Liocarcinus vernalis*	Shallow-water crab	749.70	517.10	1,266.80
28	*Arca granosa*	Blood cockle	156.80	298.00	454.80
		Median	432.05	291.13	653.70

DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid. ⁎Calculated by Nurnadia et al. [27] and Wan Rosli et al. [28].

2.6. Health Risk Assessment of Mercury from Fish and Seafood Consumption

2.6.1. Estimated Weekly Intake (EWI) and Maximum Safe Weekly Consumption (MSWC)

The EWI of total Hg and/or meHg was calculated using the following equation:(1)EWI=concofmeHgmg/kgWW×

weekly consumption WCg body weight BWkg.

The average body weights of 60 and 45 kg were used for adults and adolescents, respectively [3, 18]. The PTWI used for inorganic meHg is 1.6 μg/kg body weight/week [32].

The calculation for estimating the MSWC at PTWI 1.6 μg/kg body weight/week is as follows:(2)PTWI=conc of meHg mg/kgWW×MSWCgBWkg,MSWCg=1.6 µg/kg body weight/week×BWkgconc of meHgmg/kgWW.

2.6.2. Hazard Quotient (HQ)

Risk assessment is a tool to estimate the probability of health effects due to exposure to a hazard; this study is the exposure through the consumption of fish. USEPA developed the oral reference doses (RfDs) for Hg at 1×10^{-4} (mg/kg/day) (risk information system (IRIS) [33]. The HQ meHg was calculated based on the following equation [11, 34]:(3)HQ=EF×ED×FIR×C×10−3RfD×BW×AT,where EF = exposure frequency at 350 days/year; ED = duration of human exposure for children and adults at 6 and 30 years, respectively; FIR = seafood ingestion rate (total intake per day in gram); C = meHg concentration in the seafood (mg/kg wet weight); RfD = oral reference dose (IRIS, USEPA); BW = average body weight of population group; and AT = average time of human exposure to noncarcinogen (AT = ED × 365 days).

Target hazard is a ratio of the determined dose of a contaminant to the oral reference dose considered detrimental. When HQ ≥ 1, there is potential for noncarcinogenic health risks from the intake of meHg through the consumption of seafood by the studied population.

2.7. Statistical Analysis

Statistical analysis in this study was conducted using IBM SPSS Statistics 26. The distribution of locations covered during the study was mapped using ArcGIS Desktop [22]. The analysis began with data checking and filtering where tHg data were cleaned and verified for inconsistencies. The final dataset used was households with complete information, and those with missing values were filtered out from the analysis. These included merging incomplete addresses into the same locality, district, and state. Nonparametric statistics such as median and interquartile range (IQR) were applied to the data due to nonnormal distribution. Mann–Whitney U and Kruskal–Wallis tests also were analyzed to compare the difference between groups of fish and seafood. A 5% significance level was used for all tests in the study.

3. Results

3.1. DHA and EPA in Fish and Seafood

Average concentrations of DHA and EPA from selected fish samples (mg/100 g) collected from the east and west coasts of Peninsular Malaysia are shown in Table 1. Overall, the median concentration of DHA and EPA content in pelagic fish is the highest (907.00 mg/100 g) compared to the other groups: demersal fish (234.00 mg/100 g) and shellfish (653.70 mg/100 g). The ratio between DHA and EPA was also the highest in these fish (3.6) compared to the demersal fish (1.2) and shellfish (1.5). Pelagic fish with high content of both DHA and EPA are yellowstripe scad (*Selaroides leptolepis*) (1,101.73 mg/100 g), slender scad (*Decapterus russelli*) (1,516.30 mg/100 g), torpedo scad (*Megalaspis cordyla*) (1,009.85 mg/100 g), long-tail tuna (*Thunnus tonggol*) (1,006.50 mg/100 g), and kawakawa (*Euthynnus affinis)* (1, 114.7 mg/kg) while, for demersal fish, both DHA and EPA were at high concentrations in the Japanese threadfin bream (*Nemipterus japonicus*) (1,015.85 mg/100 g), red-banded grouper (*Epinephelus fasciatus*) (1,141.00 mg/100 g), and long-tail shad (*Hilsa macrura*) (2,210.50 mg/100 g). Shallow-water crab (*Liocarcinus vernalis*) (1,266.80 mg/100 g) also showed a high concentration of these PUFA compared to other shellfish. Among different fish and seafood species, DHA was the highest in slender scads (*Decapterus russelli*) (1,162.50 mg/100 g), while nine of the other species showed DHA levels of more than 500 mg/100 g samples. The EPA was highest in long-tail shad (*Hilsa macrura*) (2,041.80 mg/100 g) and was the only species that could be considered an excellent source of EPA compared to other species studied.

When long-chain PUFA in samples were positioned into 3 categories, as reported by Weaver et al. [35] (Figure 2), results revealed that 27% of fish and seafood studied were in category 1, with half of the samples containing PUFA less than 150 g/100 g edible portion (category 3) (Figure 2(a)). PUFA was found to be predominant in the pelagic fish and mollusks (Figure 2(b)), and among demersal fish studied, only 31% were categorized in category 1, with nearly

half of the demersal species studied containing less than 150 mg/100g PUFA.

[figure(s) omitted; refer to PDF]

3.2. Fish and Seafood meHg Concentration Level

Table 2 presents the tHg and meHg concentrations in fish and seafood in median and IQR. Sixteen fish and seafood species were identified, and the meHg levels ranged from 0.0426 to 0.4576 mg/kg of wet weight (WW). A species of cephalopods, crustaceans, freshwater fish, five demersal fish, and eight pelagic fish were included as they represented the group of fish and seafood. The meHg levels significantly varied in fish and seafood groups ($\chi KW2 = 71.385$; $p \leq 0.001$). The median concentration for meHg was at 0.0621 ± 0.0573 mg/kg WW. All demersal fish showed meHg levels at ≥0.1 mg/kg WW but not long-tail tuna (*Thunnus tonggol*) (0.3329 mg/kg WW) of pelagic fish, which showed higher levels of this metal. Similar results were shown for the crustacean and rainbow shrimp (*Metapenaeus affinis*) (0.1400 mg/kg WW).

However, for cephalopods (*Sepia officinalis*) and freshwater fish (*Clarias batrachus*), the concentration levels were very much lower. Comparison of meHg levels for different groups of pelagic and demersal fish showed significant differences at $\chi MW2 = 1145.000$ and $p \leq 0.001$. Median concentrations of meHg for pelagic fish were significantly lower (0.0597 ± 0.0392 mg/kg WW) compared to the demersal fish (0.1718 ± 0.1332 mg/kg WW) at $p \leq 0.001$. Moreover, looking at median concentrations of meHg in fish and seafood groups in descending orders, demersal fish (0.1718 mg/kg WW) > crustaceans (0.1400 mg/kg WW) > cephalopods (0.0777 mg/kg WW) > pelagic fish (0.0597 mg/kg WW) > freshwater fish (0.0578 mg/kg WW). Results of the meHg levels in fish and seafood groups from this study do not exceed both the national and international guidelines.

3.3. Fish and PUFA Intake (g/day) by Adults and Adolescents in Peninsular Malaysia

A summary of fish and PUFA (DHA and EPA) daily intake by adults and adolescents in Peninsular Malaysia is shown in Tables 3 and 4. The fish consumed was expressed in grams daily, while PUFA intake was expressed in milligrams per day (mg/day). The median intake of fish among adult populations was 24.91 g/day, ranging between 11.11 and 66.39 g/day for pelagic fish, while for demersal fish, the average intake was at a higher rate (41.28 g/day) that ranges between 26.67 and 72.67 g/day.

Table 3

Intake of PUFA, meHg, and health risk assessment data from consumption of fish/seafood by adult populations in Peninsular Malaysia.

No.	Groups/family/species	Common name	meHg (mg/kg WW)	Fish intake (g/day)	PUFA intake (g/day)	EWI (μgkg^{-1})	EWI/PTWI (%)	MSWC (kg/week)	HQ
Pelagic fish									
1	*Selaroides leptolepis*	Yellowstripe scad	0.0480	66.39	879.20	0.3721	23.3	2.00	0.5098
2	*Decapterus russelli*	Slender scad	0.0459	27.00	409.40	0.1445	9.0	2.09	0.1980
3	*Megalaspis cordyla*	Torpedo scad	0.0748	37.52	443.73	0.3273	20.5	1.28	0.4483
4	*Parastromateus niger*	Black pomfret	0.0529	11.11	102.70	0.0686	4.3	1.82	0.0939

5	*Rastrelliger kanagurta*	Indian mackerel	0.0450	36.67	202.79	0.1926	12.0	2.13	0.2639
6	*Scomberomorus guttatus*	Indo-Pacific king mackerel	0.0587	20.00	195.19	0.1370	8.6	1.63	0.1877
7	*Thunnus tonggol*	Long-tail tuna	0.0966	22.83	229.78	0.2572	16.1	0.99	0.3523
8	*Euthymus affinis*	Kawakawa	0.0667	22.83	254.49	0.1775	11.1	1.44	0.2432
		Median	0.0600	24.91	242.14	0.1851	11.6	0.88	0.2536
–									
Demersal fish									
9	*Lutjanus argentimaculatus*	Mangrove red snapper	0.1927	72.67	170.05	1.6333	102.1	0.50	2.2374
10	*Lutjanus russellii*	John’s snapper	0.1628	33.33	8.63	0.6330	39.6	0.59	0.8671
11	*Lates calcarifer*	Giant-sea perch	0.1094	26.67	851.04	0.3403	21.3	0.88	0.4662
12	*Gymnura poecilura*	Long-tail butterfly ray	0.0956	41.28	4.83	0.4606	28.8	1.00	0.6309
13	*Nemipterus japonicus*	Japanese threadfin bream	0.0997	53.97	636.09	0.6276	39.2	0.96	0.8598
		Median	0.1176	41.28	170.05	0.6276	39.2	0.88	0.8598
–									
Freshwater fish									
14	*Clarias batrachus*	Walking catfish	0.0711	35.00	98.79	0.2905	18.2	1.35	0.3979
–									

Cephalopods									
15	*Sepia officinalis*	Common cuttlefish	0.0422	44.17	512.53	0.2174	13.6	2.28	0.2978
–									
Crustaceans									
16	*Metapenaeus affinis*	Rainbow shrimp	0.0273	19.63	183.62	0.0625	3.9	3.52	0.0856

Adult age ≥18 years old; body weight (BW) for adult = 60 kg; WW: wet weight; EWI: estimated weekly intake (μg/kg BW/week); provisional tolerable weekly intake (PTWI) for MeHg = 1.6 μg/kg BW/week [32]; MSWC: maximum safe weekly consumption (kg); HQ: hazard quotient.

Table 4

Intake of PUFA, meHg, and health risk assessment data from consumption of fish/seafood by adolescents in Peninsular Malaysia.

No.	Groups/family/species	Common name	meHg (mg/kg WW)	Fish intake (g/day)	PUFA intake (g/day)	EWI (μgkg^{-1})	EWI/PTWI (%)	MSCW (kg/week)	HQ
Pelagic fish									
1	*Selaroides leptolepis*	Yellow-stripe scad	0.0480	53.64	710.35	0.4009	25.1	1.50	0.5491
2	*Decapterus russelli*	Slender scad	0.0459	22.67	343.75	0.1618	10.1	1.57	0.2216
3	*Megalaspis cordyla*	Torpedo scad	0.0748	37.52	443.73	0.4363	27.3	0.96	0.5977
4	*Parastromateus niger*	Black pomfret	0.0529	39.07	361.16	0.3214	20.1	1.36	0.4403
5	*Rastrelliger kanagurta*	Indian mackerel	0.0450	24.33	134.54	0.1704	10.7	1.60	0.2335
6	*Scomberomorus guttatus*	Indo-Pacific king mackerel	0.0587	35.53	346.76	0.3246	20.3	1.23	0.4446
7	*Thunnus tonggol*	Long-tail tuna	0.0966	31.30	315.03	0.4701	29.4	0.75	0.6440

8	*Euthymus affinis*	Kawakawa	0.0667	31.30	348.90	0.3245	20.3	1.08	0.4446
		Median	0.0600	33.42	347.83	0.3245	20.3	1.29	0.4446
–									
Demersal fish									
9	*Lutjanus argentimaculatus*	Mangrove red snapper	0.1927	101.69	237.95	3.0474	190.5	0.37	4.1746
10	*Lutjanus russellii*	John's snapper	0.1628	39.46	10.22	0.9992	62.4	0.44	1.3687
11	*Lates calcarifer*	Giant-sea perch	0.1094	25.33	808.28	0.4309	26.9	0.66	0.5903
12	*Gymnura poecilura*	Long-tail butterfly ray	0.0956	54.37	6.36	0.8088	50.5	0.75	1.1079
13	*Nemipterus japonicus*	Japanese threadfin bream	0.0997	43.83	516.58	0.6796	42.5	0.72	0.9310
		Median	0.1176	43.83	273.95	0.8088	50.55	0.66	1.1079
–									
Freshwater fish									
14	*Clarias batrachus*	Walking catfish	0.0711	40.56	114.48	0.4488	28.0	1.01	0.6148
–									
Cephalopods									
15	*Sepia officinalis*	Common cuttlefish	0.0422	39.24	455.32	0.2575	16.1	1.71	0.3527
–									
Crustaceans									

16	*Metapenaeus affinis*	Rainbow shrimp	0.0273	21.00	196.43	0.0892	5.6	2.64	0.1222

Adolescents age 10–17 years old; BW for adolescents = 45 kg; WW: wet weight. EWI: estimated weekly intake (μg/kg BW/week); provisional tolerable weekly intake (PTWI) for MeHg = 1.6 μg/kg BW/week [32]. MSWC: maximum safe weekly consumption (kg); HQ: hazard quotient.

Freshwater fish and other seafood intake fall within the range of these two fish groups (Table 3). In addition, fish and seafood intake patterns were similar for adolescents, but the amount of intake for all categories of fish and seafood was higher than that of adults (Table 4). The adolescent populations also consumed more freshwater fish and cephalopods than the adult population. Total fish consumed by adolescents were 84.7 ± 103.7 g/day which is at the margin of statistical significance (p=0.069) compared to the adult population (90.5 ± 100 g/day) with the presence of one extreme seafood intake of outliers (Figure 3(a)). Reanalyzed fish consumption data without the outlier resulted in data close to statistical significance at p=0.058 (Figure 3(b)). The subsequent analysis was based on the data, which included the outlier of a girl aged 17 years old who consumed a bowl of prawn tom-yam during dinner.

[figure(s) omitted; refer to PDF]

Higher intake of PUFA was contributed from the consumption of yellow-stripe scad (*Selaroides leptolepis*), giant-sea perch (*Lates calcarifer*), Japanese threadfin bream (*Nemipterus japonicus*), and common cuttlefish (*Sepia officinalis*) for both population groups (Tables 3 and 4). In addition, for the adult population, the consumption of slender scad (*Decapterus russelli*) (409.4 mg/day) and torpedo scad (*Megalaspis cordyla*) (443.73 mg/day) also contributed to a high amount of PUFA. The intake of PUFA through consumption of black pomfret *(Parastromateus niger)* and Indo-Pacific king mackerel *(Scomberomorus guttatus)* by adolescents was nearly two times or more when compared to the consumption by the adult population. The highest intake of PUFA for both population groups was from the cephalopods (455.32 mg/day and 512.53 mg/day, respectively), followed by the pelagic fish (347.83 mg/day) for the adolescent population. For both groups, the adults and adolescents, the pelagic (242.14 mg/day; 347.83 mg/day) fish contributed higher amounts of PUFA for daily consumption compared to the demersal (170.05 mg/day; 273.95 mg/day). The last source of PUFA for both population groups was from the consumption of John's snapper (*Lutjanus russellii*), long-tail butterfly ray (*Gymnura poecilura*), and walking catfish (*Clarius bathachus*). Overall, additional calculations showed that although adolescents consume more fish and shellfish (40.05 g/day) than adults (35.66 g/day), the final intake of PUFA for both groups was comparable at 334.37 mg/day and 323.93 mg/day, respectively.

3.4. Health Risk Assessment for Adults and Adolescents

Tables 3 and 4 show the health risk assessment (EWI and HQ) for adults and adolescents in Peninsular Malaysia, resulting from the consumption of fish and seafood data. The level of EWI was calculated in micrograms per unit of body weight per week (μg/kg BW/week). These EWI estimated values showed results below 1.6 μg/kg BW/week, the acceptable or tolerable levels recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for all different fish and seafood species. However, for adult populations, the results exceeded the EWI recommended levels for consuming Mangrove red snapper (*Lutjanus argentimaculatus*) (1.6333 μg/kg BW/week). The adolescents showed EWI values more than adults, with even double the values at 3.0474 μg/kg BW/week. The EWI values for both population groups corresponded to 102.1% and 190.5% of the PTWI value from the consumption of this fish, respectively. Despite this, the EWI values for demersal fish other than this species showed lower than the recommended value for both population groups. Compared to the values from other fish and seafood consumption, these values were higher by two to five times in both population groups.

A summary of the HQ values for meHg from the consumption of fish and seafood by different population in Peninsular Malaysia was also studied (Tables 3 and 4). The HQ is a risk index that compares the quantity of meHg consumed to a specified reference dosage. For adults, the consumption risk is from mangrove red snapper (*Lutjanus argentimaculatus*); unlikely for adolescents, the risk was from the exposure through the consumption of all demersal fish studied, except the giant-sea perch (*Lates calcarifer*) (HQ = 0.5903). Despite this, the HQ values for

other fish and seafood groups were below one, assuming that daily exposure is not likely to cause adverse health effects during the lifetime of adults and adolescents in Malaysia. In addition, lower HQ values were shown from the consumption of the other seafood groups, and the lowest values were from the consumption of crustaceans for both population groups.

3.5. Maximum Safe Weekly Consumption (MSWC) of Fish and Seafood among Adults and Adolescents

MSWC values in kilograms are reported for each fish and seafood category, as shown in Tables 3 and 4. The proportion of demersal fish from Peninsular Malaysia that all population groups should consume to fulfil the PTWI for meHg would be less than 1 kg/week (range 500 to 1 kg/week) for all population groups and much less for adolescents. For all forms of fish, the adolescents were permitted to consume up to 1 kilogram per week (range: 370 g to 750 g/week), with a more significant amount for seafood (range: 1.71 to 2.64 kg). Conversely, adults were permitted to consume more marine and freshwater fish each week (790 g to 1.67 kg).

4. Discussion

This study was carried out to optimize the balance between the health benefits of omega-3 fatty acids and risks of meHg contamination from consuming freshwater fish and marine organisms by Malaysian adults and adolescents. We used the recommended intake of fish and fish oils worldwide [31] to compare the minimum intake of long-chain omega-3 PUFA (LC ω-3 PUFA) with targeted health benefits. The Joint FAO/WHO Expert Consultants [36] recommended a 250 g EPA and DHA daily to improve adults' health while the International Society for the Study of Fatty Acids and Lipids recommended a minimum intake of 500 mg of EPA and DHA daily to reduce CHD risk in healthy adults [37]. We expended an approach of estimating health risk assessment from intake of meHg among adults and adolescents who resided in Peninsular Malaysia using the PTWIs established by the WHO [32] and the HQ values by the USEPA [33]. The PTWI represents the amount of meHg that can be ingested over a lifetime without appreciative risks, while the HQ value is an integrated index that compares the ingested amount of meHg with the standard reference dose. The USEPA signifies the value of HQ

Oily fish is the primary dietary source of LC ω-3 PUFA and the content of fish species and their environments are shown in Table 1. Results of the referred studies showed variabilities of PUFA content in seafood caught from both the east coast (South China Sea) and the west coast (the Straits of Malacca) of Peninsular Malaysia [28, 29]. Examples of a few pelagic species reported to contain high content of both PUFA are several Carangidae scads (the yellow-stripe scads, slender scads, and torpedo scads) and the long-tail tuna. Higher PUFA content was shown in the Japanese threadfin bream, red-banded grouper, and long-tail shad for demersal fish. Among species of shellfish studied, the shallow-water crab contained the highest concentration of these PUFA. Results also showed that DHA was the highest in slender scads, with another 9 species showing DHA levels of more than 500 mg/100 g samples (the yellow-stripe scad, torpedo scad, black pomfret, Indo-Pacific king mackerel, long-tail tuna, kawakawa, giant-sea perch, Japanese threadfin bream, and red-banded grouper). The long-tail shad was the only species that could be considered as an excellent source of EPA compared to other species studied.

LC ω-3 PUFA content in fish has been linked to their dietary niche, variety of dietary sources, and seasonal variations in food availability. Pelagic fish often have the largest concentration of these fatty acids, especially the DHA, because they inhabit cold water [38]. Researchers used an ecological factor that divided fish species into different habitats (pelagic, benthopelagic, and demersal), standard fish sizes as a proxy of trophic level, as well as the water temperature of their habitat (cold water, temperate, and warm water) to organize fish species by their EPA and DHA values [39]. Yet, these substitutions are not perfect. Significant differences in the fatty acids profile within species occurred due to other factors as well, for instance, fish species, genetics, harvesting seasons, reproductive cycle, and environmental characteristics [31, 38, 40–42]. Mahaffey [43] reported on a general coherent pattern of fatty acid composition for fish and shellfish species, but the relationship between ω-3 fatty acids and the fat content of all species is not a simple linear association. The study also provided an overview of the EPA and DHA contents in various fish and shellfish species from 1975 to 2000, represented on a fresh-weight basis. Results showed that the EPA concentration ranged from less than 0.01 g to 1.5 g/100 g, and the DHA concentrations ranged from less than 0.01 g/100 g to more than 2.00 g/100 g of species studied [43]. A study by Williams et al. [44] summarized fatty

acids in fillets from freshwater fish sampled from the US water of all 5 Great Lakes and inland lakes and rivers in the Great Lakes region. The studies determined that fatty acid content in freshwater species varies across spatial, biological, physical, and chemical gradients. They highlighted all taxonomic families from Great Lakes species and in-land salmonid fillets containing ≥250 mg/227 g fillet (or ≥110 mg/100 g EPA + DHA). In contrast, Dellinger et al. [41] listed a few Great Lakes species (walleye, rainbow smelt, lake herring, yellow perch, whitefish, and lake trout) that contained a high concentration of these fatty acids at a range between 244.2 and 2,395.3 mg/100 g. Gribble et al. [39] summarized data on EPA and DHA across fish populations in their review. They discovered that order Clupeiformes (e.g., sardine, *Sardinops sagax*), followed by order Salmoniformes, has the highest concentrations of *ω*-3 PUFA. At the same time, lower quantities of these long-chain fatty acids were found in other order groups, including the Perciformes, Scorpaeniformes, and Gadiformes. Earlier studies by Weaver et al. [35] showed that the most widely farmed fish have relatively high amounts of *ω*-3 PUFA in trout and Atlantic salmon but not in tilapia and catfish. Still, there are differences in levels of this LC *ω*-3 PUFA in different species of tilapia, and research revealed that *O. niloticus* contained varieties of fatty acids and *ω*-3 PUFAs compared to other species: *L. niloticus*, *T. zilli*, and *R. argentea*. It is also helpful to value significant and relatively higher levels of EPA and DPA in *R. argentea* [42]. A study conducted among species captured from the Jamaican marine environment revealed the mean concentration of EPA and DHA at 123.1 ± 93.6 mg/100 g, and species with high content of these omega-3 fatty acids were codfish (*Gadus morhua*), pickled mackerel (*Scomberomorus regalis*), sea trout (*Macrodon ancylodon*), and winchman (*Pristipomoides aquilonaris*) (EPA and DHA range between 213 and 298 mg/100 g) [45]. A more significant amount of these long-chain *ω*-3 polyunsaturated fatty acids was found in some commercially significant New Zealand species, ranging from 2.01 to 20.5 mg/g [46], consistent with research from Malaysian waterbodies.

Elemental fatty acid composition, which is cumulative in fish, is influenced by the ingredients in their diet, mainly on microalgae [38, 39, 47–50] that actively synthesize and accumulate high amounts of LC *ω*-3 PUFA. These EPA and DHA occur widely in many unicellular species, especially marine ones. The microalgae form the bottom of a food web, with their long-chain *ω*-3 PUFA finally accumulating in the lipids of the fish that consume them [50]. Studies have shown that the Cryptophyceae, Dinophyceae, and flagellate algae were the highest sources of EPA and DHA for zooplankton, and another different species of *Bacillariophyceae* with a high EPA concentration was a better food source for Daphnia [40, 48, 51]. Additional examples of aquatic microorganisms that accumulate high amounts of EPA are the diatom, *Phaeodactylum tricornutum*, and the heterotrophic marine algae, *Crypthecodinium cohnii*, which were identified as species rich in DHA [48, 50]. Further, epilithic algae in freshwater ecosystems, especially the diatoms, are comparatively rich in EPA but low in DHA. There are also advantages for some freshwater fish; in the case of *Oncorhynchus mykiss* and *Salvelinus alpinus*, they can transform dietary alpha-linolenic acid (ALA) via stearidonic acid (SDA) and EPA to DHA [47]. Major lipid classes in algae are membrane components and storage lipids, but the proportion of polar membrane lipids may vary depending on algae species. The overall fatty acids composition and its patterns for individual lipid classes of algae depend on their growth condition, seasonal variations, and developmental stages [48].

This study identified LC *ω*-3 PUFA was predominant in some pelagic fish and mollusk species. It also attempted to document the most consumed seafood species potential providers of these fatty acids to our population, categorized into groups I and 2 with EPA and DHA content ≥150 mg/100 g [35]. Total fish consumption between both population groups (adults = 84.7 ± 103.7 g/day; adolescents = 90.5 ± 100 g/day) was a marginally significant difference at p=0.058 , despite the amount of seafood consumed by the adolescents being higher for all seafood categories than the adults. In this dataset, consumption of four species of seafood, mainly the yellow-stripe scad, giant-sea perch, Japanese threadfin bream, and common cuttlefish had fulfilled the requirement established by the International Society for the Study of Fatty Acids and Lipids for a minimum intake of 500 mg EPA and DHA per day for reducing CHD risk in healthy adults [37]. While intake of another 3 species of slender scad, torpedo scad, and kawakawa is adequate per day, which contributed to ≥250 g of EPA and DHA, a daily adequate intake as proposed by The Joint FAO/WHO Expert Consultants [36] to gain healthiness for adults [31]. All listed pelagic fish species could contribute to the intake of ≥250 g of EPA and DHA for adolescents, except for an Indian mackerel. It may be accurate to

suggest an intake of double amounts of species such as Indian mackerel, Indo-Pacific king mackerel, long-tail tuna, and rainbow shrimp to Malaysian adults to meet the adequate daily intake of these PUFA.

Dietary intake levels and sources of *ω*-3 PUFA vary among countries. For Malaysians, other than fatty fish (e.g., Hoven's carp, seabass, tuna, and sardines), the basal RNI for omega-3 fatty acids of 0.3% energy may be achieved from the intake of local foods, namely, vegetables such as soybean/soybean products (tofu, tofu skin, and bean sprout), legumes, fortified foods (omega-3 eggs) and ready to drink omega-3 milk/soybean milk [52]. Similarly, in Japan, fish and shellfish, edible fats, and oils are the most essential sources of n-3 PUFAs, with the prior food group providing ranges between 1 and 2 g of n−3 PUFA/person/day. About 60% of this fat is from fresh fish, especially horse mackerels, sardines, and tuna, with an average seafood consumption rate of approximately 80 g per day among them [53]. Other findings from a population-based cohort study among the Japanese indicated an estimated daily fish intake of 111 g, 307 mg of EPA, or 123 mg of DPA, which could be related to a lower risk of depression [54]. For the US population, the most common species of fish and shellfish as sources of *ω*-3 fatty acids are salmon and shrimp, and researchers recognizing sources of *ω*-3 fatty acids present in foods in addition to fish are eggs and chicken [55]. Australian kids between the ages of 2 and 16 ate an average of 13 g of fish or shellfish daily, but only 50 to 60% of them got the recommended amount of LC *ω*-3 PUFA. Findings also showed that these kids ingested eight times as much meat as fish or seafood, which accounted for 33% of the LC *ω*-3 PUFA in these meals [56]. Malaysian adolescents appeared to have consumed roughly seven times more fish and seafood than indicated by this published data. Studies also revealed that estimations of the global dietary intake of DHA from 64% of 175 developed and developing nations were less than 200 mg/day [57]. In this study, we expanded the list of fish and seafood species that contain highly LC *ω*-3 PUFA to fully comply with the recommendations for improving health or lowering the risk of CHD in our populations.

A benefit-risk assessment was performed on LC *ω*-3 PUFA and meHg; this study found scenarios where consumption of some species of seafood, especially the pelagic fish, would confer the benefit of these fatty acids and meHg exposure would remain below the tolerable intake for the Malaysian population. Moreover, the findings demonstrated that adults and adolescents could consume more than one kilogram of pelagic, freshwater, and shellfish per week, but the risk index of HQ still assumes that daily exposure is not likely to cause negative health effects. The percent ratio of meHg exposure in adolescents towards the PTWI guideline value per week for pelagic fish is 20.3% compared to the demersal fish at 50.6%, which is higher when compared to the ratio for the adult population at 11.6% and 39.2%, respectively. The exposure by consumption of the crustacean is the lowest for both groups indicating protection of meHg accumulation by the most negligible permeability of the exoskeleton or several molting processes in the life cycle of crustaceans when new bioaccumulation starts in new exoskeleton formation [58]. In some cases, consumption of the demersals (*Lates calcarifer* and *Nemipterus japonicus*) not only contributed to a high intake of LC *ω*-3 PUFA but also increased the HQ to 1. However, these findings are contradicted by the consumption of cephalopods. Double the intake of cephalopod may increase the intake of LC *ω*-3 PUFA, with a lower risk compared to the guideline. Even a threefold increase in crustacean consumption would minimize the rise in meHg risk and help people achieve their daily LC *ω*-3 PUFA dietary requirements. Cressey et al. [46] used two approaches to compare the risk due to meHg and the benefits due to EPA and DHA from the consumption of six important commercial New Zealand fish species (the barracouta, black oreo, gemfish, ling, orange roughy, and smooth oreo). They calculated the dose-response equations for the impact of meHg and LC *ω*-3 PUFA on off-springs IQ and a hazard index from the dietary exposure estimation as a proportion of a health-based guidance value. The analysis indicates a different balance between risks and benefits for fish species studied and only one species; Ling showed the narrowest margin between the benefits and risks at 10 or more servings of this fish per week. Research on the nutritional benefits and mercury risk of commercial fish collected in the upper Laurentian Great Lakes concluded that six ounces of caught fish might benefit cardiovascular health and neonatal neurodevelopment [59]. Before this, Dellinger [60] reported that tribal members in the Upper Great Lakes region had moderately elevated mercury exposure, which is not high enough to call for widespread dietary restrictions. This was based on ten years of data collection and exposure assessment for the Ojibwe Health Study. The HQ values for all

demersal fish species in this study were less than 1, indicating that the mercury toxicity risk is modest compared to the health advantages of LC ω-3 PUFA for Malaysian adolescents and adults. Similar outcomes were also observed in Jamaicans who ate typical deep slope and reef finfish species such as snappers, grunts, and parrotfish [45].

5. Conclusions

Quantitative assessments of the meHg risk must be matched with the benefits of PUFA to establish the significance of fish and shellfish as food sources. This study emphasized the species-specific variation in the LC ω-3 PUFA (EPA and DHA) concentration from the consumption of fish and seafood by adolescents and adults in Peninsular Malaysia in relation to meHg, which harms human health. This study revealed that meHg levels ranged from 0.0426 to 0.4576 mg/kg of wet weight in 16 fish and seafood species comprised of cephalopods, crustaceans, freshwater fish, and marine fish (the demersal and pelagic fish). meHg levels showed significant variations between all species at a median concentration of 0.0621 ± 0.0573 mg/kg WW. The content of DHA and EPA differed substantially by species, with PUFA found to be predominant in the pelagic fish and mollusks at the highest ratio in pelagic fish (3.6) compared to the demersal fish (1.2) and shellfish (1.5). Pelagic fish, including long-tail tuna, yellow-stripe shad, slender shad, and long-tail shad from the demersal fish, have been found to contribute to higher PUFA intake than other types of fish and seafood. These fish also contributed to a low HQ value level, lowering the risk of health effects. It is advised to consume mangrove red snapper less frequently since the PUFA content is relatively low, but the HQ value was the highest. Results from this study were used to justify a new project proposal entitled "Assessment of Heavy Metal Risks for the Optimization of Nutrient Benefits from Fish and Seafood in Malaysia" (NMRR ID: 22-01573-PGU 22-029). One of the objectives of this new study is to analyze fatty acid content in varieties of fish and seafood species distributed throughout Malaysia.

Ethical Approval

The Medical Research and Ethics Committee, MOH Malaysia, reviewed and approved the proposal.

Consent

Informed consent forms were obtained from the subjects beforehand.

Authors' Contributions

Ahmad NI substantively contributed to the conception of the work and data collection. Equally, contributions from both authors were made to the data analysis and interpretation, drafting and critical revision of the article, and final approval for publication.

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DETAIL

Subjek:	Cardiovascular disease; Pollutants; Seafood; Fishing; Ethnicity; Brain research; Teenagers; Moisture content; Questionnaires; Fatty acids; Fish; Food safety; Health risks; Mollusks; Mercury; Crustaceans; Bioaccumulation; Polyunsaturated fatty acids; Adolescents; Methylmercury
Lokasi:	Malaysia
Perusahaan / organisasi:	Nama: Environmental Protection Agency--EPA; NAICS: 924110
Judul:	Evaluating Benefits and Risks of Polyunsaturated Fatty Acids and Methyl Mercury from Fish and Seafood Consumption in Peninsular Malaysia
Pengarang:	Ahmad, Nurul Izzah1 ; Noraishah Mohammad Sham11 Environmental Health Research Centre (EHRC), Institute for Medical Research (IMR), National Institutes of Health (NIH), Ministry of Health Malaysia (MOH), No. 1, Jalan Setia Murni U13/52, Seksyen U13, Setia Alam, Shah Alam 40170, Selangor, Malaysia
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| Terakhir diperbarui: | 2024-07-17 |
| Basis data: | Public Health Database; Publicly Available Content Database |

Retracted: Apprehending the Effect of Internet of Things (IoT) Enables Big Data Processing through Multinetwork in Supporting High-Quality Food Products to Reduce Breast Cancer

Quality Journal of Food.

Link dokumen ProQuest

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(5) Incoherent, meaningless and/or irrelevant content included in the article

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References

[1] S. K. Shukla, B. M. Kumar, D. Sinha, V. Nemade, S. Mussiraliyeva, R. Sugumar, R. Jain, "Apprehending the Effect of Internet of Things (IoT) Enables Big Data Processing through Multinetwork in Supporting High-Quality Food Products to Reduce Breast Cancer," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/2275517, 2022.

DETAIL

Subjek:	Research; Data processing; Big Data; Food products; Food quality; Internet of Things
Ketentuan indeks bisnis:	Subjek: Big Data Internet of Things
Judul:	Retracted: Apprehending the Effect of Internet of Things (IoT) Enables Big Data Processing through Multinetwork in Supporting High-Quality Food Products to Reduce Breast Cancer
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Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Investigation and Strategy Research on Dietary Nutrition Knowledge, Attitude, and Behavior of Athletes

Food Quality Journal of.

Link dokumen ProQuest

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[1] B. Feng, Y. Yuan, "Investigation and Strategy Research on Dietary Nutrition Knowledge, Attitude, and Behavior of Athletes," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/7323680, 2022.

DETAIL

Subjek:	Research
Judul:	Retracted: Investigation and Strategy Research on Dietary Nutrition Knowledge, Attitude, and Behavior of Athletes
Pengarang:	Food Quality Journal of
Judul publikasi:	Journal of Food Quality; Cairo
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| **Terakhir diperbarui:** | 2024-03-08 |
| **Basis data:** | Public Health Database; Publicly Available Content Database |

Retracted: An Empirical Investigation in Analysing the Critical Factors of Artificial Intelligence in Influencing the Food Processing Industry: A Multivariate Analysis of Variance (MANOVA) Approach

Quality Journal of Food.

Link dokumen ProQuest

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References

[1] G. S. Raghavendra, S. S. C. Mary, P. B. Acharjee, V. L. Varun, S. N. H. Bukhari, C. Dutta, I. A. Samori, "An Empirical Investigation in Analysing the Critical Factors of Artificial Intelligence in Influencing the Food Processing Industry: A Multivariate Analysis of Variance (MANOVA) Approach," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/2197717, 2022.

DETAIL

Subjek:	Research; Multivariate analysis; Food processing industry; Artificial intelligence; Variance analysis
Ketentuan indeks bisnis:	Subjek: Food processing industry Artificial intelligence
Judul:	Retracted: An Empirical Investigation in Analysing the Critical Factors of Artificial Intelligence in Influencing the Food Processing Industry: A Multivariate Analysis of Variance (MANOVA) Approach
Pengarang:	Quality Journal of Food
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Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Modified Atmosphere Packaging of Chicken Thigh Meat: Physicochemical and Sensory Characteristics during the Frozen Storage Period

Quality Journal of Food.

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References

[1] A. R. Al-Hilphy, M. H. Al-Asadi, J. H. Khalaf, A. Mousavi Khaneghah, M. Faisal Manzoor, "Modified Atmosphere Packaging of Chicken Thigh Meat: Physicochemical and Sensory Characteristics during the Frozen Storage Period," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/8876638, 2022.

DETAIL

Subjek:	Research
Judul:	Retracted: Modified Atmosphere Packaging of Chicken Thigh Meat: Physicochemical and Sensory Characteristics during the Frozen Storage Period
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
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Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Hybrid Feature-Based Disease Detection in Plant Leaf Using Convolutional Neural Network, Bayesian Optimized SVM, and Random Forest Classifier

Quality Journal of Food.

Link dokumen ProQuest

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References

[1] A. K. Singh, S. Sreenivasu, U. S. B. K. Mahalaxmi, H. Sharma, D. D. Patil, E. Asenso, "Hybrid Feature-Based Disease Detection in Plant Leaf Using Convolutional Neural Network, Bayesian Optimized SVM, and Random Forest Classifier," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/2845320, 2022.

DETAIL

Subjek:	Research; Neural networks
Judul:	Retracted: Hybrid Feature-Based Disease Detection in Plant Leaf Using Convolutional Neural Network, Bayesian Optimized SVM, and Random Forest Classifier
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
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Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Improved Support Vector Machine and Image Processing Enabled Methodology for Detection and Classification of Grape Leaf Disease

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References
[1] A. S. Ansari, M. Jawarneh, M. Ritonga, P. Jamwal, M. S. Mohammadi, R. K. Veluri, V. Kumar, M. A. Shah, "Improved Support Vector Machine and Image Processing Enabled Methodology for Detection and Classification of Grape Leaf Disease," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/9502475, 2022.

DETAIL

Subjek:	Research; Support vector machines
Judul:	Retracted: Improved Support Vector Machine and Image Processing Enabled Methodology for Detection and Classification of Grape Leaf Disease
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
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Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Improving the Oxidation Stability and Shelf-Life of Peanut Oil by Addition of Rosemary Extract Combined with Vitamin C and Ascorbyl Palmitate

Quality Journal of Food.

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References

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DETAIL

Subjek:	Research; Oxidation; Vitamin C
Judul:	Retracted: Improving the Oxidation Stability and Shelf-Life of Peanut Oil by Addition of Rosemary Extract Combined with Vitamin C and Ascorbyl Palmitate
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
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Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Machine Learning Model-Based Applications for Food Management in Alzheimer’s Using Regression Analysis Approach

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In addition, our investigation has also shown that one or more of the following human-subject reporting requirements has not been met in this article: ethical approval by an Institutional Review Board (IRB) committee or equivalent, patient/participant consent to participate, and/or agreement to publish patient/participant details (where relevant).

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] S. H. Kumhar, P. R. Kapula, H. Kaur, R. R. Krishna, M. M. Kirmani, V. A. Athavale, M. W. Ahmad, "Machine Learning Model-Based Applications for Food Management in Alzheimer's Using Regression Analysis Approach," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/1519451, 2022.

DETAIL

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Retracted: Integration of Artificial Intelligence and Blockchain Technology in Healthcare and Agriculture

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TEKS LENGKAP

This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article’s content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

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We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] S. Vyas, M. Shabaz, P. Pandit, L. R. Parvathy, I. Ofori, "Integration of Artificial Intelligence and Blockchain Technology in Healthcare and Agriculture," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/4228448, 2022.

DETAIL

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Listeria Contamination in Milk-Processing Chain and Proficiency in Listeria monocytogenes Decontamination of Small-Scale Milk Retailers

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ABSTRAK (ENGLISH)

Listeria spp. is an important foodborne bacterium. This microorganism can be discarded from milk using high temperatures such as pasteurization. The milk-processing methods of many small-scale retailers lack quality control. This study was to survey *Listeria* contamination at the farm and retailer levels. The retailers were to be interviewed for knowledge, attitude, and practice as well. Finally, we were to determine the heating processes employed to decontaminate microorganisms by the retailers using a reference strain of *L. monocytogenes*. Milk samples were collected from milk-collecting centers and small-scale retailers. In clinical trial, the processing measures were proved for the proficiency in *L. monocytogenes* decontamination. One out of 99 farms presented *Listeria* contamination, confirmed to *L. marthii*. Fifty small-scale retailers participated in the second part, including 13 males and 37 females. No *Listeria* spp. but *Staphylococcus* spp. and *Bacillus* spp. were identified in the processed milk. Data analyses revealed that the location of the retailer was significantly associated with the volume they routinely ordered per lot and the milk-processing time the retailers used to treat milk. Knowledge on raw milk contamination is significantly associated with the stocking or processing of the whole milk lot. Processing measures presale were significantly influenced by the gender of the retailer. The male retailer reportedly spent less time treating milk compared to their female counterparts. To assess the efficacy of the processing methods, a trial using *L. monocytogenes* as a reference strain was conducted. Interestingly, no *L. monocytogenes* was detected after sample treatment, but other microorganisms such as S. epidermidis, S. warneri, and Escherichia coli were found, suggesting potential issues with cross-contamination. In conclusion, while the trial implied that the retailers' processes were effective in *L. monocytogenes* decontamination, the study highlighted inappropriate practices and the risk of cross-contamination. Continuous monitoring of product safety in small-scale milk retailers is imperative to ensure consumer well-being.

TEKS LENGKAP

1. Introduction

Foodborne diseases are a major global problem. International institutes such as the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) are concerned about listeriosis because of the infectious and severe nature of the disease [1, 2]. Listeriosis is a fatal foodborne disease that can be caused by *Listeria* spp., especially *L. monocytogenes*. However, listeriosis has a low incidence in humans and animals [3–5]. *Listeria* spp. most likely contaminate raw milk, cheese, seafood, and frozen foods. At the farm level, *Listeria* spp. are contagious bacteria. Transmission normally occurs during milking and from the environment [6–8]. The prevalence

of *Listeria* spp. in raw milk was reported in a wide range such as 3–7% in North America, 0–50% in Europe, and about 5% in Iran [9–11]. In Thailand, there was not the report of *Listeria* contamination in raw milk [12]. Unpasteurized or improper processed milk caused three out of 36 outbreaks of listeriosis. The illness has many forms, gastroenteritis, septicaemia, and meningitis, for example. Pregnant women and their neonates, 65 years or older people, and immunosuppressed patients were susceptible groups for the disease [1, 2]. According to WHO, the risk group should become aware of consumption of fresh or improperly treated milk [3, 13, 14].

Raw milk is a substantial risk to human health; therefore, milk should be heated before consumption [9]. Several small-scale retailers presently buy untreated milk directly and treat it at home with processes that might not follow the protocol or be substandard. The chance of *Listeria* spp. infection increases owing to inefficient processes [15]. Size of dairy farms in Thailand varies from less than 10 up to over 100 milking cows per farm. Almost all farms were crossbred with a high percentage of Holstein Friesians. The annual raw milk averaged 1,200 tons between 2017 and 2022, related to the production of 11 kg/cow/day, approximately [16] The whole raw milk has supported the consumption within the country. Milk normally is sold as pasteurization, UHT, and sterilization products, but some are uncontrollably heated and sold in small retailers. The product from milk retailers is highly risky because the quality of milk processing is not controlled. *Listeria* spp. can contaminate in all processes to which the condition is proper for bacterial growth, even packaging or storage after heat treatment [7, 17].

This study was to operate into three sections. The first and second objectives were to survey *Listeria* contamination in fresh milk from farms and milk from small-scale retailers. The retailers were additionally interviewed for knowledge, attitude, and practice. For the final part, we were to determine the heating processes employed to decontaminate microorganisms by the retailers using a reference strain of *L. monocytogenes*.

2. Materials and Methods

2.1. Study Sample Description

Milk was collected from three collecting milk centers, Kamphaeng Saen Dairy Co-operative, Nakhon Pathom Dairy Co-operative, and Nong Pho Dairy Co-operative at the farm level. These three centers are in the central part of Thailand and received fresh milk from 3,000 farms in total. The raw milk, which passes quality check, is to be distributed to large-scale commercial dairy manufacturers or processed by the milk-collecting centers. Both the large-scale dairy manufacturer and the milk-collecting center treat milk by the standard measures and sell it as commercial products. However, there is still the small-scale milk retailer, which produces and sells unpasteurized products in the local area. They usually buy raw milk from dairy farms directly and treat milk with their own measures.

2.2. Study Design

This study was designed to explore the possibility of the contamination of *L. monocytogenes* in the small milk-processing line. The first part focused on the raw milk from dairy farms. The next step explored the product from the small-scale milk retailers. Additionally, the perception of milk safety was interviewed by the questionnaire of knowledge, attitude, and practice. Their practices in treating milk were concluded to design the treatment groups in the trial. The last step was to confirm the retailer's treatments to see if the methods were able to decontaminate *L. monocytogenes* from milk.

2.3. Sample Size and Sampling Techniques

The sample number was run by ProMESA 2.3.0.2 (INTA &Massey University, Castelar, Argentina). The sample size in the farm level was calculated based on the disease detection at 3% prevalence of *Listeria* spp. [8] and population size of 3,000 farms. The sample numbers, weighted by the total number of registered farms, of Kamphaeng Saen Dairy Co-op, Nakhon Pathom Dairy Co-op, and Nong Pho Dairy Co-op, were 33, 23, and 43 samples, respectively. A simple random sampling method was used for selecting the dairy farms.

In the retailer level, the number of samples was 50 retailers, which was based on the population size of 500 shops and 5% of prevalence in pasteurized milk [18]. The retailers were selected by a purposive sampling method from five provinces, which surrounded the positive farm from the first survey. The samples were in Nakhon Pathom, Ratchaburi, and Bangkok for 11 retailers each, whereas those from Kanchanaburi and Suphanburi were nine and

eight retailers, respectively.

2.4. Sample Collection and Transportation

Both farm and retailer levels collected at least 30 mL milk in a sterile container. The samples were kept in a cool box during transportation to the Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Thailand. Milk was to be kept at 4°C and analysis of *Listeria* spp. within 24 h.

2.5. Questionnaire Survey Description

The retailers were asked for consent to an interview. The questionnaire was about their knowledge, attitude, and practice on milk safety. The retailers who responded to the questionnaires included 72% women and 28% men. The qualification of approximately 80% of the respondents fell under a bachelor's degree. The data from the questionnaire were to be used in data analysis and design of the treatment groups in the trial.

The retailers treated milk without monitoring temperature and time. Their processes were completed by noticing the appearance of heated milk. The retailer's processes were classified into (1) using double boiling until bubbles formed, (2) using double boiling until a film layer formed, (3) using double boiling for 2 min (in 100°C water), (4) using direct heating until bubbles formed, and (5) using direct heating until a film layer formed. Table 1 shows the data of temperature and time of each process from the trial.

Table 1

Milk temperature during process and processing time for each treatment.

Measures*	Temperature (°C)	Processing time (min)
Double boiling until bubbles formed	78	19
Double boiling until a film layer formed	83	24
Double boiling for 2 min (in 100°C water)	75	2
Direct heating until bubbles formed	88	14
Direct heating until a film layer formed	90	35

*Each treatment operated in 1 liters of milk.

2.6. Experiment Description

The trial consisted of five treatments, following the retailer's processes (Table 1), and control groups as pasteurization of 63°C for 30 min and 72°C for 15 s. Sterile milk with added *L. monocytogenes* (ATCC® 51414™, American Type Culture Collection, VA, USA) were used in the experiment. Each treatment was assessed with 10^5, 10^3, and 10^1 colony forming units [CFU]/mL of *L. monocytogenes.* The sample was collected in duplicates at three time points: pretreatment, post-treatment, and 30 min post-treatment.

2.7. Bacteriological Test

Vidas® LDUO (bioMérieux, Marcy-l'Étoile, France), based on an enzyme-linked fluorescent assay (validated by AFNOR/ISO16140 (BIO 12/12–07/04)), was used in qualitatively screening *Listeria* spp. and *L. monocytogenes* contamination in the farm level. The positive samples from Vidas® LDUO and the samples from the retailer level were cultured on ALOA® One Day (bioMérieux, Marcy-l'Étoile, France). ALOA® One Day is the alternative method to detect the contamination of *Listeria* spp. in foods and environment samples, based on chromogenic agar. This analysis technique follows ISO 11290-1:2017.

The samples from the experiment were cultured on ALOA® One Day and brain heart infusion (BHI) agar for identifying and enumerating *Listeria* spp. and *L. monocytogenes*. The typical appearance of *Listeria* spp. and *L. monocytogenes* on ALOA® One Day was blue-green colonies and blue green colonies with opaque, respectively.

Listeria spp. grow on BHI agar as white colonies.

The typical colonies from both ALOA® and BHI agar were cultured on Trypticase Soy Agar (TSA) to prepare the colonies for VITEK® MS (bioMerieux, Marcy-l'Etoile, France), based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technology. VITEK® MS reported as genus with/without species according to their mass-to-charge (m/z) comparing the database. The confidence level presented the certainty of the result. *E. coli* ATCC 8739 was used as a positive control.

2.8. Data Analysis

Milk volume per lot between provinces was compared by using the Kruskal–Wallis test. The associations between variables were analyzed by the chi-squared test and Fisher's exact test using Stata 13.1 (Stata Corp LLC, College Station, TX, USA). The statistical significance was set at the significance level of 0.05.

3. Results

3.1. Farm Level

Ninety-nine samples of bulk tank milk were collected from three locations. All samples were negative for *L. monocytogenes*, but 1% was positive for *L. marthii* from the Nong Pho milk-collecting center, Ratchaburi province. The contaminating species were confirmed as *L. marthii* at a confidence value of 98.7%.

3.2. Retailer Level

Fifty milk samples from small-scale retailers were detected for the contamination of *Listeria* spp. however, VITEK® MS reported *Staphylococcus* spp. and *Bacillus* spp. contamination.

The questionnaire results suggested that 56% of individuals had knowledge regarding the severity of raw milk consumption, bacterial contamination in raw milk, and the necessity for treatment before consumption. Almost all retailers identified diarrhea as the most common illness associated with untreated milk. Other consequences included vomiting, fever, headache, convulsions, and flatulence. Notably, the results showed that 4% of individuals lacked awareness on the health impacts of untreated milk.

Regarding milk treatment, 90% of respondents knew that bacteria could be killed by heating milk. Less than 10% understood the pasteurization process, but 25% of respondents could explain it correctly. Unfortunately, 10% of milk retailers believed that milk did not require processing before sale.

The data on the practices revealed that approximately half of the responders ordered milk daily, and 6% ordered weekly or at longer intervals. Dairy farms were the primary source of raw milk for 42% of retailers, and the rest received milk from cooperations or intermediaries. The median milk volume ordered for each lot was approximately 20 kg, and Q1 and Q3 were 10 and 40 kg, respectively. Thirty-five percent of retailers processed whole milk on the day of reception. Over 50% stored the nonprocessed milk under cold conditions and 10% froze milk. A mere 10% of the processed milk was subjected to pasteurization at controlled temperature and time presale. The remaining retailers only checked the appearance of milk whilst heating to finish the treatment; they did not follow a time-controlled heating.

The data from questionnaires suggested that 62% of retailers heated milk for less than 30 min. The daily leftover-processed milk was not resold by 20% of retailers. Over 50% of the retailers kept previously heated milk under cold storage, and approximately 15% froze milk. The major health concern associated with this process was gastrointestinal illness. However, only a few respondents were aware of fever and neurological problems associated with the illness.

The results of the association test are presented in Table 2. The location of retail was associated with the volume of milk ordered. Retails in Bangkok significantly stocked a larger volume ($p<0.05$).

Table 2

Test of association using the chi-squared test and Fisher's exact test.

Variables	Explanatory variables	Chi^2	P value

Raw milk management	Sex	—	0.32
Province	—	0.07	Knowledge of milk contamination
7.26	0.007	Knowledge of bacterial virulence	—
0.70	-		
Milk treatment	Sex	10.38	0.001
Province	9.88	0.04	Knowledge of milk contamination
0.004	0.95	Knowledge of bacterial virulence	—
0.15	Knowledge of pasteurization	—	0.10
–			
Processed milk management	Sex	—	0.13
Province	—	0.39	Knowledge of milk contamination
—	0.73	Knowledge of bacterial virulence	—

Additionally, knowledge about contamination was significantly associated with the measures for managing milk after reception ($p<0.05$). The knowledge about the methods by which retailers processed whole milk in a lot instead of stocking raw milk was six-fold higher than those who lacked relevant knowledge. The sex of the retailer was not associated with the knowledge that milk treatment could reduce contamination, but the decontamination practices did. Men were 18 times less likely to heat milk for less than 30 min than women ($p<0.01$). The practices significantly depended on the location of the retailers ($p<0.05$). Retailers in Bangkok were five times more likely to treat milk

improperly compared to those in other locations.

None of the retailers sold unprocessed milk. The products were treated by double boiling or direct heating before selling. The retailers prepared milk by double boiling and direct heating without checking the temperature and processing time. The appearances, i.e., bubbles or layers formed after heating were used to notice that the products were ready to sell.

3.3. Trial Level

In the trial, the typical colony of *L. monocytogenes* was not shown from any processes post-treatment and 30 min post-treatment; however, the white colonies grew on BHI agar (Table 3). Neither of colonies on BHI agar confirmed the presence of *L. monocytogenes* contamination. Vitek® MS showed *S. epidermidis*, *S. warneri*, and *E. coli* at the confidence level of 99.9%.

Table 3

Concentration (CFU/mL) of bacteria, growing on BHI agar after treatment.

L. monocytogenes added in milk (CFU/mL)	Time[a]	Processes[b]				
1	2	3	4	5	10	T1
15	0	0	0	0	T2	0
10	0	0	10	·		
10^3	T1	0	15	0	15	0
T2	0	0	5	5	75	·
10^5	T1	0	5	0	0	0

[a]T1: post-treatment, T2: 30 min post-treatment. [b]1: using double boiling until bubbles formed, 2: using double boiling until a film layer formed, 3: using double boiling for 1–2 min, 4: using direct heating until bubbles formed, and 5: using direct heating until a film layer formed.

4. Discussion

This study reported 1% of *Listeria* contamination in bulk tank milk and identified as *L. marthii*. The first isolation of this bacterium was in 2010 from the environmental samples [19]. This microorganism was classified to be “Listeria sensu strictu” as same as *L. monocytogenes*. The bacteria in this group share common characteristics. *L. marthii* was not globally distributed; however, the members in the “Listeria sensu strictu” group probably identified from healthy animals and in animal-origin food [20, 21]. The presence of this microorganism might imply that the environment of dairy farms in the study area was suitable for the survival of *Listeria* spp. *L. marthii* was reported to be of no risk to human and animal health. This species was just found in 2010. Its characteristic and severity should be concerned continually [20].

Listeria spp. contamination incidence in bulk tank milk was notably low in this study; however, the contamination occurrence differed depending on the location [8, 9]. *L. monocytogenes* appearance in raw milk was reported to spread from indigenous silage. Therefore, farmers should carefully consider hygiene practices and high-quality feeds [8, 22]. Improper practices during milking and postmilking on the farm level (especially for small-size farms) could cause differences in these occurrences [8, 14, 23]. Fresh milk from farms could be the source of the health risk from *Listeria* spp. if people consumed raw or unpasteurized milk [13, 14, 24].

Additionally, bacteria could remain in milk in cases of improper cooling, crosscontamination during handling, packaging, or storage [13, 25]. *Listeria* spp. can still exist in food even after refrigeration because the organism can

survive at low temperatures [25].

The leukocytes in milk would degrade if the raw or improper pasteurized milk were stored at 4°C for over 3 days. As a result of this deterioration, the number of heat-resistant *L. monocytogenes* could increase [26]. Even though the small-scale retailers were suspected of treating milk improperly, the result in this study was not to identify *Listeria* spp. contamination. The current result differed from the earlier articles. The authors in [15] reported approximately 18% *Listeria* spp. contamination in boiled milk. *Listeria* prevalence, even in pasteurized milk, ranged from 5 to 40% [27, 28]. Raw milk consumption or improper processing is the cause of the contamination in ready-to-drink milk [15, 29].

We cultured *Staphylococcus* spp. and *Bacillus* spp. at the retailer level. The *Bacillus* spp. were heat-resistant species that could be killed in a wide range of temperatures and time [30]. *Bacillus* spp. spores may resist heat treatment. Spores from some bacilli species can be isolated in milk, even after sterilization [30, 31]. Additionally, spores are commonly found in the environment, including soil, dust, air, and surfaces [32].

Staphylococcus spp. are normal microbiota on the human skin and mucous membranes [33]; however, its contamination possibly resulted in crosscontamination. Many studies have elucidated the contamination of *Staphylococcus* spp., especially *S. aureus*, and its severity on human health [17, 34, 35]. Pasteurization products were identified as the source of *S. aureus* contamination. The incidence of *S. aureus* contamination in pasteurization was 4% in China, while that in South Africa was high, up to 20% [36, 37].

Milk from the retailers needed to be concerned about safety, even with no *Listeria* spp. being shown from milk. The bacteria in milk might be the result of crosscontamination from the environment and human or improper processing steps. The sample collection was the limitation in this part. Milk from retailers was analyzed only one time in the different period of the sample collection at the farm level. The result was like the snapshot situation of *Listeria* contamination.

The location was associated with the milk volume of each lot that the retailer ordered and their pasteurization practices. The order volume is related to the number of dairy farms in the area. Retailers in Bangkok may have a larger stock than other locations because of the transport limitations. The magnitude of milk treatment was unexpectedly related to location. People with unawareness of the risk of milk-borne pathogens were twice as likely to contract abdominal illness than those who were aware [38].

Earlier articles showed the risk of consuming raw milk as well as the products produced from unpasteurized milk on human health [4, 8, 39]. With quality-uncontrolled processes, public perceptions of the product safety were certainly doubtful. Surprisingly, we had reason to say that the five milk-processing measures effectively destroyed *L. monocytogenes* because of no appearance of *L. monocytogenes*. The trial revealed that all processes reached the pasteurization condition including temperature and time; however, the temperature and processing time in this study might not be the same as the retailers because each person might consider stopping heating differently.

L. monocytogenes would be reduced to greater than $\log_{10} 6.9\,mL^{-1}$ after heating at 65.5°C for 15 s [26]. However, the authors in [40] reported that low temperature, long-time pasteurization at 63°C for 30 min or high temperature, and short time could make a negligible $\log_{10} 2\,mL^{-1}$ reduction. This variation implied the possibility of failure in milk treatment. The low pathogen load following treatment could also be attributed to the lack of detectable *Listeria* spp. in the culture.

In the experiment, we isolated *S. epidermidis, S. warneri,* and *E. coli,* which may result from crosscontamination. *Staphylococcus* spp. is usually found on the skin and surfaces [32]. *E. coli* is a human pathogen that causes gastrointestinal illness. Typically, the source is the host intestine; however, *E. coli* can persist on surfaces [1, 41]. Pasteurized milk was the source of up to 9% *E. coli* contamination [42]. Milk processed with inadequate measures presented approximately two-fold higher *E. coli* numbers than adequate treatment measures [43]. According to crosscontamination suspected, the processes after heating treatment were strictly hygienic [2, 17, 42, 44].

This study collected samples from farms and retailers only one time. The incidence of *Listeria* contamination might be underestimated. The retailers notice the bubble or film layer to complete the milk heating process that the temperature and time might highly deviate from person to person. The temperature and processing time in the trial

might differ from the retailers used. *Listeria monocytogenes* in the experiment were lastly interrupted by other bacteria from crosscontamination.

5. Conclusions

In conclusion, on a farm level, which was the main source of raw milk, we observed 1% *Listeria* incidence, whereas no identification of *Listeria* spp. was observed on the retailer level and postprocessed milk in the trial. The presence of *Listeria* spp. at the farm level warns people about the risk of raw or improperly treated milk to human health, even low incidence of *Listeria*. Milk should be processed following the method of pasteurization. The retailers should prevent crosscontamination during other steps after treatment as well. Finally, the responsible organizations should incessantly educate the milk retailers in adequate protocols as well as routinely check their product safety.

Disclosure

The preprint is readily available from https://www.researchsquare.com/article/rs-1998662/v1 [45].

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DETAIL

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Retracted: A Comparative Analysis of Blockchain in Enhancing the Drug Traceability in Edible Foods Using Multiple Regression Analysis

Quality Journal of Food.

TEKS LENGKAP

This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article’s content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.
The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] K. Suresh Kumar, V. K. Nassa, D. Uike, A. kalra, A. K. Sahu, V. A. Athavale, V. Saravanan, "A Comparative Analysis of Blockchain in Enhancing the Drug Traceability in Edible Foods Using Multiple Regression Analysis," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/1689913, 2022.

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Pesticide Residues and Associated Public Health Risks in Vegetables from Irrigated Farms Adjacent to Rift Valley Lake Ziway, Ethiopia

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ABSTRAK (ENGLISH)

The overuse of pesticides has resulted in the accumulation of harmful residues in vegetables, which requires monitoring to assess the risks to human health. This article presents the levels of 35 pesticide residues in 15 composite vegetable samples from irrigated farmlands adjacent to Lake Ziway, Ethiopia, using the QuEChERS extraction method (Quick, Easy, Cheap, Effective, Rugged, and Safe) and then analyzes them using GC-MS. The study also estimated the health risks associated with the consumption of contaminated vegetables in children and adults, including carcinogenic and noncarcinogenic risks. The predominant pesticide residues found in tomatoes were α-endosulfan (0.58 mg/kg), β-BHC (0.04 mg/kg), heptachlor (0.02 mg/kg), and malathion (0.03 mg/kg), all of which were above the safety limits. Similarly, the mean concentration of heptachlor epoxide (0.04 mg/kg) and propargite (0.11 mg/kg) was higher than the allowed levels of the safety limits for onions. The concentration of pesticide residues detected in 10.6% and 7.9% of tomato and onion samples was above the maximum residual limits of the European Commission (EU-MRLs), respectively. Noncarcinogenic health risk estimates show that onion heptachlor epoxide had THQ>1, indicating the possibility of systemic health risk in both adult and child consumers. The carcinogenic health risk (CHR) showed that heptachlor epoxide in adults and children and only heptachlor in children had CHR>acceptable limit (10^{-4}) for tomato and onion. Therefore, it is critical to raise awareness among stakeholders while simultaneously implementing sound monitoring policy actions to protect the ecosystem and the health of the population in the study area and beyond.

TEKS LENGKAP

1. Introduction

Pesticides are natural or synthetic substances that are often used to control plant pests, weeds, and diseases [1, 2]. They are critical in modern agriculture; without them, up to 50% of crops in tropical warm-climate zones could be destroyed [3]. The agroclimatic conditions of the Ethiopian Rift Valley, particularly around Lake Ziway, are suitable for the production of fruits and vegetables; however, the area is highly affected by the infestation of pests and diseases during vegetable production and storage, which significantly reduces the yield and quality of agricultural products (Pesticide Action Nexus Ethiopia [4]). Therefore, the application of pesticides is mandatory in modern agriculture because it significantly reduces yield losses and maintains the quality of fruit and vegetables by controlling the infestation of pests and diseases [5].

Pesticide application is more severe in the Ethiopian Rift Valley than in other Ethiopian areas. Several studies have found that farmers overuse pesticides in their vegetable fields every other day, or even every day, due to a lack of knowledge and the lack of available sustainable alternatives [6–9]. Furthermore, reports revealed widespread use of pesticides in Ethiopia's Central Rift Valley and also poor pesticide management during storage, application, and empty container handling [8–10]. Furthermore, after pesticide application, farmers and farm workers in the region reported symptoms of acute poisoning: headache, nausea, and vomiting, in addition to using empty containers for food and beverage storage [4].

Pesticide residues in fresh fruits and vegetables pose serious health risks to consumers [11]. As a result, the identification and quantification of pesticides in the food matrix is becoming a public concern [12]. Although a previous study [13] found metalaxyl, λ-cyhalothrin, *p*,*p*′-DDT, *p*,*p*′-DDE, and α and β-endosulfan pesticides in vegetables grown on irrigated farmland surrounding Lake Ziway, only profenofos residues exceeded EU MRL in tomato and onion, with widespread cultivation and sale in nearby cities, including the capital, Addis Ababa. However, no research has been conducted on the health risks associated with the consumption of pesticide-contaminated vegetables on the irrigated farmlands of the Ethiopian Rift Valley, particularly in the Ziway district. Thus, more research is needed to determine the actual scenario of pesticide residues present in vegetables grown by irrigation in Ethiopia's Rift Valley's Ziway district, as well as the risks to consumer health. The current study aims to determine the concentration of 35 pesticide residues in tomatoes and onions grown on irrigated farmlands adjacent to Lake Ziway. The pesticides chosen for this study are commonly applied to manage pests at various stages of vegetable production in irrigated farmlands near Lake Ziway, in particular, those pesticides formulated by Adami Tulu Pesticide Processing Share Company located in the Central Rift Valley of Ethiopia [5]. This study raises public awareness and assists policymakers in taking the necessary steps to reduce human health risks.

2. Materials and Methods

2.1. Description of the Study Area

The study was carried out on three irrigated farmlands located between Meki and Ziway in three villages: Abenea-Girmama, Wellibulla, and Girrissa, all of which are located near the western side of Lake Ziway in Ethiopia's Central Rift Valley (CRV) basin. The location is between latitudes 07°57′N and 08°07′N and longitudes 038°43′E and 038°48′E, with an elevation of 1643–1655 meters above sea level. It is a notable vegetable-growing zone located 135 kilometers south of Addis Ababa in the Oromia regional state of the East Shewa Zone.

2.2. Study Design and Period

In a cross-sectional laboratory-based study, the concentration and type of pesticide residues in tomato (*Lycopersicon esculentum* L.) and onion (*Allium cepa* var. *aggregatum*) from three irrigated farmlands adjacent to Lake Ziway were examined. The samples were collected during the rainy season in late August 2021. It is worth mentioning that pesticide contamination is considerably higher during the wet season compared to the dry season [14]. As described by the authors, this is due to the fact that pesticides from various sources can be washed into existing ones, leading to increased contamination levels.

2.3. Sampling Site Selection and Sample Collection

Three sample sites (*S*1–*S*3) were chosen from three villages: Abenea-Girmama, Wellibulla, and Girrissa, based on intensive and extensive irrigation activities, proximity to pesticide stores, and proximity to a water source (Lake Ziway). Additionally, each site is approximately 1 hectare in size and has been in cultivation for more than 20 years. Tomato (*Lycopersicon esculentum* L) ($n=9$) and onion (*Allium cepa* var. *aggregatum*) ($n=6$, because the onion was fully harvested at the third site) samples were collected with the permission of the farmers of the three irrigated farms. From each site, seven subsamples were collected in triplicate. The sample (roughly 1 kg for each type) was taken using the zigzag method with 1 m apart and homogenized to represent the bulk sample. Fifteen composite vegetable samples (tomatoes and onions) were collected in triplicate from three and two sampling sites, respectively. The sample was individually packed in ziplock polyethylene bags, labelled and brought to the laboratory in an insulated icebox, and then stored in the dark at 4°C until further analysis.

2.4. Chemicals and Reagents Used

All standards (99% purity) and chemicals and solvents (HPLC grade 99.9%) were obtained from Sigma-Aldrich (St. Louis, USA), including ethyl acetate (EtOAc), acetonitrile (MeCN), and glacial acetic acid. BDH (British Drug Houses) also offered sodium acetate (NaAc) (purity 99%), magnesium sulfate, and primary secondary amine (PSA).

2.5. Sample Preparation, Extraction, and Clean-Up of Samples

2.5.1. Sample Preparation

Each tomato and onion sample was chopped using a stainless steel knife and then blended to obtain a homogenous composite. After each sample was chopped, the chopping board and blender were washed to avoid cross-contamination. The homogenous composite samples were stored in labelled bags and kept refrigerated at 4°C until further analysis.

2.5.2. Extraction and Clean-Up of Samples

The Quick, Easy, Cheap, Effective, Rugged, and Safe extraction method (QuEChERS) was used for the extraction of pesticides in vegetable samples as indicated in the Association of Official Analytical Chemists (AOAC) Method 2007.01 with slight modifications [15]. Method optimization with its basic steps of the experimental procedure was done as described in Romniou et al. [16]. 15 g of homogenized sample matrices weighed in a 50 ml Teflon tube and 15 ml of acetonitrile (MeCN) containing 1% acetic acid, 6 g of anhydrous $MgSO_4$, and 1.5 g of NaAc were added and the sample was shaken for 1 minute with Vortex (IKA® Vortex Geniw3) to facilitate contact between the solvent and the sample before being centrifuged at 4000 rpm for 5 minutes (Eppendorf 5804 R, Hamburg, Germany). To clean the extract, the upper organic layer 4 ml was taken into a dispersive solid phase extraction tube (d-SPE) containing 150 mg $MgSO_4$ and 50 mg PSA. The extracted sample was agitated for 30 seconds before being centrifuged for 5 minutes at 4000 rpm. A 4 ml supernatant was filtered through a 0.45 μm PTFE filter (polytetrafluoroethylene polymer) and transferred to clean GC vials for further analysis.

2.6. Quality Control and Pesticide Instrumental Analysis Method

2.6.1. Pesticide Standard Solution Preparation Methods

Standard pesticide stock solutions of the 35 target pesticides were prepared separately in acetonitrile (MeCN) using a method of Nisha et al. [17] at a concentration of 1000 mg/L. Then, working solutions of 0.1, 0.2, 0.5, 1, 2, 3, and 5 mg/L in MeCN were prepared. The matrix-matched standard for the preparation of the calibration curve was made by adding multiple standard working solutions to the blank extracts of both matrices separately [17] and kept in the dark at −20°C.

2.6.2. Method Validation

To create calibration curves for peak area versus pesticide concentrations, standard working solutions were made by dissolving required volumes of stock solution in acetone (9 : 1, v/v). The analytical performance of these solutions was tested for linearity (expressed as a correlation coefficient), accuracy (represented as the relative standard deviation of repeatability), and mean recovery/reliability (as a measure of trueness). Table 1 summarizes the results of these tests. Before conducting the real analysis, we validated the method as described in [18]. The validation results met the SANTE/12682/2019 guidelines with LOQ set at 0.010 mg/kg for all analytes.

Table 1

Method validation parameter results in vegetable residue analysis.

Pesticides	%Recovery	%RSD	Linearity (r2)	LOD (μg/kg)	LOQ (μg/kg)
Aldrin	101.2	11.6	0.9952	0.008	0.025
Alpha-BHC	89.0	1.53	0.9986	0.005	0.014
Alpha-endosulfan	100.5	2.7	0.9978	0.006	0.019
Bendiocarb	98.2	6.73	0.9945	0.018	0.053
Beta-BHC	**112.8**	1.63	0.999	0.004	0.011
Beta-endosulfan	104.4	11.2	0.9972	0.006	0.017
Bromophos-ethyl	89.4	5.31	0.9981	0.002	0.006
Chlordane	106.3	1.92	0.9977	0.006	0.017
Chlorpyrifos-methyl	85.3	5.59	0.9987	0.002	0.005
Cyfluthrin	80.6	3.14	0.9982	0.005	0.015
Cypermethrin (zeta)	96.4	4.13	0.9927	0.018	0.0562
p,*p*′-DDD	106.3	11.2	0.9954	0.008	0.245
p,*p*′-DDE	**75.8**	10.23	0.9961	0.008	0.025
p,*p*′-DDT	97.6	6.73	0.9937	0.009	0.029
Dieldrin	92.0	6.65	0.9977	0.009	0.02812
Diazinon	94.3	4.49	0.998	0.003	0.009
Dichlobenil	91.2	4.13	0.9972	0.004	0.011
Endrin	100.4	3.41	0.9995	0.006	0.01945
Ethion	94.7	5.96	0.9962	0.003	0.008
Famphur	101.1	2.11	0.9989	0.002	0.006

Fenitrothion	110.9	5.76	0.9982	0.003	0.009
Fenthion	104.2	5.12	0.9987	0.002	0.006
Heptachlor	80.6	10.26	0.9978	0.006	0.017
Heptachlor epoxide	108.9	6.97	0.9983	0.005	0.015
Hexachlorobenzene	80.2	10.62	0.9906	0.006	0.018
Indoxacarb	94.7	14.64	0.9969	0.004	0.012
Lindane	106.2	9.45	0.9966	0.014	0.04159
Malathion	104.8	4.48	0.9974	0.013	0.03799
Methoxychlor	103.2	8.26	0.9969	0.005	0.016
Parathion	97.7	10.3	0.996	0.004	0.013
Piperonyl butoxide	95.9	8.24	0.9963	0.007	0.02203
Profenofos	91.7	8.44	0.9991	0.002	0.006
Propargite	83.7	6.38	0.9963	0.007	0.02203
Propoxur	93.1	8.42	0.9987	0.002	0.007
Thionazin	76.9	13.83	0.9957	0.004	0.013

Values in bold indicate the lower and the upper recovery.

2.6.3. Limits of Detection (LOD) and Determining the Quantity

The LOD and LOQ were calculated using the International Conference on Harmonization [19] suggested guidelines (LOD = 3.3 × δ/m and LOQ = 10 × δ/m), based on the standard deviation of the response and the slope of the calibration curves, where m is the slope of the calibration curve and δ is the standard deviation. The standard deviation of the result was used as the standard deviation of the *y*-intercepts of the regression lines (Table 1).

2.6.4. Instrumental Pesticide Analysis

Gas chromatography-mass spectrometry (GC-MS) (Agilent 7890B Turbo MSD 5977A, Agilent, Santa Clara, USA) was used to determine levels of pesticide residue. The GC-MS system was equipped with triple quadruple MS operated in electron impact (EI) mode, Triple-Axis HED EM employed as detector, and an HP-5 MS 30 m × 0.32 mm × 0.25 μm column (Agilent, Santa Clara, USA). The injection volume was 2 μL in splitless mode at 180°C, with helium used as a carrier gas at a flow rate of 1.2 ml/min. The oven temperature started at 60°C and remained at this temperature for 1 min, increasing to 120°C at 40°C min^{-1} for a ramp rate of 2.5 min and then to 310°C at a ramp rate of 5°C min^{-1}, holding at 300°C for 40.5 minutes. All instrumental analyses were performed at the Bless Agri-Food Laboratory, which is an approved laboratory of the Ethiopian National Accreditation Office (ENAO) (FA: T0030).

2.7. Potential Risk Assessment Method

A previously described model proposed by Fakhri et al. [20] and USEPA [21] was used to assess the carcinogenic and noncarcinogenic risks of identifying pesticides to adults and children in the monitored region.

2.7.1. Noncarcinogenic Risk (NCR)/Hazard Quotient

The target hazard quotient (THQ) and the hazard index (HI) were used to evaluate noncarcinogenic health risks based on the results of the pesticide analysis and the exposure assumptions, according to the US Environmental Protection Agency [22]. THQ is calculated by comparing the chronic daily intake (CDI) with the reference dose (RFD) [23] using the following equation:(1)THQ=CDIRFD,where THQ is the target hazard quotient, CDI is the chronic daily intake, and RFD is the oral reference dose obtained from the integrated risk information system, and equation (2) was used to calculate the CDI of pesticide-contaminated vegetables [20, 24].(2)CDI=CvegxIRixEFixEDi BWixAT,where Cveg is the concentration of pesticides (mg/kg) in vegetables (mean and 95% confidence interval detected concentrations); IR is the ingestion rate of vegetable food for adults and children, which is 240 g/person/day and 160 g/person/day, respectively, according to Ethiopian Food Based Dietary Guidelines [25]; EF_i is the frequency of exposure (from 365 days/year when consuming vegetables seven times a week to 52 days/year for people who eat vegetables once a week) according to the Food and Agriculture Organization (FAO, 2011); ED_i, the duration of exposure (for children and adults is 6 and 65 years, respectively) (FAO, 2011) [26]; BW_i, the default average body weight used by FAO/WHO (for children and adults is between 15 kg and 60 kg, respectively); AT is the average exposure time for noncarcinogens (365 days/year × ED) (for children and adults are 2190 and 23,725 days, respectively) (Kumar et al., 2013) [27].

The HI of a pesticide mixture was calculated by the sum of THQ for each component (equation (3)). According to the USEPA [28], HI 1 indicates a chance of noncancer effects.(3)HI=∑i=1nTHQi.

2.7.2. Target Carcinogenic Risk (TCR)

The possible target cancer risk to the population due to the intake of specific potentially cancer-causing pesticides was assessed using equation (4), and the TCR was estimated for adults and children based on their lifetime exposure to pesticides in this study [29].(4)TCR=CDIxCSFxADAF.

In equation (4), CSF is the cancer slope factor for carcinogenic pesticides in vegetables (mg/kg/day), the probability that a single substance increases the risk of cancer through an oral exposure pathway, and ADAF is an age-dependent adjustment factor (for children, it is 1, and for adults, it is 3) [22]. CSF (mg/kg/d) for targeted pesticides: heptachlor epoxide = 9.1; heptachlor = 4.5; hexachlorobenzene = 1.6; and not available for *α*-endosulfan, malathion, and propargite. If TCR −6, cancer risks are considered negligible; however, if CR > 10^{-4}, cancer risks are considered unacceptable by most international regulatory agencies [29, 30]. Acceptable risk limits for carcinogens range from 10^{-4} (where a person's lifetime risk of developing cancer is 1 in 10,000) to 10^{-6} (risk of developing cancer over a human lifetime is 1 in 1,000,000) [30].

2.8. Data Analysis

Data were analyzed using SPSS software version 26.0. One-way analysis of variance (ANOVA) was used to compare site-wise differences in mean values of pesticide residues at $\alpha = 0.05$ level of significance. When significant differences were obtained, means were tested using Tukey's multiple comparison test at $\alpha = 0.05$. The result was statistically significant when the probability was less than 0.05 ($P<0.05$). A one-sample *t*-test was used to assess the statistical significance of the sample pesticide residues with respect to the trading standards established by international agencies (e.g., Codex Alimentarius and the EU) to ensure that residues are regulated in the global food trade.

3. Results and Discussion

3.1. Method Validation Result

Ensuring the safety of pesticide use requires analyzing residues in food particularly vegetables, which pose a significant challenge to public health [17]. Table 1 shows that the validation results satisfied the SANTE/12682/2019 guidelines. The calibration curves for a collection of 35 pesticide standards, including isomers and degradation products, have a correlation coefficient (r2) greater than 0.9906. The average recovery for both vegetables was between 75 and 113%, which was within the analytical range permitted [31]. The LOD and LOQ for the pesticides

tested ranged from 0.002 to 0.018 μg/kg and 0.005 to 0.245 μg/kg, respectively. The average relative standard deviation (% RSD) is less than 10%. These results indicate that the technique is accurate since most of the collected pesticides were within the allowed analytical range (70–120%) and precise, as the percentage RSD

3.2. Pesticide Residue Concentration in Vegetables

Following validation of the QuEChERS method, the concentrations of pesticide residues in fifteen composite samples of tomatoes and onions were determined. The results show that 22 (62.9%) pesticide residues were detected in both vegetables, with 21 (60%) and 20 (57.1%) pesticide residues detected in tomato and onion, respectively (Tables 2 and 3), while the concentrations of the remaining 14 pesticides in tomato and 15 in onion were found to be below the detection limit. Tomatoes contain 8 different types of pesticides, which are organochlorines (α-endosulfan, chlordane,4-4(DDT, DDD, and DDE), dieldrin, lindane, and methoxychlor), 2 types of carbamates (bendiocarb and indoxacarb), 1 type of benzodioxole (pipronyl butoxide), and 3 types of pyrethroids (cyfluthrin, cypermethrin, and deltamethrin). Similarly, onions contain 10 different types of pesticides, which are aldrin, β-endosulfan, chlordane,4-4(DDT, DDD, and DDE), dieldrin, lindane, hexachlorobenzene, and methoxychlor; 1 type of carbamates (bendiocarb); 1 type of benzodioxole (pipronyl butoxide); and 3 types of pyrethroids (cyfluthrin, cypermethrin, and deltamethrin).

Table 2

Concentration (mg/kg) of pesticide residues in tomato samples collected from irrigated farmlands in the vicinity of Lake Ziway (n=9).

Category	Pesticides detected	Lowest value	Highest value	Mean ± SD	EU-MRL	Mean-difference(mean-EU-MRL)	P=value. sig. (2-tailed)
OC	α-BHC	0.002	0.005	0.003 ± 0.001	0.01	−0.0066	≤0.001
β-BHC	0.0001	**0.04**	**0.024 ± 0.005**	0.01	0.01 39	**0.034**	Heptachlor epoxide
0.0001	0.01	0.005 ± 0.003	0.01	−0.0063	0.02 4	Heptachlor	0.01
0.02	**0.012 ± 0.005**	0.01	0.0017	0.468	α-Endo sulfa n	0.003	**0.63**
0.331 ± 0.220	0.05	0.3225	**0.003**	Aldrin	0.00 04	0.01	0.003 ± 0.003
0.01	−0.0069	0.015	Hexachlor obenzene	0.000	0.**15 2**	0.001 ± 0.0005	0.01
−0.009	0.025	-					

OP	Bromophos-ethyl	0.000	0.003	0.001 ± 0.001	0.01	−0.0086	≤0.001
Chlorpyrifos-methyl	0.0002	0.003	0.001 ± 0.0007	0.01	−0.0089	≤0.001	Diazinon
0.0000	0.001	0.0004 ± 0.0004	0.01	−0.0095	≤0.001	Ethion	0.0001
0.001	0.001 ± 0.0004	0.01	−0.0092	≤0.001	Famphur	0.0006	0.003
0.001 ± 0.001	0.01	−0.0085	≤0.001	Fenitrothion	0.000	0.003	0.001 ± 0.001
0.01	−0.0090	≤0.001	Fenthion	0.001	0.003	0.002 ± 0.001	0.01
−0.0081	≤0.001	Malathion	0.008	**0.048**	**0.02 ± 0.02**	0.02	0.0112
0.304	Parathion	0.0005	0.004	0.001 ± 0.0001	0.05	−0.0087	≤0.001
Profenofos	0.0004	0.002	0.001 ± 0.0006	10	−9.9996	≤0.001	Thionazin
0.0002	0.0005	0.0002 ± 0.0001	0.01	−0.0099	0.014	-	
C	Propoxur	0.01	0.038	0.023 ± 0.00001	0.05	−0.0275	≤0.001
–							
OS	Propargite	0.001	**0.792**	**0.032 ± 0.012**	0.01	0.0221	0.162
–							
NA	Dichlobenil	0.0002	0.002	0.001 ± 0.0003	0.01	−0.0089	≤0.001

Note. OC = organochlorine; OP = organophosphate; *C* = carbamate; OS = organosulfite; NA = not assigned. Values in bold indicate the conc. above acceptable EU-MRL.

Table 3

Concentration (mg/kg) of pesticide residues in onion samples collected from irrigated farmlands in the vicinity of

Lake Ziway (n=6).

Category	Pesticides detected	Lowest value	Highest value	Mean ± SD	EU-MRL	Mean-difference(mean-EU-MRL)	P=valuesig. (2 tailed)
OC	α-BHC	0.002	0.005	0.004 ± 0.001	0.01	−0.0065	≤0.001
β-BHC	0.0001	0.01	0.001 ± 0.002	0.01	−0.0086	≤0.001	Hepta-epoxide
0.006	**0.038**	**0.023 ± 0.014**	**0.01**	**0.0132**	0.069	Heptachlor	0.0004
0.01	0.003 ± 0.004	0.01	−0.0017	0.083	α-Endosulfan	0.001	0.003
0.002 ± 0.001	0.1	−0.0473	≤0.001	-			
OP	Bromophos-ethyl	0.00004	0.003	0.0004 ± 0.0004	0.01	−0.0097	≤0.001
Chlorpyrifos-methyl	0.0002	0.001	0.0005 ± 0.0002	0.01	−0.0095	≤0.001	Diazinon
0.00004	0.0012	0.0001 ± 0.001	0.05	−0.0099	0.005	Ethion	0.0004
0.001	0.0007 ± 0.0004	0.02	−0.0093	0.017	Famphur	0.0001	0.001
0.0009 ± 0.0003	0.01	−0.0091	≤0.001	Fenitrothion	0.0002	0.0005	0.0003 ± 0.0001
0.01	−0.0097	≤0.001	Fenthion	0.001	0.003	0.002 ± 0.0005	0.01
−0.0082	≤0.001	Malathion	0.001	**0.02**	**0.016 + 0.007**	0.02	**0.0059**

0.440	Parathion	0.001	0.001	0.001 ± 0.0003	0.05	−0.0091	≤0.001
Profenofos	0.0002	0.001	0.001 ± 0.0002	0.02	−0.0194	≤0.001	Thionazin
0.00001	0.001	0.0003 ± 0.0001	0.01	−0.0097	≤0.001	-	
C	Indoxacarb	0.0003	0.004	0.003 ± 0.002	0.02	−0.0170	0.006
Propoxur	0.01	0.025	0.02 ± 0.01	0.05	−0.0345	≤0.001	·
OS	Propargite	**0.0004**	**0.112**	**0.042 ± 0.025**	0.01	0.0321	0.295
–							
NA	Dichlobenil	0.0001	0.001	0.001 ± 0.0003	0.01	−0.0091	≤0.001

Note. OC = organochlorine; OP = organophosphate; *C* = carbamate; OS = organosulfite; NA = not assigned. Values in bold indicate the conc. above acceptable EU-MRL.

3.2.1. Pesticide Residue Concentrations in Tomato

As shown in Table 2, five pesticide residues in tomatoes exceeded the default EU-MRL 0.01 mg/kg standards. Only food items with pesticide residues exceeding the default EU-MRL of 0.01 mg/kg were considered for substantial pollution and food safety concerns. The mean residue of *β*-BHC in tomatoes was 0.024 mg/kg, which was twice that of EU-MRL (0.01 mg/kg) and Codex Alimentarius (FAO/WHO) (0.01 mg/kg). A sample *t*-test revealed statistically significant differences ($P<0.05$) between the mean *β*-BHC content of tomatoes and the Codex Alimentarius and EU-MRL standards (see Table 2). As a result, eating tomatoes in the current study area may be unsafe due to *β*-BHC contamination.

The mean concentration of heptachlor was found to be higher than EU-MRL (0.01 mg/kg), while the difference was not statistically significant ($P>0.05$) (Table 2). This indicates that the average heptachlor concentration was close to the acceptable standard of EU-MRL. According to the Agency for Toxic Substances and Disease Registry [32], heptachlor can accumulate in the soil and be passed on to vegetables. The mean residue concentrations of *α*-endosulfan (0.331 mg/kg) were six times higher than the EU-MRL limit of 0.05 mg/kg but less than the FAO/WHO standard of 0.5 mg/kg. The difference in mean concentrations of *α*-endosulfan and EU-MRL was statistically significant ($P<0.05$). Therefore, the tomato in the current study may be unsafe to consume because of *α*-endosulfan contamination. In this particular study, the highest concentration of *α*-endosulfan recorded was similar to the findings of Sheikh et al. [37] in tomato samples from the Pakistani Sindh market, where the values ranged from null to 0.68 mg/kg. The average concentration of this study is also consistent with the results of Essumang et al. [38] from Ghana (0.3 mg/kg) and Mahugija et al. [39] from Tanzania (0.3 mg/kg). However, the current finding was higher than the results of López-Dávila et al. [40] (0.01 mg/kg) from Cuba, Oyeyiola et al. [41] (0.0016 mg/kg) from Nigeria, and Loha et al. [13] (0.006 mg/kg) from Ethiopia. The difference in results may be due to the difference in research settings. Nonetheless, the high concentration of endosulfan in tomatoes in this study could be attributed to the hyper-accumulating nature of tomatoes as stated by Kumar et al. [27].

Malathion residues were in concentrations ranging from less than the detection limit to 0.048 mg/kg (Table 2). The average recorded malathion concentration was 0.02 mg/kg, comparable to EU-MRL but less than FAO/WHO Codex Alimentarius (0.5 mg/kg). Consuming tomatoes according to the FAO/WHO standard could be safe with regard to malathion residues. This finding was lower than the 0.33 mg/kg reported by Fakhri et al. [20] from Bangladesh and comparable to the data obtained by Akoto et al. [42] from Ghana (0.027 ± 0.021 mg/kg). The residual concentrations of propargite were determined to be 0.154 mg/kg, higher than the EU-MRL limit of 0.01 mg/kg, but less than the Codex Alimentarius standard [26] of 2 mg/kg. According to FAO/WHO standards, the tomatoes at the current study site were safe in terms of contamination by propargite residues. This result was comparable to the 0.06 mg/kg reported by Marrez et al. [43] from Egypt. Generally, the order of pesticide residues in tomatoes was as follows: *α*-endosulfan > propargite > *β*-BHC > malathion > heptachlor (Table 2).

3.2.2. Pesticide Residue Concentration in Onion

As shown in Table 3, the present results indicated that the levels of heptachlor epoxide (0.023 ± 0.014 mg/kg) and propargite (0.042 ± 0.025 mg/kg) in onions were higher than the maximum residue limit (MRL) of (0.01 mg/kg) suggested by the European Union, while the remaining were detected below the EU-MRL standards. The concentration of identified heptachlor epoxide and propargite residues in onion exceeded the EU-MRL twice and five times, respectively. But the difference was not statistically significant ($P>0.05$). As a result, onion in the present study area was safe for human consumption with respect to heptachlor epoxide and propargite residue contamination.

3.2.3. Pesticide Residual Concentrations in Vegetables Compared by Sites

The pesticide residue concentrations in both vegetables across sites are summarized in Table 4. In tomatoes, the pesticide residue loads of *β*-BHC, *α*-endosulfan, and heptachlor decreased in the following order: Site 1 (Abenea-Girmama) > Site 3 (Girrissa) > Site 2 (Wellibulla). Similarly, the average concentration of malathion in tomatoes was classified as follows: Site 3 > Site 1 > Site 2. In onion, the sequence reversed for heptachlor and malathion: Site 2 > Site 1. Propargite concentrations in tomatoes are found in the following decreasing order: Site 2 > Site 3 > Site 1; similarly, for onion: Site 2 dominates Site 1. The concentration of *α*-BHC in onions was summarized in the following decreasing order: Site 1 > Site 2, but for the heptachlor epoxide, the sequence reversed: Site 2 > Site 1.

Table 4

Pesticide concentrations (mg/kg, wet weight) in tomato and onion from the study sites (mean ± SD, *n* = 15).

Vegetables	Pesticides	Site 1 (Abenea-Girmama)	Site 2 (Wellibulla)	Site 3 (Girrissa)	MRL (mg/kg)	
CA	EU	Tomato	*β*-BHC	**0.037 ± 0.006[a]**	**0.016 ± 0.011[c]**	**0.026 ± 0.01[b]**
0.01	0.01	Heptachlor	**0.015 ± 0.003[a]**	BDL	0.01 ± 0.004[b]	NA
0.01	*α*-Endosulfan	**0.575 ± 0.08[a]**	0.264 ± 0.12[c]	0.346 ± 0.248[b]	0.5	0.05
Malathion	0.01 ± 0.001[b]	BDL	**0.031 ± 0.02[a]**	0.5	0.02	Propargite
BDL	**0.053 ± 0.004[a]**	**0.012 ± 0.002[b]**	2	0.01	·	

Onion	*α*-BHC	0.01 ± 0.005[a]	0.004 ± 0.002[b]	*	0.01	0.01
Heptachlor epoxide	0.01 ± 0.004[b]	**0.04 + 0.004[a]**	*	NA	0.01	Heptachlor
BDL	0.01 ± 0.004	*	NA	0.01	Malathion	BDL
0.02 ± 0.01	*	1	0.02	Propargite	**0.04 ± 0.01[b]**	**0.11 ± 0.001[a]**

Note. Mean values with different superscript letters in a row are different from each other. Values in bold are those above the maximum residue limit (MRL) in the diet of humans according to the EU-MRL standards and CA = Codex Alimentarius. BDL = below detection limit. Values in bold indicate values above the EU-MRL. * = sample from 3rd site was not available.

Although pesticide residue concentrations vary between sites, one-way ANOVA did not reveal statistically significant difference ($P>0.05$) among sites in mean pesticide residue concentrations in tomato and onion samples from these sites (Table 4). According to direct conversations with farmers in all areas, all get their seedlings from the same company called Flora Vege and use the same pesticide from vendors found in the market area, which is the most obvious explanation for this finding.

3.3. Comparison of Pesticide Residue with the MRL Set by International Authorities

The pesticide residues in tomato and onion were compared with the corresponding MRLs of each pesticide and are indicated in Tables 5 and 6. Ethiopia does not have a national MRL for any pesticide but relies on Codex Alimentarius as a member country [44]. However, due to the lack of available data on the present pesticides tested, we consider the MRL set by the EU. The current study found residues in 71.4% and 73.8% of the tomato and onion samples, respectively. Only 10.6% and 7.9% of the tomato and onion samples, respectively, exceeded the EU's maximum residue limit (MRL) (see Tables 5 and 6). According to this study, the use of pesticides in the study area is excessive. Additionally, the detected pesticides that exceeded the maximum residue limit (MRL) were outdated. The levels of heptachlor and heptachlor epoxide surpassed the MRL banned by the Stockholm Convention. Despite being a signatory to the Stockholm Agreement, Ethiopia continues to use obsolete chemicals in agriculture. The report is in line with the UNEP report of 2019. Although Ethiopia has ratified the Basel, Stockholm, and Rotterdam Conventions, the laws and regulations regarding hazardous chemicals and environmental protection are still insufficient to prevent the unauthorized use of outdated chemicals. Overall, pesticide concentrations that exceed the maximum permissible limit (MPL) may have acute or chronic health consequences if consumed regularly. Pesticides, for example, have been documented to cause nausea, dizziness, vomiting, migraines, stomach discomfort, rashes, and even death [45]. Pesticides have a wide range of long-term health effects, including respiratory and cognitive difficulties, cancer, diabetes, cardiovascular disease, neurological diseases such as Parkinson's disease, autism, infertility, congenital birth defects, and DNA damage [46–45].

Table 5

Pesticide residue concentration (*μ*g/kg) in tomato samples collected from irrigated farmlands in the vicinity of Lake Ziway.

Site	*α*-BHC	*β*-BHC	H-epoxy	H-chlor	*α*-Endo	Aldrin	HCB	Bro-E	Chlor	Diazin	Ethion	Famphur	Fenitrothion	Fenthion

*S*1 (Abunea-Germama)	*R*1	1.75	BDL	7.32	14.02	BDL	BDL	1.01	1.98	1.66	BDL	BDL	2.60	2.86	2.71
*R*2	5.04	42.06	BDL	12.99	518.49	BDL	1.70	0.38	0.69	BDL	0.65	BDL	0.16	1.70	*R*3
3.75	32.87	3.41	18.22	630.52	BDL	BDL	0.80	1.12	BDL	1.14	1.26	0.74	1.95		
*S*2 (Wellibulla)	*R*1	2.24	BDL	BDL	BDL	189.04	0.39	BDL	3.29	2.60	1.19	BDL	2.90	2.39	2.70
*R*2	3.91	23.71	BDL	BDL	402.77	1.20	BDL	1.05	0.34	0.07	BDL	0.79	0.06	1.33	*R*3
2.90	7.79	BDL	BDL	200.34	BDL	BDL	0.96	1.12	0.05	BDL	1.47	0.69	1.93		
*S*3 Girrissa	*R*1	2.13	20.81	BDL	7.27	258.49	5.87	BDL	0.14	0.51	0.48	BDL	0.65	0.18	1.43
*R*2	3.91	33.84	0.12	BDL	626.12	4.87	BDL	0.06	0.20	BDL	0.21	1.01	0.04	1.44	*R*3
5.24	6.26	4.12	6.86	153.99	BDL	BDL	1.66	1.04	0.44	1.12	1.65	1.69	2.14		
EU-MRL		10	10	10	10	50	10	10	10	10	10	10	10	10	10
Total detected		9	8	5	5	9	5	2	9	9	5	4	8	9	9
% (+ve) sample		100	88.9	55.6	55.6	100.0	55.6	22.2	100	100	55.6	44.4	88.9	100	100
Sample>EU-MRL		0	5	0	3	8	0	0	0	0	0	0	0	0	0
% (>EU-MRL)		0	55.6	0.0	33.3	88.9	0.0	0	0	0	0	0	0	0	0

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Site	Malathion	Parathion	Profenofos	Thionazin	Propargite	Propoxur	Dichlobenil

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*S*1 (Abenea-Girmama)	*R*1	14.156	3.62	BDL	0.02	BDL	8.75	0.02
*R*2	BDL	1.52	0.53	0.46	BDL	30.88	1.34	*R*3
7.6837		1.16	0.58	BDL	BDL	14.18	1.24	
*S*2 (Wellibulla)	*R*1	BDL	0.51	1.71	BDL	50.15	16.60	BDL
*R*2	BDL	0.56	1.42	BDL	BDL	36.05	BDL	*R*3
BDL		0.45	0.37	BDL	55.35	17.79	BDL	
*S*3 (Girrissa)	*R*1	47.75	1.16	0.77	BDL	10.02	16.53	1.26
*R*2	15.16	0.72	1.64	BDL	BDL	38.46	0.51	*R*3
BDL		1.66	ND	BDL	13.01	23.43	2.36	
EU-MRL		20	50	10000	10	10	50	10
Total detected		4	9	7	2	4	9	6
Sample>EU-MRL		1	0	0	0	4	0	0
% (+ve) sample		44.44	100	77.78	22.22	44.44	100.00	66.67
% (>EU-MRL)		11.11	0	0.00	0.00	44.44	0.00	0.00

Table 6

Pesticide concentration (μg/kg) in onion samples collected from irrigated farmlands in the vicinity of Lake Ziway.

Site		α-BHC	β-BHC	Hepta-epoxide	Heptachlor	α-Endosulfan	Bromophos-ethyl	Chlorpyrifos-methyl	Diazinon	Thionazin	Ethion	Famphur
*S*1	*R*1	3.56	5.45	13.73	BDL	1.50	0.55	0.80	0.19	BDL	BDL	1.45
*R*2	BDL	1.32	5.74	BDL	3.35	BDL	0.23	0.04	0.26	BDL	BDL	*R*3

3.02	BDL	13.17	BDL	3.35	0.09	0.44	BDL	0.38	BDL	0.73		
*S*2	*R*1	2.25	0.48	37.55	BDL	BDL	0.04	0.25	BDL	0.01	BDL	0.74
*R*2	5.46	0.90	31.23	8.02	BDL	0.40	0.60	BDL	0.30	0.94	0.93	*R*3
3.02	0.09	37.55	8.48	BDL	0.09	0.44	BDL	0.39	0.43	0.73		
EU-MRL		10	10	10	10	100	10	10	50	10	20	10
Total detected		6	6	6	3	3	5	6	2	5	2	5
Sample>EU-MRL		0	0	5	0	0	0	0	0	0	0	0
% (+ve) sample		100	100	100	50	33.33	83.33	100	33.33	83.33	33.33	83.3333
% (>EU-MRL)		0	0	83.3	0	0	0	0	0	0	0	0
–												
Site		Fenitrothion	Fenthion	Malathion	Parathion	Profenofos	PBO	Propargite	Propoxur	Indoxacarb	Dichlobenil	
–												
*S*1 (Abenea-Girmama)	*R*1	0.46	1.80	BDL	1.29	0.82	BDL	45.75	8.61	BDL	BDL	
*R*2	0.30	2.75	BDL	1.05	0.54	BDL	BDL	12.73	BDL	0.10		*R*3
0.40	1.63	BDL	0.60	0.82	BDL	29.77	25.15	BDL	1.10			
*S*2 (Wellibulla)	*R*1	0.16	1.50	11.02	1.19	0.64	BDL	BDL	7.42	0.29	1.09	
*R*2	0.25	1.45	20.74	0.73	0.23	BDL	112.36	13.74	4.30	BDL		*R*3

0.40	1.63	BDL	0.60	0.82	BDL	112.36	25.15	4.30	1.10		
EU-MRL		10	10.00	20.00	50.00	20.00	0	10.00	50.00	20.00	10.00
Sample detected		6.00	6.00	2.00	6.00	6.00	0	5.00	6.00	3.00	4.00
Sample>EU-MRL		0.00	0.00	1.00	0.00	0.00	0	4.00	0.00	0.00	0.00
% (+ve) sample		100	100.00	33.33	100.00	100.00	0	83.33	100.00	50.00	66.67

3.4. Potential Health Risks from Vegetable Consumption

3.4.1. Target Hazard Quotient (THQ)

Tables 7 and 8 show the THQ results for the research areas for those who consume tomatoes and onions one to seven times a week. THQ was estimated using only residue concentrations greater than or equal to the EU-MRL standard. The THQ values for *α*-endosulfan, heptachlor, malathion, and propargite residues in adults ranged from 0.0003 to 0.12, while in children they varied from 0.001 to 0.32 (Table 7). THQ values less than one (THQ

Table 7

Target hazard quotient (THQ) and hazard index (HI) of pesticide residues from consumption of tomato produced in the study sites at different levels (days per week) of exposure.

Sites	Levels of exposure (d/w)	Target hazard quotient (THQ)								Hazard index (HI)	
α-Endosulfan		Heptachlor		Malathion		Propargite		*A*	*C*	*A*	*C*
A	*C*	*A*	*C*	*A*	*C*	*S*1	1	0.016	0.044	0.017	0.046
0.0003	0.001	—	—	0.034	0.090	2	0.033	0.088	0.034	0.091	0.001
0.002	—	—	0.068	0.181	3	0.049	0.131	0.051	0.137	0.001	0.002
—	—	0.102	0.271	5	0.082	0.219	0.086	0.229	0.001	0.004	—
—	0.169	0.451	7	0.115	0.307	0.120	0.320	0.002	0.005	—	—
0.237	0.632	-									
*S*2	1	0.008	0.020	—	—	—	—	0.002	0.004	0.009	0.024

2	0.015	0.040	—	—	—	—	0.003	0.008	0.018	0.048	3
0.023	0.060	—	—	—	—	0.005	0.012	0.027	0.072	5	0.038
0.101	—	—	—	—	0.008	0.020	0.045	0.121	7	0.053	0.141
—	—	—	—	0.011	0.028	0.063	0.169	.			
*S*3	1	0.010	0.026	0.011	0.030	0.001	0.002	0.000	0.001	0.023	0.060
2	0.020	0.053	0.023	0.061	0.002	0.005	0.001	0.002	0.045	0.120	3
0.030	0.079	0.034	0.091	0.003	0.007	0.001	0.003	0.068	0.180	5	0.049
0.132	0.057	0.152	0.004	0.012	0.002	0.005	0.113	0.301	7	0.069	0.185

Note. *A*=adult; *C*=children.

Table 8

Target hazard quotient (THQ) and hazard index (HI) of pesticide residues from consumption of onion produced in the study sites at different levels (days per week) of exposure.

Sites	Levels of exposure (d/w)	Target hazard quotient (THQ)								Hazard index (HI)	
Heptachlor epoxide		Heptachlor		Malathion		Propargite		*A*	*C*	*A*	*C*
A	*C*	*A*	*C*	*A*	*C*	*S*1	1	0.440	1.172	—	—
—	—	0.001	0.003	0.441	**1.175**	2	0.879	**2.344**	—	—	—
—	0.002	0.006	0.881	**2.350**	3	**1.319**	**3.516**	—	—	—	—
0.003	0.009	**1.322**	**3.526**	5	**2.198**	**5.861**	—	—	—	—	0.006

0.015	**2.204**	**5.876**	7	**3.077**	**8.205**	—	—	—	—	0.008	0.021
3.085	**8.226**	-									
*S*2	1	**1.758**	**4.689**	0.011	0.030	0.001	0.002	0.003	0.008	**1.773**	**4.729**
2	**3.516**	**9.377**	0.023	0.061	0.001	0.003	0.006	0.017	**3.547**	**9.458**	3
5.275	**14.066**	0.034	0.091	0.002	0.005	0.009	0.025	**5.320**	**14.187**	5	**8.791**
23.443	0.057	0.152	0.003	0.008	0.016	0.042	**8.867**	**23.645**	7	**12.308**	**32.821**

Note. Values in bold (>1) indicate potential noncarcinogenic health risk for humans. *A* = adult; *C* = children.

Regarding the site, the estimated THQ levels in onion for heptachlor epoxide were higher than one (THQ > 1) for all exposure periods for adults and children at Site 2, while at Site 1, adults were exposed more than three days a week and children were exposed two days a week.

3.4.2. Hazard Index (HI)

The estimated hazard index is shown in Tables 7 and 8 as the sum of THQ for tomato and onion consumption at the sample sites one to seven times per week. The HI values obtained from tomato consumption at all three sites were less than unity (HI

[figure(s) omitted; refer to PDF]

3.4.3. Carcinogenic Health Risks (CHRs)

According to the Agency for Toxic Substances and Disease Registry [32], the EPA and the International Agency for Research on Cancer (IARC) listed heptachlor as a probable human carcinogen. Furthermore, the EPA classified heptachlor epoxide as a possible human carcinogen. Tables 9 and 10 summarize the CHRs due to the consumption of tomatoes and onions one to seven times a week. The highest CHR value obtained for seven-day exposure to heptachlor through tomato consumption was $2.16E-03$ for tomato intake from Site 1 (Table 9), which means that two cancer cases occur per 1000 children, while the lowest was $5.20E-05$ for onion consumption from Site 2 (Table 10) for adults with one exposure per week (approximately three cancer cases occur per 100,000 adult individuals) (Figure 2(a)). Similarly, for heptachlor epoxide, the highest CHR value was $1.16E-02$ from Site 2 for children to seven-day exposure per week, which means that one cancer case occurs per 100 children, and the lowest was $5.2E-05$ from Site 1 for adults to once exposure per week (5 cancer cases per 100,000 adult individuals) (Figure 2(b)). The study revealed that the cancer health risk (CHR) for heptachlor was within the acceptable range ($<10^{-4}$) for a dose of 2 days per week or less for Site 1 when it comes to tomato consumption and 3 days per week or less for Sites 2 and 3 with respect to adult consumption of tomatoes and onions, respectively. This indicates that at this level of exposure, there is no possible risk of developing cancer from ingesting heptachlor residues from tomato consumption for adults.

Table 9

Carcinogenic risks (CRs) of heptachlor due to consumption of tomato from the study sites.

Levels of exposure (d/w)	Site 1	Site 3

A	C	A	C	1
3.86E−05	3.09E−04	2.6E−05	2.06E−04	2
7.71E−05	6.17E−04	5.1E−05	4.11E−04	3
1.54E−04	9.26E−04	7.7E−05	6.17E−04	5
3.09E−04	**1.54E−03**	1.29E−04	**1.03E−03**	7

Note. Values in bold indicate TCR above acceptable limit (10^{-4}). *A*=adult; *C*=children.

Table 10

Carcinogenic risks (CRs) of pesticides due to consumption of onion from the study sites.

Levels of exposure (d/w)	Site 1		Site 2		Site 2	
Heptachlor epoxide		Heptachlor epoxide		Heptachlor		A
C	A	C	A	C	1	5.20E−05
4.16E−04	2.08E−04	**1.66E−03**	2.57E−05	2.06E−04	2	1.04E−04
8.32E−04	4.16E−04	**3.33E−03**	5.14E−05	4.11E−04	3	1.56E−04
1.25E−03	**6.24E−04**	**4.99E−03**	7.71E−05	6.17E−04	5	2.60E−04
2.08E−03	**1.04E−03**	**8.32E−03**	1.29E−04	**1.03E−03**	7	3.64E−04

Note. Values in bold indicate TCR above acceptable limit (10^{-4}). *A*=adult; *C*=children.

[figure(s) omitted; refer to PDF]

On the other hand, the CHR values for heptachlor exceeded the acceptable limit (>10^{-4}) in the case of children who consumed tomatoes and onions at all levels of exposure per week. Furthermore, for adults, the CHR values exceeded the acceptable limit at Site 1 with respect to tomato consumption of 3 days per week or more and at Site 3 for tomato consumption of more than 5 days per week (Table 9). Therefore, it is reasonable to conclude that children at all levels of exposure and adults who consume tomatoes more than 3 days per week may face a potential risk of developing cancer in the study area and beyond.

The CHR values for heptachlor epoxide for all levels of exposure to children from Sites 1 and 2 and for adult exposure of two or more days of consumption per week from Site 1 and at all levels of exposure from Site 2 were higher than the permissible limit (>10^{-4}) (Table 10). Therefore, it is possible to conclude that consumption of heptachlor epoxide could pose substantial cancer risks to adults and children by eating onions, which is a component of the diet of residents of the research area.

In summary, the results of this study showed that the dietary intake of heptachlor and heptachlor epoxide at average exposure levels would create the possibility of developing cancer in children through the consumption of the investigated vegetables. Furthermore, this finding is consistent with previous research indicating that children appear to be particularly vulnerable to heptachlor and heptachlor epoxide poisoning [32]. As a result, immediate measures need to be taken to control and minimize heptachlor and heptachlor epoxide exposure through vegetable

consumption in the research region.

4. Conclusion

This study found that the consumption of tomatoes and onions from all study sites at varying levels (days per week) of exposure could be safe from the noncarcinogenic risk of the toxicities of *α*-endosulfan, heptachlor, malathion, and propargite residues for adults and children. Concerning the carcinogenic risk, the consumption of tomatoes and onions from all study sites at varying degrees of exposure (days per week) may be safer in terms of residual heptachlor toxicities for adults and children (consuming <3 days per week). The carcinogenic risk of onion heptachlor epoxide was estimated to be 1.46×10^{-3} g/kg/day, which implies that the cancer risk of heptachlor epoxide in an adult is 1.46 per 1,000 individuals continuously exposed. In children, the risk was estimated to be 1.16×10^{-2} g/kg/day (1.16 per 100 individuals), with a threat multiplied by 10. The findings suggest that farmers and their families, as well as those who consume vegetables grown on soils contaminated by pesticides on a regular basis, are the most vulnerable risk group whose health must be protected. Therefore, it is critical to raise awareness among stakeholders while simultaneously implementing sound monitoring policy actions to protect the ecosystem and the health of the population.

Authors' Contributions

Asrat Fekadu designed the study, carried out data collection, designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Girma Tilahun was responsible for conceptualization, design of the experiments, review and editing, supervision, investigation, project administration, and funding acquisition. Solomon Sorsa was responsible for writing, reviewing, and editing. All authors have read and approved the manuscript to reach its final form and agreed to its submission.

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

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(4) Inappropriate citations

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The presence of these indicators undermines our confidence in the integrity of the article’s content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] V. K. S. Maddala, K. Jayarajan, M. Braveen, R. Walia, P. Krishna, S. Ponnusamy, K. Kaliyaperumal, "Multisensor Data and Cross-Validation Technique for Merging Temporal Images for the Agricultural Performance Monitoring System," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/9575423, 2022.

DETAIL

Subjek:	Research; Monitoring systems
Judul:	Retracted: Multisensor Data and Cross-Validation Technique for Merging Temporal Images for the Agricultural Performance Monitoring System
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
Tanggal publikasi:	2024
Penerbit:	Hindawi Limited
Tempat publikasi:	Cairo
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-multisensor-data-cross-validation/docview/2921795511/se-2?accountid=211160

| **Terakhir diperbarui:** | 2024-03-08 |
| **Basis data:** | Public Health Database; Publicly Available Content Database |

Retracted: Minimizing the Error Gap in Smart Framing by Forecasting Production and Demand Using ARIMA Model

Quality Journal of Food.

Link dokumen ProQuest

TEKS LENGKAP

This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

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References

[1] S. G. Wawale, M. Jawarneh, P. N. Kumar, T. Felix, J. Bhola, R. Raj, S. Eswaran, R. Boddu, "Minimizing the Error Gap in Smart Framing by Forecasting Production and Demand Using ARIMA Model," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/1139440, 2022.

DETAIL

Subjek:	Research
Judul:	Retracted: Minimizing the Error Gap in Smart Framing by Forecasting Production and Demand Using ARIMA Model
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
Tanggal publikasi:	2024
Penerbit:	Hindawi Limited
Tempat publikasi:	Cairo
Negara publikasi:	United Kingdom, Cairo
Subjek publikasi:	Food And Food Industries
ISSN:	01469428
e-ISSN:	17454557
Jenis sumber:	Jurnal Akademik
Bahasa publikasi:	English
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-minimizing-error-gap-smart-framing/docview/2921795500/se-2?accountid=211160

| **Terakhir diperbarui:** | 2024-03-08 |
| **Basis data:** | Public Health Database; Publicly Available Content Database |

Retracted: Analysis Method of Agricultural Total Factor Productivity Based on Stochastic Block Model (SBM) and Machine Learning

Quality Journal of Food.

Link dokumen ProQuest

TEKS LENGKAP

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References

[1] Y. Li, C. Chen, F. Liu, J. Wang, "Analysis Method of Agricultural Total Factor Productivity Based on Stochastic Block Model (SBM) and Machine Learning," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/9297205, 2022.

DETAIL

Subjek:	Research; Machine learning
Ketentuan indeks bisnis:	Subjek: Machine learning
Judul:	Retracted: Analysis Method of Agricultural Total Factor Productivity Based on Stochastic Block Model (SBM) and Machine Learning
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
Tanggal publikasi:	2024
Penerbit:	Hindawi Limited
Tempat publikasi:	Cairo
Negara publikasi:	United Kingdom, Cairo
Subjek publikasi:	Food And Food Industries
ISSN:	01469428
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DOI:	https://doi.org/10.1155/2024/9890401
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-analysis-method-agricultural-total/docview/2921795497/se-2?accountid=211160

| Terakhir diperbarui: | 2024-03-08 |
| Basis data: | Public Health Database; Publicly Available Content Database |

Retracted: Identifying Smart Strategies for Effective Agriculture Solution Using Data Mining Techniques

Quality Journal of Food.

Link dokumen ProQuest

TEKS LENGKAP

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References

[1] A. J. B. Suarez, B. Singh, F. H. Almukhtar, R. Kler, S. Vyas, K. Kaliyaperumal, "Identifying Smart Strategies for Effective Agriculture Solution Using Data Mining Techniques," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/6600049, 2022.

DETAIL

Subjek:	Research; Data mining
Ketentuan indeks bisnis:	Subjek: Data mining
Judul:	Retracted: Identifying Smart Strategies for Effective Agriculture Solution Using Data Mining Techniques
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
Tanggal publikasi:	2024
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Tempat publikasi:	Cairo
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Subjek publikasi:	Food And Food Industries
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Basis data:	Public Health Database; Publicly Available Content Database

Dokumen 71 dari 77

Retracted: The Emerging Role of Implementing Machine Learning in Food Recommendation for Chronic Kidney Diseases Using Correlation Analysis

Food Quality Journal of.

TEKS LENGKAP

This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

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References

[1] S. Gupta, N. Garg, D. Sinha, B. Yadav, B. Gupta, S. Miah, "The Emerging Role of Implementing Machine Learning in Food Recommendation for Chronic Kidney Diseases Using Correlation Analysis," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/7176261, 2022.

DETAIL

Subjek:	Research; Machine learning; Correlation analysis; Kidney diseases
Ketentuan indeks bisnis:	Subjek: Machine learning
Judul:	Retracted: The Emerging Role of Implementing Machine Learning in Food Recommendation for Chronic Kidney Diseases Using Correlation Analysis
Pengarang:	Food Quality Journal of
Judul publikasi:	Journal of Food Quality; Cairo
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Negara publikasi:	United Kingdom, Cairo
Subjek publikasi:	Food And Food Industries
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-emerging-role-implementing-machine/docview/2921795357/se-2?accountid=211160
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Basis data:	Public Health Database; Publicly Available Content Database

Retracted: A System of Remote Patients’ Monitoring and Alerting Using the Machine Learning Technique

Quality Journal of Food.

Link dokumen ProQuest

TEKS LENGKAP

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(2) Discrepancies in the description of the research reported
(3) Discrepancies between the availability of data and the research described
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(5) Incoherent, meaningless and/or irrelevant content included in the article
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References

[1] M. Dhinakaran, K. Phasinam, J. Alanya-Beltran, K. Srivastava, D. V. Babu, S. K. Singh, "A System of Remote Patients' Monitoring and Alerting Using the Machine Learning Technique," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/6274092, 2022.

DETAIL

Subjek:	Research; Machine learning
Ketentuan indeks bisnis:	Subjek: Machine learning
Judul:	Retracted: A System of Remote Patients' Monitoring and Alerting Using the Machine Learning Technique
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
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Penerbit:	Hindawi Limited
Tempat publikasi:	Cairo
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-system-remote-patients-monitoring/docview/2921795340/se-2?accountid=211160

Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Forecasting the Applied Deep Learning Tools in Enhancing Food Quality for Heart Related Diseases Effectively: A Study Using Structural Equation Model Analysis

Food Quality Journal of.

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References

[1] S. L. Bangare, D. Virmani, G. R. Karetla, P. Chaudhary, H. Kaur, S. N. H. Bukhari, S. Miah, "Forecasting the Applied Deep Learning Tools in Enhancing Food Quality for Heart Related Diseases Effectively: A Study Using Structural Equation Model Analysis," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/6987569, 2022.

DETAIL

Subjek:	Research; Deep learning; Food quality; Structural equation modeling
Judul:	Retracted: Forecasting the Applied Deep Learning Tools in Enhancing Food Quality for Heart Related Diseases Effectively: A Study Using Structural Equation Model Analysis
Pengarang:	Food Quality Journal of
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
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Penerbit:	Hindawi Limited
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Subjek publikasi:	Food And Food Industries
ISSN:	01469428
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Jenis sumber:	Jurnal Akademik
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DOI:	https://doi.org/10.1155/2024/9769363
ID dokumen ProQuest:	2921795305
URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-forecasting-applied-deep-learning-tools/docview/2921795305/se-2?accountid=211160

Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Cont ent Database

Retracted: Multidimensional Attention-Based CNN Model for Identifying Apple Leaf Disease

Quality Journal of Food.

TEKS LENGKAP

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(2) Discrepancies in the description of the research reported

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(4) Inappropriate citations

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References

[1] K. Perveen, S. Kumar, S. Kansal, M. Soni, N. A. Alshaikh, S. Batool, M. N. Khanam, B. Osei, "Multidimensional Attention-Based CNN Model for Identifying Apple Leaf Disease," Journal of Food Quality, vol. 2023,DOI: 10.1155/2023/9504186, 2023.

DETAIL

Judul:	Retracted: Multidimensional Attention-Based CNN Model for Identifying Apple Leaf Disease
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
Tanggal publikasi:	2024
Penerbit:	Hindawi Limited
Tempat publikasi:	Cairo

Negara publikasi:	United Kingdom, Cairo
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DOI:	https://doi.org/10.1155/2024/9769786
ID dokumen ProQuest:	2921795285
URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-multidimensional-attention-based-cnn/docview/2921795285/se-2?accountid=211160

Terakhir diperbarui:	2024-02-06
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Performance of Machine Learning and Image Processing in Plant Leaf Disease Detection

Quality Journal of Food.

TEKS LENGKAP

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References

[1] A. S. Zamani, L. Anand, K. P. Rane, P. Prabhu, A. M. Buttar, H. Pallathadka, A. Raghuvanshi, B. N. Dugbakie, "Performance of Machine Learning and Image Processing in Plant Leaf Disease Detection," Journal of Food Quality, vol. 2022,DOI: 10.1155/2022/1598796, 2022.

DETAIL

Subjek:	Research; Machine learning
Ketentuan indeks bisnis:	Subjek: Machine learning
Judul:	Retracted: Performance of Machine Learning and Image Processing in Plant Leaf Disease Detection
Pengarang:	Quality Journal of Food
Judul publikasi:	Journal of Food Quality; Cairo
Volume:	2024
Tahun publikasi:	2024
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Tempat publikasi:	Cairo

Negara publikasi:	United Kingdom, Cairo
Subjek publikasi:	Food And Food Industries
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URL Dokumen:	https://www.proquest.com/scholarly-journals/retracted-performance-machine-learning-image/docview/2921795245/se-2?accountid=211160

Terakhir diperbarui:	2024-03-08
Basis data:	Public Health Database; Publicly Available Content Database

Retracted: Empirical Analysis for Improving Food Quality Using Artificial Intelligence Technology for Enhancing Healthcare Sector

Quality Journal of Food.

TEKS LENGKAP

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The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

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Optimal Food Matrix Model for Digestibility and Bioavailability of Calcium and Zinc

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ABSTRAK (ENGLISH)

The nutrient deficiency resulting from inappropriate dietary intake leads to major risk factors of malnutrition and poses many serious threats and challenges to human health and capabilities. Malnutrition can be prevented through efficient accessibility and bioavailability from different food matrices. The objective of this study was to assess the digestibility and bioavailability of calcium and zinc with food matrices such as casual food (bread curry mixture), yogurt (plain and fruited), juices (orange, apple, carrot, and tomato), coffee, water (water and sparkling water), and smoothies after digestion with saliva, gastric, duodenal, and small intestine juices. 20 mg calcium and 3 mg zinc were mixed with the above food matrices and digestibility and bioavailability were determined. The result showed that the highest calcium digestibility (49.75%) was observed with plain yogurt and the lowest digestibility (10.10%) was observed with sparkling water. The highest (22.80%) and lowest (6.20%) calcium bioavailability were observed with fruit yogurt and carrot juice, respectively. The highest (13.55%) and lowest (10.20%) zinc digestibility were observed with coffee and orange juices, respectively. The highest (4.85%) and lowest (1.05%) zinc bioavailability were observed with fruit yogurt and bread sauce, respectively. Thus, this study helps to determine the optimal food matrix model for the best digestibility and potential bioavailability of calcium and zinc from vitamin-mineral products.

TEKS LENGKAP

1. Introduction

The nutrient deficiency resulting from inappropriate dietary intake leads to major risk factors of malnutrition. This results in many serious threats and challenges to human health and potential. Nutrient deficiencies can be rectified by making nutrients accessible and bioavailable through improved supplement formulations and nutrient-drug interaction [1]. Supplementation and fortification are the most cost-effective techniques to address nutrient deficiency in communities. Nutrient deficiencies occur as a result of reduction in calories and increase of calorie-dense foods in contemporary diets [2]. The availability of nutrients with the food matrix is a key element of fortification, determining the bioavailability of nutrients in the gut besides the nutrient composition and processing conditions. Besides the abovementioned conditions, the available fraction of nutrients from the food mixture has a major importance in the determination of the bioavailability of nutrients. Bioavailability can be influenced by several factors such as the chemical state of the nutrient, its release from the food matrix, possible interactions with other food components, the presence of suppressors or cofactors, and the formation of stable compounds that are slowly metabolized *in vivo* [3]. Bioavailability or digestibility is the fraction of nutrients released from the food matrix after digestion and available for absorption. The fraction of nutrients in a food mixture that is absorbed and utilized by a body is called bioavailability [4]. The bioavailability of divalent cations is low with all food matrices. In the last two decades, despite a lot of awareness campaigns, calcium intake is still suboptimal and needs careful supplementation and intake models. Today, calcium is still singled out as a major public health concern due to its critical importance in bone health and other physiological functions [5].

The average digested available calcium from ingested calcium load ranges from 25% to 35% with net bioavailability of 10% to 12%. In general, the bioavailability of calcium is inversely proportional to the loaded quantity. For example, for a specific matrix, if the loaded dose is 20 mg, the bioavailability reaches 80% and if, the loaded dose is 1000 mg, the bioavailability decreases to 20% for the same matrix. Thus, from a nutritional point of view, the bioavailable contents are more important than the loaded fraction to understand the quantity and matrix model for nutritional and therapeutic purposes [6]. Most of the studies indicate that food matrix is the major factor in the bioavailability of calcium compared to an empty stomach and increases twofold when incorporated with various food, supplement, or beverage matrices [2, 7]. Calcium is less soluble and precipitates immediately in intestinal pH after making a

complex with dietary phytate, and zinc remains soluble. Zinc is another important mineral that has a global deficiency impact affecting billions of people across the globe. It is normally observed that the bioavailability of zinc from fortified foods or supplements is higher in an empty stomach than in the fed state showing that food matrix and/or digestive processes can reduce zinc bioavailability [8, 9]. Besides that, the bioavailability of zinc depends on the metabolic quality of the natural composition of food. Zinc oxide is most commonly used as a fortificant or supplement, however, the bioavailability of zinc oxide in a fasted state is less as compared to zinc citrate or zinc gluconate and sometimes, observed undetectable absorption may be linked with stomach acid quantity [10]. Some animal studies show controversial opinions on zinc absorption with calcium and calcium-fortified foods. Calcium in the form of calcium carbonate with dietary phytate hinders the absorption of zinc, while Miller and his colleagues reported a significant favorable effect of calcium on zinc absorption [11, 12]. Thus, the discordances in calcium bioavailability highlight the reality that bioavailability cannot be predicted based on the current chemical knowledge of the source nor can the matrix be extrapolated to other untested matrices. Similarly, the bioavailability of zinc dynamics has not been clearly established in any study as per our knowledge. The objective of this study was to sort out the optimal food matrix model for digestibility and bioavailability of calcium and zinc from mineral products.

2. Materials and Methods

2.1. Procurement of Materials

Calcium and zinc supplements were purchased from the local market and brand selection was made after careful consideration of the reputation of the brand, peer recommendation, and annual consumption. Further selected food matrices i.e., casual food (bread curry mixture), yogurt (plain and fruited), juices (orange, apple, carrot, and tomato), coffee, water (mineral water and sparkling water), and smoothies were procured from the local market in highest possible quality standards. Analytical grade chemicals or reagents, enzymes, and secretions, i.e., pepsin, mucin, glucuronic acid, glucose, glucosamine, uric acid, and pancreatin along with lipase, bile salt, and bovine serum albumin, were also supplied by Chem-Tech, Pakistan, Aldrich Sigma Pakistan. The study was conducted at the Department of Nutritional Sciences, Government College University, Faisalabad.

2.2. Food Matrix and Supplement Treatment

Two supplements of calcium and zinc having the highest annual sales in Pakistan from the previous five years were selected and subjected to be fortified with chosen food matrices as shown in Table 1. On the basis of recommendation and nutrient reference values (NRV) as per the recommendation of the manufacturer, calcium was recommended at 400 mg and zinc, at 6 mg. However, for easy handling and availability of reagents and chemicals, we reduced the recommended dose to half and took 200 mg of calcium powder, 3 mg of zinc, and half the quantity of each matrix to attain the recommended doses.

Table 1

Food matrix and supplement treatment.

Calcium (200 mg) and zinc (3 mg) supplement					
Coffee	Water	Yogurt	Bread sauce	Juice	
Smoothie	Fruit	Vegetable	Plain	Sparkling	Plain

2.3. Sample Preparation for Digestion

Different food matrices such as bread sauce (100 g bread + 150 g sauce), coffee (120 mL), water (250 mL), plain and fruited yogurt (150 g), fruit juices (apple and orange, 150 mL), vegetable juices (carrot and tomato, 150 mL), and smoothie (carrot + orange) of fruit and vegetable (150 mL) were prepared as reported earlier [13]. The samples were mixed separately by employing a conventional mixer primarily to mimic the food fragmentation. The food matrices

were homogenized with calcium and zinc separately. The samples were drawn as mentioned below and subjected to digestion by adding saliva, gastric, intestinal, and bile juices at successive steps at 37 ± 0.3°C [14].

2.4. Oral and Gastric Digestion

The samples from each of the above mixtures (5 g) were taken and mixed with 6 mL of simulated saliva solution (pH 6.8) and then stirred for 5 min at 37°C. Furthermore, 12 mL of simulated gastric juice (pH 1.5) was added and the mixture was stirred for 2 hr at 37°C.

2.5. Intestinal Digestion

After completion of gastric digestion, 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of 70% bicarbonate solution (pH 8.0) were added and the mixture was stirred for 2 hr at 37°C. After small intestine digestion, incubated samples were subjected to targeted nutrient bioavailability.

2.6. Bioavailability of Nutrients

The dialysis tube of cutoff 50 kDa, flat (Membrane Filtration Products, Inc., Seguin, TX, USA) containing 10 mL of phosphate buffer (pH 7) was placed in the 250 mL flask containing the digested mixture and stirred for 2 hr at 37°C. The samples from dialysis tubes and flasks were taken to determine the calcium and zinc concentrations. Dialyzable nutrients will be available for absorption in the small intestine.

2.7. Mineral Analysis

Calcium and zinc levels in different food matrices were examined by the atomic absorption spectrophotometry technique at a high-tech lab facility at the University of Agriculture, Faisalabad, Pakistan. Wet digestion was carried out as described earlier [13]. Samples were filtered and subjected to calcium and zinc quantification by using a Hitachi Polarize Zeeman Atomic Absorption Spectrometer at 214 nm and 624 nm wavelength, respectively.

2.8. Statistical Analysis

A descriptive analysis of means was performed by using Microsoft Excel (Microsoft Corporation, Redmond, USA) and computed as mean ± error and are presented in simple graphs. The digestibility/solubility and absorption/bioavailability of minerals were calculated by using the following equation:(1)Digestibilitysolubility%=Solublecontentsof CalciumTotalcontentsofthesampleX100,AbsorptionBioavailability%=Calciumcontentsindialysate Totalcalciumcontentsofthesample X100.

3. Results

3.1. Digestibility and Bioavailability of Calcium with Different Food Matrices

The calcium and zinc contents originally present in food matrices are shown in Table 2. The highest calcium contents were present in yogurt matrices (118.04 mg/100 g) and lowest, in water (4.58 mg/100 mL). The highest zinc contents were observed in bread + sauce (6.10 mg/100 g) and remained nondetected in coffee and water (see Table 3).

Table 2

Composition and concentrations of digestive juices.

Secretion	Saliva	Gastric juice	Duodenal juice	Small intestine juice
Reagents	3 M NaCl 0.41 M urea 0.09 mM uric acid	1.0 M HCl 0.15 M $CaCl_2$ 1 g BSA*	0.83 M KCl 0.15 M $CaCl_2$ 1.0 g BSA*	1.0 M $NaHCO_3$ 0.15 M $CaCl_2$ 1.8 g BSA* 30 g bile
Enzymes	90 mg α-amylase 25 mg mucin	2.5 g pepsin 3 g mucin	9 g pancreatin 1.5 g lipase	——
pH	6.8 ± 0.2	1.50 ± 0.02	8.0 ± 0.2	7.0 ± 0.2

*BSA = bovine serum albumin.

Table 3

Calcium and zinc contents (mg/100 mL or 100 mg).

Food matrix	Coffee	Water		Yogurt		Bread sauce	Juice				Smoothie
Fruit		Vegetable		Plain	Sparkling	Plain	Fruited	Apple	Orange	Carrot	Tomato
Calcium	7.78	4.58	4.69	118.04	105.30	11.31	5.33	10.01	49.66	32.91	31.28
Zinc	ND	ND	ND	0.57	0.54	6.10	0.04	0.03	0.30	0.12	0.04

The digestibility and potential absorption/bioavailability of calcium with liquid matrices such as coffee and water (sparkling and plain) are presented in Figure 1. Calcium supplement (200 mg) was subjected to digestion with gastrointestinal enzymes. The results showed that calcium digestibility with coffee was 13.15% and potential bioavailability was 7.14%. Similarly, the digestibility of calcium with sparkling and simple water was 10.10% and 10.55%, respectively. The potential absorption of calcium with sparkling and plain water was 8.65% and 8.95%, respectively.

[figure(s) omitted; refer to PDF]

The digestibility and potential bioavailability of calcium with solid food matrices such as bread sauce, fruit yogurt, and plain yogurt are presented in Figure 2. The results showed that the digestibility of calcium with bread sauce was 31.85% and potential bioavailability was 10.35%. The digestibility and bioavailability with fruit yogurt were 34.60% and 22.80%, respectively. The calcium digestibility with plain yogurt was 49.75% and potential bioavailability was 22.20%.

[figure(s) omitted; refer to PDF]

The digestibility and bioavailability of calcium with fruit and vegetable matrices such as apple, carrot, orange, and tomato juices and smoothies (apple, orange, and carrot) are presented in Figure 3. The results showed that the digestibility and bioavailability of calcium with apple juices were 14.65% and 9.0%, respectively. Calcium digestibility and bioavailability with carrot juice were 12.35% and 6.2%, respectively. The digestibility and bioavailability with orange juice were 24.15% and 11.55%, respectively. The digestibility and bioavailability with tomato juice were 21.40% and 14.30%, respectively. Smoothie matrix showed 13.50% and 7.6% digestible and bioavailable calcium, respectively.

[figure(s) omitted; refer to PDF]

3.2. Digestibility and Bioavailability of Zinc with Different Food Matrices

The digestion and bioavailability of zinc with liquid matrices such as coffee, sparkling water, and plain water are presented in Figure 4. Zinc supplement (3 mg) was subjected to digestion with gastrointestinal enzymes by using the above liquids, and the results showed that the digestibility of zinc with coffee was 13.55% and its bioavailability was 4.45%. Zinc digestibility with sparkling water was 12.30% and its bioavailability was 1.45%. Zinc digestibility with simple water was 12.9% and bioavailability was 1.15%.

[figure(s) omitted; refer to PDF]

Zinc digestibility and bioavailability with solid matrices such as bread sauce, fruit yogurt, and plain yogurt are presented in Figure 5. The results showed that zinc digestibility with bread sauce was 12.95% and its bioavailability was 1.05%. Similarly, the amount of digested zinc with fruit yogurt was 13.15% and its bioavailability was 4.85%. The amount of digested zinc in plain yogurt was 13.70% and its bioavailability was 4.65%.

[figure(s) omitted; refer to PDF]

The digestibility and bioavailability of zinc with fruit and vegetable matrices such as apple, carrot, orange, and tomato juices and smoothies (apple, orange, and carrot) are presented in Figure 6. The results showed that the digestibility and bioavailability of zinc with apple juices were 13.55% and 1.20%, respectively. The digestibility and bioavailability with orange juice were 10.20% and 1.10%, respectively. Zinc digestibility and bioavailability with carrot juice were 11.05% and 1.45%, respectively. The digestibility and bioavailability with tomato juice were 12.31% and 1.55%, respectively. Similarly, the smoothie matrix showed 10.20% and 2.60% digestible and bioavailable zinc, respectively.

[figure(s) omitted; refer to PDF]

4. Discussion

The fortification and supplementation is one of the main strategies to meet the recommended level of calcium; however, the food matrix and nature of calcium salt is the key consideration in the processing and bioavailability. It has been shown that milk and milk products serve as the best food matrix for absorption of calcium with most of the salt except tricalcium phosphate. However, information is lacking regarding the provision of calcium from pure salt and supplements and food/beverage vehicles posing a challenge in the identification of suitable vehicles for the absorption of calcium [16]. We observed that among eleven tested food matrices, yogurt was the best matrix for digestibility (49.75%) and bioavailability (22.80%) of calcium. It was reported earlier that all available formulations of calcium in the form of excipients, encapsulation, and granulation showed 7.5%–39% calcium availability [17]. The natural composition synchronizing with salt ingredients can strongly influence the bioavailability of that mineral as yogurt has naturally the highest content of calcium which may influence the bioavailability and help in determining the precise additional dose of calcium for fortification because above a certain quantity, additional calcium fortification leads to a reduced bioavailability of calcium [6]. This required a precise extrapolation of salt, supplement, and food matrix/beverage without generalization of only calcium sources for pharmaceutical preparation.

Besides the original calcium present in the food matrix, several other factors can enhance or inhibit the bioavailability. For example, citrus juice can enhance the bioavailability of calcium and bread can reduce it [17]. We observed that the digestibility of calcium was the highest with yogurt but the bioavailability was low. In contrast, higher dietary intake increases the intestinal absorption of zinc from foods [18] but we did not observe that higher concentration increases the bioavailability of zinc. The digested zinc contents were similar in all food matrices but coffee and yogurt have 4 times higher bioavailability of zinc than the other eight tested matrices. This indicates that food matrix and nutritional contents are the key factors in determining the bioavailability of zinc. The higher phytate and phosphorus contents inhibit zinc absorption, while the protein matrix enhances the intestinal intake of zinc [19]. Similarly, the other minerals may influence the absorption of zinc. Calcium and cadmium are considered major inhibitors of zinc [20].

5. Conclusion

Thus, the main challenge for better digestibility and bioavailability is associated with the prevention of interaction with other minerals of food matrix and processing additives. We observed that high protein content in food matrices such as yogurt can be better for the absorption of calcium and zinc. This study will help to fortify the products intended for mass or specific consumption, including cereals, fruits, and dairy-based products.

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