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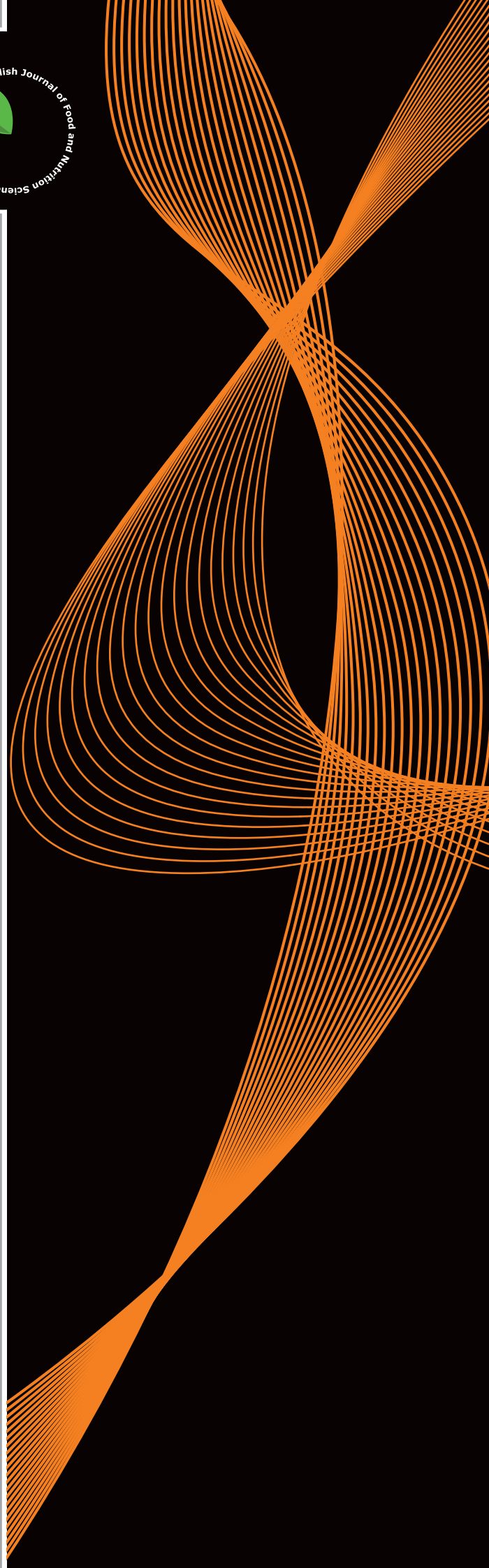

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Impact of Lupin Inclusion on the Rheological and Qualitative Characteristics, and Sensory Acceptability of Baked Rolls

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Lupin represents a high-protein and high-fibre non-traditional raw material that can improve the nutritional value of baked goods. This study investigated the effect of the incorporation of lupin flour on the rheological characteristics of wheat dough as well as qualitative characteristics, sensory acceptability and nutritional value of baked rolls. The rheological properties of blended flours enriched with various weight proportions of lupin flour (5–25%) were analysed *via* Mixolab. Compared with the control sample (wheat flour), lupin flour increased water absorption from 55.00% to 68.05% and decreased dough stability from 9.32 to 6.86 min. Qualitative assessment of baked rolls revealed a significant reduction in volume (from 252.50 mL to 123.75 mL) and specific volume (from 285.19 mL/100 g to 142.30 mL/100 g) with increasing lupin addition to flour blends. Instrumental color analysis revealed darker, more yellow hue with *L** values decreasing from 74.94 to 69.95. Nutritional analysis demonstrated higher protein (19.14 g/100 g) and total dietary fibre contents (13.24 g/100 g) in the rolls from the blend enriched with 25% lupin flour. Sensory evaluation indicated that baked goods produced from the flour blends containing up to 15% lupin maintained acceptable sensory qualities, while higher lupin levels adversely affected flavor, porosity, and overall acceptability of the rolls. Correlation analysis showed strong associations between dough stability and starch content (r=0.941) and the C2 parameter of the Mixolab (r=0.916). These results suggest that while lupin flour enhances the nutritional profile of baked rolls, optimal incorporation levels (up to 15% in the flour blend) are necessary to maintain product quality and consumer acceptance.

Keywords: baked rolls, fibre, lupin, Mixolab, protein, rheology

INTRODUCTION

The demand for cereal-based products that are rich in plant-derived nutrients and bioactive compounds is currently growing. This trend is driven not only by economic and environmental considerations but also by the increasing interest in new, safe, and healthy foods [Spina *et al*., 2024]. Owing to their complementary amino acid profiles, legume proteins are considered excellent supplements for cereal-based foods. They represent an abundant source of lysine but lack sufficient sulfur-containing amino acids, whereas cereal proteins are rich in sulfur amino acids but deficient in lysine. This complementary relationship makes the combination of legume and cereal proteins nutritionally beneficial, enhancing the overall amino acid balance of the food. Beyond their nutritional advantages, pulse proteins possess remarkable functional properties, including solubility, gelation, and water-binding capacity, all of which play a vital role in contributing to the texture and sensory attributes of final products. Various legumes, including soybean, chickpea, pea, and lupin, have been investigated as protein-enriching ingredients in bakery products, *via* flours and different protein preparations

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such as protein concentrates and isolates [Shrestha *et al*., 2021; Spina *et al*., 2024].

Lupin has garnered worldwide interest because of its nutritional value, absence of genetic modification in commercial cultivation, enhanced sustainability, and lower costs than current production methods do [Shrestha *et al*., 2021]. Lupin seeds are an excellent gluten-free source of protein (29–40 g/100 g), dietary fibre (7.2–16 g/100 g), and lipids (5.5–19 g/100 g). They also provide essential minerals, including zinc, iron, and manganese, as well as vitamins such as niacin, thiamine, tocopherols, and riboflavin, along with antioxidants [Pleming *et al*., 2021]. Furthermore, consuming lupin is associated with numerous health benefits, including improved bowel function and reductions in cholesterol levels, blood glucose, and the glycemic index [Shrestha *et al*., 2021]. A limiting factor for the consumption of legumes and their products is the presence of antinutritional factors. The main antinutritional factors found in lupin are alkaloids (lupinine, lupanine, sparteine, lupinidine, hydroxylupanine), which can be removed through processes such as heat treatment or soaking lupin seeds in water [Süli *et al*., 2017].

These qualities position lupin as an appealing and economically viable option for the production of innovative cereal-based products [Pleming *et al*., 2021]. Lupin flour is made by removing the hulls from whole lupin seeds, isolating the kernels, and grinding them into a fine powder. Compared with refined wheat flour, lupin kernel flour contains approximately 40 g protein and 40 g dietary fibre *per* 100 g, offering a higher nutritional density and lower energy value and is characterized by its pale-yellow coloration and slight beany flavor [Pereira *et al*., 2024].

Previously, several authors have investigated the impact of various legume flours, such as chickpea, bean, lentil, and soybean, on the rheological properties of wheat dough as well as qualitative and sensory characteristics, and proximate composition of formulated baked goods [Belc *et al*., 2021; Bojňanská *et al*., 2021; Calderón *et al*., 2022; Rizvi *et al*., 2022]. However, only a few studies have focused on improving the nutritional composition of common bakery products by including lupin flour as a protein-rich and fibre-rich raw material [Calderón *et al*., 2022; Pleming *et al*., 2021; Plustea *et al*., 2022]. Therefore, the aim of the present study was to provide a comprehensive assessment of the thermomechanical behavior of wheat flour blends with different weight proportions of lupin flour (5, 10, 15, 20, and 25% substitution of wheat flour), baking performance, sensory acceptability, and nutritional characteristics of formulated baked rolls.

MATERIALS AND METHODS

Materials

Lupin flour (LF) (Sobo Naturkost, Köln, Germany), obtained from the Slovak market with a focus on healthy nutrition, contained 7.5 g of lipids, 13 g of carbohydrates, 39 g of protein, 32 g of fibre, and 0.04 g of salt *per* 100 g of product. Commercial fine wheat flour (marked as a control) (Mlyn Pohronský Ruskov a.s., Slovakia) (1.25 g of lipids, 74.58 g of carbohydrates, 10.37 g of protein, 2.89 g of fibre *per* 100 g of product) and other ingredients (vegetable oil, sugar, salt, yeast) were purchased from Slovak local markets. The nutritional information was mentioned by the manufacturer on the product's label.

Flour blends were prepared by blending wheat flour with different portions of lupin flour at levels of 5, 10, 15, 20 and 25% by weight (LF5–LF25).

Rheological properties of dough

The rheological behavior of the dough was studied using a Mixolab 2 device (Chopin Technologies, Villeneuve-la-Garenne, France) applying the "Chopin+" protocol with a constant mixing speed of 80 rpm. An amount of 75 g of flour was used, with a target consistency (C1) of 1.1±0.05 Nm. Initially, the dough was mixed for 8 min at 30°C. The mixture was subsequently heated for 15 min at a rate of 4°C/min until it reached 90°C. The dough was maintained at 90°C for 7 min before being cooled to 50°C at a rate of 4°C/min. Finally, the mixture was mixed for 5 min at 50°C. The determined Mixolab parameters were as follows: water absorption (WA, %), dough stability (DS, min), C2 (Nm) – weakening of the protein during mechanical stress at increasing temperature, C3 (Nm) – starch gelatinization, C4 (Nm) – stability of the formed starch gel, C5 (Nm) – starch retrogradation during the cooling stage, C1–C2 (Nm) – protein network strength under increasing heating, C3–C2 (Nm) – starch gelatinization rate, C3–C4 (Nm) – amylase activity, and C5–C4 (Nm) – anti-stalling effects, which represent the shelf-life of the end products [Guardado-Félix *et al*., 2020].

Rolls preparation

Baked rolls were prepared according to the recipe described by Holkovičová *et al*. [2024]. The dough was prepared from 300 g of wheat flour/flour blends which were first dry-mixed in a farinographic mixing bowl. Next, 5.63 g of salt, 3.22 g of sugar, 7.5 g of vegetable oil and 12.06 g of yeast previously dissolved in water were added followed by the addition of water up to a farinographic consistency of 400 BU (Brabender units). After being kneaded for 6 min, the resulting dough was fermented at 27°C for 20 min. Consequently, the dough was portioned into 100 g loaves and shaped using a dough former (Extensograph Brabender, Duisburg, Germany). After the second 45-min proofing, the dough was baked at 230°C for 15 min with steam (250 mL), cooled for 2 h and packed into plastic bags.

Determination of qualitative properties of baked rolls

The qualitative parameters of the baked rolls were assessed 2 h after baking. The volume of the rolls was determined using the rapeseed displacement method. The specific volume (mL *per* 100 g of loaf) was established by dividing the measured volume by the weight of the rolls. The cambering of the baked rolls was determined by the ratio of the loaf's height to its width [Minarovičová *et al*., 2018].

Color analysis

The color of the crumb of the baked rolls was evaluated instrumentally using a Cary 300 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) with a sphere diffuse

reflectance accessory (DRA-CA-30I). The crumb of the baked rolls was dried and ground in a grinder mill (Model 0010, Eta, Hlinsko, Czech Republic) before analysis. The individual color values were established using CIELab. The color parameters were: L^* (lightness, 0 – black, 100 – white), a^* ($-a^*$ – green, $+a^*$ – red), b^* ($-b^*$ – blue, $+b^*$ – yellow), chroma (C), and hue angle (h). The spectrophotometer was calibrated with a white plate [Mazumder *et al*., 2021]. All the determinations were carried out in five replicates, and the average value was used. The total color difference (ΔE) with respect to the control sample was calculated according to Equation (1) [Pathare *et al*., 2013]:

$$\Delta E = \sqrt{(L_i^* - L_c^*)^2 + (a_i^* - a_c^*)^2 + (b_i^* - b_c^*)^2} \quad (1)$$

where: i represents a different level of lupin substitution, and c represents the control sample.

The yellowness index (YI), whiteness index (WI), and browning index (BI) were computed following Equations (2), (3), (4) and (5) adopted from Pathare *et al*. [2013]:

$$YI = \frac{142.86 \times b^*}{L^*} \quad (2)$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (3)$$

$$BI = \frac{100 \times (x - 0.31)}{0.17} \quad (4)$$

$$x = \frac{a^* + 1.75 \times L^*}{5.645 \times L^* + a^* - 3.012 \times b^*} \quad (5)$$

Chemical composition analysis

The proximate analysis of the final products involved the determination of moisture according the International Association for Cereal Science and Technology (ICC) standard No. 110/1 [ICC, 1976], lipids by the Soxhlet method [Stanković *et al*., 2018], and ash by gravimetric method (ICC standard no. 104/1) [ICC, 1990]. The protein content was assessed as the total nitrogen content by the Kjeldahl method with a factor of 6.25 (lupin flour and flour blends) for nitrogen conversion to crude protein [Rizvi *et al*., 2022]. The starch content was assessed by the Ewers polarimetric method measuring optical rotation [Omar *et al*., 2016]. The total dietary fibre content (TDF) was determined by the enzymatic gravimetric method using the Megazyme assay kit [Lauková *et al*., 2019]. The results of chemical composition were expressed in g *per* 100 g of baked rolls. The total carbohydrate content was calculated based on the difference obtained by subtracting the sum of moisture, lipid, protein and ash content in 100 g of the baked rolls from the weight of 100 g of product.

The energy value of the products was determined by taking into account the conversion factors reported by Plustea *et al*. [2022], *i.e.*, 9 for lipids and 4 for carbohydrates and proteins and expressed in kcal *per* 100 g of the product.

Evaluation of sensory acceptability

The baked rolls were subjected to a preliminary sensory acceptability evaluation using a 5-point hedonic scale, with the following rates: 1 – extremely dislike, 2 – slightly dislike, 3 – neither like nor dislike, 4 – slightly like, and 5 – extremely like [Plustea *et al*., 2022]. This initial evaluation was conducted with a small group (11 members) of semi-trained participants, including staff and students from the Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia. The evaluated attributes included color, flavor and taste of baked rolls, adhesiveness to palate, springiness, porosity and overall acceptability.

Statistical analysis

All analyses were performed in triplicate, with results reported as the mean and standard deviation. Analysis of variance (ANOVA) was used to identify significant differences ($p<0.05$, $p<0.01$, $p<0.001$). Fisher's least significant difference (LSD) test was applied to assess the significance of differences between the control sample and samples with various levels of lupin flour. Pearson's correlation test was used to evaluate the relationships between rheological parameters and qualitative properties of the baked rolls. Statistical analyses were performed using Statgraphics version 19 (Statsoft-Inc., The Plains, VA, USA).

RESULTS AND DISCUSSION

Rheological properties of dough

Previously it has been documented that incorporation of legume flours into wheat dough affects the viscoelastic and mixing properties of the dough. Mixolab is a rheological instrument that subjects dough to both mechanical and thermal stress, allowing for the assessment of changes in the protein-starch network under conditions that closely simulate real bakery processes [Bojňanská *et al*., 2021]. The Mixolab parameters of wheat flour (control sample) and flour blends with different proportions of LF are presented in **Table 1**.

WA provides information about the percentage of water required for the dough to produce a torque of 1.1 Nm. WA significantly increased ($p<0.001$) with the addition of LF to flour blends from 55.00% in the case of the control to 68.05% in the flour blend containing 25% LF (**Table 1**). This enhancement in WA was likely attributable to the hygroscopic properties of LF, which facilitate higher water retention within the dough matrix. This phenomenon may be due to lupin's high protein and fibre contents, which can interact with water molecules [Calderón *et al*., 2022]. Previously, several authors [Belc *et al*., 2021; Maradudin *et al*., 2019] confirmed an increased WA of flour blends including various legume flours (pea, soybean, bean, and chickpea) in comparison with that of wheat flour.

DS, a dough characteristic that indicates dough resistance to mixing and kneading [Manano *et al*., 2021], was reduced with the increasing levels of LF in the flour blends (**Table 1**). These findings indicate that incorporating LF disrupted the starch-protein matrix, reducing dough elasticity and leading to its weakening during prolonged mixing [Calderón *et al*., 2022; Kohajdová *et al*., 2011].

C2 and C1–C2 are parameters related to protein weakening in the second stage of Mixolab measurement. C2, which is the

Table 1. Mixolab parameters of wheat flour (control) and blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively).

Parameter	Control	LF5	LF10	LF15	LF20	LF25
WA (%)	55.00±0.00	56.75±0.05***	58.95±0.15***	61.20±0.00***	64.90±0.10***	68.05±0.05***
DS (min)	9.32±0.10	9.23±0.09	9.18±0.08	8.55±0.23*	7.82±0.30***	6.86±0.01***
C2 (Nm)	0.53±0.01	0.51±0.00*	0.49±0.00**	0.48±0.01**	0.45±0.00***	0.43±0.00***
C3 (Nm)	2.03±0.03	1.90±0.01**	1.76±0.00***	1.61±0.01***	1.56±0.03***	1.49±0.02***
C4 (Nm)	2.21±0.04	2.05±0.02**	1.89±0.00***	1.75±0.00***	1.55±0.02***	1.41±0.03***
C5 (Nm)	3.53±0.00	3.26±0.01***	2.96±0.02***	2.66±0.00***	2.29±0.03***	1.99±0.01***
C1–C2 (Nm)	0.59±0.01	0.60±0.01	0.61±0.01	0.65±0.02	0.65±0.02	0.67±0.02
C3–C2 (Nm)	1.50±0.01	1.40±0.00**	1.27±0.00***	1.13±0.00***	1.11±0.04***	1.05±0.01***
C3–C4 (Nm)	0.18±0.01	0.15±0.01	0.13±0.05*	0.14±0.01*	0.06±0.01***	0.08±0.01***
C5–C4 (Nm)	1.32±0.04	1.21±0.01*	1.08±0.02***	0.91±0.00***	0.74±0.05***	0.59±0.01***

Results are presented as mean ± standard deviation (n=3). *, **, and *** indicate a significant difference at p<0.05, p<0.01, and p<0.001, respectively, compared to control. WA, water absorption; DS, dough stability; C2, weakening of the protein during mechanical stress; C3, starch gelatinization; C4, stability of starch gel; C5, starch retrogradation; C1–C2, protein network strength; C3–C2, starch gelatinization rate; C3–C4, amylase activity; C5–C4, anti-stalling effect.

minimum value of torque observed after the initial mixing phase, is an indicator of the quality and stability of wheat protein network in response to thermal weakening [Plustea *et al*., 2022]. The combined effect of mechanical stress and increasing temperature induced a significant decrease in the C2 torque of samples from 0.53 Nm in the control to 0.43 Nm in LF25 (**Table 1**). Similar results as those in this study were also obtained by Maradudin *et al*. [2019] and Calderón *et al*. [2022] when bean and sweet lupin flours were incorporated into wheat dough. This indicates that the presence of LF increases protein weakening, possibly due to its interaction with gluten proteins, disrupting their network and reducing dough strength. C1–C2, reflecting the extent of protein weakening, slightly increased with increasing LF levels (**Table 1**). However, since there were no significant differences observed, this parameter indicates only minor initial protein weakening, likely due to the initial dilution of gluten by LF proteins.

During the third phase of Mixolab measurement, the temperature further increases from 60°C to 90°C. At this point, starch granules swell as a result of WA, which increases the viscosity of the dough and requires greater torque for mixing. Torque point C3 expresses the starch gelatinization and the viscosity of the dough during heating [Plustea *et al*., 2022]. Wheat flour contained more starch compared to flour blends, which gave control sample higher C3 value with a torque of 2.03 Nm (**Table 1**). The reduction in starch gelatinization observed with the addition of LF may be attributed to the decreased starch content in the samples or the interference of lupin components, such as fibres and proteins, with starch granule swelling, potentially hindering the gelatinization process. This phenomenon has been previously discussed in the context of similar interactions [Abdel-Samie & Abdulla, 2016].

C4 represents the torque at the end of the heating phase, indicating the extent of enzymatic activity and starch breakdown [Plustea *et al*., 2022]. The results suggest that as the substitution level of LF increases, consistency decreases due to enhanced amylolytic activity, leading to reduced hot gel stability. This implies that LF may either enhance amylase activity or promote starch hydrolysis, resulting in greater starch breakdown during heating. A similar pattern was observed by Maradudin *et al*. [2019], who reported increased starch degradation with the incorporation of bean flour. The difference between C3 and C4 is the rate of amylase activity in the dough. The greater the difference between C3 and C4, the higher the amylase activity and lower stability of hot starch paste. The lower value of this parameter (C3–C4) was recorded with the control sample, amounting to 0.18 Nm (**Table 1**).

The decrease in temperature during the final cooling process from 90°C to 50°C is associated with starch retrogradation. In this phase, there is an increase in dough resistance, the starch hardens, thereby increasing its consistency (C5 value) [Plustea *et al*., 2022]. Starch retrogradation (C5 and C5–C4) has been shown to be the primary cause of baked goods firming, and is considered as an indicator of final product shelf-life [Abdel-Samie & Abdulla, 2016]. The C5 values of samples decreased significantly, from 3.53 Nm in the control to 1.99 Nm in LF25 (**Table 1**). The study results indicate that the incorporation of lupin flour into baked products can prolong their shelf-life and delay their staling. This phenomenon may be attributed to the high lipid content of lupin flour, which interacts with starch amylose in the dough, reducing starch retrogradation. The complex formed between amylose and lipids is insoluble in water, preventing amylose from leaching out of starch granules, and the starch retrogradation process is delayed [Codină *et al*., 2019]. These results were in accordance with those reported by Calderón *et al*. [2022] who documented that the addition of 20% lupin flour delayed bread firming.

Qualitative parameters of baked rolls

The baking quality is primarily influenced by the physical characteristics of the baked goods, including loaf volume, specific volume, and shape retention [Rodrigues *et al*., 2014]. The volume

Table 2. Qualitative parameters of baked rolls prepared using wheat flour (control) and blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively).

Parameter	Control	LF5	LF10	LF15	LF20	LF25
Loaf volume (mL)	252.5±9.8	210.0±5.5**	181.3±3.7***	153.8±3.7***	138.8±4.9***	123.8±1.9***
Loaf specific volume (mL/100 g)	285.2±12.3	237.2±8.5**	201.2±5.6***	173.9±5.3***	156.7±7.1***	142.3±2.6***
Cambering	0.60±0.03	0.54±0.02*	0.52±0.01*	0.49±0.00**	0.45±0.01***	0.44±0.01***

Results are presented as mean ± standard deviation (n=3). *, **, and *** indicate a significant difference at p<0.05, p<0.01, and p<0.001, respectively, compared to control.

Table 3. Color parameters of baked rolls prepared using wheat flour (control) and blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively).

Parameter	Control	LF5	LF10	LF15	LF20	LF25
L^*	74.94±0.09	73.87±0.25***	72.47±0.30***	72.09±0.16***	70.52±0.02***	69.95±0.08***
a^*	2.83±0.07	2.55±0.01***	2.50±0.01***	2.30±0.01***	1.89±0.02***	1.52±0.02***
b^*	14.24±0.07	14.56±0.07	14.81±0.04**	16.60±0.02***	16.64±0.06***	18.19±0.13***
C	14.52±0.08	14.78±0.07	15.02±0.04**	16.76±0.35***	16.75±0.07***	18.26±0.13***
h (°)	80.08±0.05	80.34±0.06**	81.46±0.04***	82.42±0.04***	82.80±0.01***	84.14±0.05***
ΔE	0.00 ± 0.00	1.01±0.02***	2.73±0.06***	3.70±0.06***	5.17±0.01***	6.61±0.07***
WI	71.04±0.05	69.98±0.19***	68.64±0.25***	67.44±0.08***	66.10±0.03***	64.84±0.12***
YI	27.14±0.11	28.15±0.05**	29.19±0.10***	32.90±0.64***	33.71±0.12***	37.16±0.29***
BI	23.42±0.10	24.04±0.14**	24.93±0.09**	27.99±0.18***	28.33±0.15***	31.07±0.07***

Results are presented as mean ± standard deviation (n=3). *, **, and *** indicate a significant difference at p<0.05, p<0.01, and p<0.001, respectively, compared to control. L^*, lightness; a^*, red-green spectrum; b^*, yellow-blue spectrum; C, chroma; h, hue angle; ΔE, total color difference; WI, whiteness index; YI, yellowness index; BI, browning index.

of baked goods is influenced mainly by the generation and retention of gas throughout processing. The wheat dough's unique capacity to hold gas is attributed primarily to the presence and functionality of the viscoelastic gluten network, as well as the role of water-extractable arabinoxylans. Higher loaf volumes are typically associated with better dough fermentation and gas retention properties, leading to a lighter and airier texture [Verdonck *et al*., 2023].

The data presented in **Table 2** show that the control rolls exhibited a volume of 252.5 mL and a specific volume of 285.2 mL/100 g. In comparison, the rolls produced using flour blends enriched with more than 5% LF showed significant decreases (p<0.001) in both volume and specific volume. Michalak-Majewska *et al*. [2017] proposed that increasing the proportion of non-gluten flours in dough negatively affects its ability to rise and retain gas. This effect is likely due to the dilution of gluten content and the presence of fibres, which disrupt the formation of a stable gluten network.

Cambering, the width/height ratio of the central slice, is another crucial parameter influencing the aesthetic appeal of bread and bread-like products such as rolls and buns. Higher cambering values indicate a more desirable, arched loaf shape, whereas values between 0.60 and 0.70 are considered favourable, while values below 0.50 are deemed inadequate [Holkovičová *et al*., 2024]. In our study, the control rolls had a cambering value of 0.60, which was within the favourable range. However, when the LF in the roll production was used, the cambering values were significantly lower competed to the control, with LF5 at 0.54 and LF25 at 0.44 (**Table 2**). The observed reductions in cambering suggest that a higher LF content results in flatter loaves, likely due to a weakened gluten network and diminished gas retention capacity in LF-enriched dough. Similar findings regarding the impact of alternative flours on loaf structure and gas retention have been reported by Verdonck *et al.* [2023].

Instrumental color of baked rolls

Color is a critical quality attribute in baked goods, as it influences consumer perception and acceptance. A consistent and appealing color is often associated with freshness, proper baking, and high-quality ingredients [Pathare *et al*., 2013].

The incorporation of LF into the baked rolls resulted in significant changes in the crumb color parameters, as outlined in **Table 3**. The L^* value, which represents lightness, decreased significantly (p<0.001) with the addition of LF, indicating that the rolls became darker. For instance, the L^* value dropped from 74.94 in the control sample to 69.95 in the LF25 sample. According to Kolarič *et al*. [2020], acceptable values for the L^* parameter are those higher than 60, suggesting that despite the darkening effect, all samples remained within the desirable range for consumer acceptability. The a^* values, representing the red-green spectrum, also decreased significantly (p<0.001) with increasing levels of LF (**Table 3**), indicating a shift toward

Table 4. Nutritional composition of baked rolls prepared using wheat flour (control) and blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively).

Parameter	Control	LF5	LF10	LF15	LF20	LF25
Moisture (g/100 g)	5.48±0.04	4.86±0.08**	4.73±0.04**	3.95±0.15***	4.31±0.17***	4.43±0.10***
Lipids (g/100 g)	1.41±0.07	1.93±0.05**	2.56±0.16***	2.80±0.02***	3.75±0.11***	4.12±0.06***
Carbohydrates (g/100 g)	81.15±0.27	79.70±0.23**	77.42±0.15***	75.88±0.12***	72.82±0.01***	70.72±0.30***
Proteins (g/100 g)	11.05±0.23	12.52±0.23**	14.24±0.05***	16.15±0.28***	17.65±0.03***	19.14±0.27***
Ash (g/100 g)	0.91±0.00	0.99±0.04*	1.05±0.02**	1.22±0.00***	1.47±0.02***	1.59±0.02***
Starch (g/100 g)	70.85±0.30	67.39±0.23***	64.65±0.02***	62.82±0.40***	59.81±0.20***	57.59±0.03***
Dietary fibre (g/100 g)	3.93±0.03	8.08±0.18***	10.06±0.05***	11.80±010***	12.68±0.18***	13.24±0.12***
Energy value (kcal/100 g)	365.79±0.38	353.97±0.26**	349.46±1.27***	346.15±0.07***	344.94±1.85***	343.57±0.17***

Results are presented as mean ± standard deviation (n=3). *, **, and *** indicate a significant difference at p<0.05, p<0.01, and p<0.001, respectively, compared to control.

less red and more green. Similarly, the b* values, representing the yellow-blue spectrum, increased with higher LF content, which means that the yellow hue of the samples intensified [Atudorei *et al*., 2022]. These color changes were caused by the presence of natural pigments in the raw material. LF is naturally yellower than wheat flour owing to its high carotenoid content (544.78 µg/100 g), mainly lutein and zeaxanthin, which are characterized by intense yellow-orange color [Villacrés *et al*., 2020]. Comparable results have been reported in several studies focused on the incorporation of lupin into baked goods [Atudorei *et al*., 2022; Jayasena & Nasar-Abbas, 2011; Yaver & Bilgiçli, 2021].

The C parameter serves as a quantitative measure of color intensity. Higher chroma values indicate greater color vividness as perceived by the human eye [Pathare *et al*., 2013]. LF increased the C parameter of the products (**Table 3**), indicating more saturated and intense colors. This is indicative of the higher pigment concentration from the LF, contributing to the overall color intensity [Villacrés *et al*., 2020].

The hue angle, which represents the property of the color, can vary from 0° (pure red) to 270° (pure blue), where 90° is pure yellow and 180° is pure green. In our study, the hue angle of the rolls produced using flour blends was significantly higher compared to the control (**Table 3**), reflecting slight shifts in the perceived color type. All the baked rolls exhibited an h value of approximately 80°, indicating their yellowish hue. This color characteristic aligns with findings reported by Mazumder *et al.* [2021].

ΔE represented the overall color difference compared to the control sample. The ΔE values were significantly (p<0.001) higher than zero (**Table 3**), indicating that the color difference between the LF-incorporated baked rolls and the control was visually noticeable. This aligns with the criteria outlined by Pathare *et al.* [2013], where a ΔE value exceeding the threshold is perceptible to the human eye.

The yellowness index (YI) provides a numerical measure of the extent of yellowness in a material. It characterizes color shifts from near-white opaque surfaces to yellowish tones [Yeo & Sung, 2021]. The whiteness index (WI) integrates lightness and the yellow-blue color spectrum into a single value. It reflects the overall whiteness of food products and can be used to assess discoloration occurring during processes such as drying [Pathare *et al*., 2013]. The addition of LF to the rolls resulted in a significant increase in the YI and a notable decrease in the WI (**Table 3**). Combined with the changes in L*, it can be stated that the overall color of baked rolls with LF addition ranged from bright yellow to brownish-yellow. However, the light-brown color that develops during the baking process could also be a result of the Millard reaction, the reaction between reducing sugars and amino acids [Jayasena & Nasar-Abbas, 2011]. Therefore, in our study, browning index (BI) was determined. It reflects color changes typically associated with non-enzymatic browning reactions, such as the Maillard reaction and caramelization, which occur during processing or cooking. The addition of LF led to a significant increase in BI of rolls compared to the control (**Table 3**). In lupin-enriched backed rolls, more Maillard reaction products could appear deepening the yellow-brown color of the products, due to the presence of free amino acids and proteins in lupin flour, which participate in this reaction. These findings align with those of Schouten *et al*. [2023], who reported a similar increase in BI with the addition of LF in baked goods.

The proximate composition of baked rolls

The nutritional composition of baked rolls substituted with different levels of LF is presented in **Table 4**. Moisture content is a crucial parameter as it affects the texture, shelf-life, and microbial stability of bread [Holkovičová *et al*., 2024]. It was observed that values of this parameter decreased significantly with the addition of LF, ranging from 4.86 g/100 g (LF5) to 4.43 g/100 g (LF25). The reduction in moisture content can be attributed to the high protein and fibre contents of lupin, which bind water more effectively, reducing the free moisture content in the rolls. This behavior is consistent with the findings of Kohajdová *et al*. [2011], who reported that the addition of legume flours resulted in a lower moisture content of baked products.

It was determined that the baked rolls prepared from flour blends contained a higher amount of lipids, with LF25 containing up to 3 times more lipids than wheat flour baked rolls

(1.41 g/100 g) (**Table 4**). A comparable lipid content in lupin bread (1.55 g/100 g) was also described in a study by Pereira *et al*. [2024]. From a nutritional point of view, unsaturated fatty acids are especially important, and represent up to 90% of the total amount of fatty acids in lupin. These are primarily oleic acid (32–50%), linoleic acid (17–47%) and linolenic acid (3–11%) [Rybiński *et al*., 2018].

The replacement of wheat flour with LF significantly (p<0.001) decreased the starch content in baked rolls (**Table 4**). As was previously documented, lupin flour contains significantly less starch than wheat flour (1–4 g/100 g *vs.* 70–77.1 g/100 g) [Kohajdová *et al*., 2011].

Ash content increased with the addition of lupin, from 0.91 g/100 g in the control to 1.59 g/100 g in the LF25 sample (**Table 4**). Lupin seeds are rich in essential minerals like calcium, magnesium, and potassium, which contribute to the increased ash content in the rolls [Plustea *et al*., 2022].

Protein content is a crucial nutritional parameter that contributes to the structure and nutritional value of bakery products. A significant increase in protein content of the rolls was observed (from 11.05 g/100 g to 12.52–19.14 g/100 g) following the substitution of the wheat flour with LF. Previously, Yaver & Bilgiçli [2021] also documented an enhanced protein content (approximately 10%) in the wheat bread incorporated with ultrasound-treated lupin. Lupin is rich in high-quality proteins such as albumins and globulins, which enhance the protein content of the rolls. These proteins not only improve the nutritional profile but also contribute to the functional properties of the dough [Shrestha *et al*., 2021].

The total content of dietary fibre in the baked rolls also significantly (p<0,001) increased with the use of LF for their production, from 3.93 g/100 g in the control to 13.24 g/100 g in the LF25 sample (**Table 4**). The predominant fibre component in lupin seeds is insoluble fibre (89%), which is mainly composed of cellulose, hemicellulose and lignin [Parmdeep & Singh, 2017]. These findings are in line with those reported by Villarino *et al*. [2015], who studied the impact of the addition of lupin varieties to wheat bread and reported that the total dietary fibre content ranged between 14.6–16.2 g/100 g.

The results of this study demonstrated that the baked rolls incorporated with lupin flour containing more than 10% lupin can be considered, according to European Commission Regulation (EC) No 1924/2006 [Regulation EC, 2006], as a foodstuff high in fibre and protein (products containing at least 6 g of fibre *per* 100 g and at least 20% of the energy value of the rolls was provided by proteins).

According to Wójcik *et al*. [2021], the total carbohydrates include all digestible carbohydrates, primarily starches and sugars, which serve as a significant source of energy for the consumer. The total carbohydrate content was significantly lower in the LF baked rolls (70.72–79.70 g/100 g) than in the control (81.15 g/100 g). This trend aligns with Wójcik *et al*. [2021] who observed similar reductions in carbohydrate content when 10% pea flour was added to bread formulations.

The energy value indicates the number of calories that baked rolls provide to the body when consumed [Sedláková *et al*., 2016]. The energy value of the products decreased with the addition of lupin flour, from 365.79 kcal/100 g in the control to 343.57 kcal/100 g in the LF25 sample. The reduction in energy value is due to the lower carbohydrate content and higher fibre content of the lupin-enriched rolls, which are less calorie--dense. Furthermore, Sedláková *et al*. [2016] reported that lupin consumption caused feelings of satiety and affected the energy balance. These findings suggest that, compared with wheat bread, baked rolls enriched with lupin seed flour may decrease appetite for a short time.

Correlations between the assessed parameters

Correlation analysis revealed high positive correlations between WA and the protein content of the baked rolls (correlation coefficient, r=0.988) and TDF (r=0.898), indicating that the increased WA is closely connected to the enhanced protein and fibre contents contributed by the lupin flour. Comparable findings have been reported by Plustea *et al*. [2022], who investigated lupin-fortified wheat bread with a replacement of lupin flour at levels of 10, 20, and 30%.

Among the rheological parameters, DS also showed strong positive correlations with starch content and the C2 parameter (r=0.941 and r=0.916, respectively) and negative correlations with C1–C2 and protein content (r=–0.945, r=–0.929). Previously it was documented, that DS, C2, and C2–C1 were parameters related to the development and strength of the gluten matrix [Calderón *et al*., 2022].

Strong positive correlations were found between C3, C4, and C5 parameters (r in the range of 0.897–0.961). A strong positive correlation between C3 and C5 (r=0.942) was also documented by Belc *et al*. [2021] for wheat dough incorporated with legume protein concentrates.

Moreover, the content of starch in the samples was strongly positively correlated with the difference between C3 and C2 (r=0.953). The decrease in the viscosity peak (C3) and C3–C2 parameter with the addition of fewer starch components was in line with the findings by Abdel-Samie & Abdulla [2016]. The starch content also showed a strong positive correlation with the C5 parameter of Mixolab (r=0.946) and specific volume values (r=0.949). The C5 parameter is closely connected to the staling process of baked goods. Due to this fact, these results support the importance of the starch phase during the baking process [Abdel-Samie & Abdulla, 2016].

High positive correlations were also observed between loaf volume, specific volume, and the cambering of samples (r in the range of 0.884–0.9215). The relationships between the mentioned parameters underscores the importance of dough expansion and gas retention and that denser loaves (lower specific volume) tend to have poorer shapes [Monteiro *et al*., 2021]. Moreover, WA was negatively correlated with loaf volume (r=–0.943), specific volume (r=–0.939), and cambering (r=–0.957). In general, the higher the WA ability of a sample is, the greater the specific volume is, up to a certain point. Beyond this point, the weak structure of the dough may cause it to collapse during fermentation or baking. Such a high WA can lead

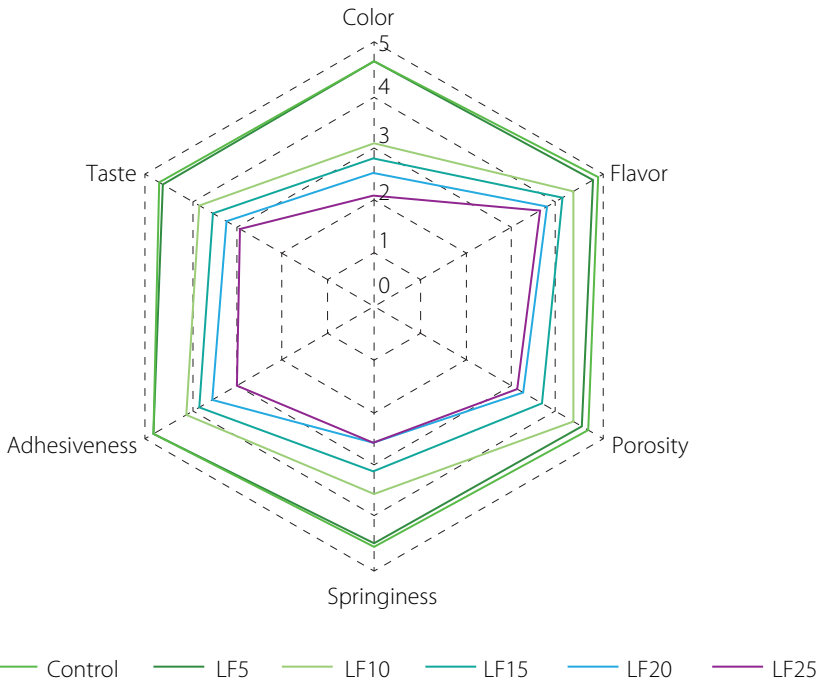

Figure 1. Sensory acceptability of baked rolls prepared using wheat flour (control) and flour blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively).

to heavier, denser dough, which impacts the loaf's ability to rise and maintain a good shape [Monteiro *et al*., 2021].

In the case of the other Mixolab parameters, the specific volume showed the highest positive correlation with C2 (r=0.971) among all the Mixolab parameters, followed by C5 (r=0.965), C3 (r=0.960) and dough stability (r=0.815). Similar results were also reported by Manano *et al*. [2021], who studied the properties of wheat-cassava composite dough.

Furthermore, the loaf volume and specific volume were negatively correlated with the protein content (r=–0.977 and r=–0.975, respectively). A higher protein content could lead to stronger dough that may not expand as much, resulting in lower volumes.

Sensory acceptability of baked rolls

Sensory analysis is crucial for assessing the quality, acceptability, and consumer preference of food products [Ruiz-Capillas & Herrero, 2021]. **Figure 1** shows the effects of the incorporation of LF to wheat baked rolls on their color, flavor, porosity, springiness, adhesiveness, and taste acceptability. The sensory scores for color did not differ significantly (p≥0.05) between the control sample and LF5. However, with increasing LF levels, the baked rolls became darker and yellowish, and hence less attractive for the panellists. These results are also supported by correlation analysis, which revealed strong correlations (r=0.841–0.913) between the sensory color scores and instrumentally measured b* value and YI.

The taste and flavor scores of the baked rolls were significantly affected at a lupin substitution level of 20% (**Figure 1**). The decline might be due to the beany flavor associated with lupin flour. Likewise, taste and flavor in other bakery products were also affected by higher lupin contents (over 30%) because of their pronounced beany flavor and aftertaste [Jayasena & Nasar-Abbas, 2011; Plustea *et al*., 2022]. The overall aroma of lupins is described as beany or legume-like, green, or earthy [Schlegel *et al*., 2019]. One of the main compounds associated with the distinctive beany flavor in lupins, peas, beans, and soy is the alkyl aldehyde hexanal [Schlegel *et al*., 2019]. 2-Isopropyl-3-methoxypyrazine, 3-*sec*-butyl-2-methoxypyrazine, and 3-isobutyl--2-methoxypyrazine are other compounds mainly responsible for beany-like aroma impressions in lupin.

Up to 10% substitution of wheat flour with LF did not affect the porosity of the baked rolls (**Figure 1**). At ≥15% levels, however, the porosity scores were significantly lower than those of the control. Rathnayake *et al*. [2018] highlighted the importance of protein quality in achieving good porosity. Porosity refers to the size and distribution of air cells within a crumb. The higher protein and fibre contents of lupin can negatively influence the gluten network, reducing gas retention and resulting in denser crumb structures.

The sensory score for the springiness of baked rolls decreased gradually with increasing replacement levels of LF (**Figure 1**). This effect may be attributed to the weaker gluten network, which affects the product elasticity. Analogous trends as in this study were observed by Villacrés *et al*. [2020] when wheat flour was replaced with different levels (10, 15, 20%) of fermented lupin flour.

Adhesiveness indicates how much the baked goods stick to surfaces such as teeth or the mouth [Sugiura, *et al*., 2017]. The present study results showed that the adhesiveness was higher

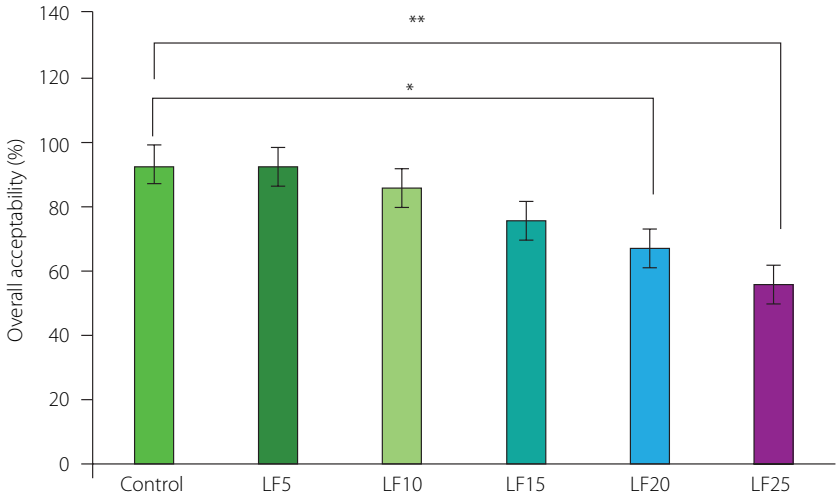

Figure 2. Overall acceptability of baked rolls prepared using wheat flour (control) and flour blends containing 5–25% by weight of lupin flour as a wheat flour substitute (LF5–LF25, respectively). Asterisk brackets denote a significant difference between samples at $p<0.05$ (*) and $p<0.01$ (**).

in the control sample and decreased significantly with increasing levels of lupin in the samples. This may be due to the higher fibre content in lupin, which can reduce adhesiveness and contribute to a less sticky mouthfeel.

The overall acceptability indicates general palatability of the product. The baked rolls prepared by replacing wheat flour with up to 15% lupin flour were not significantly ($p\geq0.05$) different from the control sample (**Figure 2**). However, at the 20% and 25% replacement levels, a significant decrease ($p<0.05$ and $p<0.01$, respectively) was observed in the overall acceptability score.

Sensory acceptability evaluation revealed that replacing wheat flour with LF up to 15% did not negatively affect the sensory quality of the studied baked rolls.

CONCLUSIONS

The results demonstrated that lupin flour incorporation into the wheat dough could significantly modify its rheological properties resulting in increased WA and reduced DS. These changes in the dough rheological characteristics were also reflected in the final baked rolls, which exhibited reduced volume, specific volume, and cambering. Among the tested formulations, the rolls prepared from the blended flour containing 15% lupin flour were evaluated as the most acceptable for assessors. Products containing higher levels of lupin presented a significant decrease in the overall acceptability due to their beany aftertaste. From a nutritional standpoint, the lupin-enriched rolls produced from the flour blend containing more than 10 % lupin can be considered as "high in protein and fibre".

In conclusion, the incorporation of lupin flour into baked rolls offers a promising way to enhance their nutritional profile without compromising their sensory acceptability, provided that the substitution level is less than 15%. This balance allows for the production of baked goods that can meet the dietary needs of consumers seeking higher protein and fibre contents in their diets.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

ADDITIONAL INFORMATION

The research was carried out in accordance with the Code of Ethics of the Slovak University of Technology in Bratislava. The panellists were informed of the conditions for participating and gave informed consent for the sensory analysis. The principle of voluntary participation, confidentiality and anonymity was applied in the sensory analysis. Before the sensory evaluation was conducted, each participant was informed of the conditions for participating, the purpose of research and sensory evaluation, the process of evaluation, the evaluated products, the possible risks, and the possible benefits of the study. The procedure of sensory evaluation was performed in compliance with International Standard ISO 13299.

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Development and Evaluation of *Piper sarmentosum*-Based Kombucha: Fermentation, Bioactivity, and Sensory Acceptance

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Piper sarmentosum, renowned for its bioactive properties, was explored as a material for kombucha production. This study investigated *P. sarmentosum*-based kombucha (PSK), focusing on its phytochemical content, antioxidant and antibacterial activities, anti-amylase effects, and sensory attributes during a 21-day fermentation period. An increase was found in the total flavonoid content (TFC) of PSK from 6.02 mg QE/L at the start to 34.07 mg QE/L after 21 days of fermentation. Similarly, the total phenolic content (TPC) increased from 108.2 mg GAE/L to 266.2 mg GAE/L during the same period. Antioxidant activity of PSK, assessed through DPPH and ABTS assays, reached 0.33 µmol TE/mL and 0.51 µmol TE/mL, respectively, by day 21. While PSK did not show significant antibacterial activity against *Escherichia coli* and *Salmonella typhi*, it exhibited increased inhibition against *Vibrio cholerae* (inhibition zone dimeter of 18.3 mm) and *Staphylococcus aureus* (inhibition zone dimeter of 23.2 mm) by day 14, with a decrease by day 21. Additionally, α-amylase inhibition increased from 10.7% on day 0 to 15.4% on day 21. Sensory evaluations indicated that a 14-day fermentation period optimally balanced sweetness, sourness, and astringency of kombucha. Our study results indicate that *P. sarmentosum* is a promising substrate for preparing kombucha.

Keywords: biological activities, functional beverage, *Piper sarmentosum*, SCOBY, sensory evaluation

INTRODUCTION

The use of plants with bioactive properties has a rich historical backdrop and continues to play a crucial role in contemporary health practices. Among such plants, *Piper sarmentosum*, commonly known as betel leaf, stands out for its diverse therapeutic benefits. Native to Southeast Asia, *P. sarmentosum* has been used in traditional treatments for its anti-inflammatory, antimicrobial, and antioxidant properties [Sun *et al.*, 2020]. Phytochemical analysis of *Piper sarmentosum* reveals the presence of various bioactive compounds, including flavonoids and phenolic acids, which contribute to its health-promoting effects [Adib *et al.*, 2024]. These compounds are known to exhibit a range of beneficial activities, including anti-cancer, anti-diabetic, and liver-protective effects [Adib *et al.*, 2024; Othman *et al.*, 2022].

Kombucha, a fermented tea beverage, has gained global popularity for its potential health benefits [Kapp & Sumner, 2019]. Originating from Asia, kombucha is produced through the fermentation of a sweetened tea by a symbiotic culture of bacteria and yeast (SCOBY). The fermentation process results in the formation of organic acids such as acetic, gluconic, and lactic acids, as well as bioactive compounds like phenolic acids and flavonoids, which are credited with the drink's health-promoting properties [Coelho *et al.*, 2020]. These include antioxidant and antimicrobial effects, which contribute to its potential in supporting gut health and enhancing overall immunity [Coelho *et al.*, 2020; Kitwetcharoen *et al.*, 2023].

While traditional kombucha is typically made using tea from *Camellia sinensis*, recent innovations have explored the use

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of alternative substrates to create diverse kombucha varieties [Bortolomedi *et al.*, 2022]. Herbs, fruits, and vegetables have been employed to enrich the nutritional profile and therapeutic potential of kombucha [Emiljanowicz & Malinowska-Pańczyk, 2020]. *P. sarmentosum*, with its rich profile of beneficial phytochemicals, presents a promising candidate for this purpose [Sun *et al.*, 2020]. Incorporating *P. sarmentosum* into kombucha could potentially offer a functional beverage that combines the probiotic benefits of kombucha with the bioactive properties of *P. sarmentosum*.

This study aimed to develop and evaluate a novel kombucha variant using *P. sarmentosum* as a primary ingredient. The phytochemical composition, bioactivity including antioxidant, antibacterial, and anti-amylase activities, and sensory acceptability of *P. sarmentosum*-based kombucha (PSK) were investigated. By exploring this innovative approach, we sought to enhance the functional food and beverage market with a product that offers synergistic health benefits, thus contributing to the advancement of nutritionally enriched beverages.

MATERIALS AND METHODS

Preparation of *Piper sarmentosum*-based kombucha

P. sarmentosum was grown and harvested from experimental garden of the Department of Biotechnology, NTT Hi-tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Viet Nam. The harvested leaves were dried at 40°C in a hot air oven until the moisture content was reduced to below 10 g/100 g, ensuring suitability for kombucha preparation. The starter cultures, provided by Foodplus Ltd., Hanoi, Vietnam, included a sour broth and a cellulosic pellicle stored at 4°C. This SCOBY comprises various microorganisms, including the yeast species: *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*, as well as the acetic acid bacteria (AAB): *Komagataeibacter pomaceri* and *Komagataeibacter rhaeticus*. The microbial content was verified at approximately 5×10^6 CFU/g by the Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi (VNU), Vietnam.

Dry *P. sarmentosum* leaves were used to prepare the tea infusion. To prepare this infusion, 10 g of the leaves were steeped in 1,000 mL of hot water for 20 min. The infusion was then filtered using a sterile sieve. Sucrose was added at a concentration of 100 g/L and fully dissolved in the tea infusion. After cooling to ambient temperature, the mixture was divided into small bottles (90 mL each). To each bottle, 3 g of SCOBY (3%) and 10 mL of sour broth (10%) were added. Kombucha beverages were fermented at 30°C and observed at 0, 7, 14, and 21 days to represent the commonly studied stages of fermentation [Valiyan *et al.*, 2021]. Three independent batches were prepared and analyzed.

Fermentation monitoring by measuring SCOBY growth, sugar content, pH, and total acidity

During the fermentation period (days 0, 7, 14, and 21), samples of the cellulosic pellicle were collected from each batch to measure SCOBY production (g/L). The sugar content (°Brix) of each sample was measured using an RA500 KEM refractometer (Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan) at 20°C. The pH values of the kombucha beverages were measured with a Mettler Toledo J12683 pH meter (Mettler-Toledo International, Inc., Greifensee, Switzerland). Total acidity was assessed through potentiometric titration and expressed as g of acetic acid equivalents *per* 100 mL. This was achieved by titrating with 0.1 M NaOH until the pH reached 8.1. All analyses were performed in triplicate.

Determination of total phenolic and total flavonoid contents

The total phenolic content (TPC) in PSK was measured using the colorimetric assay with the Folin–Ciocalteu reagent [Singleton & Rossi, 1965]. In this method, 100 µL of the fermentation samples were combined with 900 µL of distilled water and 500 µL of 10% Folin–Ciocalteu reagent. Then, 500 µL of a 7.5% Na_2CO_3 solution was added. The final mixture was incubated at 40°C for 30 min. The absorbance of the reaction mixture was measured at a wavelength of 765 nm by a C-7000UV spectrometer (Peak Instruments Inc., Houston, TX, USA). The TPC was quantified using a gallic acid standard curve, with results expressed as mg of gallic acid equivalents (GAE) *per* L of kombucha (mg GAE/L).

The total flavonoid content (TFC) was quantified using a colorimetric method with $AlCl_3$ [Amjadi *et al.*, 2023]. For this assay, 0.5 mL of each kombucha beverage was diluted in 1.5 mL of 99% ethanol and allowed to stand for 5 min. Next, 0.1 mL of 10% $AlCl_3$ was added, and the mixture was left at room temperature for 5 min. Subsequently, 0.1 mL of 1 M CH_3COOK and 2.8 mL of distilled water were introduced to the solution and incubated for 45 min at ambient temperature. The absorbance of the final reaction mixture was measured at 415 nm by a C-7000UV spectrometer (Peak Instruments Inc.). A quercetin standard solution was used to construct the standard curve for TFC quantification, with results expressed as mg of quercetin equivalents (QE) *per* L of kombucha (mg QE/L).

Assessment of antioxidant activity

The assessment of free radical scavenging activity in PSK was conducted using spectrophotometric methods, specifically the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay.

The DPPH assay was conducted following the method developed by Brand-Williams *et al.* [1995] with slight modifications. The portion of 50 µL of each diluted PSK or Trolox solution was mixed with 1 mL of a DPPH radical solution (40 mg/L, TCI Chemical Co., Japan). After incubating the mixture in a dark environment at ambient temperature for 30 min, its absorbance was measured at a wavelength of 517 nm by a C-7000UV spectrometer (Peak Instruments Inc.).

The ABTS assay followed the methodology outlined by Re *et al.* [1999]. The ABTS (Cool Chemical Science and Technology, Beijing, China) solution at a concentration of 7 mM was mixed with a 2.45 mM $K_2S_2O_8$ solution and stored in darkness for 16 h before use. For the assay, 1 mL of the prepared ABTS radical cation solution was combined with diluted PSK or Trolox. After incubating the mixture in the dark at room temperature for 6 min, its absorbance was measured at 734 nm by a C-7000UV spectrometer (Peak Instruments Inc.).

The results of both assays were expressed as µmol of Trolox equivalent (TE) *per* mL of kombucha.

Evaluation of antibacterial efficacy

The antibacterial efficacy of PSK was assessed using the agar diffusion method against several pathogenic bacterial strains: *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, and *Staphylococcus aureus*. These bacteria were first cultured overnight in Mueller Hinton Broth (HiMedia Laboratories, Mumbai, India). The bacterial cultures were then suspended in phosphate-buffered saline (PBS) and diluted to achieve a concentration of 1×10^7 CFU/mL.

For the antibacterial assay, 100 µL of each bacterial suspension were evenly spread on Mueller Hinton Agar (MHA) plates. Sterile metallic tubes were used to create three wells, each 5 mm in diameter, on the agar plates. Following this, 100 µL of each *P. sarmentosum* tea kombucha sample were added to the wells to enable diffusion into the agar. Sterile distilled water served as the control. The plates were then incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the zones of inhibition around each well.

Assessment of α-amylase inhibition activity

The inhibition of α-amylase activity was assessed *via* the starch-iodine test, following the methodology described in a previous study [Do *et al.*, 2024]. In this assay, 400 µL of the PSK were mixed with 400 µL of a 4 µM α-amylase solution (Sigma-Aldrich, St Louis, MO, USA) in a 0.04 M phosphate buffer solution at pH 6.9. This mixture was incubated at 36°C for 10 min. Subsequently, 400 µL of a 1% starch solution was added, and the mixture was incubated again at 36°C for another 10 min. The reaction was halted by adding 100 µL of 1 M HCl. To develop the colour, 100 µL of a 5 mM iodine solution was added to 200 µL of the reaction mixture, followed by dilution with 1 mL of distilled water. The absorbance was measured at 570 nm using a C-7000UV spectrometer (Peak Instruments Inc.). Phosphate-buffered saline (PBS) served as the negative control, while acarbose (1 mg/mL) was used as the positive control. The inhibition of α-amylase activity was calculated using Equation (1):

$$\text{Inhibition (\%)} = \frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)} \times 100 \quad (1)$$

where: *Ac* is the absorbance of the negative control (enzyme + substrate, no inhibitor), *Acb* is the absorbance of the control blank (substrate without enzyme or inhibitor), *As* is the absorbance of the test sample (enzyme + substrate + inhibitor), and *Asb* is the absorbance of the sample blank (substrate + inhibitor, no enzyme).

Sensory evaluation

The sensory properties of PSK were evaluated by a panel of 20 trained assessors (10 men and 10 women, aged 22–35 years), selected from graduate students and faculty members of the Department of Food Technology, Institute of Applied Technology and Sustainable Development, Nguyen Tat Thanh University, Ho Chi Minh City. The panelists had prior experience with sensory evaluation and underwent an additional training session to familiarize themselves with the evaluation process and the specific attributes of the kombucha. During the training, the meaning and evaluation criteria for each sensory attribute (appearance, color, odor, sweetness, sourness, astringency, and overall sensory acceptability) were explained using reference samples to standardize interpretations and avoid misjudgments. A 9-point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely), was used to rate the intensity of each characteristic [Panda *et al.*, 2017].

The assessments were conducted in a sensory evaluation laboratory designed to minimize external distractions, equipped with uniform lighting and air circulation. The kombucha samples, representing three batches of PSK for 7, 14, and 21 days, were coded with three-digit random numbers to ensure unbiased evaluation. Samples were served in clean, transparent tumblers. Between assessments, panelists were provided with water and unsalted crackers to cleanse their palates. Panelists were instructed not to discuss their scores during the assessment sessions.

Statistical analysis

The data obtained from the analysis were processed and reported as the means and standard deviation (SD) from three experimental replicates (representing three different fermentation batches of PSK, fermented for 7, 14, and 21 days). Statistical significance between different fermentation times was determined using one-way analysis of variance (ANOVA) followed by Tukey's test with a significance threshold set at $p \leq 0.05$. All statistical analyses and graph generation were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Changes in SCOBY mass, pH, acetic acid content, and sugar content during fermentation

During the fermentation of the sweetened infusion of *P. sarmentosum* leaves, the indicators including SCOBY mass, pH, total acidity, and sugar content were monitored and recorded at days 0, 7, 14, and 21 (**Figure 1**). The weight of the SCOBY layer showed a significant ($p \leq 0.05$) increase beginning from day 7 and continuing throughout the 14 days of fermentation. The wet SCOBY weight increased from 27.3 g/L on day 7 to 95.3 g/L by day 14, remaining steady at 95.2 g/L on day 21. A similar trend was observed for the dry SCOBY. This accumulation of microbial biomass indicates that sweetened infusion of *P. sarmentosum* was an effective substrate for SCOBY formation. Previous studies demonstrated that the co-fermentation of whole-plant cassava with increasing levels of *P. sarmentosum* fostered a diverse microbial community increasing lactic acid bacteria count and shaping the predicted functional roles of the bacteria [Li *et al.*, 2024]. Concurrently, fermentation leads to a rapid decrease in reducing sugar content in kombucha, as observed in our studies where sugar levels content diminished significantly ($p \leq 0.05$) over time (**Figure 1B**). This substantial reduction in sugar content suggests efficient microbial activity in PSK, beneficial for consumers seeking lower sugar intake.

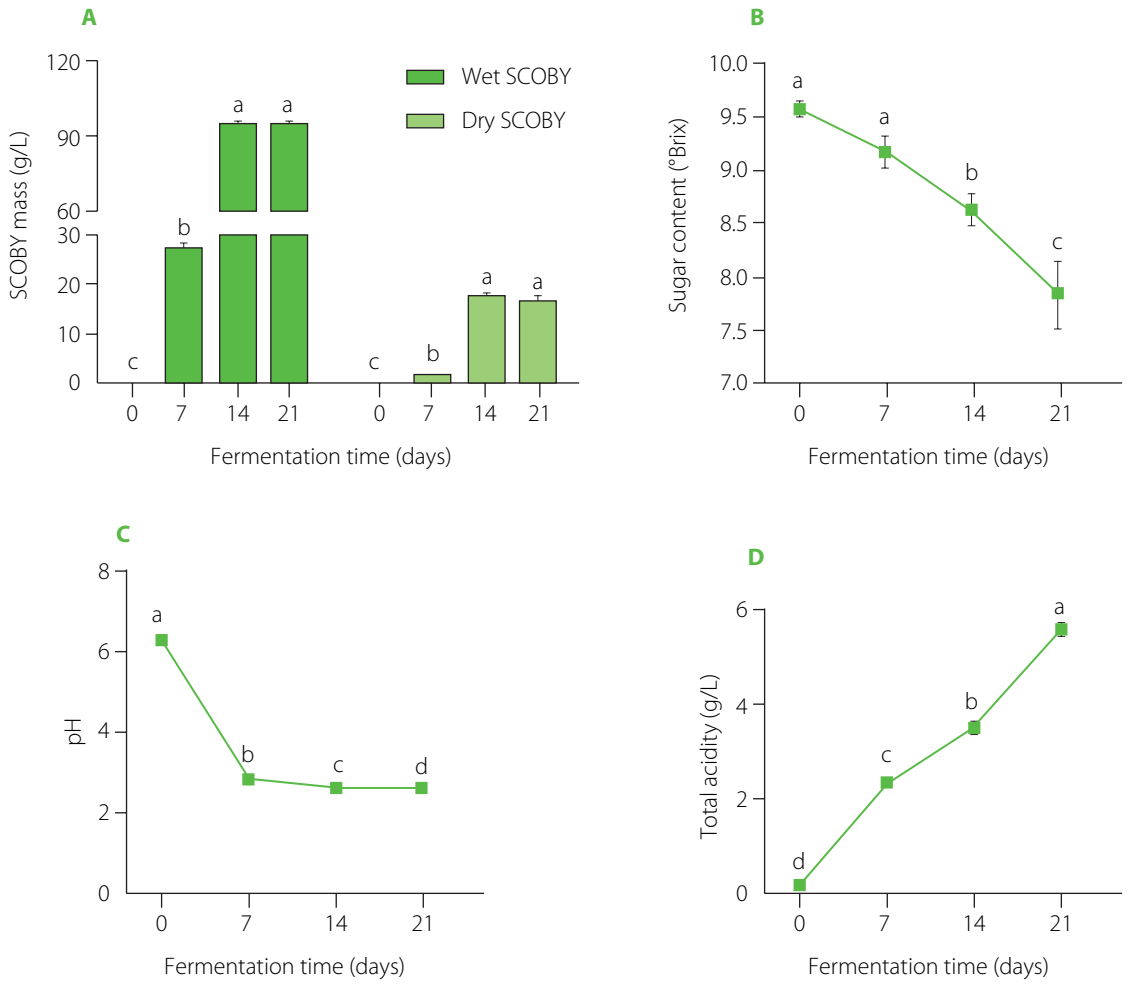

Figure 1. Mass of symbiotic culture of bacteria and yeast (SCOBY) (**A**), sugar content expressed by Brix scale (**B**), pH (**C**) and total acidity (**D**) during production of *Piper sarmentosum*-based kombucha. Data are presented as the means and standard deviations (n=3). Different letters above bars/points indicate significant differences over fermentation time ($p \leq 0.05$).

The initial pH of the sweetened *P. sarmentosum* leaf infusion was 6.33, which decreased markedly over the fermentation period (**Figure 1C**). At the end of the fermentation period (day 21), the pH of PSK was approximately 2.55. The pH values of the PSK were within the standard safe range for human consumption, which is between 2.5 and 4.2 [Nummer, 2013]. The increase in the total acidity (**Figure 1D**) corresponded with the observed decrease in pH, indicating effective fermentation and robust acid production in PSK, essential for the microbiological safety and stability of kombucha [Mo *et al.*, 2008].

Total phenolic and total flavonoid contents of kombucha

Phenolics are well-known for their antioxidant properties, which contribute significantly to the health benefits of kombucha [Kitwetcharoen *et al.*, 2023]. Recent findings by Ware *et al.* [2024] revealed a rich profile of over 150 metabolites in *P. sarmentosum* leaves, predominantly consisting of flavonoids, which play a crucial role in the bioactive potential of the plant. During fermentation of the sweetened *P. sarmentosum* leaf infusion, the TFC started at approximately 6.02 mg QE/L on day 0, increasing to 7.96 mg QE/L and 10.28 mg QE/L on days 7 and 14, respectively, with no significant (p>0.05) changes observed during this period. The TFC then increased sharply, reaching a peak of 34.07 mg QE/L by day 21. Similarly, TPC in PSK also increased significantly throughout the fermentation period (**Figure 2B**). Initially determined at approximately 108.2 mg GAE/L on day 0, the TPC increased significantly to 221.4 mg GAE/L by day 7. It further increased notably to 251.27 mg GAE/L by day 14 and 266.2 mg GAE/L by day 21. Our data on PSK align with findings from kombucha made with black tea and alternative substrates like papaya leaves and wheatgrass, showing a significant increase in TFC and TPC during fermentation [de Noronha *et al.*, 2022; Do *et al.*, 2024; Sun *et al.*, 2015]. Microbial enzymes-including glucosidase, pectinase, xylanase, cellulase, and glucanase, particularly from acetic acid bacteria and yeasts, hydrolyze the oligomeric and polymeric polyphenols and glycoside forms of phenolics in tea to simple phenolics and aglycons, such as catechin and epicatechin, thereby increasing the total phenolic and flavonoid contents [Chu & Chen, 2006; de Noronha *et al.*, 2022; Kim *et al.*, 2023]. While the increases in total phenolic and flavonoid content were notable, comprehensive metabolomic studies are crucial for the accurate identification and quantification of these bioactive compounds.

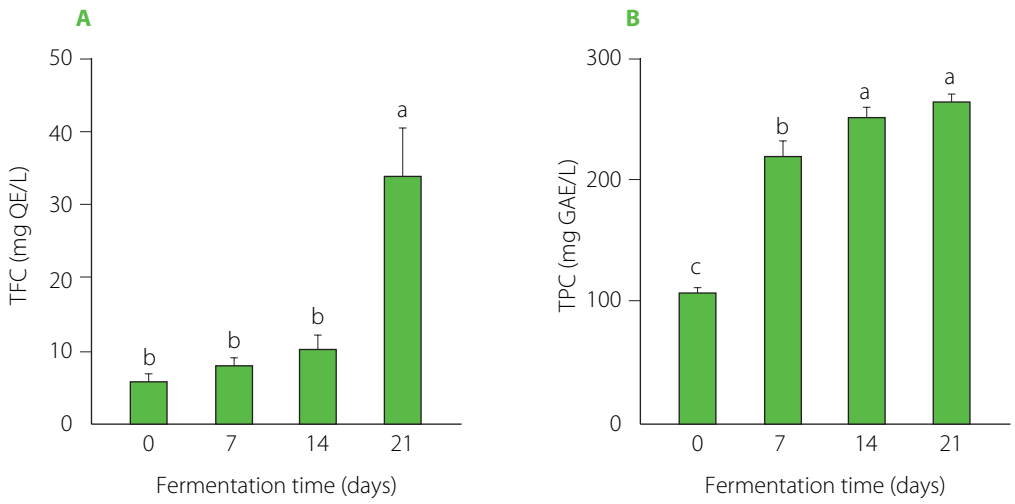

Figure 2. Total flavonoid content (TFC) (**A**) and total phenolic content (TPC) (**B**) in *Piper sarmentosum*-based kombucha at various fermentation times. Data are presented as the mean and standard deviation (n=3). Different letters indicate significant differences over fermentation time ($p \leq 0.05$). GAE, gallic acid equivalent; QE, quercetin equivalent.

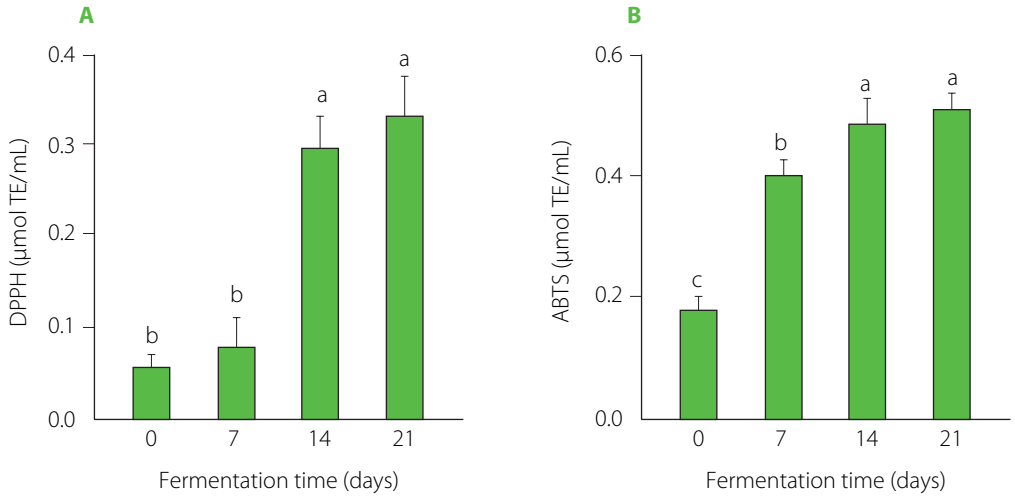

Figure 3. DPPH radical scavenging activity (**A**) and ABTS radical cation scavenging activity (**B**) of *Piper sarmentosum*-based kombucha at various fermentation times. Data are presented as the mean and standard deviation (n=3). Different letters denote significant differences over fermentation time ($p \leq 0.05$).

Antioxidant activity

Antioxidants play a crucial role in scavenging free radicals that cause oxidative stress and damage to cells [Ifeanyi, 2018]. Kombucha is rich in such antioxidants, which are primarily phenolic compounds [Kitwetcharoen *et al.*, 2023]. In our study, the DPPH assay results indicated an increase in antioxidant activity of the sweetened *P. sarmentosum* leaf infusion over the fermentation period (**Figure 3A**). At day 0, the antioxidant capacity was approximately 0.06 µmol TE/mL and increased significantly to 0.29 µmol TE/mL by day 14 and 0.33 µmol TE/mL by day 21. Similarly, the ABTS assay results show a significant ($p \leq 0.05$) increase in antioxidant activity throughout the fermentation period (**Figure 3B**). At day 0, the antiradical activity against ABTS cation radical was approximately 0.18 µmol TE/mL. It increased significantly to 0.40 µmol TE/mL by day 7, and further rose to 0.49 µmol TE/mL and 0.51 µmol TE/mL by days 14 and 21, respectively. This enhancement was likely a result of the increased content of phenolics including flavonoids during fermentation, as shown in **Figure 2**. A study by Huang *et al.* [2024] determined that the DPPH radical and ABTS radical cation scavenging activities of kombucha produced from black tea were the highest (2 and 3 µmol TE/mL, respectively) after 9 days of fermentation. In comparison, kombucha produced from *P. sarmentosum* exhibited lower DPPH radical and ABTS radical cation scavenging activates. This difference may primarily be due to the lower baseline antioxidant content in *P. sarmentosum* compared to *C. sinensis*. For example, prior to fermentation, the TPC of sweetened *P. sarmentosum* leaf infusion in our study was approximately 108 mg/L, while that of *C. sinensis* infusions, including green and black tea, was around five times higher [Hsieh *et al.*, 2021]. While PSK had lower antioxidant activity than traditional kombucha, the substantial increase observed during fermentation highlights its potential as a functional beverage.

Table 1. Antibacterial activity of *Piper sarmentosum-based kombucha* (PSK) at various fermentation times determined by agar diffusion assay.

Bacterium	Inhibition zone diameter (mm)			
	Day 0	Day 7	Day 14	Day 21
Vibrio cholerae	–	11.2±1.2^{b}	18.3±0.8^{a}	11.3±1.0^{b}
Staphylococcus aureus	–	17.4±1.7^{b}	23.2±2.3^{a}	18.3±4.2^{b}

Data are presented as the mean ± standard deviation (n=3). Dashes indicate no inhibition. Different letters denote significant differences over fermentation time (p≤0.05).

Table 2. α-Amylase inhibition activity (%) of *Piper sarmentosum*-based kombucha (PSK) at various fermentation times and acarbose at concentration 1 mg/mL as a positive control.

Fermentation time	PSK	Acarbose
Day 0	10.7±2.6^{b}	69.1±2.5
Day 7	13.8±1.2^{a}	
Day 14	15.2±1.3^{a}	
Day 21	15.4±1.0^{a}	

Data are presented as the mean ± standard deviation (n=3). Different letters indicate significant differences over fermentation time (p≤0.05).

Antibacterial activity

Previous studies have shown that traditional kombucha from black tea [Battikh *et al.*, 2013] and kombucha fermented from substrates of various herbs, such as thyme, fennel, rosemary, and mint, exhibited potential antimicrobial properties through the agar diffusion method [Battikh *et al.*, 2012]. Extracts from *P. sarmentosum* leaves contain various secondary metabolites, such as glycosides, flavonoids, terpenoids, alkaloids, and phenolics, which have shown inhibitory effects against *S. aureus* [Yusof *et al.*, 2019]. For the first time, we demonstrated the antimicrobial activity of kombucha derived from *P. sarmentosum*. We conducted tests against four common pathogenic bacteria: *E. coli*, *S. typhi*, *V. cholerae*, and *S. aureus*.

PSK showed no significant (p<0.05) antibacterial effect against *E. coli* and *S. typhi* throughout the fermentation period. The lack of effect on *E. coli* and *S. typhi* suggests that the antibacterial compounds produced during the fermentation of sweetened *P. sarmentosum* leaf infusion are not effective against these strains. Conversely, the antibacterial activity against *S. aureus* and *V. cholerae* exhibited a substantial increase beginning on the 7th day (**Table 1**). The inhibition zone further increased to 18.3 mm for *V. cholerae* and 23.2 mm for *S. aureus* by day 14, before decreasing to 11.3 and 18.3 mm, respectively, by day 21. The pH decrease observed during fermentation (**Figure 1C**) could contribute to the increased antibacterial activity, as acidic conditions are known to inhibit the growth of many bacterial pathogens [Sanwal *et al.*, 2023]. Additionally, the phenolic compounds in kombucha are widely recognized for their antimicrobial properties [Nyiew *et al.*, 2022]. Hence, the significant increase in TFC and TPC during fermentation likely contributed to the enhanced antimicrobial activity of PSK. However, we did not observe a consistent correlation between TFC and TPC with antimicrobial activity across three fermentation periods. This suggests that the inhibitory effect of PSK on the pathogenic bacteria may not predominantly rely on its TFC and TPC. Another study also verified the existence of antimicrobial compounds other than organic acids that were generated during fermentation [Mo *et al.*, 2008]. The antimicrobial activity observed in kombucha likely results from a combination of inherent properties from raw materials, as well as organic acids and antimicrobial compounds such as bacteriocins produced by microorganisms during fermentation [Mo *et al.*, 2008; Nyiew *et al.*, 2022; Su *et al.*, 2023]. Further studies are needed to discover the exact mechanisms behind the antibacterial effects of PSK, including the role of pH and the specific compounds involved.

Inhibition of α-amylase activity

The inhibition of α-amylase activity by PSK is a significant area of research, particularly in the context of managing diabetes and obesity. Our previous and other studies have shown that kombucha tea has hypoglycemic effects in both *in vivo* and *in vitro* settings [Permatasari *et al.*, 2021; Phan-Van *et al.*, 2024]. Our current data show that inhibition of α-amylase activity of sweetened *P. sarmentosum* leaf infusion before fermentation was approximately 10.7% (**Table 2**). By day 7 of fermentation, this inhibition level significantly (p≤0.05) increased to around 13.8%. Although the inhibition percentage increased to 15.2% by day 14 and 15.4% by day 21, there was no significant (p>0.05) difference between days 7, 14, and 21, indicating that the primary increase occurred early in the fermentation process. The inhibitory effect of kombucha on α-amylase is attributed to the combined influence of organic acids formed during fermentation and the enhanced activity of tea-derived compounds activated by the fermentation process [Dickmann *et al.*, 2017]. The findings support the development of PSK as functional food that may aid in preventing conditions related to high blood sugar, such as obesity and hypertension. Given that PSK shows promise as an α-amylase inhibitor, further investigation is required to fully elucidate its effects in animal models.

Sensory evaluation

While kombucha is traditionally produced with black tea, using herbal and floral infusions can provide a healthier alternative to sugary soft drinks while also introducing a wider range of flavors [Zhang *et al.*, 2021]. In addition, the sensory profile of kombucha is significantly influenced by fermentation time, which affects perceived turbidity, aroma (including vinegar, citric fruit, and alcoholic notes), and flavor (sour, bitter, and vinegar), highlighting the importance of controlling fermentation duration [Dartora *et al.*, 2023]. In our study, significant changes were observed in the results of the evaluation of sensory attributes of the sweetened *P. sarmentosum* leaf infusion during the 21-day fermentation period. The optimal fermentation period for achieving a balanced and enjoyable sensory profile in PSK was around 14 days. After this period, there was a decline in sweetness along with a minor decrease in acceptance of sourness and astringency, which influenced overall acceptability (**Figure 4**). This could

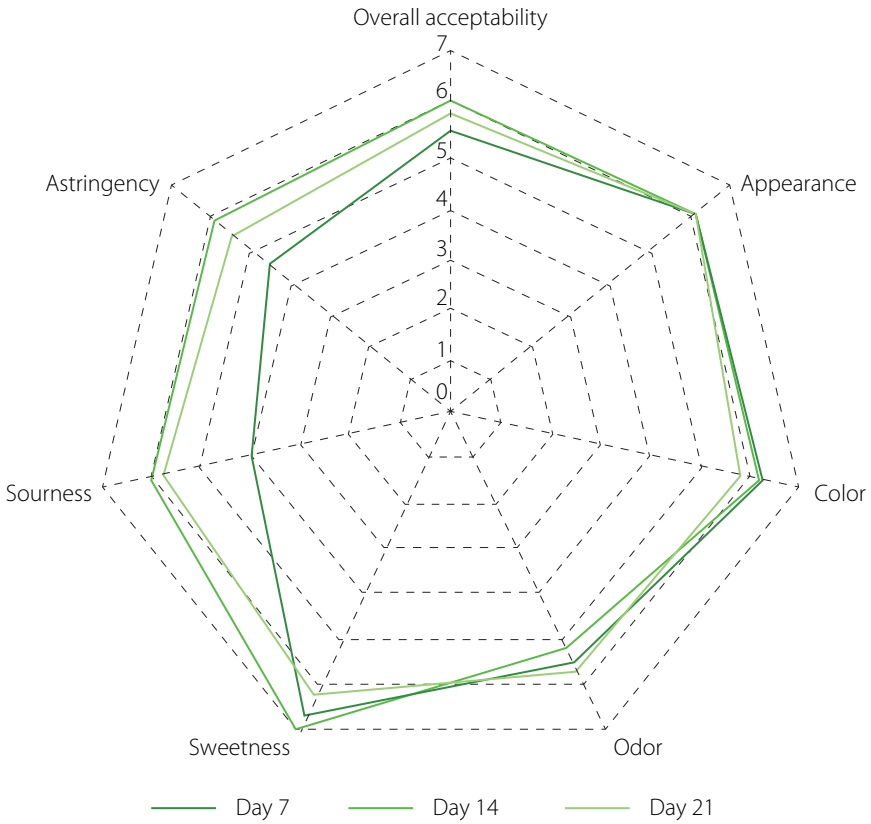

Figure 4. Sensory assessment of *Piper sarmentosum*-based kombucha after 7, 14, and 21 days of fermentation.

be explained by the lower pH value and higher total acidity at 21 days, as shown in **Figure 1**. Studies have consistently demonstrated that longer fermentations (15–20 days) of yerba maté-based kombucha may lead to higher acidity and a "vinegar taste", impacting overall sensory acceptance [dos Santos *et al.*, 2024]. Therefore, our findings can guide the production process to ensure a kombucha beverage with high sensory quality.

CONCLUSIONS

The use of *P. sarmentosum* in kombucha fermentation results in enhanced SCOBY growth, notable pH reduction, increased total acidity, and effective sugar utilization, indicating that *P. sarmentosum* could serve as an alternative substrate for this process. The significant increase in antioxidant and antibacterial activities, as well as α-amylase inhibition, highlights PSK's potential as a functional beverage with enhanced health benefits. The sensory evaluation indicates that a 14-day fermentation period was optimal for achieving a balanced and enjoyable sensory profile. In summary, *P. sarmentosum* shows potential as a raw material for the development of a non-alcoholic functional kombucha beverage, offering promising bioactivities and sensory qualities. Subsequent investigations will examine the physiological effects of PSK in animal models.

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CONFLICT OF INTERESTS

The group of authors has no conflicts of interest to disclose.

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Elevating the Bioactive Potential of Strawberries with Apple Pomace Extract-Infused Chitosan Edible Coating

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Postharvest shelf life has always been one of the main shortcomings in the cultivation of perishable fruit, of which strawberries are one of the most important. Hence, this study aimed to maintain the postharvest quality of fully ripe strawberries with the utilization of sustainable and edible coatings made of chitosan and chitosan infused with apple pomace extract. Fruit quality was assessed on days 0, 2, 4, and 9 following the treatment. A sensory evaluation was also performed on day 0 and day 4 and this included visual (freshness, color and glossiness) and organoleptic traits (hardness, texture, flavor, juiciness and sugar/acid ratio perception). Overall, the coating application resulted in the fruit quality improvement in terms of bioactive compound contents and antioxidant capacity. Furthermore, a decrease was recorded in the number of yeasts and moulds, and in the aerobic mesophilic count. Most differences were recorded on days 4 and 9 when the juice of treated strawberries had significantly higher contents of total phenolic compounds, total flavonoids and total anthocyanins, as well as antioxidant capacity. The total anthocyanin content and total flavan-3-ol content determined in the residue after juice separation were also maintained throughout the storage period. Sensory evaluation tests conducted on day 4 resulted in coated strawberries rated with higher scores for all of the sensory parameters when compared to the control.

Keywords: fruit quality, perishable fruit, phenolic compounds, postharvest shelf-life, sensory evaluation, sustainability

INTRODUCTION

In recent times, biodegradable edible coatings have gained significant attention as environmentally friendly alternatives to conventional food packaging. These coatings not only act as a protective barrier against microbial contamination and oxidation but also prevent physical damage. Furthermore, as they are usually made from natural compounds, such as proteins, lipids, and polysaccharides, their use promotes environmental sustainability. By reducing dependence on non-renewable resources edible coatings offer a sustainable approach to postharvest fruit treatment [Miteluț *et al*., 2021].

Chitosan is the preferred polysaccharide coating for a range of fruits, mainly due to its ability to minimize moisture evaporation, and reduce browning and fungal infections [Obianom *et al*., 2019]. Numerous investigations have been conducted to evaluate the potential of chitosan as an edible coating for strawberry fruit, providing both physical and microbial protection [Khodaei & Hamidi-Esfahani, 2019; Saleem *et al*., 2021]. For example, Jiang *et al.* [2020] investigated the impact of chitosan coating with varied molecular weights (5, 19, and 61 kDa) on the shelf life of strawberries. A polymer with a molecular weight of 61 kDa substantially decreased

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respiration rate and water loss in treated strawberries when compared to the untreated fruits.

While the chitosan preservation technique is progressively advancing, innovative approaches, like the addition of natural additives in the coating, remain relatively underexplored. These additives can improve coatings in terms of their antimicrobial and antioxidant properties, thus extending the shelf life of the food product and improving its safety [Priyadarshi *et al.,* 2024]. Martínez-González *et al.* [2020] formulated a chitosan-based coating incorporating nanostructured chitosan particles and propolis extract. They evaluated its impact on strawberry quality and antioxidant capacity during postharvest storage and found that after 8 days of storage, the coated strawberries exhibited higher contents of total phenolics and total flavonoids, and antioxidant capacity compared to the uncoated fruits. Another aspect to consider is that the addition of these natural components can amplify the sensory attributes of the coated products, influencing their color, flavor, and aroma, thus making them more appealing to the consumer [Pérez-Santaescolástica *et al*., 2022]. Furthermore, the use of naturally derived fruit extracts has also shown potential in fortifying edible coatings, particularly in slowing oxidation and controlling enzyme activity [Kumar *et al*., 2021].

It is estimated that several million metric tons of apple pomace are produced globally each year. Approximately 70–75% of apples are consumed fresh, while the remaining 25–30% is processed into value-added products such as wine, juice, preserves, and dried foods [Plaza *et al*., 2013]. Unfortunately, a significant portion of apple pomace goes unrecovered, often being discarded as waste. Due to its high content of acids and sugars and low protein content, it is unsuitable for animal feed or landfill disposal. As a result, it is often labelled as food waste. However, apple pomace is rich in nutrients and bioactive compounds, such as carbohydrates, phenolics, dietary fibres, and minerals. The predominant phenolic compounds identified in apple pomace across various cultivars include benzoic acids (such as gallic acid), hydroxycinnamic acids (notably chlorogenic acid), flavan-3-ols ((+)-catechin), flavonols (rutin), and chalcones (phloridzin) [Grigoras *et al*., 2013]. The phenolic compounds found in apples are recognised for their significant health benefits and their capacity to mitigate oxidative damage caused by free radicals. Therefore, it can be utilised directly or after minimal processing as a functional ingredient in the development of edible coatings [Lyu *et al*., 2020]. Green techniques for extracting bioactive compounds from apple pomace including minimally processed methods like ultrasound-assisted extraction, cold pressing, supercritical fluid extraction, minimise the use of harmful solvents and energy consumption [Reis *et al*., 2012]. These environmentally friendly approaches enhance the yield and quality of bioactive compounds while promoting sustainability and reducing waste.

Strawberries (*Fragaria* × *ananassa* Duchesne) are hailed as "fruit queen" due to their distinct taste and aroma. They stand out as a highly sought-after fruit across the globe. Their nutritional value is signified by the content of vitamin C, β-carotene, vitamin E, along with other beneficial compounds [Priyadarshi *et al*., 2024]. However, strawberries have a brief post-harvest lifespan and are highly perishable, primarily due to moisture loss, mechanical injury, and both physical and microbial decay [Riaz *et al*., 2021].

Given the aforementioned, the application of extract-infused edible coating can simultaneously be used to improve or sustain the nutritional quality of strawberries and contribute to the utilization of the agro-industrial by-product, apple pomace. Although some studies have reported the properties of chitosan-based films with extracts from apple peels and their potential for use in coating strawberries [Riaz *et al*., 2018, 2021], this research represents the first application of an eco-friendly edible coating made from chitosan infused apple pomace aqueous extract with unique application to fully ripe strawberries. This approach sought to enhance the nutritional quality of strawberries, extend their shelf life, and promote sustainability by utilising an agro-industrial by-product, apple pomace, in a novel and practical application. Fully ripe strawberries have a very limited storage potential period and were selected to test the coating effect in the most challenging conditions.

MATERIALS AND METHODS

Plant material and chemicals

"Albion" strawberries were harvested (22nd May 2023) from the greenhouse of the private company Jagodar-HB in Donja Lomnica, Zagreb County, Croatia. Fruits were immediately transferred to the laboratory. According to visual strawberry maturity detection based on additional fruit color development, these strawberry fruits were at the stage of full ripeness and had a very limited storage potential period. This was done intentionally to test the coating effect in the most challenging conditions. Apple pomace was collected from different local apple juice producers in the Zagreb county. High molecular weight chitosan (CAS Number: 9012-76-4, molecular weight 310,000–375,000 Da; viscosity 800–2,000 cP in a 1% (*w/w*) solution in 1% acetic acid at 25°C) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other chemicals were of analytical grade and used as received without further purification.

Ultrasound-assisted apple pomace extraction

Before the preparation of aqueous apple pomace extract, the freeze-dried samples were crushed and sifted using a 0.45 mm metal sieve to ensure uniform particle distribution. Based on the preliminary results and technical simplicity, the aqueous apple pomace extract was prepared by suspending exactly 6 g of apple pomace powder (size <0.45 mm) in 200 mL of distilled water. Ultrasound-homogenization-assisted extraction was performed using UP200St-Sonotrode S26d14 equipment (Hielscher, Teltow, Germany). Amplitude was set to 75% and extraction was carried out for 15 min. The parameters were set based on the preliminary results of the extraction kinetics and the point of inflection of the curve, relative to the highest total phenolic content. Following the ultrasound-assisted

process, the extracted solution was filtered through Whatman No. 4 filter paper using vacuum filtration.

Preparation of edible coatings

A chitosan solution (1%, *w/v*) was prepared by dissolving chitosan in a sterile citric acid solution (2%, *w/v*) [Saleem *et al*., 2021]. The solution was stirred and left overnight to ensure full dissolution. The same procedure was used for preparing chitosan infused with aqueous apple pomace extract, which had a total phenolic content of 181.2 mg gallic acid equivalents *per* L. Citric acid (2%, *w/v*) was dissolved in the aqueous apple pomace extract. Then, a chitosan solution (1%, *w/v*) was prepared by dissolving chitosan in the obtained extract solution. The chitosan solution was stirred and left overnight to ensure full dissolution.

Coating application

Two treatments – (i) chitosan coating (chitosan), (ii) chitosan coating infused with apple pomace extract (chitosan/xtr.) – and control (uncoated) were used in this research. Each treatment consisted of three repetitions. Each repetition had 30 strawberries. Before being immersed in the coatings, strawberries were washed using tap water and left to air dry. Three sets (for each treatment) of randomly chosen strawberries (90 fruits *per* treatment) were submerged in the respective coating solutions for 3 min [Jurić *et al*., 2023]. After the dipping process, the strawberries were removed from the solutions, well-drained, and left to air dry. Each repetition included one plastic container that is commercially used for strawberry storage. Each container held 30 strawberries. Subsequently, all containers were placed in cold storage conditions (3±1°C, relative humidity of 95%). The samples were examined at intervals of 0, 2, 4, and 9 days.

Weight loss determination

The weight of six fruits *per* repetition (18 fruits *per* treatment) was measured on all sampling days using a digital analytical balance (OHAUS Adventurer AX2202, Ohaus Corporation, Parsippany, NJ, USA) with a precision of 0.01 g. Weight loss was calculated according to Equation (1):

$$\text{Weight loss (\%)} = \frac{a - b}{a} \times 100 \quad (1)$$

where: a is the weight of strawberries at previous inspection day and b is the weight of fruits on the inspection date.

Total weight loss was calculated as a difference in weight loss between the first and the last inspection day (9th day).

Firmness, total soluble solids, and titratable acidity measurements

The measurements of firmness, total soluble solids (TSS), and titratable acidity (TA) were performed on five randomly selected strawberries from each repetition (15 fruits *per* treatment). Firmness was assessed using a PCE PTR-200 penetrometer (PCE Instruments, Jupiter/Palm Beach, FL, USA) equipped with a 6 mm diameter plunger, with results expressed in kg/cm^2. Measurements were taken at two opposite equatorial positions on each fruit. TSS was determined using a handheld digital refractometer (Atago, PAL-1, Tokyo, Japan) and expressed in °Brix. TA was measured through titration with 0.1 M NaOH, and results were expressed as g citric acid equivalents *per* 100 g [Mitcham *et al*., 1996].

Chemical analysis

Preparation of samples for chemical analysis

Strawberries were crushed and homogenised using a FOSS 2094 equipment (FOSS, Hillerød, Denmark). The resulting homogenates were centrifuged at 1,467×*g* (9,000 rpm) for 20 min using a NUVE NF 800R multi-purpose centrifuge (NUVE, Ankara, Turkey). The supernatants were then filtered through Whatman No. 4 filter paper and utilised for further analysis, with the juice diluted as needed. Residue after centrifugation was saved for specific chemical analyses. All chemical analyses were performed using a UV-1900i spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of total phenolic content

The total phenolic content (TPC) was determined using a modified spectrophotometric method based on Singleton *et al*. [1999]. For the analysis, 100 µL of strawberry juice was combined with 7.9 mL of distilled water and 0.5 mL of the Folin-Ciocalteu's reagent (diluted 1:2, *v/v*, with distilled water). The mixture was then combined with 1.5 mL of 20% (*w/v*) Na_2CO_3 and vortexed thoroughly. After a 2-h incubation, absorbance was measured at 765 nm, and the results were expressed as mg of gallic acid equivalents *per* L of juice (mg GAE/L).

Determination of total flavonoid content

Total flavonoid content (TFC) was determined using a spectrophotometric method described by Ivanova *et al.* [2010]. A 1 mL aliquot of strawberry juice was transferred to a 10 mL volumetric flask containing 4 mL of distilled water. To this, 300 µL of an $NaNO_2$ solution (0.5 g/L) was added, followed by 300 µL of $AlCl_3$ (1 g/L) after 5 min. After additional 6 min, 2 mL of an NaOH solution (1 M) was introduced to the mixture. The final volume was adjusted to 10 mL with distilled water. Absorbance was measured at 360 nm, and the results were expressed as mg of quercetin equivalents *per* L of juice (mg QE/L).

Determination of contents of total anthocyanins and total flavan-3-ols

Total anthocyanin content (TAC) was determined both in the juice and residue after juice separation, while total flavan-3-ol content (F-3-O) only in the residue. Specifically, TAC was determined using 1% (*v/v*) hydrochloric acid in a 70% EtOH solution. A volume of 0.1 mL of juice was added to the solution (10 mL). For the residue, 0.1000 g was weighed and subjected to solid-liquid extraction using the same solution (10 mL) on an orbital shaker for 20 min. Suspensions were filtered through the Whatman No.4 filter paper and diluted as necessary. Absorbance was measured at 525 nm. The total anthocyanin content was quantified and expressed as mg of cyanidin 3-*O*-glucoside equivalents (C3G) *per* L for the juice samples, and *per* 100 g for the residue samples according to Vieira *et al*. [2019]. The total

flavan-3-ol content was measured using the *p*-(dimethylamino) cinnamaldehyde (*p*-DMACA) method [Di Stefano *et al.*, 1989]. In brief, 1 g of the strawberry residue was shaken with 100 mL of an EtOH (70%, *v/v*)/HCl (1%, *v/v*) solution for 20 min. An aliquot of 1 mL of the filtrate was added to a 10 mL volumetric flask. Subsequently, three drops of glycerol were introduced, followed by 5 mL of a freshly prepared 1% (*w/v*) *p*-DMACA reagent in a chilled mixture of methanol and hydrochloric acid (in a 4:1, *v/v,* ratio). The total volume was then brought to 10 mL using methanol, and after a 7-min incubation period, the absorbance was recorded at a wavelength of 640 nm. The total flavan-3-ol content was expressed as mg (–)-epicatechin equivalents *per* 100 g residue (mg EE /100 g).

Determination of antioxidant capacity

The antioxidant capacity of strawberry juice was measured using the DPPH and ABTS assays, which involve 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations, following the methods of Brand-Williams *et al*. [1995] and Re *et al*. [1999], respectively. The results were expressed as mmol Trolox equivalents *per* L of juice (mmol TE/L).

Microbiological analysis

Three strawberries from each treatment replicate were smashed and mixed thoroughly inside a sterile stomacher bag. A 10 g aliquot of the blended material was transferred to another stomacher bag, mixed with 90 mL of 0.1% buffered peptone water, and homogenised for 1 min at 250 rpm (BagMixer® 400 P, Interscience, Puycapel, France). In addition, 1 mL of the coating materials was diluted in 9 mL of 0.1% buffered peptone water and homogenised in the same manner as strawberries. Serial decimal dilutions were prepared from these initial dilutions. The Plate Count Agar (PCA; Biolife, Milan, Italy) was used for counting total mesophilic and psychrotrophic bacteria, Violet Red Bile Glucose (VRBG) agar (Biolife) for counting enterobacteria and Dichloran Rose-Bengal Chloramphenicol (DRBC) agar (Biolife,) for yeasts and moulds. The microbial loads were enumerated by spread-plating 100 µL of each dilution into the DRBC and pour-plating 1 mL of each dilution into VRBG and PCA. The aerobic mesophilic count (AMC) was estimated after incubation of PCA Petri dishes for 48 h at 37°C, *Enterobacteriaceae* were kept for 24 h at 35°C, DRBC Petri dishes were incubated for 7 days at 20°C whereas growth of psychrotrophic bacteria was estimated after incubating PCA plates at 4°C for 7 days [Brasil *et al*., 2012]. The microbial populations were detected on separate days during the experimental period (9 days), and the results were captured in the form of a log of colony-forming units *per* g (log CFU/g).

Sensory analysis

Sensory analysis was conducted on day 0 and day 4, given that fully ripe strawberries are generally acquired and consumed within three days post-harvest in commercial settings. Eleven panellists had no previous experience in the assessment of strawberries but were sensory trained. Panellists were of various age groups (22–53 years), genders (8 F and 3 M), and different ethnicities. Panellists were chosen using the following criteria: (a) no strawberry aversions, allergies, or intolerances; (b) no smoking; (c) ages 22 to 65; (d) normal perceptive abilities; (e) availability for all sessions; and (f) interest in participation. The panellists were given a brief explanation to the technique (with question marks). All samples were examined in partitioned booths within the laboratory, with white light illumination at room temperature. Question marks included visual (freshness, color, and glossiness) and organoleptic (hardness, texture, flavor, juiciness and sugar/acid ratio perception) traits with following rating scores: 1 (unsatisfactory), 1.5 (acceptable to unsatisfactory), 2 (acceptable), 2.5 (moderate), 3 (good), 3.5 (good to very good), 4 (very good), 4.5 (very good to excellent), and 5 (excellent). Each panellist was physically separated from the other, and all samples were coded. Firstly, visual attributes were scored based on the whole strawberry and afterward, organoleptic traits were scored based on strawberry slices. Between each tasting, water and white bread were provided for the taste neutralisation.

Statistical analysis

The obtained dataset was analyzed using IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA) and XLSTAT add-on (Addinsoft, Paris, France) for Microsoft Office 2016 (Microsoft, Redmond, WA, USA). An analysis of variance (ANOVA) was employed for results of chemical, sensory and microbiological analyses to determine differences between treatments and storage times. The chemical analysis data are represented as means with standard deviations obtained from three replicates. The significance ($p<0.05$) was established using the *post hoc t*-tests with Bonferroni adjustment. The microbiological data are shown as means with standard deviations from three replicates. Before statistical analysis, the normal distribution of microbiological data was assessed using the Shapiro-Wilk test, and the homogeneity of variance using Levene's test. Given the non-normal distribution and heterogeneous variance of the data, statistically significant differences in microbial purity of the edible coatings were determined using the Kruskal-Wallis test, while the microbial counts of different microbial groups on strawberries were analyzed using the Scheirer-Ray-Hare test. To determine statistically significant differences between the groups, the multiple pairwise comparisons were performed using *post-hoc* Dunn's test with a Bonferroni adjustment of *p*-values. Differences with $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Ultrasound-assisted extraction of apple pomace

Minimising food loss and waste is essential for sustainable development, and hence shelf-life extension technologies are exploring innovative solutions. This research utilised aqueous apple pomace extract obtained *via* ultrasound-assisted extraction to enrich a 1% chitosan solution for the development of edible coatings on fully ripe strawberries. Ultrasound-assisted extraction is a non-thermal process that applies acoustic energy to increase

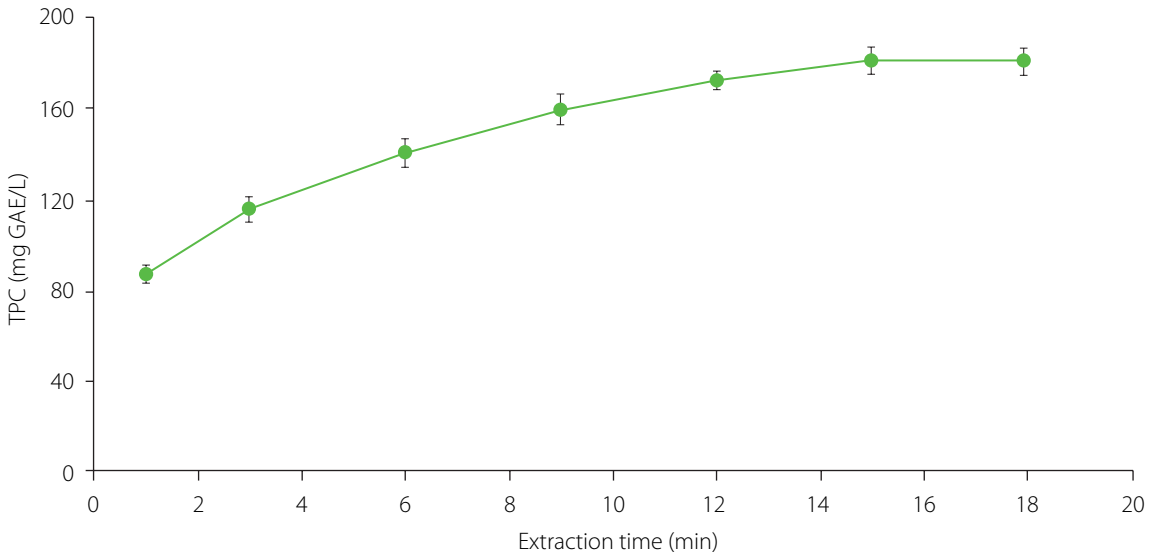

Figure 1. Total phenolic content (TPC) in extracts obtained at different times of aqueous ultrasound-assisted extraction of apple pomace.

Table 1. The weight loss, firmness, total soluble solids (TSS), and titratable acidity (TA) of untreated strawberries (control), strawberries coated with chitosan (chitosan), and strawberries coated with chitosan/apple pomace extract (chitosan/xtr.) over storage time (0–9 days).

Parameter	Treatment	Day 0	Day 2	Day 4	Day 9
Weight loss (%)	Control	–	5.12±1.13	4.51±1.34	11.84±3.00
	Chitosan	–	6.40±0.76	5.44±0.89	12.55±1.90
	Chitosan/xtr.	–	5.74±1.11	5.00±1.20	13.02±3.26
Firmness (kg/cm^2)	Control	0.38±0.08	0.34±0.09	0.30±0.07	0.36±0.12
	Chitosan	0.37±0.08	0.38±0.08	0.28±0.09	0.37±0.10
	Chitosan/xtr.	0.37±0.07	0.37±0.12	0.30±0.10	0.40±0.12
TSS (°Brix)	Control	9.75±0.85	9.73±1.00	9.37±0.91	10.31±1.15
	Chitosan	9.23±0.78	9.59±0.75	9.78±0.80	10.39±0.91
	Chitosan/xtr.	9.52±0.75	9.73±0.80	10.08±0.89	10.88±0.86
TA (g citric acid eq/100 g)	Control	0.82±0.07	0.83±0.08	0.87±0.06	0.78±0.06
	Chitosan	0.79±0.13	0.81±0.07	0.83±0.08	0.77±0.09
	Chitosan/xtr.	0.81±0.10	0.84±0.14	0.87±0.07	0.83±0.08

Results are shown as mean ± standard deviation. There were no significant differences between treatments during storage ($p \geq 0.05$).

the release and diffusion rates of bioactive compounds by breaking down the cell walls *via* cavitation of the solvent [Misra *et al*., 2018]. In the preliminary experiment, the ultrasound-assisted aqueous extraction time of apple pomace was optimised to achieve the highest content of phenolic compounds in the extract The optimal extraction time for the highest yield of phenolic compounds was 15 min, as shown in **Figure 1**. Finally, the total phenolic content of extract was 181.2 mg GAE/L corresponding to 6.04±0.20 mg GAE/g apple pomace powder.

■ Weight loss, firmness, total soluble solids, and titratable acidity of strawberries

Through this research, chitosan, a natural biodegradable polysaccharide extracted from marine natural sources, was used as an edible coating, while an aqueous apple pomace extract was used to potentially enhance edible coating performance. The application of edible coatings did not have a significant effect ($p \geq 0.05$) on any of the investigated fruit quality parameters (weight loss, firmness, TSS, and TA) throughout the storage (**Table 1**). Total weight loss varied from 21.05% for the control group, 22.15% for the chitosan/xtr. treatment, to 22.26% for the chitosan-coated strawberries. Possible explanations for the non-significant effect of the coatings may be attributed to the relatively brief duration of storage for fully ripe strawberries. Similar results were found previously where from day 3–6, the weight loss of coatings-treated strawberries showed no significant differences as compared with the control [Liu *et al*., 2021]. Furthermore, in the same study, it is worth noting that after longer periods of storage (8 days),

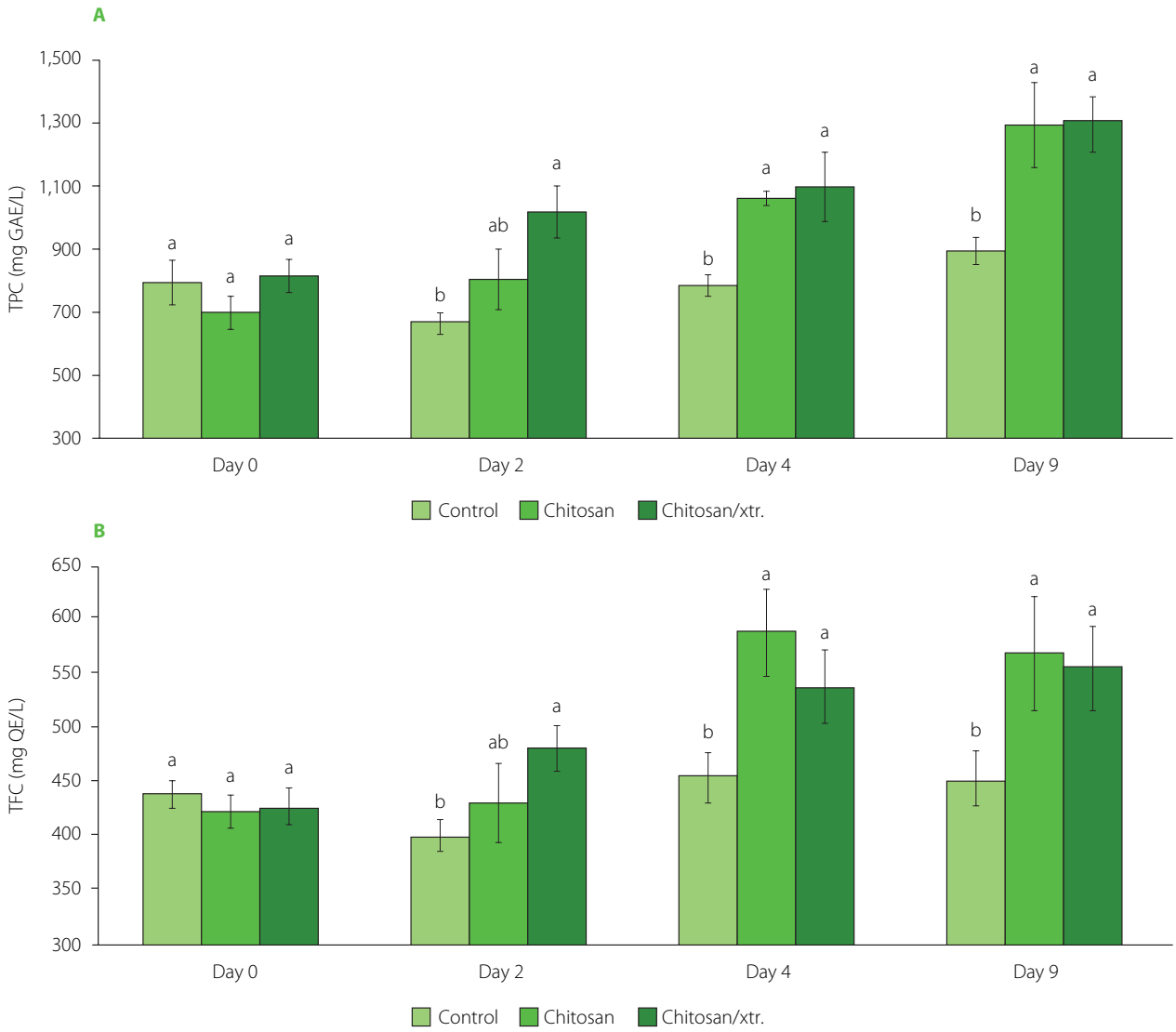

Figure 2. Total phenolic content (TPC) (**A**) and total flavonoid content (TFC) (**B**) of untreated strawberries (control), strawberries coated with chitosan (chitosan), and strawberries coated with chitosan/apple pomace extract (chitosan/xtr.), over storage time (0–9 days). Values marked by a different letter within the same sampling time are significantly different ($p<0.05$) according to the post hoc *t*-tests with Bonferroni adjustment. GAE, gallic acid equivalent; QE, quercetin equivalent.

a coating composed of the hydroxyethylcellulose/sodium alginate blend infused with asparagus waste extract significantly decreased weight loss. Nevertheless, the treatment with only hydroxyethylcellulose infused with extract significantly increased the weight loss of strawberry fruit [Liu *et al.*, 2021]. These results highlight the importance of composition when preparing blends of biopolymers as coating materials. Also, in this case, it can be observed that the weight loss is primarily related to the edible coating and not the additive (*i.e.*, natural extract). Generally, the weight loss of fresh strawberries is mainly due to the water loss. This can be attributed to the varied pore sizes and pore numbers present in the coatings, influencing the moisture and gas exchange.

There was no significant difference ($p\geq0.05$) recorded regarding the treatment effect on strawberry firmness or TSS levels. Similarly, an outcome was observed before, where chitosan coating infused with apple peel polyphenols was applied [Riaz *et al.*, 2021]. Related to TSS, another study on quality attributes and sensory tests [Azodanlou *et al.*, 2003], reported that strawberries were always highly appreciated if their sweetness and aroma intensity were high. In the cited study strawberries that had on average 8.3°Brix belonged to a very good quality class in contrast to those with lower TSS values. Accordingly, it can be considered that strawberries used in this study had excellent TSS levels for consumer appreciation and that their TA levels were around the maximum proposed by Kader [2005] who found that strawberries should have minimum of 7°Brix SSC and maximum of 0.8% TA. In this study TA levels were around or slightly above the proposed aforementioned maximum. Slightly and partially alleviated TA levels do not indicate not-optimal taste, since SSC levels were much higher than the proposed ones. The primary organic acids in strawberry fruit are citric and malic acids, which are important flavor compounds [Azodanlou *et al.*, 2003]. Although no significant differences were recorded, TA was elevated in chitosan/xtr. treated strawberries in contrast to the control ones on day 9 of cold storage (**Table 1**). Moreover, on the last sampling day, TA decrease was highest in the control and lowest in the chitosan/xtr. samples. A similar finding was reported by [Riaz *et al.* 2021], who observed greater differences in strawberries

coated with apple peel polyphenol-infused chitosan (0.78%) when compared to only chitosan coating (0.68%).

Bioactive compound content and antioxidant capacity of strawberries

Bioactive compounds are part of the natural plant defence mechanisms. Specifically, phenolics are organic compounds that have gained significant attention in the nutritional domain over the past years. An increasing number of studies suggest that the intake of phenolics might be crucial for health, influencing metabolism, weight management, chronic illness prevention, and cellular growth [Cory *et al*., 2018]. The effects of coating on the strawberry's total phenolic content and total flavonoid content are depicted in **Figure 2**. Interestingly, juice from chitosan/xtr. treated strawberries had significantly (p<0.05) higher TPC, relative to the control after 2, 4, and 9 days of storage. On days 4 and 9, both coatings (chitosan and chitosan/xtr.) significantly (p<0.05) influenced TPC in juice when compared to the control. Respectively, the TPC showed an increasing trend over storage time in the treated samples. Similar findings in TPC were reported before where authors utilised chitosan infused with apple peel polyphenols as a coating for strawberry fruits [Riaz *et al*., 2021]. Fagundes *et al.* [2013] suggested that tomato fruit coated with hydroxypropyl methylcellulose-lipid exhibited an increased phenolic content over time. Liu *et al*. [2021] also observed an increase in the total phenolic content at the end of an 8-day storage period (25°C) in strawberry fruits coated with hydroxyethyl cellulose incorporating asparagus waste extract. It has to be noted that there are some contradictory results reported, where strawberry fruits coated with chitosan-based coatings and carboxymethyl cellulose show a decreasing trend in TPC during the storage period [Khodaei & Hamidi-Esfahani, 2019].

A similar trend to the TPC was observed for TFC (**Figure 2B**). It was previously reported that chitosan coatings effectively stimulate the production of flavonoids in mandarin fruits [Jurić *et al*., 2023]. This stimulation might be a reaction to the direct application of the acidic chitosan solution onto the surface of mandarin fruit. Such an environmental change (introduction of an acidic medium) may trigger the fruit to enhance its flavonoid biosynthesis, leading to elevated flavonoid levels after storage. Herein, on day 4 and day 9, juices of the treated strawberries had significantly (p<0.05) higher TFC than the control (**Figure 1B**). Again, similarly to the TPC, on the second sampling day, the difference was significant (p<0.05) only for juice of the chitosan/xtr. treated strawberries. In addition, the TFC of the control during storage showed a trend without change. Herein we report that at the end of storage, both coatings (chitosan and chitosan/xtr.) resulted in significantly higher TFC in strawberry juices with values of 568.3 mg QE/L and 556.9 mg QE/L, respectively. However, the highest TFC (587.9 mg QE/L) was determined for the chitosan-coated strawberries at day 4 of storage. The findings regarding total phenolic and total flavonoid contents further align with the observations made by Liu *et al.* [2021]. They noted that hydroxyethyl cellulose coatings might have difficulties forming dense layers because of their inherently porous structure. Conversely, coatings like hydroxyethyl cellulose/sodium alginate blend and sodium alginate proved more effective in forming barriers against oxygen, subsequently slowing down the deterioration of phenolics, including flavonoids, in strawberries.

Anthocyanins, water-soluble pigments, typically located in plant cell vacuoles, belong to the group of flavonoids. The red color of strawberries is due to the presence of anthocyanin pigments. In strawberry fruits, the main anthocyanins include pelargonidin 3-glucoside, while pelargonidin 3-rutinoside and cyanidin 3-glucoside are present as minor compounds [Petriccione *et al*., 2015]. In this work, TAC was spectrophotometrically analyzed, both, in the strawberry juice (**Figure 3A**) and the residue after juice separation (**Figure 3B**). TAC in juice increased during storage for both treatments, while the same was not the case for the control. On the initial day, TAC in juice ranged from 213.1 mg/L to 217.8 mg/L. At the end of storage, total anthocyanin content increased for the strawberries treated with chitosan and chitosan/xtr. to 292.1 and 259.4 mg/L, respectively, and decreased for the control to 207.20 mg/L. Riaz *et al*. [2021] investigated the efficacy of chitosan-based coatings with added apple peel polyphenols during strawberries storage, and observed a reduction in the anthocyanin content of the coated fruits. A noticeable uptick in anthocyanin content was observed in the extract-infused chitosan-treated strawberries on the second day, which might be attributed to the ongoing production of these compounds post-harvest [Sogvar *et al*., 2016]. As reported by Riaz *et al*. [2021], throughout storage, a decline in anthocyanin levels in treated strawberries (after day 2) might result from the increased enzyme activity, specifically polyphenol oxidase. The composite coatings functioned as a barrier to gaseous exchange, potentially altering fruit biochemical processes responsible for anthocyanin production. However, this was not the case in our work where TAC in juice increased after 4 and 9 days of storage and only for the treated strawberries. This can be attributed to the fact that only one biopolymer (chitosan) was used and not a blend (combination of biopolymers), signifying the importance of coating composition and porosity which should be finely regulated if the goal is to increase anthocyanin content. Considering the TAC in the residue (**Figure 3B**), a similar trend was observed as in juice, except on day 2 of storage where relatively highest values were recorded for all samples.

Flavan-3-ols are common compounds found in seeds of strawberry fruit. In this study, the content of flavan-3-ols was determined in strawberry residue (**Figure 3C**). On day 0 and day 2, there were no significant differences (p≥0.05) between treatments in the content of total flavan-3-ols. Similarly, as for other recorded parameters (TPC, TFC, TAC, antioxidant capacity), at day 4 and 9, the residue of the treated strawberries maintained a significantly higher total flavan-3-ol content than the control. The content of flavonoids (flavan-3-ols and anthocyanins) generally decreases due to oxidation reactions during storage, and the application of chitosan coating maintained flavan-3-ol levels due to the reduction in oxygen availability [Saleem *et al*., 2021].

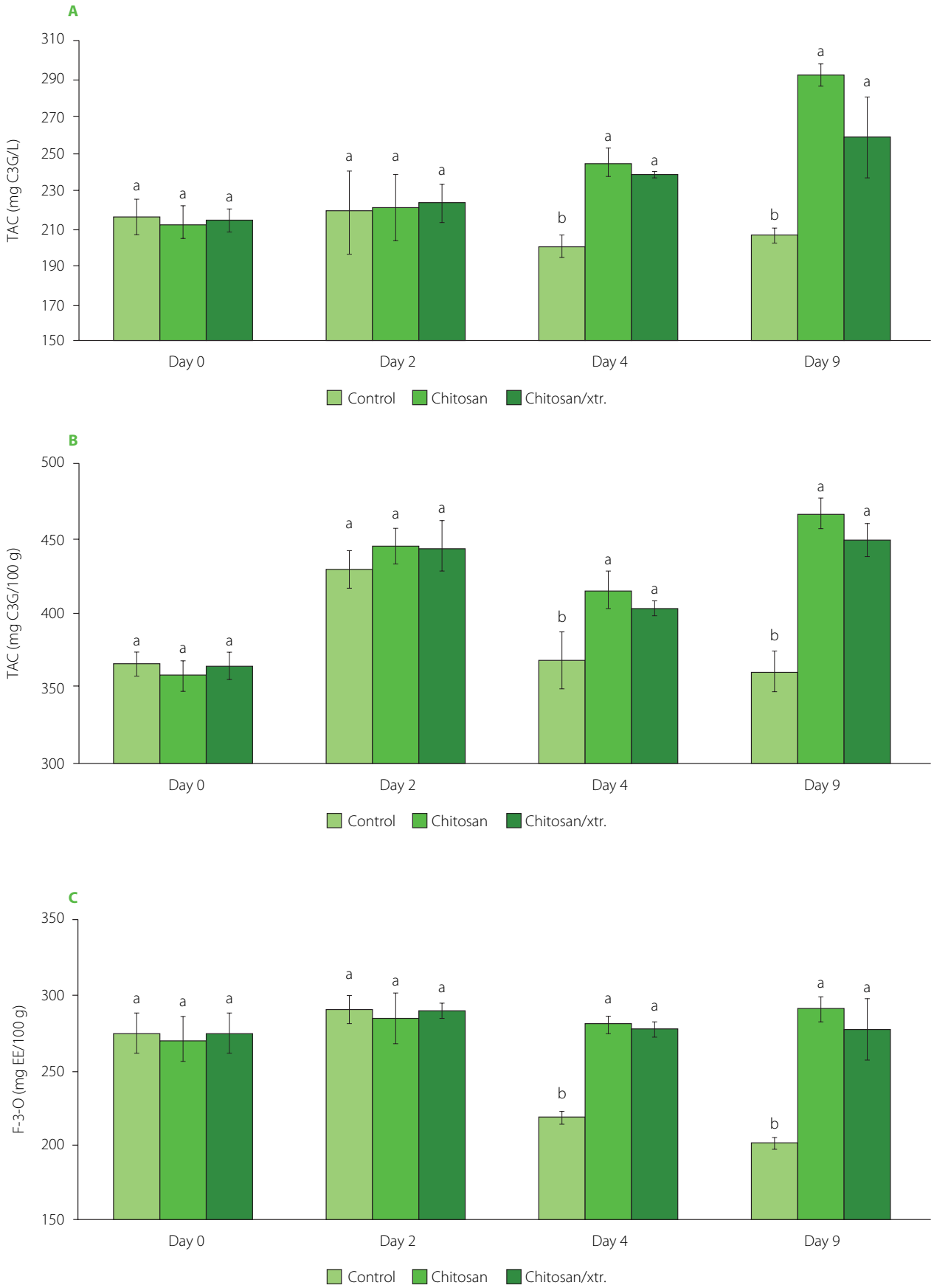

Figure 3. Total anthocyanin content (TAC) in juice (**A**), TAC in residue after juice separation (**B**), and total flavan-3-ols content (F-3-O) in residue (**C**) of untreated strawberries (control), strawberries coated with chitosan (chitosan) and strawberries coated with chitosan/apple pomace extract (chitosan/xtr.), over storage time (0–9 days). Values marked by a different letter within the same sampling time are significantly different ($p<0.05$) according to the post hoc *t*-tests with Bonferroni adjustment. C3G, cyanidin 3-*O*-glucoside equivalent; EE, (–)-epicatechin equivalent.

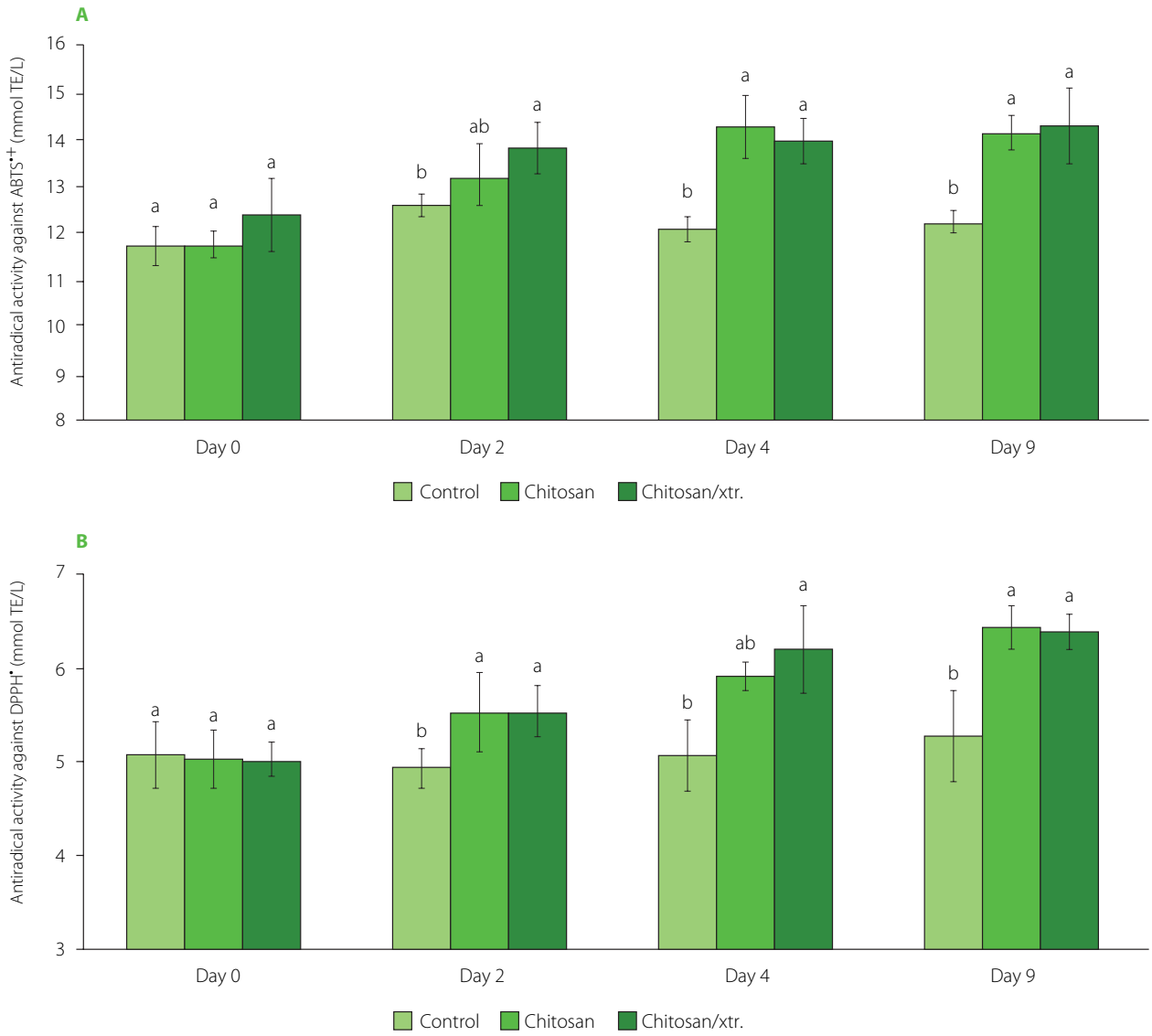

Figure 4. Antioxidant capacity of juices from untreated strawberries (control), strawberries coated with chitosan (chitosan), and strawberries coated with chitosan infused with apple pomace extract (chitosan/xtr.) determined using ABTS assay (**A**) and DPPH assay (**B**) over storage time (0–9 days). Values marked by a different letter within the same sampling time are significantly different ($p<0.05$) according to the post hoc *t*-tests with Bonferroni adjustment. TE, Trolox equivalent.

Regarding the antioxidant capacity of strawberries determined with the ABTS and DPPH assays, we have obtained a high correlation with a correlation coefficient (r) of 0.89 between the methods. The antioxidant capacity of the treated strawberries was found to be significantly higher than of the control on day 4 and day 9 (**Figure 4**). It is also worth noting that the results of ABTS assays for only chitosan/xtr. treated strawberries were significantly ($p<0.05$) higher than for the control on day 2, while chitosan-only treated strawberries did not significantly differ ($p\geq0.05$) in their antioxidant capacity (both assays) at that time. Generally, antioxidant capacity of strawberries highly correlates with the total phenolic content and total flavonoid content [Chen *et al*., 2023], which is following the results presented in this study. Furthermore, Tulipani *et al.* [2008] have linked antioxidant capacity of different strawberry genotypes with the presence of effective oxygen-radical scavengers, such as phenolic compounds and ascorbic acid. Antioxidants were relatively stable in the coated fruits as compared to the uncoated ones. The coating helps reduce enzyme activity and preserve the fruit quality, which slows down the breakdown of antioxidant compounds, especially those that act as effective oxygen radical scavengers [Riaz *et al*., 2021].

■ Microbiological quality of strawberries and coatings

The AMC and yeast and mould count in the untreated and the coated strawberries are shown in **Table 2**. Yeasts and moulds were not detected in the coating materials, while AMC was 3.97±2.78 and 4.27±2.42 log CFU/g in chitosan and chitosan/xtr., respectively. *Enterobacteriaceae* and psychrotrophic bacteria were not detected in any of the samples tested.

In the untreated fruit (control), AMC was constant and ranged from 4.45 log CFU/g (day 0) to 4.47 log CFU/g (day 9), while yeast and moulds were present only in the control samples during the first two days of storage (2.82 log CFU/g for day 0, and 2.94 log CFU/g for day 2) (**Table 2**). Contrary to the expected

Table 2. The aerobic mesophilic count (AMC) and yeast and mould count (log CFU/g) of untreated strawberries (control), strawberries coated with chitosan (chitosan), and strawberries coated with chitosan/apple pomace extract (chitosan/xtr.) over storage time (0–9 days).

Microorganism	Treatment	Day 0	Day 2	Day 4	Day 9
AMC	Control	4.45±0.03^{a}	4.44±0.02^{a}	4.46±0.01^{a}	4.47±0.01^{a}
	Chitosan	5.37±0.02^{a}	4.22±0.02^{b}	3.43±0.01^{c}	3.54±0.02^{c}
	Chitosan/xtr.	4.51±0.01^{a}	3.97±0.04^{b}	3.31±0.01^{c}	4.12±0.02^{b}
Yeasts and moulds	Control	2.82±0.11	2.94±0.08	<1	<1
	Chitosan	<1	<1	<1	<1
	Chitosan/xtr.	<1	<1	<1	<1

Results are shown as mean ± standard deviation. Different letters (a–c) within rows and columns indicate statistically significant differences, as determined by the *post-hoc* Dunn's test with Bonferroni correction of the *p*-value ($p<0.05$).

increase in AMC, yeast, and mould counts typically observed in untreated strawberries during storage [Khan *et al*., 2019; Martínez *et al*., 2018], in our study, AMC remained stable throughout the storage, while yeasts and moulds could no longer be detected in the uncoated strawberries after the second day. The absence of yeasts and moulds can be attributed to several factors. Firstly, the low initial yeast and mould loads on strawberries at the beginning of the experiment, which may be explained by the low contamination levels during the ripening and harvesting of the strawberries, as these phases determine the post-harvest fungal incidence [Contigiani *et al*., 2018]. Furthermore, the storage conditions similar to those used in our experiment (3°C, humidity level between 90 and 95%) have been reported to slow down the metabolic activity of yeasts and moulds, lowering their proliferation, and reducing spore viability, thus reducing overall fungal counts [Ansiska *et al*., 2023; Feliziani & Romanazzi, 2016; Trinetta *et al*., 2020]. Additionally, the bioactive compounds inherent in strawberries, mainly various phenolic compounds with antifungal activity, can effectively inhibit the mycelial growth and slow down the spore germination [El-Seedi *et al*., 2012; Morales *et al*., 2017; Pott *et al*., 2020], further reducing fungal counts. Finally, competition for niche and nutrients with other microorganisms that are part of natural strawberry microbiota [Sretenović *et al*., 2024], may also account for the absence of yeast and moulds and the sustained stable AMC.

The increased AMC in coated strawberries compared to the control at day 0 was most likely associated with the bacteria introduced by the coating and extracts. Although chitosan and chitosan-based edible polymers are known to be antimicrobial [Khan *et al*., 2019], they can also be sources of microorganisms, which was mostly not considered in previous studies. Despite the introduction of microorganisms by the coating materials, the AMC and level of yeasts and moulds in the coated strawberries in our study was lower than in the control samples, which is in agreement with the study by Khan *et al*. [2019] on chitosan-based edible coatings for strawberries.

Later in the storage period, a decrease in AMC was observed in the chitosan-coated fruit (from 5.37 log CFU/g, day 0, to 3.54 log CFU/g, day 9) and in the chitosan/xtr. fruit samples (from 4.51 log CFU/g, day 0 to 4.12 log CFU/g, day 9) (**Table 2**). The AMC was significantly lower in the coated strawberries compared to the uncoated ones, regardless of the type of coating and the storage time (Dunn's test with Bonferroni correction, $p<0.05$). While chitosan tended to suppress AMC more effectively than chitosan/xtr. for most of the 9-day storage period, this difference was statistically significant only at day 9, suggesting that the efficacy of chitosan in suppressing AMC becomes more pronounced later in the storage period. Yeasts and moulds were not detected in the coated fruit samples (<1 log CFU/g), regardless of the day of storage. The antimicrobial effect observed in this study is most likely due to the antimicrobial properties of chitosan, which is consistent with previous studies on strawberries treated with chitosan-based edible coatings [Khan *et al*., 2019; Martínez *et al*., 2018]. While phenolic compounds from apple pomace are also known for their antimicrobial properties [Riaz *et al*., 2018; Wang *et al*., 2015], our results suggest that the addition of apple pomace extract to chitosan coating did not significantly improve the microbiological quality or shelf-life of the strawberries compared to the chitosan coating alone.

Regardless of the day of storage, no visible signs of defect were observed on any of the fruit analyzed. AMC was generally 3–4 log lower than the value of 7.0–8.0 log CFU/g suggested by Ragaert *et al*. [2006] as critical for organoleptic alteration in foods.

Our results indicate a good microbiological quality of the tested fruit and are in agreement with the results of studies on the microbiological quality of berries in the EU and the USA [Macori *et al*., 2018; Quansah *et al*., 2019]. For example, the AMC ranged from 1.7 to 6.9 log CFU/g in most of the samples analyzed by Macori *et al*. [2018] and averaged 3.89 log CFU/g in the study by Quansah *et al*. [2019]. Enterobacteriaceae were detected in the range of 1 to 4 log CFU/g in 13% of the samples analyzed by Macori *et al*. [2018], and the total yeast and mould counts and the total coliform counts in the berries studied by Quansah *et al*. [2019] were 4.42 and 1.42 log CFU/g, respectively.

Overall, our results have shown that chitosan-based edible coatings represent an effective and sustainable approach for controlling the growth of undesirable microbiota and extending the shelf-life of strawberries while preserving organoleptic properties during storage.

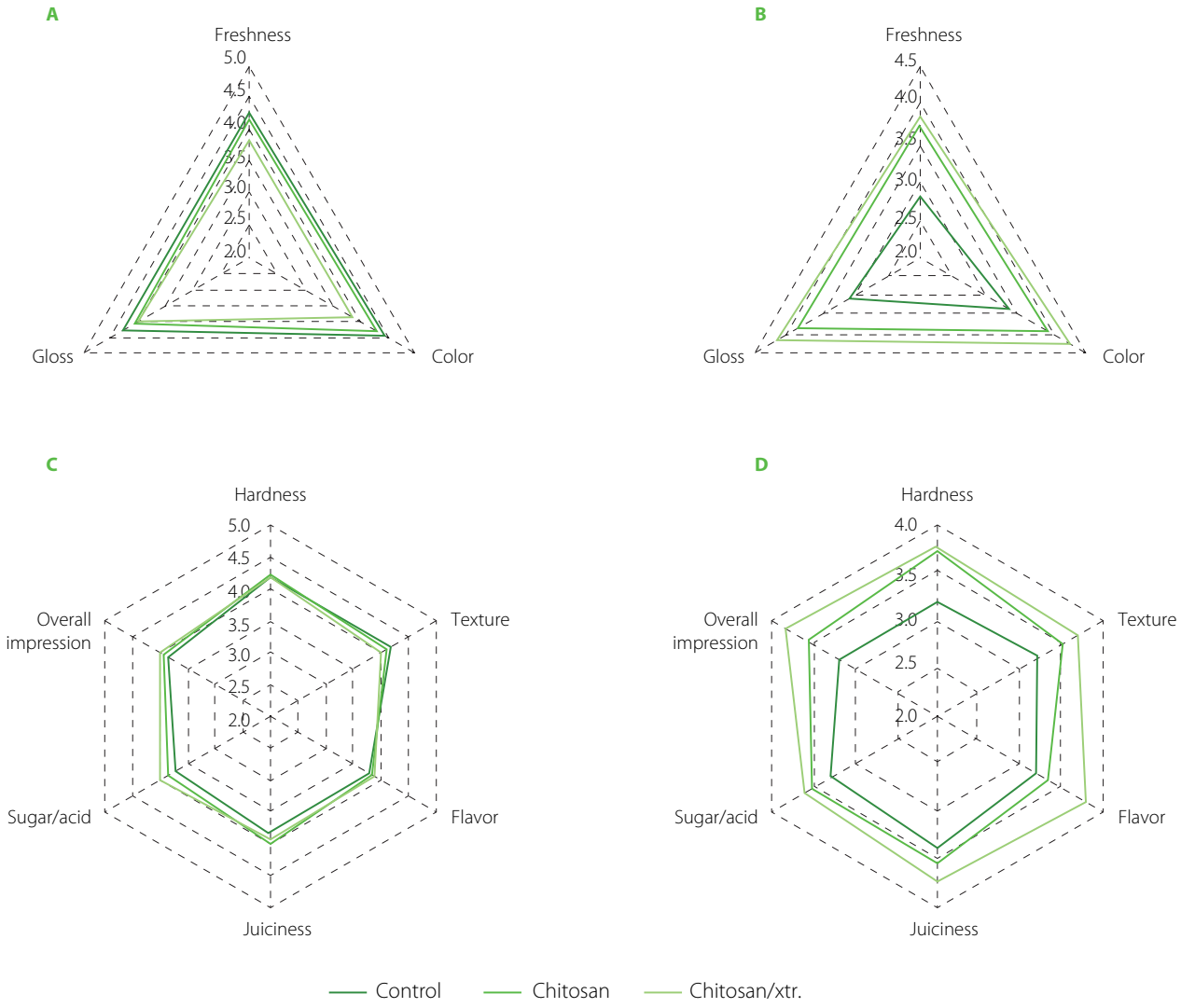

Figure 5. Sensory evaluation of untreated strawberries (control), strawberries coated with chitosan (chitosan), and strawberries coated with chitosan infused with apple pomace extract (chitosan/xtr.) in terms of visual impressions at day 0 (**A**) and day 4 (**B**), as well as in terms of taste on day 0 (**C**) day and day 4 (**D**).

Sensory evaluation of strawberries

Sensorial characterisation plays an important role in consumer food acceptance [Khodaei *et al*., 2021]. Sensory characteristics of the strawberries were evaluated on 0 and 4 days of the study, and the results are presented in **Figure 5**. Initially, the highest score in freshness was given to the strawberries coated with chitosan/apple pomace extract, while the lowest color score was given to the chitosan-coated strawberries. Regarding the organoleptic parameters, juiciness, sugar/acid perception, and overall impression were rated the lowest for the control strawberries. However, differences in terms of values were negligible. On day 4, interestingly, the relatively lowest visual parameters (gloss, color, and freshness) were found in the control samples. Hence, both treated strawberries can be characterised by positively increased visual perception. The significant increase in the total anthocyanin content in the coated strawberries during storage life may explain the preference of the panellists for these samples. Since at the time of initial purchase consumers judge the quality of the fresh fruit based on appearance (including "freshness") [Kader, 2005], this may have a crucial role in the customer's decision to buy strawberries. The relatively lowest organoleptic scores (hardness, texture, flavor, juiciness, and sugar/acid ratio perception) were noted at day 4 for the control samples, whereas the treated strawberries showed superiority. The sensory attributes of strawberries that have undergone treatment are crucial from an economic standpoint for producers. This is because repeat purchases are largely contingent on consumer contentment, particularly concerning the taste and overall eating quality of the product. Ensuring that the strawberries meet or exceed consumer expectations in these areas can significantly influence their market success and profitability.

CONCLUSIONS

The developed chitosan and apple pomace extract-infused chitosan edible coatings improved, maintained, and effectively enhanced the bioactive properties, sensory attributes, and microbiological quality of fully ripe strawberries during storage. The study demonstrates that these coatings increase the total

phenolic content, the total flavonoid content, and antioxidant capacity while reducing microbial counts. Additionally, the coatings maintain the strawberries' visual and organoleptic quality, extending their shelf-life in a sustainable and eco-friendly manner. Throughout the 9-day storage period, the fully ripe strawberries exhibited no significant variations in weight loss, firmness, the total soluble solids, or titratable acidity. No significant differences were observed between the chitosan coating and the extract-infused chitosan coating in terms of the total phenolic content, the total flavonoid content, and antioxidant capacity in strawberries after 4 days of storage. Conversely, on day 2, the extract-infused chitosan coating exhibited a significant impact, leading to increased levels of total phenolic compounds and flavonoids, and antioxidant capacity when compared to the plain chitosan coating. Moreover, both chitosan and apple pomace extract-infused chitosan edible coatings effectively suppressed microbial growth in strawberries, and significantly reduced AMC compared to the untreated controls over a 9-day storage period. In addition, no yeasts or moulds were detected in the coated samples during the entire storage period. While the addition of apple pomace extract did not significantly enhance antimicrobial efficacy of chitosan, chitosan-based edible coatings offer a promising approach for preserving strawberry quality and extending shelf-life. Herein we point out that there are some limitations to this research work. One of them is the relatively low bioactive content (total phenolic content) in the initial edible coating due to the simple ultrasound-assisted extraction method with the use of only water as a solvent. This can be further mitigated by applying different green extraction techniques and green solvents to achieve a higher initial content of bioactive compounds. Overall, chitosan proved its potential in various studies as an edible coating, and here its influence was confirmed. However, there is still a need for additional research dealing with chitosan concentration, application of additives and preparation of adequate blends (composites) with desirable porosity and functionality.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest in the publication.

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Impact of Combined Ultrasound and Citric Acid Treatments on the Quality and Safety of Natural Horse Casings Over Storage Durations

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This study investigated the combined effects of ultrasound (US) and citric acid (CA) treatments on the microbiological safety and mechanical integrity of natural horse casings during storage. Derived from horse intestines, these casings are susceptible to microbial contamination that can impact food safety and quality. Samples underwent treatment with ultrasound (35 kHz, 200 W for 5 or 10 min), citric acid at a concentration of 0.5% and 1%, and their combination. Results showed that the treatment combination of 1% CA with US for 5 min yielded the lowest initial total plate count (TPC) at 1.63 log cfu/g and maintained effective microbial control, with TPC at 3.24 log cfu/g by day 13 of storage at 4°C. This combination also preserved the mechanical properties of the casings, achieving tensile strength of 5.7 MPa and elongation at break at 58%. The findings suggest that combining US and CA significantly enhances the microbial safety and mechanical resilience of horse casings, offering a promising approach for the meat processing industry.

Keywords: horse intestine, mechanical properties, microbiological properties, natural casings

INTRODUCTION

Proper management of meat by-products can unlock significant economic value through appropriate processing and utilization [Alao *et al.,* 2017; Toldrá *et al.,* 2023]. These products include an extensive variety of items, many of which are abundant in vital nutrients like vitamins, minerals, and amino acids [Henchion *et al.,* 2016; Mora *et al.,* 2019; Seong *et al.,* 2014]. The extent to which these by-products are accepted as food varies widely across cultures and geographical areas; what is considered a delicacy in one country might be deemed not edible in another [Alao *et al.,* 2017]. Significantly, organ meats such as liver, heart, kidney and spleen that are rich in nutrients have been adopted into the cuisines and eating habits of different societies all over the world indicating their global importance [Nollet & Toldrá, 2011]. The use of horse by-products is deeply entrenched in Kazakhstan's culinary and cultural traditions [Atambayeva *et al.,* 2023; Zhumanova *et al.,* 2019], illustrating a meticulous approach to utilizing every aspect of the animal. This tradition highlights both the pragmatism and the deep respect the Kazakh people have for horses, creatures that have been essential to their history and daily lives.

In recent years, microbial safety and quality preservation in meat products have become critical research areas due to heightened consumer demand for safe and high-quality food.

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Various studies have explored advanced preservation methods, such as organic acid treatments, high-pressure processing, and ultrasound, to extend shelf-life and reduce microbial contamination [Woldemariam *et al.,* 2019]. Organic acids like citric acid are commonly used for their antimicrobial effects, especially when combined with ultrasound, which enhances microbial inactivation through cavitation [Turhan *et al.,* 2022]. These combined methods are particularly effective in inactivating pathogens and preserving meat quality without compromising the product's sensory attributes, thus meeting the industry's stringent safety standards. However, despite these advancements, natural casing materials like those derived from horse intestines present unique challenges in microbial management due to their complex structure and susceptibility to contamination.

Multiple factors can affect the quality and characteristics of natural casings, including the age, breed, and food of the animal, as well as the ambient conditions in which the animal was raised. In order to be suitable for practical use, these casings must possess sufficient strength to withstand the forces associated with filling, stuffing, and processing [Bakker *et al.,* 1999; Ockerman & Hansen, 2000]. Bacteria are naturally present in casings with a typical concentration of 4 to 7 log cfu/g [Bakker *et al.,* 1999; Byun *et al.,* 2001; Hammou *et al.,* 2010; Wijnker *et al.,* 2019] and can also contain viral particles within their structure. The study conducted by Çağlar *et al.* [2018] detected the presence of *Escherichia coli* in nine out of the 21 casing samples analyzed. Among these samples, two showed notably high levels of contamination, with 5.77 log cfu/g for *E. coli* and 5.81 log cfu/g for *Staphylococci*. Another strain of *Salmonella* spp. was found in two of the casings. Research has indicated that the count of bacteria present in hog and sheep casings can be significant at the beginning. Bakker *et al.* [1999] reported overall aerobic counts of 6.3 and 5.9 log cfu/g, as well as halophilic bacteria counts of 4.5 and 5.2 log cfu/g. In contrast, Rebecchi *et al.* [2015] counted, isolated, and described a number of *Staphylococcus*, *Lactobacillus*, *Bifidobacterium*, *Vagococcus*, and *Clostridium* bacteria. More detailed bacteriological investigations, such as those carried out by Fahmy *et al.* [2021] on 90 natural sheep casings (both local and imported) found that imported casings had greater bacterial levels than the local products. Similarly, initial studies by Byun *et al.* [2001] revealed large numbers of aerobic bacteria, *Enterococci*, and coliforms in pork and lamb casings.

Ultrasound technology is vital for the safe processing of intestines and the production of high-quality natural casings, such those used for sausages. Utilizing ultrasound during the cleaning and preparation stages of these casings can significantly decrease the presence of microorganisms. According to research published by Lauteri *et al.* [2023], the use of ultrasonic cavitation can effectively remove biofilms and other contaminants, significantly lessening the risk of impurity. The microbiological safety of beef products is improved by ultrasonic-assisted procedures in addition to their cleaning effects [de Lima Alves *et al.,* 2018; Morild *et al.,* 2011]. For example, after 30 min of sonication at frequencies of 20 and 40 kHz, Sienkiewicz *et al.* [2017] showed that *Salmonella* spp. were completely inactivated in both low and high bacterial populations. Moreover, integrating ultrasound with other sanitation methods, such as hypochlorite, mild heat, pressure, steam, or organic acids, has been shown to significantly increase the antibacterial efficacy in meat processing, as evidenced by studies like those conducted by Arroyo *et al.* [2011], Diez *et al.* [2008], and Kang *et al.* [2018]. The capacity of ultrasound treatment to increase the permeability and uniformity of the casings is another reason why it is highly regarded. This ability has the potential to enhance the overall processing qualities as well as the appearance of the finished product. Not only does this application contribute to the safety of food, but it also enhances the functional features of the casings, which is in line with the expectations of the industry for food products that are of high quality and non-hazardous. Combining ultrasound with other antimicrobial methods results in an increase in the effectiveness of ultrasound as an antibacterial agent. Hence, a number of researchers have investigated the synergistic effects of ultrasound in conjunction with other antimicrobial techniques in order to enhance its capabilities in deactivating microorganisms and enzymes. This has been documented by Nicolau-Lapeña *et al.* [2019], Silveira *et al.* [2018], and Singla & Sit [2021]. The utilization of organic acids in combination has proved highly effective in deactivating foodborne pathogens [Diez *et al.,* 2009; Irazoqui *et al.,* 2024]. The combined effect of these therapies greatly enhances microbiological safety by utilizing the mechanical force generated by ultrasound-induced cavitation in conjunction with the antibacterial characteristics of organic acids. Combining these two methods not only helps remove biofilms and other microbiological pollutants more thoroughly, but it also improves the acid's ability to penetrate the casings, resulting in a more even and efficient decrease in pathogen levels. Research has shown that combining both procedures results in more effective elimination of microorganisms compared to using either strategy individually [Görgüç *et al.,* 2021; Singla & Sit, 2021]. Building upon existing research, studies by Morild *et al.* [2011] and Lauteri *et al.* [2023] demonstrated the efficacy of ultrasound in reducing microbial load in meat products. Our study employs similar ultrasound frequencies (35 kHz) but examines the combined effect with citric acid, a relatively less explored synergy in this context. Unlike prior studies that used ultrasound independently, we explored how combining it with citric acid may offer a more potent antimicrobial effect, potentially enhancing both microbial safety and mechanical resilience. The outcome is not only more secure, but also of superior quality shells with enhanced sensory and mechanical characteristics, complying with industry benchmarks for both safety and product uniformity. This combined approach represents a great development in food processing technology since it maximizes meat product functional qualities and safety.

The purpose of this research was to completely analyze the microbiological safety and mechanical integrity of natural horse casings following treatment with ultrasound, citric acid, and a combination of both. This inquiry attempted to establish the usefulness of these strategies, both individually

Table 1. Process variables for the treatment of natural horse casings.

Treatment ID		Treatment type	Citric acid concentration	Duration (min)	Storage time (day)
A1	Control, 5 min	Water	–	5	1, 5, 9, 13
A2	Control, 10 min	Water	–	10	1, 5, 9, 13
B1	CA (0.5%), 5 min	Citric acid	0.5% (*v/v*)	5	1, 5, 9, 13
B2	CA (0.5%), 10 min	Citric acid	0.5% (*v/v*)	10	1, 5, 9, 13
C1	CA (1%), 5 min	Citric acid	1% (*v/v*)	5	1, 5, 9, 13
C2	CA (1%), 10 min	Citric acid	1% (*v/v*)	10	1, 5, 9, 13
D1	US, 5 min	Ultrasound	–	5	1, 5, 9, 13
D2	US, 10 min	Ultrasound	–	10	1, 5, 9, 13
E1	US + CA (0.5%), 5 min	Combined	0.5% (*v/v*)	5	1, 5, 9, 13
F1	US + CA (1%), 5 min	Combined	1 %(*v/v*)	5	1, 5, 9, 13

CA, citric acid; US, ultrasound.

and in combination, in boosting the quality and safety of casings used in sausage manufacture. By examining the individual and combined impacts of ultrasonic and citric acid treatments, this study intended to establish optimal processing settings that ensure the highest standards in product safety, functional performance, and customer acceptability. This technique could potentially set new criteria for industry procedures, enhancing the overall quality and marketability of beef products.

This study focused on short-term storage durations (up to 13 days) to assess the immediate impact of ultrasound and citric acid treatments on microbial safety and mechanical integrity of natural horse casings. The chosen timeframe was designed to capture early-stage microbial reductions, which are critical for initial product stability and quality. Although long-term total plate count (TPC) testing over several months would provide valuable insights into the durability of microbial safety and structural improvements, it was outside the scope of this study due to resource and time constraints. Future research will aim to explore these extended effects, offering a more comprehensive understanding of treatment efficacy over prolonged storage periods.

MATERIALS AND METHODS

Horse intestine collection

Horse intestines (the transverse and small colons with a diameter of 50 to 120 mm) from horses aged 3–4 years and weighing approximately 300 to 360 kg were randomly collected from various retail markets in the Astana provinces, Kazakhstan. The horses were slaughtered following standard procedures that involved severing major blood vessels, bleeding, skinning, and evisceration. Immediately after slaughter, the horse intestine samples were harvested, cleared of visible fats and connective tissues, and rinsed with tap water to eliminate any residual food and feces. Subsequently, horse intestine (about 100 kg) was segmented into roughly 0.5 m long fragments for treatments involving ultrasound, citric acid, or a combination of both.

Experimental design

Samples were collected and treated with citric acid (CA), ultrasound (US), or a combination of both. Details of the treatment parameters are outlined in **Table 1**. Post-treatment, the samples were stored in sterile conditions at 4°C for assessment across various durations (1, 5, 9, and 13 days). Key metrics assessed include TPC, content of thiobarbituric acid reactive substances (TBARS), pH, tensile strength, and elongation at break. All studies were performed in the Laboratory of Microorganisms Genetics and Biochemistry at the National Center for Biotechnology (Astana, Kazakhstan) based on the international standards.

Procedures for treating horse intestines and their storage

Throughout the ultrasound treatment, a refrigeration unit maintained the water temperature at 20°C, regulated by a temperature control system. The experiment utilized a 10 L ultrasound tank (Sapphire UZV-28 TTC, Sapphire LLC, Moscow, Russia), filled with 8 L of distilled water, operating at a frequency of 35 kHz and a power of 200 W. The full experimental set up is given in **Table 1**. The treatments were conducted as follows: control (water) – intestines were soaked in distilled water for 5 and 10 min; CA treatment – intestines were soaked in citric acid solutions (0.5% or 1.0%, *v/v*) for 5 and 10 min; US treatment – intestines were immersed in distilled water and subjected to ultrasound at 35 kHz and 200 W for 5 and 10 min; combined treatment – intestines were first soaked in citric acid solutions (0.5% or 1.0%, *v/v*) for 5 min and subsequently subjected to ultrasound under the same conditions described for the US treatment.

In all treatments, the solution temperature was maintained at 20°C, and the solution-to-intestine ratio was consistently kept at 8:1 (8 L of solution *per* 1 kg of intestine) to ensure uniform and effective treatment conditions. After treatment, the samples were briefly washed with tap water for 1 min and then dried using wiper papers. Subsequently, the samples were packed in plastic pouches, approximately 0.5 kg *per* bag, sealed, and stored at

4°C for 1, 5, 9, and 13 days. Each 500 g portion was treated as an experimental unit.

Determination of total plate count

The TPC in horse intestines was determined following the standard procedure [GOST 10444.15-94] maintained by the Euro-Asian Council for Standardization, Metrology, and Certification (EASC). Initially, 25 g of the intestine samples were mixed with 225 mL of peptone water (1 g/L peptone) and homogenized using an HG-202 laboratory dispersant (HT Machinery Co., Ltd., Tokyo, Japan) for 1 min. Subsequent to homogenization, the samples underwent serial dilution in peptone water (from 1:10 to 1:100, *v/v*). Then, 1 mL of each diluted sample was spread onto the plates of an aerobic plate count equipment (Test-2, Food Eyes, Beijing, China) and incubated at 37°C for 48 h. After incubation, red colonies visible on the plates were counted as TPC, and the results were recorded in logarithmic colony forming units *per* g (log cfu/g).

pH measurement

pH measurements were conducted by blending 5 g of each sample with 45 mL of distilled water in a homogenizer (HG-202, HT Machinery Co., Ltd., Tokyo, Japan). The pH levels were then assessed using a pH meter (MP220, Mettler Toledo Intl, Greifensee, Switzerland).

Instrumental color analysis

The surface color of the horse intestines treated with CA solutions, US and a combination of CA (1%) and US for 5 min was quantified using a chroma-meter (CR-300, Konica Minolta, Inc., Marunouchi, Chiyoda-ku, Tokyo, Japan), which was calibrated using a white plate (Y=93.5, X=0.3132, y=0.3198). Five distinct measurements were made at various points on the exterior of the samples. The CIE color space was followed in recording the results, which included values for L^* (lightness), a^* (redness), and b^* (yellowness).

Lipid oxidation measurement

The content of TBARS was measured in the samples following each specific treatment – control (water only), CA treatments, US treatment, and combined CA (1%) and US treatment for 5 min – to determine the levels of lipid oxidation, following the approach published by Kang *et al*. [2018]. In each test, 5 g of the sample were mixed with 15 mL of distilled water, 50 µL of saturated butylated hydroxyanisole, and 20 mL of a solution containing thiobarbituric acid (0.02 M) and trichloroacetic acid (15%, *w/v*) in a 1:1 (*v/v*) ratio (TBA/TCA). The mixture was then mixed at 11,000 rpm for 15 s with an HG-202 homogenizer (HT Machinery Co., Ltd., Tokyo, Japan). The total volume of each homogenized sample was increased to 50 mL with an additional TBA/TCA solution and promptly chilled on ice. The samples were soaked in a 90°C-water bath for 15 min. After heating, the samples were rapidly cooled on ice for 20 min before being centrifuged at 3,000×*g* for 10 min in a Z-216-M centrifuge (Hermle Labortechnik GmbH, Wehingen, Baden-Württemberg, Germany). Approximately 1.5 mL of the supernatant was then collected from each sample, and its absorbance was determined at 531 nm using a UV-visible spectrophotometer (UV-VIS-AgilentCary-60, Agilent Technologies Inc., Santa Clara, CA, USA). TBARS levels were calculated based on the weight of the sample and expressed in mg of malondialdehyde *per* kg of sample (mg MDA/kg). This procedure was repeated three times for each sample in each treatment.

Determination of mechanical properties of the horse intestine casings

The mechanical properties for both the treated casings (with CA solutions, US and a combination of CA (1%) and US) and the control were assessed using a tabletop testing machine (Instron, Model-1026, Norwood, MA, USA) immediately after each treatment session of 5 min. Samples of the casing were prepared by cutting them into strips measuring 120 mm in length and 50 mm in width, corresponding to the width of an open casing, aligned longitudinally. These were then secured within the grips of the equipment, set at an initial distance of 40 mm. Throughout the traction tests, which progressed at a rate of 12 mm/min, data on tension and relative deformation were continuously gathered. Tensile strength (MPa) and elongation at break (ε, %) for each film were determined based on five replicated measurements of the tension/deformation profiles [Okamoto, 1978].

Statistical analysis

The experiment was performed in triplicate, with three independent trials conducted for each treatment condition. After each treatment and at each storage time interval, two samples *per* trial were analyzed, resulting in a total of six samples *per* condition *per* storage time. A mixed-effects model was used to evaluate the effects of citric acid concentration, ultrasound treatment, treatment duration, and storage interval, as well as their interactions, on the response variables: TPC, TBARS, and pH. This model accommodated the complexity of the design, allowing for repeated measures on the same samples over time. Fixed effects included the main factors and their interactions, while random effects accounted for within-sample variability. Analysis of variance (ANOVA) was applied to determine the significance of each main effect and interaction on the response variables. Post-hoc comparisons were conducted using Duncan's multiple range test at a 0.05 significance level to identify significant differences among treatment combinations. All statistical analyses were performed using SAS software (Version 8.1, SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Total plate counts and pH

Table 2 outlines the variations in TPC of horse intestine subjected to ultrasound treatment, varying concentrations of citric acid washes, and their combined effects during cold storage. Over time, TPC significantly increased ($p<0.05$) with longer storage durations. By the final day of storage (day 13), TPC levels

Table 2. Total plate counts (log cfu/g) in horse intestine subjected to various treatments during storage at 4°C.

Treatment	Storage time (day)				*p*-Value
	1	5	9	13	
A1; Control, 5 min	2.19 ± 1.25^{bB}	3.32 ± 2.11^{bAB}	3.34 ± 1.44^{bB}	5.24 ± 2.15^{aA}	**
A2; Control, 10 min	2.22 ± 1.18^{bB}	3.36 ± 1.22^{bAB}	3.35 ± 1.11^{bB}	5.54 ± 2.13^{aA}	**
B1; CA (0.5%), 5 min	2.12 ± 0.09^{bB}	2.83 ± 1.27^{bB}	3.43 ± 2.16^{bAB}	4.58 ± 1.65^{aAB}	**
B2; CA (0.5%), 10 min	2.01 ± 1.08^{bB}	2.67 ± 1.57^{bB}	3.25 ± 2.13^{aAB}	4.29 ± 1.32^{aB}	**
C1; CA (1%), 5 min	2.43 ± 1.17^{cA}	3.64 ± 1.12^{bA}	3.28 ± 1.34^{bB}	3.72 ± 2.27^{aBC}	**
C2; CA (1%), 10 min	2.40 ± 1.11^{bA}	3.49 ± 1.09^{aA}	3.05 ± 1.38^{bB}	3.76 ± 1.85^{aBC}	**
D1; US, 5 min	2.11 ± 1.24^{bB}	2.32 ± 1.44^{bB}	4.25 ± 2.37^{aA}	3.41 ± 1.33^{aBC}	**
D2; US, 10 min	2.21 ± 1.17^{bB}	2.34 ± 1.06^{bB}	3.95 ± 1.28^{aA}	3.45 ± 1.16^{aBC}	**
E1; US + CA (0.5%), 5 min	2.38 ± 1.29^{cB}	3.92 ± 2.01^{aA}	3.20 ± 1.55^{bB}	3.87 ± 1.68^{aB}	**
F1; US + CA (1%), 5 min	1.63 ± 1.45^{bB}	1.94 ± 1.36^{bB}	2.35 ± 1.56^{bB}	3.24 ± 1.81^{aC}	**

Results are shown as mean ± standard deviation (n=6). Lowercase letters within each row indicate significant differences across storage time for the same treatment (p<0.05). Capital letters within each column indicate significant differences between treatments for the same storage day (p<0.05). **p<0.01; CA, citric acid; US, ultrasound.

in the control samples (A1, A2) were significantly higher (p<0.05) than those in the treated samples, with the exception of some citric acid-only treatments, where no significant differences were observed. This indicates that while citric acid effectively reduced microbial counts initially, different concentrations and durations did not significantly alter its long-term efficacy. The combination of citric acid (1%) with ultrasound treatment (F1) consistently resulted in the lowest microbial counts throughout storage, demonstrating the potential of the combined treatment to provide enhanced microbial control. While all treatments showed statistically significant changes in TPC (log cfu/g) during cold storage at 4°C (p<0.01), treatment F1 (US + CA (1%), 5 min) yielded the most effective control over microbial growth. On day 1, the treatment F1 showed a trend of lower TPC (1.63 log cfu/g) compared to the other treatments; however, these differences were not statistically significant (p≥0.05), except in comparison to C1and C2, as indicated in **Table 2**. Over the storage period, F1 consistently maintained lower microbial counts compared to the other treatments, but these results highlight observed trends rather than definitive differences, emphasizing the importance of analyzing subsequent storage days for more pronounced effects. The observed trend suggests that citric acid concentration was a critical factor in combination with ultrasound in controlling microbial growth. Combination treatment US + CA (1%) was generally more effective than single treatments (either citric acid or ultrasound alone). These insights could guide optimization strategies for food preservation processes, particularly for products susceptible to microbial spoilage. Our findings align with previous studies that highlight the microbial reduction capability of ultrasound at similar frequencies and intensities. For instance, Morild et *al.* [2011] reported effective reduction in bacterial loads in pork using ultrasound. In turn, Lauteri *et al.* [2023] found similar effects in beef products. However, our combined ultrasound and citric acid treatment achieved enhanced microbial control compared to ultrasound alone, consistent with recent research by Silveira *et al.* [2018], who noted the synergistic effects of ultrasound with organic acids on microbial deactivation. This combined treatment approach appears to significantly increase the efficacy of citric acid for pathogen control in casings, presenting a promising sanitization strategy for fresh animal by-products in food processing. Moreover, it is known that citric acid and its salts, in addition to their preservative effect, have a positive effect on the organoleptic properties of natural casings, improve the color and odor of intestines, and an aqueous solution of citric acid is used to remove the unnatural musty odor of casings if it appears during the storage of intestinal raw materials [Sidorova, 2011].

It is advisable to promptly remove internal organs from animals within 30 min post-slaughter to uphold quality standards. According to Galarz *et al.* [2016], higher temperatures can notably shorten the shelf-life of chicken breasts, varying from 10 to 26 days at 2°C and from 4 to 8 days at 10°C. Furthermore, Hanna *et al.* [1982] observed changes in bacterial counts in beef, pork, and lamb organs over a 5-day storage period at 2°C. In turn, Kang *et al.* [2014, 2018] stressed the importance of thorough washing of the small intestine to eliminate blood, indigestible feed, and feces, emphasizing the critical role in reducing bacterial loads to enhance shelf-life and maintain freshness.

Ultrasound treatment applied to fresh intestines resulted in significant changes in TPC over the storage period, but it did not consistently inhibit TPC. The reduction in microorganisms is largely attributed to cavitation, a physical phenomenon described by several authors [Alarcon-Rojo *et al.,* 2019; Bhargava *et al.,* 2021; Zupanc *et al.,* 2019]. Cavitation involves the formation and oscillation of vapor bubbles within a liquid at pressures below its vapor pressure, which grow and implode powerfully enough to detach and deactivate pathogens on submerged surfaces. Our study findings indicated no significant difference

Table 3. pH of horse intestine subjected to various treatments during storage at 4°C.

Treatment	Storage time (day)				*p*-Value
	1	5	9	13	
A1; Control, 5 min	7.1±0.01^{aA}	7.0±0.03^{aA}	6.9±0.11^{aA}	6.9±0.15^{aA}	ns
A2; Control, 10 min	7.1±0.12^{aA}	6.9±0.14^{aA}	7.0±0.02^{aA}	6.9±0.07^{aA}	ns
B1; CA (0.5%), 5 min	6.8±0.03^{aA}	6.5±0.04^{aA}	6.7±0.06^{aA}	6.6±0.09^{aA}	ns
B2; CA (0.5%,) 10 min	6.7±0.11^{aA}	6.6±0.16^{aA}	6.6±0.01^{aA}	6.5±0.13^{aA}	ns
C1; CA (1%), 5 min	6.4±0.10^{aB}	6.1±0.12^{aB}	5.9±0.19^{aB}	5.7±0.18^{bB}	*
C2; CA (1%), 10 min	6.3±0.07^{aB}	6.0±0.02^{aB}	5.8±0.12^{bB}	5.6±0.11^{bB}	*
D1; US, 5 min	7.0±0.01^{aA}	6.9±0.01^{aA}	6.9±0.14^{aA}	6.7±0.18^{aA}	ns
D2; US, 10 min	7.0±0.01^{aA}	6.9±0.13^{aA}	6.8±0.06^{aA}	6.8±0.09^{aA}	ns
E1; US + CA (0.5%), 5 min	6.05±0.02^{aC}	5.62±0.15^{abC}	5.58±0.12^{bC}	5.52±0.11^{bC}	**
F1; US + CA (1%), 5 min	5.65±0.03^{bC}	5.69±0.10^{bC}	5.47±0.02^{aC}	5.26±0.13^{aC}	**

Results are shown as mean ± standard deviation (n=6). Different lowercase letters within the same row indicate significant differences across storage time for the same treatment (p<0.05). Different capital letters within each column indicate significant differences between treatments for the same storage day (p<0.05). *p<0.05; **p<0.01; ns, not significant (p≥0.05); CA, citric acid; US, ultrasound.

in microbial reduction between 5-min and 10-min ultrasound treatments, suggesting the most effective microbial inhibition within the first 5 min, with no substantial increase in effectiveness thereafter. This suggests that ultrasound alone might not suffice for food industry standards due to its limited sanitizing effectiveness and the relatively long treatment times required. Therefore, to develop ultrasound as a viable, short-duration food processing intervention, it should be combined with other methods such as heat, pressure, or organic acids to effectively inactivate and detach foodborne pathogens [Earnshaw *et al.,* 1995; Onyeaka *et al.,* 2021; Silveira *et al.,* 2018]. Our findings are consistent with those of Aguilar *et al.* [2021], who reported the inactivation of three types of microorganisms using a 10-min ultrasound treatment on raw meat emulsion.

The citric acid treatments (*e.g.*, B1, B2, C1, C2, E1, F1) demonstrated varying degrees of effectiveness in lowering TPC, with significant reductions observed (p<0.01). Reductions in microbial counts were evaluated by comparing each treatment group to the control samples, which were intestines soaked in water. **Table 2** provides details on the significant and non-significant differences between treatments at each storage interval. For both concentrations, the 10-min treatments (B2 and C2) showed slightly better control of TPC than the 5-min treatments (B1 and C1). However, the differences between 5-min and 10-min treatments were not substantial. The effectiveness of organic acids in inhibiting microbial growth is influenced by several factors, including pH, the proportion of undissociated acid, the acid chain length, and the cellular physiology and metabolism [Ji *et al.,* 2023]. Lipophilic organic acids, such as sorbic acid, benzoic acid, and lauric acid, can penetrate cell plasma membranes, lowering the internal pH and disrupting cellular function, which contributes to their antimicrobial properties [Ben Braïek & Smaoui, 2021; Sullivan *et al.,* 2020; Ullah *et al.,* 2012]. The reduction in TPC became more pronounced as the pH decreased over time, especially in treatments with higher concentrations of citric acid (from 0.5% to 1.0%), indicating an enhanced antimicrobial effect. However, microbial reduction levels stabilized at a 1.0% CA concentration, consistent with findings from Dan *et al.* [2017] and Yılmaz Eker *et al.* [2024].

Table 3 shows the alterations in pH values for horse intestines under various treatment conditions during cold storage. Significant decreases in pH were observed only for the treatments C1, C2, E1, and F1, particularly in those with higher concentration of citric acid (1%) and/or combined ultrasound and citric acid treatment. Other treatments did not produce statistically significant changes over time. This pattern indicates that higher citric acid concentrations and certain treatment combinations were more effective in achieving and maintaining lower pH levels, which contribute to the antimicrobial properties of the treatments. These treatments not only resulted in the lowest pH levels but also demonstrated significant statistical differences, with some showing highly significant reductions (p<0.01). Increasing the concentration and duration of citric acid treatment led to more pronounced pH reductions, as evident from the differences between B1/B2 (CA (0.5%) treatments) and C1/C2 (CA (1%) treatments). The CA (1%) treatments were more effective in lowering pH than the CA (0.5%) treatments. This finding aligns with observations made by Kang *et al.* [2018], who reported similar pH stability in pig small intestine after seven days of cold storage, highlighting the stringent requirements for thorough washing processes in animal intestines to maintain quality during cold distribution. We can conclude that both combinations of citric acid concentration and ultrasound treatment effectively reduce the pH of horse intestines during cold storage, with the CA (1%) treatment showing a more pronounced and consistent acidification effect. This trend is crucial for the food processing industry, as

Table 4. Parameters of color of horse intestines subjected to various treatments during storage at 4°C.

Parameter	Treatment, 5 min	Storage time (day)				*p*-Value
		1	5	9	13	
*L**	CA (0.5%)	65.73±2.81^{aA}	61.58±3.41^{bB}	64.32±1.23^{aA}	64.33±2.35^{aA}	*
	CA (1%)	64.21±0.81^{aA}	67.35±1.32^{bA}	60.54±0.61^{cB}	61.47±2.11^{cB}	**
	US	65.53±1.21^{aA}	62.95±2.01^{aA}	61.39±0.18^{bB}	64.55±1.11^{aA}	*
	US + CA (1%)	64.89±0.52^{aA}	63.08±1.36^{aA}	60.88±0.96^{bB}	63.95±2.54^{aA}	*
*a**	CA (0.5%)	18.21±1.45^{aA}	16.35±0.91^{aA}	18.59±1.40^{aA}	17.61±1.61^{aAB}	ns
	CA (1%)	17.56±2.21^{aA}	16.78±1.83^{aA}	17.99±1.78^{aAB}	16.53±2.22^{aB}	ns
	US	18.32±1.84^{aA}	17.54±2.48^{aA}	16.77±2.44^{abB}	17.33±1.81^{aAB}	ns
	US + CA (1%)	18.10±2.65^{aA}	17.41±1.92^{aA}	17.30±2.04^{aAB}	18.45±2.35^{aA}	ns
*b**	CA (0.5%)	8.12±1.77^{aA}	10.62±2.05^{bA}	10.02±2.43^{bAB}	9.83±1.81^{abB}	*
	CA (1%)	8.07±2.58^{aA}	11.33±1.88^{bA}	9.56±1.68^{aB}	10.44±2.56^{abAB}	*
	US	7.85±2.65^{aA}	10.14±1.89^{bA}	10.09±1.79^{bAB}	11.14±2.01^{abA}	*
	US + CA (1%)	8.43±2.11^{aA}	9.65±1.86^{aB}	10.57±2.26^{aA}	10.69±2.71^{abAB}	*

Results are shown as mean ± standard deviation (n=6). Different lowercase letters within the same row indicate significant differences across storage time for the same treatment (p<0.05). Different capital letters within each column, separately for each parameter, indicate significant differences between treatments for the same storage day (p<0.05). *p<0.05; **p<0.01; ns, not significant (p≥0.05); CA, citric acid; US, ultrasound; *L**, lightness; *a**, redness; *b**, yellowness.

lower pH levels can help extend the shelf-life of natural casings by inhibiting the growth of spoilage and pathogenic microorganisms. The consistent reduction in pH levels demonstrates that these treatment methods can effectively ensure the safety and quality of sausage casings in commercial settings, providing critical insights for optimizing food preservation processes.

Shelf-life refers to the duration for which a product remains safe for consumption while retaining acceptable sensory qualities. Meat spoilage is primarily driven by microbial activity, oxidative changes, and enzymatic breakdown [Soladoye *et al.,* 2024]. While specific microorganisms are known to cause spoilage [Odeyemi *et al.,* 2020; Zhu *et al.,* 2022], it is often the result of complex interactions among diverse microbial populations. Notably, spoilage can occur even in the absence of microbial activity; for instance, vacuum-sealed sterile meat can deteriorate and develop a bitter taste due to the proteolytic breakdown of meat proteins by endogenous enzymes [Abril *et al.,* 2023]. The progression of meat spoilage is often indicated by changes in odor, which serve as a useful measure for assessing spoilage in various meats, including pork, beef [Djordjević *et al.,* 2019; Yu *et al.,* 2022], and chicken [Katiyo *et al.,* 2020]. These odorous characteristics, ranging from "fresh" to "spoiled", reflect similar patterns of microbial and chemical changes across different types of meat. Moreover, lowering the pH has been shown to effectively inhibit microbial growth, thereby enhancing the preservation quality of food [Atasoy *et al.,* 2024]. This aligns with our findings that reducing pH, particularly through the use of citric acid in combination with ultrasound, significantly improves the shelf-life of horse intestines during cold storage. In the meat industry, preserving meat and its products is critical. Various techniques such as a gamma irradiation, e-beam irradiation, high-pressure processing, and pulsed electric field have been explored for their bactericidal effects in packaged products, aiming to extend both shelf-life and safety [Amit *et al.* 2017]. In this study, the combination of citric acid solutions and ultrasound treatment proved to be an effective method for improving both the shelf-life and safety of horse intestines during cold storage.

Color and thiobarbituric acid reactive substances

The results presented in **Table 4** reveal changes in the color parameters of horse intestines during cold storage at 4°C following treatments with citric acid, ultrasound, and their combination. The *L** values, representing the lightness of the samples, showed significant variation over the storage period and minimal differences between the treated horse intestines. This suggests that the treatments did not noticeably affect the light reflectance properties of the samples, and the perceived lightness remained largely unchanged. The *a** values, representing redness, exhibited minimal variation across treatments and no significant changes during storage, indicating stable redness throughout the study. The most noticeable changes were found in the yellowness (*b**) values. The observed increase in *b** values, which indicates a rise in yellowness, can be attributed to several underlying biochemical processes. One primary factor influencing color changes during storage is lipid oxidation, commonly observed in meat products, which leads to the formation of yellowish pigments, including compounds like aldehydes. Carotenoids, naturally yellow pigments in meat, may also contribute to higher *b** values [Amaral *et al.,* 2018]. Furthermore, ultrasound treatment, known for inducing cavitation, can cause protein denaturation, altering the structural characteristics of proteins and affecting light reflectance on the meat surface, thereby influencing color changes,

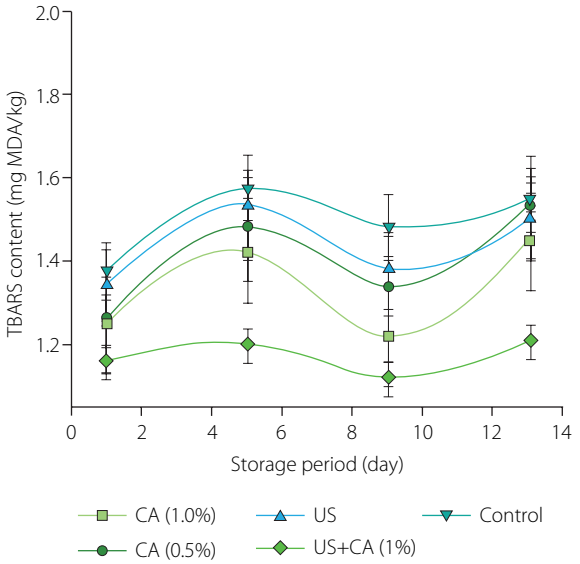

Figure 1. Content of thiobarbituric acid reactive substances (TBARS) in horse intestine subjected to various treatments for 5 min during storage at 4°C. CA, citric acid; MDA, malondialdehyde equivalent; US, ultrasound.

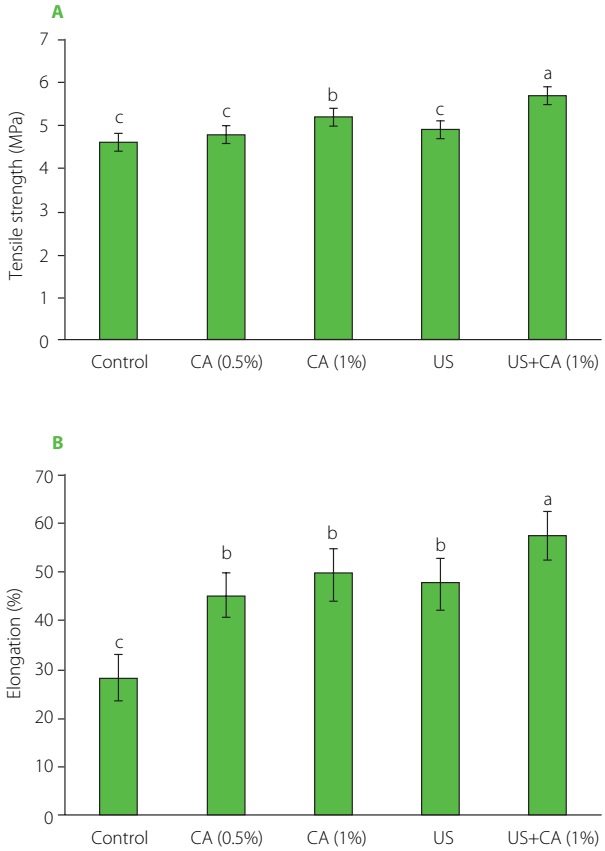

Figure 2. Tensile strength (**A**) and elongation (**B**) of control horse intestine and subjected to various treatments for 5 min. Different letters above bars indicate significant differences among treatments ($p<0.05$). CA, citric acid; US, ultrasound.

including an increase in yellowness [Roobab *et al.,* 2024]. The influence of pH, particularly in the treatments involving CA (1%), also plays a critical role; the lower pH achieved through these treatments can affect the stability of meat pigments, promoting the development of yellow hues due to the stabilization of oxidation products and altered protein structures [Renerre, 2007]. Overall, the a^* values remained stable throughout storage, while the increase in b^* values highlights the role of biochemical changes in influencing yellowness, particularly under the effects of lipid oxidation, protein denaturation, and pH modifications from the applied treatments.

Thiobarbituric acid reactive substances serve as a measure of lipid oxidation and are commonly used to assess the extent of secondary lipid oxidation in food products [Abeyrathne *et al.,* 2021]. As depicted in **Figure 1**, TBARS levels showed variations across the different treatment groups over time, with notable differences emerging as the storage period progressed. Specifically, during storage, the samples treated with both ultrasound and citric acid exhibited significantly lower TBARS values ($p<0.05$) compared to those subjected to either treatment alone. This suggests that the combination of citric acid and ultrasound was the most effective method for minimizing lipid peroxidation in horse intestines during cold storage. The combined treatment maintained the lowest and most stable TBARS values throughout the storage period, indicating its effectiveness in inhibiting lipid degradation and potentially preserving the quality of the casings better than the treatments using citric acid or ultrasound alone. This outcome is consistent with the broader context of our findings, where the combined effect of citric acid and ultrasound was also observed in maintaining pH stability and reducing microbial load, as seen in **Tables 3** and **4**. The observed combined effect between citric acid and ultrasound in preventing lipid oxidation could be due to the more uniform and effective penetration of the acid into the tissue, enhanced by the mechanical effects of ultrasound, which may disrupt cell membranes and improve the distribution and action of the citric acid. In the meat processing industry, enhancing the value of lower-priced meat by-products through such technological and scientific advancements is essential. The findings suggest that this combined treatment not only extends the shelf-life but also maintains the overall quality of the product, making it a particularly promising method for refrigerated storage of meat products. Implementing such approaches can significantly increase the economic value of these by-products, thereby benefiting the overall meat production industry [Ahmed *et al.,* 2020; Elgadir & Mariod, 2023; Jayathilakan *et al.,* 2012].

■ Mechanical properties

Figure 2 presents the results of traction tests performed on casings subjected to four distinct treatments – CA (0.5%), CA (1%), US alone, and a combination of US with CA (1%) – alongside a control sample. All treatments were administered for a duration of 5 min. This specific time frame was selected based on previous findings indicating that a 5-min combination of acid treatment and ultrasound yielded the optimal outcomes in terms of microbial stability and pH control. The data from these tests are quantified in terms of tensile strength (TS, MPa) and elongation at break (ε, %) [Srinivasa *et al.,* 2007], which reflect the chemical and physical properties of the casings. The results presented in **Figure 2a** demonstrate that the tensile strength of the casings

Table 5. Results of analysis of variance (ANOVA) for main effects and interactions.

Effect	TPC		TBARS		pH	
	F-Value	*p*-Value	F-Value	*p*-Value	F-Value	*p*-Value
Citric acid concentration (C)	13.57	<0.01**	12.11	<0.01**	10.43	<0.01**
Ultrasound treatment (U)	10.77	<0.01**	8.56	<0.05*	6.42	<0.05*
Treatment duration (D)	3.98	0.04*	5.21	<0.05*	7.25	<0.05*
Storage interval (S)	16.55	<0.01**	20.13	<0.01**	18.98	<0.01**
C×U	8.22	<0.05*	7.99	<0.05*	10.45	<0.01**
C×D	4.87	<0.05*	5.67	<0.05*	7.82	<0.01**
C×S	9.55	<0.01**	6.39	<0.05*	12.34	<0.01**
U×D	3.24	0.08	4.11	<0.05*	5.56	<0.05*
U×S	7.66	<0.05*	9.78	<0.01**	8.23	<0.05*
D×S	5.99	<0.05*	7.21	<0.05*	10.12	<0.01**
C×U×D	2.11	0.14	3.65	<0.05*	5.78	<0.05*
C×U×S	6.77	<0.05*	8.33	<0.01**	9.11	<0.01**
C×D×S	4.33	<0.05*	6.12	<0.05*	7.45	<0.01**
U×D×S	5.14	<0.05*	7.23	<0.05*	8.66	<0.01**

*Significant at $p<0.05$; **Significant at $p<0.01$; TBARS, thiobarbituric acid reactive substances; TPC, total plate count.

was significantly influenced by the treatments. The combined treatment (US + CA (1%)) showed the highest tensile strength (5.7 MPa), followed by the 1% citric acid treatment (5.2 MPa), both of which were significantly higher ($p<0.05$) than the control (4.6 MPa) and other treatments. These findings indicate that the combined treatment and 1% citric acid treatment enhanced the structural integrity of the casings compared to the control and other treatment groups. This improvement can be attributed to the synergistic effects of citric acid and ultrasound, which may have strengthened the collagen fibers within the casings. Moreover, all treated casings showed an increased elongation capacity compared to control, with an average increase of approximately 77% (from ε=28% to ε=45–58%). This increase in flexibility is typical of protein- and carbohydrate-based films that have been plasticized with substances like glycerol or sorbitol [González-Torres *et al.,* 2021]. The intestinal bands of horses, which are part of the outer longitudinal muscular layer of the large intestine and are rich in elastin and collagen fibers, demonstrate the protein-based structure's susceptibility to plasticizers [Lopes & Pfeiffer, 2000]. Among the treated samples, those subjected to the combined treatment of ultrasound and 1% citric acid exhibited the highest elongation percentage (58%), followed by the 1% citric acid treatment (50%) (**Figure 2**). The 0.5% citric acid and ultrasound-only treatments also showed moderate improvements in elongation percentages compared to the control but were not as effective as the combined treatment or the 1% citric acid treatment. These results highlight the superior performance of the combined treatment and higher citric acid concentrations in improving the mechanical flexibility of the casings. This treatment could offer significant benefits in applications where enhanced flexibility and structural integrity are critical, such as in the manufacture of natural sausage casings where both properties are essential for optimal performance during filling and handling. The data suggest that integrating ultrasound with citric acid could be a promising method for improving the quality and processing characteristics of natural casings in the food industry. However, to further validate the efficacy and durability of this treatment, additional research is needed to examine how these mechanical properties hold up under different storage conditions and varying environmental factors. Future work will help determine the long-term viability of the ultrasound and citric acid treatment, ensuring that the improvements in flexibility and strength are maintained throughout the product's shelf-life and under commercial usage scenarios.

Interactions between effects

The results from the ANOVA, detailed in Table 5, highlight the main effects and interaction terms that were statistically significant for various measurements. The significant main effects of citric acid concentration, ultrasound treatment, and storage interval on TPC, TBARS, and pH indicate that these factors individually exert a strong influence on microbial load, lipid oxidation, and the acidity of the casings.

The significant interaction terms, such as citric acid concentration and ultrasound treatment (C×U), citric acid concentration and storage time (C×S), and ultrasound treatment and storage time (U×S), indicate that the effect of one factor was influenced by the levels of another factor. For example, the interaction

between citric acid concentration and ultrasound treatment (C×U) showed that the combined application of these treatments had a more pronounced impact on TPC, TBARS, and pH levels than either treatment alone. Specifically, higher concentrations of citric acid in conjunction with ultrasound treatment result in greater reductions in TPC and TBARS, as well as more stable pH levels over time. Similarly, the interaction between citric acid concentration and storage time (C×S) demonstrates that the effectiveness of citric acid in controlling microbial growth and maintaining pH varies with the duration of storage, indicating that citric acid's effects are sustained or even enhanced over longer periods. These interactions underscore the importance of combining treatment factors to achieve optimal microbial stability and quality preservation in storage. The extended analysis elucidates the significant impacts of each treatment and their interactions on the quality and preservation of horse intestine. The clear reporting of statistical values, alongside a detailed interpretation of what these effects entail, provides a thorough understanding of how these treatments can be optimized for better food preservation outcomes. This detailed statistical approach ensures that the findings are not only statistically significant but also practically relevant, providing valuable insights for food processing industries.

CONCLUSIONS

The combined application of ultrasound and citric acid has been shown to be more effective in reducing total plate counts than citric acid alone, without compromising product quality. This dual approach enhances microbial safety, reduces acid usage, and shortens processing times, offering cost savings for the meat industry while maintaining food quality. The treatment not only lowered pathogen presence on natural casings but also extended shelf-life and improved mechanical properties. Casings processed with this method were stronger and more elastic, reducing losses and costs in sausage manufacturing. Despite significant changes in yellowness, the combined treatment of 1% citric acid with ultrasound proved to be the most effective method for microbial load reduction and quality preservation of horse intestines during storage. While some degree of color change was expected due to biochemical reactions during treatment, the enhanced microbial safety and extended shelf-life outweigh these minor visual impacts. These findings establish the combined treatment as a promising approach for managing foodborne pathogens and maintaining quality in meat products, especially in applications requiring sustainable and efficient preservation strategies. However, the efficiency of ultrasound, driven by cavitation, can be influenced by factors such as organic matter, water hardness, and dissolved gases, necessitating further trials to optimize conditions for industrial applications. This study highlights the potential for the food processing industry, particularly in sectors emphasizing natural and minimally processed solutions, to adopt ultrasound and citric acid treatments for enhanced microbial safety and optimized processing.

Future research should concentrate on a detailed investigation of the combined ultrasound and citric acid treatment to eliminate foodborne pathogens in inoculated horse intestine casings. While this approach has demonstrated significant potential in enhancing microbial safety, further studies are required to fine-tune the conditions for maximum efficacy. Furthermore, exploring the impact of this combined treatment on the quality properties and shelf stability of sausages made from treated casings will yield valuable insights. Such research should assess the effects on texture, flavor, color, overall consumer acceptance, and how mechanical properties fare under different storage conditions and environmental factors, thereby informing best practices for industrial applications. Examining the long-term stability and microbial resistance of treated casings could lead to significant advancements in food safety and quality, potentially revolutionizing meat processing techniques. The implications of these findings could extend beyond the meat industry, offering novel methods for preserving and enhancing the safety of various perishable food products.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Effects of Blanching Conditions on the Enzyme Inhibition and Antioxidant Loss in Rambutan (*Nephelium lappaceum* L.) Seeds

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Rambutan seeds – a by-product of rambutan fruit processing industry – contains various nutrients as well as phenolics and saponins with antioxidant capacities. This by-product needs to be treated before being used as a new raw material to produce value-added products. In this study, water blanching of rambutan seeds was performed and the impacts of blanching temperature and time on lipase (LP), lipoxygenase (LOX) and polyphenol oxidase (PPO) activities and antioxidant loss were investigated. At blanching temperature of 95°C, the inactivation rate constant of LP, LOX and PPO was 29.93×10^{-3}, 42.80×10^{-3} and 59.33×10^{-3} 1/min, respectively while their half-life was 23.19, 16.19 and 11.68 min, respectively. During the blanching, the total saponin content in rambutan seeds first increased and then decreased while the total phenolic content was gradually reduced. In the accelerated storage of dried rambutan seeds, the degradation rate constants of phenolics and saponins of the blanched sample were 1.7 and 1.5 times, respectively, lower than those of the unblanched counterpart; additionally, the acidic and peroxide values of oil of the blanched seeds increased more slowly and less than those of the unblanched counterpart. Blanching rambutan seeds deactivated their enzymes, resulting in lesser antioxidant loss and lipid changes in the dried seeds during the accelerated storage.

Keywords: accelerated storage, biocatalyst, fruit seed, heat treatment, phenolics, saponins

INTRODUCTION

Rambutan (*Nephelium lappaceum* L.) is a tropical fruit tree that has been widely cultivated in Southeast Asia, Oceania, South America and Africa [Jahurul *et al.*, 2020a]. Rambutan seeds are a by-product of rambutan fruit processing industry such as dried rambutan, rambutan in sugar syrup, jam, jelly, and rambutan juice [Afzaal *et al.*, 2023]. Rambutan seeds account for about 4-7% of the rambutan fruit weight [Jahurul *et al.*, 2020a]. This by-product contains various nutrients valuable for humans. The lipid content of rambutan seeds is roughly 33.4 g/100 g dry matter (dm) [Hernández-Hernández *et al.*, 2019], in which the unsaturated fatty acid level is approximately 48.1% of total lipids [Chimplee & Klinkesorn, 2015]. According to Jahurul *et al.* [2020b], the melting point of rambutan seed lipid is similar to that of cocoa butter; as a result, it can be used to partially replace cocoa butter in chocolate processing. The protein content of rambutan seeds is about 7.8 g/100 g dm [Hernández-Hernández *et al.*, 2019]; specifically, the albumin fraction of rambutan seed proteins is reported to have good functional properties including high gelation, emulsifying and foaming capacities [Vuong *et al.*, 2016]. The total carbohydrate content of rambutan seeds is approximately 46 g/100 g dm; the main components

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are starch and dietary fiber [Hernández-Hernández *et al.*, 2019]. Rambutan seeds also contain minerals like calcium, zinc, iron and manganese [Akhtar *et al.*, 2017] and vitamins such as thiamin, riboflavin, and niacin [Afzaal *et al.*, 2023]. Moreover, phenolics and saponins with antioxidant activities are reported in rambutan seeds [Chai *et al.*, 2018]. Nowadays, rambutan seeds have been roasted and used as a snack food in the Philippines [Jahurul *et al.*, 2020a]. Many studies have been performed to use rambutan seeds as a raw material to extract lipids [Sirisompong *et al.*, 2011], proteins [Vuong *et al.*, 2016] and various bioactive compounds [Sai-Ut *et al.*, 2023].

In order to use rambutan seeds as a potential raw material to produce value-added products on an industrial scale, the seeds released from rambutan fruit processing industry processes need to be dried and preserved. Rambutan seeds contain lipase (LP) which catalyzes the hydrolysis of triglyceride to yield glycerol and fatty acids, thereby increasing the free fatty acid content during the drying process of rambutan seeds [Jahurul *et al.*, 2020b]. Besides, fatty nuts and seeds contain lipoxygenase (LOX) [Shi *et al.*, 2020], which catalyzes the oxidation of free fatty acids to produce peroxides and carbonyl compounds, resulting in rancid odors and harmful effects for human health [Liburdi *et al.*, 2021]. In addition, polyphenol oxidase (PPO, EC.1.14.18.1) identified in many fruits [Villamil-Galindo *et al.*, 2020] catalyzes the oxidation of phenolic compounds to form quinones, which can be polymerized to generate brown pigments such as melanin; these compounds negatively affect sensory attributes of the product [Dibanda *et al.*, 2020]. Rambutan seeds are rich in lipids and phenolics [Jahurul *et al.*, 2020a]. Inactivation of enzymes that promote phenolic and lipid transformation in rambutan seeds is essential before using the seeds in the making of value-added products.

Blanching is a conventional method to pretreat fruits and vegetables in the food industry, mainly to inactivate their enzymes and microorganisms [Chao *et al.*, 2022]. Moreover, the blanching process can reduce the content of some anti-nutrients such as oxalate and phytate [Malhotra *et al.*, 2023], prevent quality changes and shorten drying time for fruits and vegetables [Kim *et al.*, 2020]. However, changes in enzyme activities and antioxidant contents in rambutan seeds during the blanching have not been considered.

In this research, rambutan seeds were blanched in hot water. The research objective was to investigate the effects of blanching temperature and time on the inactivation of LP, LOX and PPO as well as the loss in total phenolic and total saponin contents and antioxidant capacities of rambutan seeds. The antioxidant contents and capacities as well as the acidic and peroxide values of oil of the dried rambutan seeds during the accelerated storage were also evaluated to clarify the impacts of the blanching process on the quality of dried seeds.

MATERIALS AND METHODS

Materials

Rambutan (*Nephelium lappaceum* L., Java) fruits used in this study originated from a farm in Long khanh city, Dong nai province (Vietnam). About 100 kg of rambutan fruits were harvested after 115–120 days of flowering.

Chemicals and reagents

Linoleic acid was purchased from Thermo Fisher Scientific (Waltham, MA, USA) while vanillin, escin, catechol, gallic acid, Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman--2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, 2,4,6-tri(2-pyridyl)-*s*-triazine, NaCl, KH_2PO_4, Na_2CO_3, and glycerol were bought from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade. Gum arabic (code: GRM682-500G) originated from HiMedia (Maharashtra, India).

Blanching rambutan seeds

After separating the shell and flesh, the rambutan seeds were manually washed with tap water for 15 min. The blanching process was carried out in a thermostatic water bath (WNB14 model, Memmert, Schwabach, Germany). About 200 g of rambutan seeds were used in each batch; the ratio of rambutan seeds and water was 1/5 (*w/v*). The blanching temperature was fixed at 80, 85, 90 or 95°C, while the blanching time varied from 5 to 25 min. The blanched rambutan seeds were then cooled in 4°C water to ambient temperature for about 10 min and used for determination of LP, LOX and PPO activities as well as total phenolic and total saponin contents and antioxidant capacities. The unblanched rambutan seeds served as the control sample.

Accelerated storage of dried rambutan seeds

Rambutan seeds were blanched at 95°C for 25 min and cooled in water to about 70°C according to the blanching procedure reported above. The seeds were convectively dried at 70°C in a drier (SF30 model, Memmert) with hot air velocity of 2 m/s until the moisture content achieved 10-11%. The dried rambutan seeds put in polyethylene bags (about 200 g seeds *per* bag) were used for accelerated storage according to a reference of Lopez *et al.* [2022] with slight modifications. The dried seeds were preserved in an incubator (TH3-PE-100 model, Jeiotech, Daejeon, Korea) at 60°C, air humidity of 75% for 20 days. Every fourth day, samples were collected for determination of total phenolic and total saponin contents, antioxidant capacities, acidic and peroxide values. The control sample was unblanched rambutan seeds that were convectively dried under the same conditions.

Enzyme assays

To extract enzymes from rambutan seeds, about 10 g of seeds were added into 50 mL of a solvent; using 0.2 M sodium phosphate buffer (pH 7.0) for LP, 1/15 M sodium phosphate buffer (pH 6.0) for LOX, and 0.1 M sodium phosphate buffer (pH 5.5) for PPO. The mixture was ground for 2 min using a laboratory mill (A11 model, IKA, Freiburg, Germany) and then incubated in a thermostat-shaker (Certomat BS1 model, B Braun Biotech International, Melsungen, Germany) at 30°C and 100 rpm for 30 min. The suspension was then centrifuged (Z366K centrifuge, Hermle, Baden-Württemberg, Germany) at 4°C and 10,000×*g*

for 30 min, and the supernatant was used to determine LP, LOX and PPO activities.

The LP activity was measured following to the procedure of Mustranta *et al.* [1993] with slight modification. The substrate solution including 15 mL of olive oil and 35 mL of an emulsifying agent (1 L of an emulsifying agent contained 17.9 g of NaCl, 0.41 g of KH_2PO_4, 540 mL of glycerol, 10 g of gum arabic, and distilled water) was prepared by mixing and homogenizing (APV 2000 model, SPX Flow, Bydgoszcz, Poland) at ambient temperature; the pressure on the first and second stage was 350 and 80 bar, respectively. Then, 5 mL of the substrate solution was mixed with 4 mL of 0.2 M sodium phosphate buffer (pH 7.0), and 1 mL of the crude enzyme extract and the mixture was heated at 37°C for 10 min. Then, 10 mL of an acetone-ethanol mixture (1/1, *v/v*) was added to stop the reaction. The released fatty acids were titrated with 0.05 M NaOH solution. One LP unit is defined as an amount of enzyme that catalyzes the hydrolysis of triglyceride to release 1 µmol of free fatty acids within 1 min under the assay conditions. The results were expressed as the number of units *per* g dm of the rambutan seeds.

The LOX activity was estimated according to the procedure posited by Gökmen *et al.* [2002] with slight modification. The substrate solution consisting of linoleic acid (157.2 µL), Tween-20 (157.2 µL) and deionized water (10 mL) was prepared by homogenization at 30°C and 200 rpm for 5 min. Then, 1 mL of 1 M NaOH solution was added to the obtained mixture, which was next diluted to 200 mL with 1/15 M sodium phosphate buffer (pH 6.0), resulting in a 2.5 mM linoleic acid solution. About 29 mL of the substrate solution and 1 mL of the crude enzyme extract were added into a 100 mL Erlenmeyer flask which was put in a thermostat-shaker at 30°C and 100 rpm for 5 min. After that, 1 mL of the reaction mixture was transferred into a test tube, to which 4 mL of 0.1 M NaOH solution was added to stop the enzymatic reaction. The absorbance was then recorded at the wavelength of 234 nm. One LOX unit is defined as an amount of enzyme that catalyzes the oxidation of linoleic acid to increase absorbance of the reaction mixture by 0.001 unit at the wavelength of 234 nm *per* min under the assay conditions. The results were presented as the number of units *per* g dm of the rambutan seeds.

The PPO activity was determined following a procedure presented by Zhang *et al.* [2018]. The reaction mixture was composed of 1.5 mL of 0.1 M sodium phosphate buffer (pH 5.5) and 1 mL of a 0.2 M catechol solution. About 0.5 mL of the crude enzyme extract was then added to the reaction mixture. The reaction was performed at 30°C for 2 min, and the absorbance was measured at the wavelength of 420 nm. One PPO unit is defined as an amount of enzyme that catalyzes the oxidation of catechol to increase absorbance of the reaction mixture by 0.001 unit at the wavelength of 420 nm *per* min under the assay conditions. The results were shown as the number of units *per* g dm of the rambutan seeds.

Chemical analysis

About 30 g of rambutan seeds were ground in a laboratory mill (A11 model, IKA) for 2 min, and the milled seeds were defatted using a Soxhlet system. About 10 g of defatted rambutan seed powder was added into a 250 mL Erlenmeyer flask containing 100 mL of 80% (*v/v*) aqueous methanol; the Erlenmeyer flask was then put in a thermostat-shaker (Certomat® BS-1 model, B. Braun Biotech. International) at 30°C and 100 rpm for 30 min for extraction. At the end of the extraction, the suspension was centrifuged at 4°C and 10,000×*g* for 30 min and the supernatant was used to determine the total phenolic and total saponin contents and antioxidant capacities.

The total phenolic content was quantified by the spectrophotometric method according to a procedure reported by Le *et al.* [2024] with slight modification. About 0.2 mL of the diluted extract and 1 mL of the Folin-Ciocalteu reagent were added into a test tube. After that, 0.8 mL of a 10% Na_2CO_3 solution and 3 mL of distilled water were added, and the mixture was vortexed. The reaction occurred at ambient temperature in the dark within 30 min. The absorbance of the obtained sample was measured at the wavelength of 760 nm on a spectrophotometer (2600i model, Shimadzu Co., Kyoto, Japan). The standard curve was established using a gallic acid solution with concentrations varying from 0 to 50 mg/mL. The total phenolic content was expressed as mg gallic acid equivalent *per* 100 g dm of the rambutan seeds (mg GAE/100 g dm).

The total saponin content was determined by the spectrophotometric method following a procedure described by Hiai *et al.* [1976] with slight modification. About 0.5 mL of the diluted extract and 0.5 mL of 8% (*w/v*) vanillin in ethanol were added into a test tube; after that, 5 mL of a 72% (*v/v*) H_2SO_4 solution were added into the tube, which was then put in cold water to adjust the mixture temperature to about 70°C. The mixture was incubated at 70°C for 20 min and then cooled to ambient temperature using cold water. The absorbance of the obtained sample was measured at the wavelength of 560 nm on a spectrophotometer (2600i model, Shimadzu Co.). The standard curve was established using an escin solution with concentrations varying from 0 to 1,000 mg/mL. The total saponin content was shown as mg escin equivalent *per* 100 g dm of the rambutan seeds (mg EE/100 g dm).

Antioxidant capacities were determined by DPPH and ferric reducing antioxidant power (FRAP) assays, using procedures reported by Brand-Williams *et al.* [1995] and Benzie & Strain [1996], respectively, with slight modification. For the DPPH assay, 0.1 mL of the crude extract and 3.9 mL of the 0.1 mM methanolic DPPH radical solution were added into tubes, vortexed and incubated at ambient temperature in the dark for 30 min. The absorbance was recorded at the wavelength of 517 nm. For the FRAP assay, 0.6 mL of the diluted extract and 3.4 mL of the FRAP reagent solution were added into a test tube, vortexed and left at ambient temperature in the dark for 15 min. The absorbance was measured at the wavelength of 593 nm. Antioxidant capacity was presented as µmol Trolox equivalent *per* 100 g dm of the rambutan seeds (µmol Trolox/100 g dm).

Acidic and peroxide values were quantified by the AOAC International 940.28 and 965.33 methods, respectively [AOAC, 2000]. About 20 g of rambutan seed powder were added into

300 mL of hexane; the oil extraction was performed at about 65°C for 2 h in a Soxhlet system. The liquid phase was then subjected to vacuum evaporation at 45°C using a rotary evaporator (Model RV 3V-C, IKA) for hexane removal. The obtained seed oil was used for determination of acidic and peroxide values, which were expressed in mg KOH/g seed oil and meq/kg seed oil, respectively.

Determination of kinetic parameters of enzyme inactivation and antioxidant degradation during the blanching of fresh rambutan seeds and the accelerated storage of dried rambutan seeds

The kinetic parameters of enzyme inactivation and antioxidant degradation during the blanching of fresh rambutan seeds and the accelerated storage of dried rambutan seeds were determined using the first-order kinetics [Kayın *et al.,* 2019], according to Equation (1):

$$\frac{C_t}{C_0} = \exp(-k \times t) \quad (1)$$

where: C_t and C_0 are the LP/LOX/PPO activity or the total phenolic/total saponin content at time t and zero; k is the first-order rate constant of enzyme inactivation or antioxidant degradation; t is the blanching time (min) or the accelerated storage time (day).

Then, the logarithm of Equation (1) was used to get Equation (2):

$$\ln C_t = -k \times t + \ln C_0 \quad (2)$$

In practice, the enzyme activity C_t and C_0 expressed in U/g dry matter of the rambutan seeds can be replaced by the percentage of the initial enzyme activity.

From the experimental data, a graph showing the relationship between ln(enzyme activity or total phenolic/total saponin content) over time was established. The inactivation/degradation rate constant (k) and half-life ($t_{1/2}$) were then determined; where k is the slope of the straight lines Equation (2) and $t_{1/2}$ is calculated according to Equation (3):

$$t_{1/2} = (\ln 2)/k \quad (3)$$

The impact of temperature on the enzyme inactivation rate constant or the phenolic/saponin degradation rate constant follows a first-order Arrhenius plot between lnk and 1/T. The dependence of k on temperature (T) was determined according to the activation energy (E_a), according to Equation (4):

$$\ln k = -E_a/(R \times T) + \ln C \quad (4)$$

where: E_a is the energy activation of the reaction (kcal/mol); R is the gas constant (8.314×10^{-3} kJ/(K×mol); T is the absolute temperature (K); and C is the pre-exponential constant.

The E_a value was calculated from the slope of the straight-line Equation (4).

Statistical analysis

Each blanching and accelerated storage experiment was done in triplicate. The results were shown as means and standard deviation (n=3) and subjected to analysis of variance, using Statgraphics Centurion XIX (Manugistics Inc, Rockville, MD, USA). Mean values were considered significantly different as the probability was less than 0.05 using least significance difference (LSD) multiple range test.

RESULTS AND DISCUSSION

Impacts of blanching temperature and time on lipase, lipoxygenase and polyphenol oxidase activities in rambutan seeds

Changes in LP, LOX and PPO activities during the blanching of rambutan seeds are presented in **Figure 1**. At the four investigated temperatures, the enzyme activities in rambutan seeds gradually decreased with the blanching time. After 25-min blanching at 80, 85, 90 and 95°C, the remaining LP activity was 76, 66, 59 and 46%, respectively (**Figure 1A**); meanwhile the remaining LOX activity was 63, 48, 39 and 32%, respectively (**Figure 1B**); and the remaining PPO activity was 44, 35, 32 and 20% compared to the initial level, respectively (**Figure 1C**). It can be noted that with the same blanching time, the PPO activity was reduced more than the LP and LOX activities. Therefore, LP and LOX needed longer heat treatment time than PPO to achieve the same inactivation level at the same blanching temperature.

Our statistical analysis shows that the decrease in LP, LOX and PPO activities in rambutan seeds over the blanching time followed the Arrhenius first-order kinetic model of enzymatic reactions, as indicated by the coefficient of determination (R^2) which ranged from 0.90 to 0.99 (data not shown). Previously, the changes in PPO activity in *Agaricus bisporus* mushroom [Cheng *et al.*, 2013] and *Aruncus dioicus var kamtschaticus* samnamul [Kim *et al.*, 2020] during the water blanching were also fitted to the first-order kinetic model.

The kinetic parameters of LP, LOX and PPO inactivation are visualized in **Table 1**. The higher the blanching temperature was, the greater was the inactivation rate constant of LP, LOX and PPO in rambutan seeds. Specifically, when increasing the blanching temperature from 80 to 95°C, the inactivation rate constants of LP, LOX and PPO increased 2.7 times, 2.3 times and 2.1 times, respectively. At 90°C, the PPO inactivation rate constant in rambutan seeds was 42.8×10^{-3} 1/min; meanwhile that value in dragon fruit peel was 4.4×10^{-3} 1/s [Mai *et al.*, 2022], which is equivalent to 264×10^{-3} 1/min, whereas that value in mangosteen peel was 17.1×10^{-3} 1/min [Deylami *et al.*, 2016]. Thus, the inactivation rate constant of PPO in rambutan seeds was much lower than that in dragon fruit peel but higher than that in mangosteen peel.

In contrast to the enzyme inactivation rate constant, the half-life of LP, LOX and PPO in rambutan seeds gradually decreased with the blanching temperature (**Table 1**). The higher the blanching temperature was, the shorter was the enzyme half-life. The half-life of LP in rambutan seeds was 1.43 to 1.68 times and 1.98 to 2.65 times higher than that of LOX and PPO,

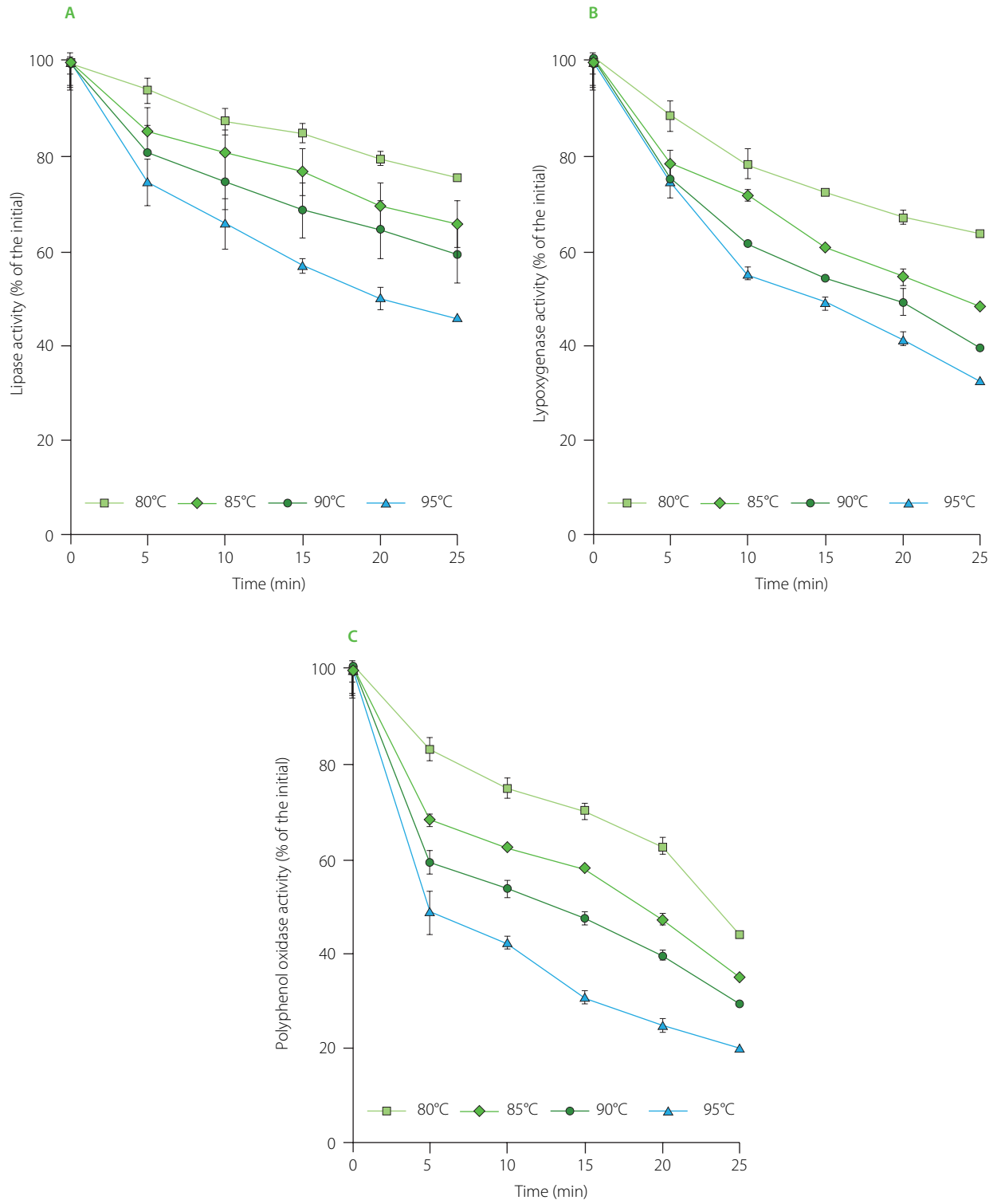

Figure 1. Changes in lipase (**A**), lipoxygenase (**B**) and polyphenol oxidase (**C**) activities in rambutan seeds during blanching.

respectively. The thermal stability of LP in rambutan seeds was higher than that of LOX while the PPO showed the lowest thermal stability.

Among the three investigated enzymes in rambutan seeds, LP showed the highest activation energy of enzyme inactivation, followed by LOX and PPO (**Table 1**). It is reported that E_a of LP inactivation in wheat germ was 21.719 kJ/mol [Xu *et al.*, 2016], which was 3.2 times lower than that of LP in rambutan seeds. Meanwhile, E_a of LOX inactivation in rambutan seeds was nearly similar to the value of LOX in lupine beans (60.5 kJ/mol) but much lower than that of soybean LOX (119 kJ/mol) [Stephany *et al.*, 2016]. Besides, E_a of PPO inactivation in rambutan seeds was higher than that in dragon fruit peel (43.63 kJ/mol) [Mai *et al.*, 2022], but much lower than that in pomegranate

Table 1. Inactivation rate constant, half-life and activation energy of lipase, lipoxygenase and polyphenol oxidase inactivation in rambutan seeds at different blanching temperatures.

Enzyme	Temperature (°C)	Inactivation rate constant (k) ($\times10^{-3}$ 1/min)	Half-life ($t_{1/2}$) (min)	Activation energy (E_a) (kJ/mol)
Lipase	80	10.93 ± 0.90^{dC}	63.68^{aA}	69.61 ± 5.94^{A}
	85	15.80 ± 0.36^{cC}	43.88^{bA}	
	90	19.20 ± 0.50^{bC}	36.05^{cA}	
	95	29.93 ± 1.40^{aC}	23.19^{dA}	
Lipoxygenase	80	18.30 ± 0.60^{dB}	37.83^{aB}	59.61 ± 2.89^{B}
	85	28.30 ± 0.26^{cB}	24.49^{bB}	
	90	34.87 ± 2.28^{bB}	19.93^{cB}	
	95	42.80 ± 0.26^{aB}	16.19^{dB}	
Polyphenol oxidase	80	28.86 ± 1.10^{dA}	24.03^{aC}	49.85 ± 3.51^{C}
	85	36.96 ± 0.50^{cA}	18.75^{bC}	
	90	42.76 ± 1.10^{bA}	16.21^{cC}	
	95	59.33 ± 0.41^{aA}	11.68^{dC}	

Data are shown as mean ± standard deviation (n=3). Values with different subscripts (a–d) in the same column are significantly different (p<0.05) for each enzyme. Values with different capital letter (A–C) in the same column are significantly different (p<0.05) for each blanching temperature.

flesh (112.97 kJ/mol) [Rayan & Morsy, 2020] and in straw mushroom (214 kJ/mol) [Cheng *et al.*, 2013]. Enzymes in various agricultural products have various activation energies of their inactivation due to difference in dimensional structure of protein molecules [Fante & Noreña, 2012]. In addition, the heat transfer rate inside agricultural products is dependent on their chemical composition, thereby affecting enzyme inactivation level [Gonçalves *et al.*, 2010].

In this study, the inactivation rate constant, half-life and activation energy of enzyme inactivation for LP, LOX and PPO in rambutan seeds were calculated and reported for the first time, contributing fundamental information to rambutan seed biochemistry.

■ Impact of blanching temperature and time on antioxidant contents and capacities in rambutan seeds

Changes in the total phenolic and total saponin contents and antioxidant capacity of rambutan seeds during the blanching are shown in **Figure 2**. The total phenolic content in rambutan seeds gradually decreased during the blanching. The greater the blanching temperature was, the lower was the remaining total phenolic content in rambutan seeds. Specifically, when the blanching temperature was increased from 80 to 95°C, the decrease in the total phenolic content after 25 min was enhanced from 11.2 to 25.6%. That could be due to partial diffusion of soluble phenolics from the rambutan seeds into the blanching water. Moreover, high blanching temperature could cause some phenolics to be hydrolyzed or oxidized [Cao *et al.*, 2021]. Previous studies also reported the loss of phenolic compounds during the blanching of carrot peels [Chantaro *et al.*, 2008], spinach, swamp cabbage and kale [Ismail *et al.*, 2004].

The reduction in phenolic content in rambutan seeds during the blanching was fit to the first-order kinetic model with R^2 varying from 0.90 to 0.99 (data not shown). Similar results were also recorded in the blanching of beetroot, eggplant, green pea, and green pepper within the temperature range of 70–90°C [Eyarkai Nambi *et al.*, 2016].

Table 2 presents the kinetic parameters of phenolic degradation during the blanching. Blanching temperature increase from 80 to 95°C enhanced their degradation rate constant 2.3 times; on the contrary, their half-life time was significantly reduced. The degradation rate constant of phenolics in rambutan seeds at 90°C was 11.06×10^{-3} 1/min, which was nearly similar to that in pitaya peel (2×10^{-4} 1/s = 12×10^{-3} 1/min) [Mai *et al.*, 2022]. Thus, the thermal stability of phenolic compounds in rambutan seeds and dragon fruit peel during the blanching process was equivalent. Similarly, the activation energy of phenolic degradation in rambutan seeds (63.33 kJ/mol) and in dragon fruit peel (59.70 kJ/mol) did not differ significantly; however, these values were 1.95 times lower than that determined for carrot (123.51 kJ/mol) [Gonçalves *et al.*, 2010].

Contrary to the changes in the total phenolic content, the total saponin content in rambutan seeds increased to the maximum during the early stages of blanching (**Figure 2B**). After 10-min blanching at 80, 85, 90 and 95°C, the total saponin content was 114, 111, 110 and 108% greater than that at the beginning

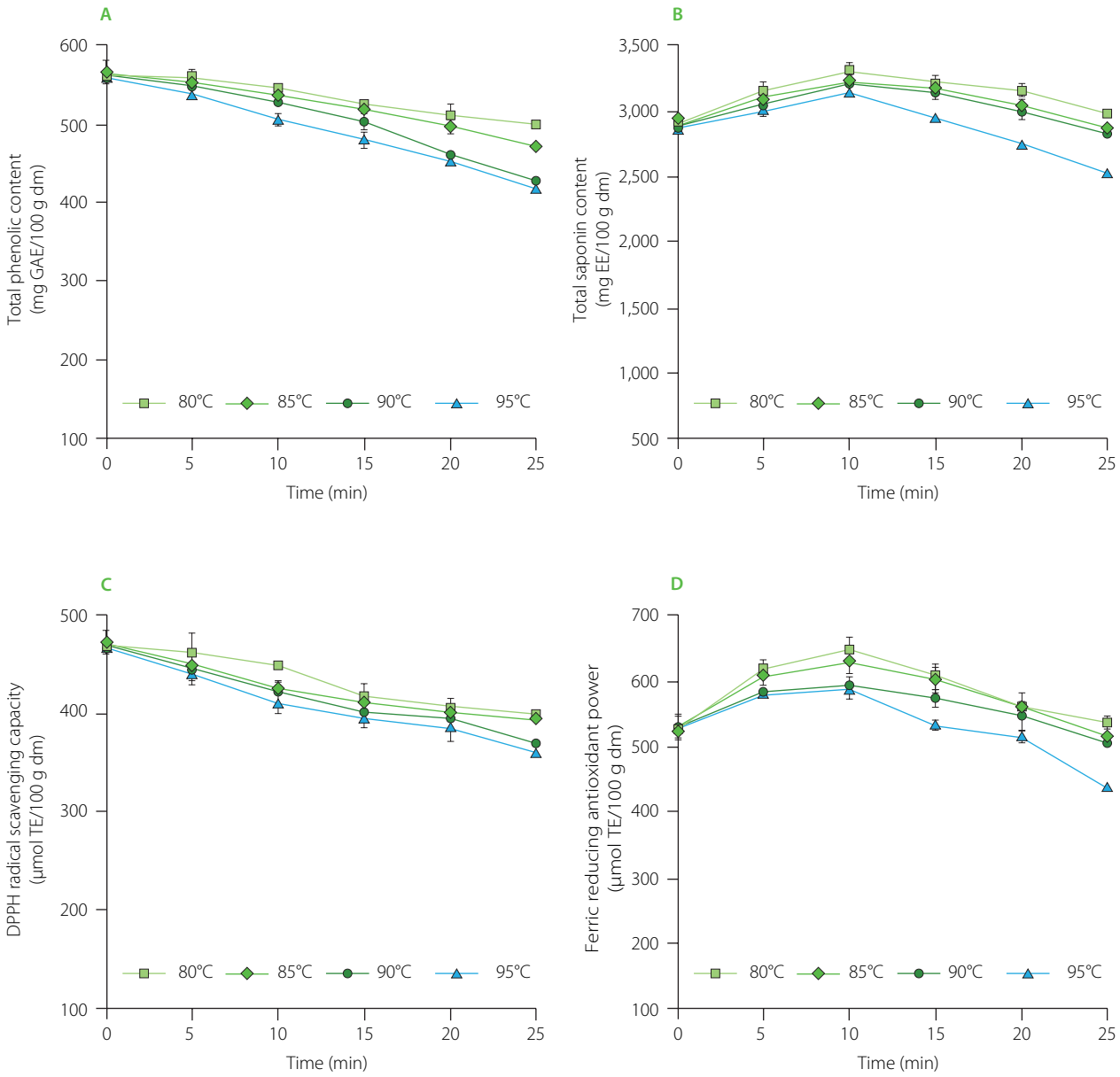

Figure 2. Changes in total phenolic (**A**) and total saponin (**B**) contents, DPPH radical scavenging capacity (**C**), and ferric reducing antioxidant power (**D**) of rambutan seeds during blanching. GAE, gallic acid equivalent; dm, dry matter; EE: escin equivalent; DPPH radical, 2,2-diphenyl-1-picrylhydrazyl radical; TE, Trolox equivalent.

Table 2. Degradation rate constant, half-life and activation energy of degradation of phenolics in rambutan seeds during the blanching at different temperatures.

Temperature (°C)	Degradation rate constant (k) ($\times 10^{-3}$ 1/min)	Half-life ($t_{1/2}$) (min)	Activation energy (E_a) (kJ/mol)
80	5.13±0.32^c	135.4^a	63.33±4.12
85	7.03±0.40^b	98.7^b	
90	11.06±1.19^a	63.1^c	
95	11.73±1.28^a	59.5^c	

Data are shown as mean ± standard deviation (n=3). Values with different subscripts in the same column differ significantly (p<0.05).

of thermal treatment. A similar increase in the saponin level has recently been reported by Zhang *et al.* [2022] when *Toona sinensis* leaves were blanched at 100°C for 30 s, upon which the total saponin content increased by 62% as compared to the initial content in the leaves. It can be explained that the high blanching temperature could damage cellular membrane in the plant tissues, improving saponin extraction [Zhang *et al.*, 2022]. Nevertheless, the saponin content in rambutan seeds decreased at the later stages of blanching. After 25 min blanching at 80, 85, 90 and 95°C, the remaining saponin content was 102, 99, 97 and 86% of the initial level, respectively. The reduction in saponin content was also reported in the noni (*Morinda citrifolia* L.) fruit [Kha *et al.*, 2021]. The main reason is due to saponin degradation at high temperature [Liu *et al.*, 2020]. Moreover, saponin loss could be a result of their release into the blanching water [Vuong *et al.*, 2015].

Figure 2C shows that the DPPH radical scavenging capacity of rambutan seeds gradually decreased with blanching time.

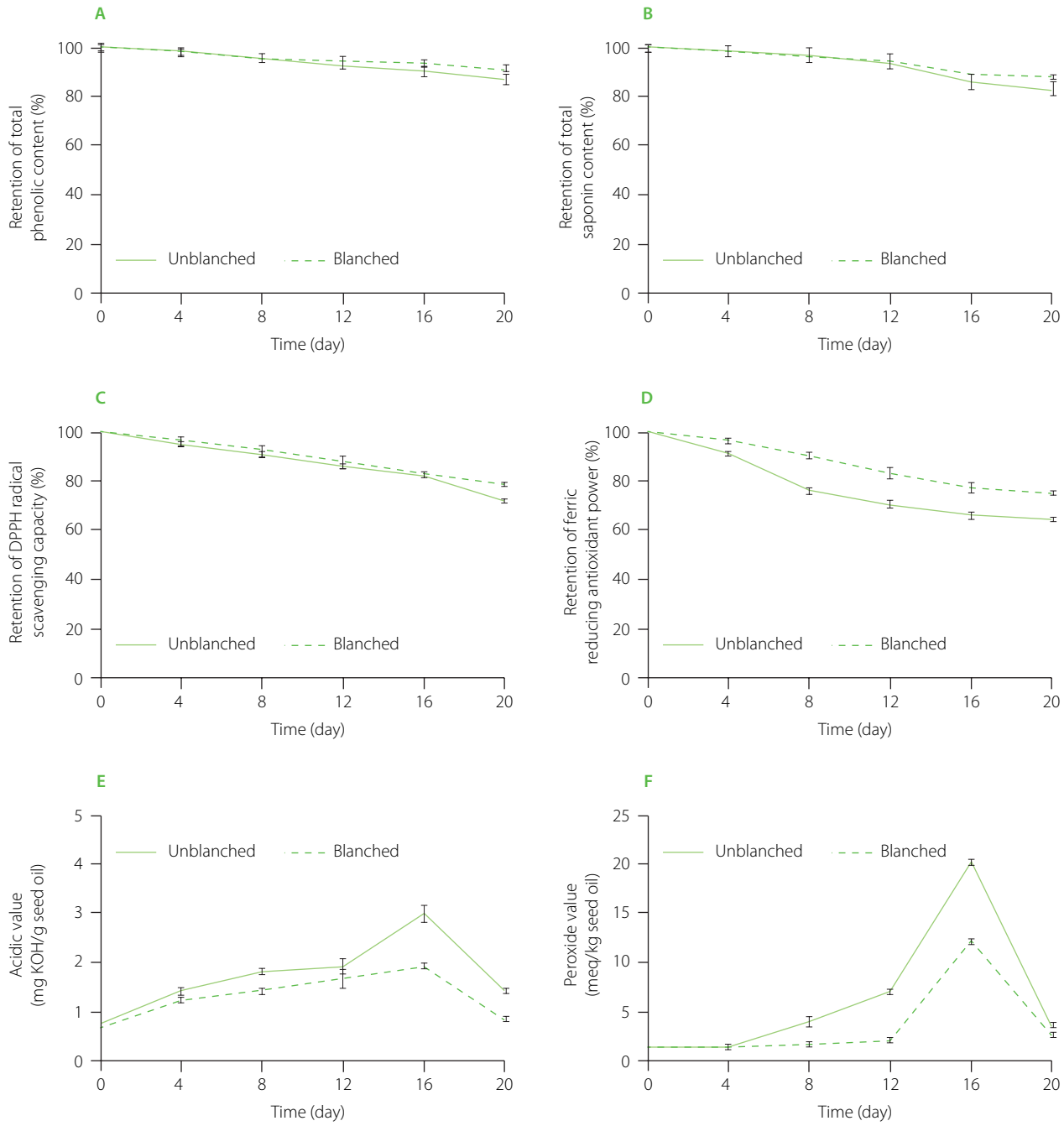

Figure 3. Changes in total phenolic (**A**) and total saponin (**B**) contents, DPPH radical scavenging ability (**C**), and ferric reducing antioxidant power (**D**) of dried rambutan seeds, and acidic value (**E**) and peroxide value (**F**) of their oil during accelerated storage. DPPH radical, 2,2-diphenyl-1-picrylhydrazyl radical.

The higher the blanching temperature was, the lower was the DPPH radical scavenging capacity of rambutan seeds. At 25 min blanching time, the increase in temperature from 80 to 95°C augmented the loss in DPPH radical scavenging capacity in rambutan seeds from 15% to 23% compared to that at the beginning of blanching. However, the ferric reducing antioxidant power of rambutan seeds increased in the early stages of blanching and then gradually decreased. The antioxidant capacity measured by the FRAP assay after 25-min blanching was 102, 98, 95 and 83% of the initial value corresponding to the blanching temperature of 80, 85, 90 and 95°C, respectively. In this study, the changes in the total phenolic content and DPPH radical scavenging capacity of rambutan seeds were the same; meanwhile, the changes in the total saponin content and ferric reducing antioxidant power were nearly similar. Recently, Do *et al.* [2022] concluded that the total saponin content of *Codonopsis javanica* roots, especially the triterpenoid saponin content, were closely correlated with their ferric reducing antioxidant power. The quantitative changes in antioxidant contents and capacities in the present study provide important information to food technologists about the need to select appropriate blanching conditions in industrial production. Further study on phenolic and saponin profiles in rambutan seeds should be done to elucidate the correlation between

Table 3. Degradation rate constant and half-life of phenolics and saponins during the accelerated storage of rambutan seeds.

Rambutan seeds	Phenolics		Saponins	
	Degradation rate constant (k) ($\times 10^{-3}$ 1/day)	Half-life ($t_{1/2}$) (day)	Degradation rate constant (k) ($\times 10^{-3}$ 1/day)	Half-life ($t_{1/2}$) (day)
Unblanched	6.9±0.40^{a}	101^{b}	9.96±0.92^{a}	70^{b}
Blanched	4.0±0.47^{b}	173^{a}	6.83±0.37^{b}	10^{a}

Data are shown as mean ± standard deviation (n=3). Values with different subscripts in the same column differ significantly (p<0.05).

the antioxidant contents and capacities of the seeds during the blanching treatment.

■ Impact of blanching on the quality of dried rambutan seeds during the accelerated storage

It can be noted that at the blanching temperature of 95°C and time of 25 min, the remaining activity of lipase, that was the most thermo-resistant enzyme in rambutan seeds, was less than 50% of the initial activity. These blanching conditions were selected to evaluate the impact of blanching conditions on the quality of dried rambutan seeds during the accelerated storage. Changes in the total phenolic and total saponin contents in the dried rambutan seeds during the accelerated storage are presented in **Figure 3**. The control sample was the unblanched rambutan seeds that were convectively dried under the same conditions.

Generally, the total phenolic and total saponin contents in the unblanched and blanched seeds gradually decreased during the accelerated storage. At the 20th day, the total phenolic and the total saponin contents in the blanched seeds remained at 91 and 88%, respectively, as compared to those at the beginning of the storage, while those values in the unblanched seeds remained at 86 and 83% of the initial values.

Table 3 shows that the degradation constant rates of phenolics and saponins in the blanched seeds were 1.7 and 1.5 times lower, respectively, than those in the unblanched counterpart; on the contrary, the half-life of phenolics and saponins in the blanched seeds were much longer. These results confirmed that the blanching significantly reduced the loss in phenolics and saponins during the accelerated storage of the dried rambutan seeds. A recent study showed that after 16-week storage, the phenolic loss in the blanched-dried pitaya peel was 20.4%, while that in the unblanched-dried counterpart was 33.9% of the initial value [Mai *et al.*, 2022].

Figures 3C and **D** show that the antioxidant capacities of the dried rambutan seeds gradually decreased during the accelerated storage. At the end of the storage, the DPPH radical scavenging capacity and ferric reducing antioxidant power of the blanched sample remained at 78 and 75% of the initial values, respectively, while the antioxidant capacities of the unblanched sample estimated by DPPH and FRAP assays were 72 and 64% of the initial values. These results were in agreement with the total phenolic and the total saponin contents in the dried seeds. The blanched-dried seeds had a lower loss in phenolics and saponins and greater antioxidant capacities than the unblanched-dried counterpart, confirming the significance of blanching in the treatment procedure of rambutan seeds.

Acidic value represents the degree of lipid hydrolysis during food preservation [Prescha *et al.*, 2014]. During the first 16 days of the accelerated storage, the acidic value of rambutan seeds gradually increased to a maximum value and was 3.7 and 2.8 times higher than the initial value for unblanched and blanched seeds, respectively (**Figure 3E**). This observation confirmed that the triglyceride hydrolysis took place during the accelerated storage [Teimouri Okhchlar *et al.*, 2024]. From the 16th to the 20th day of the storage, the acidic values in both samples gradually decreased due to enhanced oxidation of free fatty acids [Toci *et al.*, 2013].

Peroxides are a representative compounds of primary oxidation products when lipids are oxidized [Xia & Budge, 2017]. The peroxide value of oil of the blanched-dried seeds remained unchanged during the first 12 days of the accelerated storage (**Figure 3E**); this value increased from the 12th to the 16th day and then decreased on the 20th day of the storage. However, the peroxide value of oil of the unblanched-dried seeds remained constant during the first 4 days; then it increased from the 4th to the 16th day, to ultimately decrease at the end of the storage. The maximum of the peroxide value in the blanched sample was 66% lower than that in the unblanched counterpart. Thus, the acidic and peroxide values in the blanched sample increased more slowly and less during the accelerated storage than those noted in the non-blanched samples. Similar results have recently been reported during the accelerated storage of the blanched and unblanched pomegranate seeds [Kaseke *et al.*, 2021]. The reduction in the peroxide value at the later stages of the storage was due to the oxidation of peroxides into secondary oxidation products such as aldehydes, ketones, alcohols and short-chain organic acids [Choe & Min, 2006].

CONCLUSIONS

The blanching of rambutan seeds significantly diminished their LP, LOX and PPO activities. LP showed the highest thermal stability, followed by LOX and PPO. The total saponin content of rambutan seeds was enhanced during the early stages of blanching but decreased during the later stages, while the total phenolic content

decreased successively during this thermal treatment. The reduction in the total phenolic and the total saponin contents and antioxidant capacities of the dried rambutan seeds was observed during the accelerated storage. However, the antioxidant loss of the blanched sample was lesser than that of the unblanched counterpart. Additionally, the increase in acidic and peroxide values of oil of the blanched-dried seeds was slower and lesser than those of the unblanched-dried seeds. Blanching treatment deactivated enzymes in rambutan seeds and effectively protected their antioxidants during the accelerated storage. Future study on phenolic and saponin profiles in rambutan seeds is essential to elucidate the correlation between their antioxidant contents and capacities during the blanching treatment.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Phenolic Content, Microbiological and Physicochemical Qualities, and *In Vitro* Biological Activities of Two Monofloral Kinds of Honey from Edough Peninsula, Annaba, Algeria

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Since antiquity, honey has attracted interest across cultures for its nutritional and health-promoting values. Hence, this study aimed to evaluate the phenolic contents, physicochemical and microbiological qualities and biological activities of two monofloral kinds of honey (arbutus and heather) from Edough Peninsula, Annaba (northeast of Algeria). The physicochemical parameters were determined according to international regulations. Antioxidant capacity was evaluated as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity and reducing power (RP). Antimicrobial activity was tested against multi-resistant bacterial and fungal strains isolated from clinical samples, and the *in vitro* anti-inflammatory potential was examined using a protein denaturation assay. Both honey samples generally complied with quality standards laid down in international legislation, and no microbial contamination was found. Compared to heather honey, arbutus honey had a higher antioxidant capacity with a half maximal DPPH$^{\bullet}$ scavenging concentration of 25.4 mg/mL and the concentration corresponding to 0.5 absorbance in the RP assay of 8.17 mg/mL, along with a higher total phenolic content (108.3 mg GAE/100 g) and total flavonoid content (6.50 mg QE/100 g), and anti-inflammatory activity (half maximal bovine serum albumin denaturation concentration of 0.29 mg/mL). The antibacterial activities of both honey samples were similar with the minimum inhibitory concentration ranging from 62.5 to 500 µg/mL, and the *E. faecium* strain was more sensitive than the others. In conclusion, both kinds of honey meet international quality standards, with relevant potential for antioxidant, antibacterial, and anti-inflammatory purposes.

Keywords: anti-inflammatory activity, antimicrobial activity, antioxidant capacity, arbutus honey, heather honey, physicochemical parameters

INTRODUCTION

Honey, a complex biological product with great diversity, has been used in different cultures since antiquity for its nutritional and medicinal properties. Several scientific studies confirm its valuable biological activities, including antioxidant [Becerril-Sánchez *et al*., 2021; Kavanagh *et al*., 2019], anti-inflammatory [Zaidi *et al*., 2019], antibacterial [Abdellah *et al*., 2020; Chettoum *et al*., 2023], and antimicrobial [Bakchiche *et al*., 2020; Latifa *et al*., 2020] effects. These properties are linked to its constituents, such as phenolic compounds, carotenoids, vitamins, sugars, enzymes, and methylglyoxal [Cianciosi *et al*., 2018; da Silva *et al*., 2016]. The content of these compounds in honey depends on its botanical and entomological sources, as well as environmental conditions [Moniruzzaman *et al*., 2013]. Honey's physicochemical properties and phenolic content serve as quality markers, helping to identify its floral

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source and geographical origin [Kavanagh *et al*., 2019; Majewska *et al*., 2019].

The physicochemical quality and authenticity of natural honey depend on specific parameters such as its acidity, water content, electrical conductivity, hydroxymethylfurfural content, and sugar content [da Silva *et al*., 2016; Nabti & Tichati, 2022]. In addition, phenolic compounds, which are secondary metabolites transferred from nectar to honey, are mainly classified into two families' phenolic acids and flavonoids and may be used as botanical and quality markers [Kavanagh *et al*., 2019]. Previous studies have reported that honey samples with a high phenolic content often exhibit strong antioxidant activities, suggesting a causal relationship between them [Becerril-Sánchez *et al*., 2021; Otmani *et al*., 2021]. Phenolic compounds help mitigate damage caused by free radicals by acting as metal chelators, interfering with the chain reactions of free radicals, and possibly preventing their formation [Tichati *et al*., 2021].

Honey is a bacteriostatic food because of its high sugar content, low pH, and the presence of compounds with antibacterial activity [Abdellah *et al*., 2020; Latifa *et al*., 2020]. Under these conditions, the contamination of honey by pathogenic microbes can occur from several sources, primarily related to the bees' digestive tract and the natural environment, such as the hive itself, air, dust, water, pollen grains, and beekeeping practices [Valdés-Silverio *et al*., 2018].

Algeria is known for its important floral resources, thanks to its geographical location and diverse landscapes, climates, and soils. Melliferous plants are mainly spontaneous species. This spontaneous flora is considered an important food source for bees [Belaid *et al*., 2020; Hamel & Boulemtafes, 2017]. Some of these plants, such as thyme, rosemary, eucalyptus, jujube, lavender, heather, and olive, are also known for their bioactivities. These plants, used in the traditional pharmacopeia, likely enhance the health-promoting properties of honey, making it a valuable product [Khalil *et al*., 2012; Otmani *et al*., 2021] and enabling the production of various monofloral [Mesbahi *et al*., 2019; Nakib *et al*., 2024] and polyfloral honeys [Homrani *et al*., 2020; Makhloufi *et al*., 2021].

In the Edough Peninsula (Northeast Algeria), honey production has seen significant growth due to the region's vast floristic diversity and its wealth of honey species, with approximately 107 species pollinated by bees, distributed across 36 families, with Fabaceae and Asteraceae being predominant [Hamel & Boulemtafes, 2017]. The local bee species, known as the Tellian bee (*Apis mellifica intermissa*), is well-suited to this region. The Edough Peninsula produces various types of honey, including polyfloral, arbutus, eucalyptus, and heather honey. Arbutus honey, also known as bitter honey, is a monofloral honey with a distinct bitter taste, originating primarily from the Mediterranean basin. It is produced from the strawberry tree (*Arbutus unedo* L.) flower, which blooms in late autumn and early winter when other flowers are scarce [Hamel & Boulemtafes, 2017; Jurič *et al*., 2022]. This honey is well-known for its nutritional and health benefits, which are linked to its rich content of phenolic compounds and its high antioxidant potential [Jurič *et al*., 2022; Lovaković, *et al*., 2018]. Heather honey, produced from plants of the Ericaceae family, is valued for its attractive sensory properties, physicochemical quality, and biological activity [Cianciosi *et al*., 2018; Kasiotis *et al*., 2022]. In the Edough Peninsula, its derived from three species of *Erica* plants: *Erica arborea* L., *Erica scoparia* L. subsp. *scoparia*, and *Erica multiflora* L. [Hamel & Boulemtafes, 2017].

In 2023, the Edough Peninsula was declared Algeria's first natural park to protect the integrity of its ecosystems and conserve its unique biodiversity. The availability of strawberry trees (*Arbutus unedo* L.) and *Erica* species in this region facilitates the production of various types of honey. Therefore, this study aimed to evaluate and compare the physicochemical characteristics, microbiological quality, total phenolic and total flavonoid contents, and the antioxidant, anti-inflammatory, and antimicrobial activities of two monofloral honey: arbutus honey (bitter honey) and heather honey, from this area.

MATERIALS AND METHODS

Collection of honey samples

Each type of honey was taken from two experienced producers, each providing three authentic samples of one variety during the 2024 harvest (arbutus in January and heather in the spring) from two sites in the Edough Peninsula Annaba, northeast of Algeria (**Figure 1**), a region characterized by the presence of strawberry trees (*Arbutus unedo L.*) and *Erica* species, which support the production of these types of honey. The samples were identified as arbutus honey, locally known by the popular name "Lenj" or bitter honey, and heather honey, known as "Bouhadad" (as reported by the beekeepers). Arbutus honey was collected from Aïn Abdallah, Tréat, Annaba (latitude: 36°56'21.14" N and longitude: 7°26'22.54" E), while heather honey was sourced from El Manjra, Seraidi, Annaba (latitude: 36°55'23.74" N and longitude: 7°36'18.67" E) (**Figure 1**). The honey samples were placed in hermetically sealed bottles, stored at 4°C and analyzed within two months since collection.

Determination of physicochemical parameters

The water content of the honey samples was determined at 20°C using a PAL-2 ATAGO digital refractometer (ATAGO Co. Ltd., Tokyo, Japan), following the AOAC International method no. 969.38B [AOAC, 1992]. The refractive index obtained from the refractometer measurement was used to calculate the water content, which was expressed in g/100 g of honey.

The electrical conductivity (EC), pH, and density were assessed according to the harmonized methods of the European Honey Commission [Bogdanov *et al*., 1997]. A Sension+ EC71 conductivity meter (Hach, Berlin, Germany) was employed to measure the EC of a honey solution (20%, *w/v*) prepared with deionized water at 20°C. The results were expressed in mS/cm. The pH was measured using a 10% (*w/v*) solution of honey prepared in distilled water using a calibrated PHS-3BW Benchtop pH meter (Biobase Biodustry, Shandong, China). The density of each honey was ascertained using a pycnometer. It was calculated as the density of honey divided by the density of distilled water under the same conditions and expressed in g/mL.

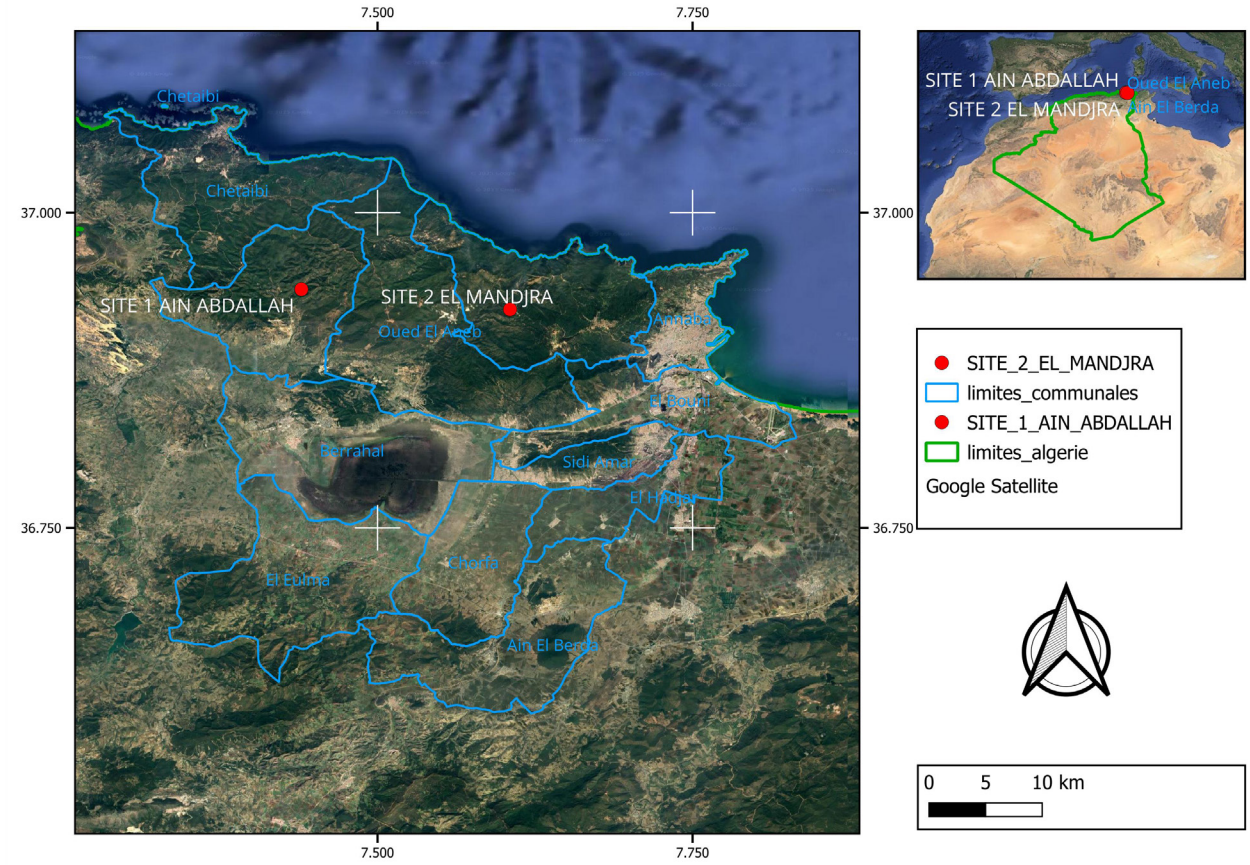

Figure 1. The geographical location of investigated honey samples. The map was created using QGIS 3.36.3-Maidenhead with Google Satellite imagery.

A titrimetric standard method no. 962.19 was employed to estimate free, combined, and total acidities [AOAC, 1990]. First, 10 g of honey were dissolved in 75 mL of pure water, and the solution was titrated with 0.05 M NaOH to reach a pH of 8.5 (free acidity); then, a volume of 10 mL of 0.05 M NaOH was added to the solution, and the pH was adjusted to 8.30 using a 0.05 M HCl solution to assess combined acidity. Total acidity (sum of free and combined acidity) was calculated and expressed in meq of acid *per* kg.

Ash content determination

The ash content of the honey samples was estimated following AOAC International method no. 920.181[AOAC, 1990]. To this end, 5 g of each honey sample and a few drops of olive oil were placed into a pre-weighed crucible and heated to evaporate the water, then placed in a muffle furnace (KEJIA 1,600°C, Zhengzhou, China) at 550°C for 4 h, during which the samples underwent calcination until reaching a constant mass. After cooling in a desiccator, the weight of the crucible with the ash was recorded. The ash content was then calculated. Results were expressed in g *per* 100 g of honey.

Determination of hydroxymethylfurfural content

The hydroxymethylfurfural (HMF) content of the honey samples was quantified by the spectrophotometric AOAC International method no. 980.23 [AOAC, 1990]. Honey (5 g) was mixed with 25 mL of distilled water. To this solution, 0.5 mL each of Carrez I and Carrez II solutions were added and stirred thoroughly. After filtration, the filtrate was diluted to a total volume of 50 mL with distilled water after discarding the first 10 mL of the filtrate. The remaining solution was divided into two tubes, each containing 5 mL. The first tube was supplemented with 5 mL of distilled water as the test sample. The other tube, which was designated as the reference, was supplemented with 5 mL of a 0.2% sodium bisulfate solution. Finally, the absorbance of the test sample was read at 284 nm (A_{284}) and 336 nm (A_{336}) against that of the reference solution using a Cary 60 UV-Vis spectrophotometer (Agilent, St. Clara, CA, USA), and the results were calculated and expressed in mg/kg using Equation (1):

$$\text{HMF content} = (A_{284} - A_{336}) \times 149.7 \times m \quad (1)$$

where: m is mass of honey taken (5 g) and 149.7 is a constant.

Determination of sugar content

The total and reducing sugar contents were estimated using the Bertrand method [Audigie *et al.*, 1984]. To estimate the total sugar content, about 0.5 g of each honey was mixed with 20 mL of distilled water containing 2 mL of 2.2 M HCl. After being heated at 65°C for 45 min in a WB14 water bath (Memmert, Schwalbach,

Germany), the solution was neutralized with a 3 M NaOH solution using phenolphthalein as an indicator. The mixture was then brought to a final volume of 100 mL with distilled water. Then, to 10 mL of the diluted sample, 10 mL each of Fehling A and Fehling B solutions were added. The mixture was heated to boiling for 3 min, forming a brick-red precipitate. The precipitate was recovered with 10 mL of a 2 M $Fe_2(SO_4)_3$ solution. Finally, the resulting green color solution was titrated with a 0.004 M $KMnO_4$ solution. Based on the volume of $KMnO_4$ solution used, the mass of precipitate copper (m_{Cu}) was calculated. The total sugar content was estimated using the table of invert sugars, which correlates m_{Cu} with the equivalent mass of invertible sugar. Results were expressed as g/100 g of honey.

Concerning reducing sugars (RS), 10 mL of a 0.5% (*w/v*) honey solution in distilled water was placed into a beaker. Then, 10 mL each of Fehling A and Fehling B solutions were added. The resulting mixture was then processed using the same procedure for the total sugar analysis. Reducing sugar content was then calculated and expressed as g/100 g of honey.

Sucrose content was calculated using Equation (2):

$$\text{Sucrose (g/100 g)} = (\text{total sugars} - \text{reducing sugars}) \times 0.95 \quad (2)$$

Determination of microbiological quality

The evaluation of microbiological quality included the quantification of total aerobic mesophilic flora (TAMF), total and fecal coliforms, sulfite-reducing anaerobes, as well as the identification of *Salmonella* spp. and the quantification of yeasts and molds.

Total aerobic mesophilic flora

The TAMF was quantified *via* plate count agar (PCA) following International Organization for Standardization (ISO) 4833-1:2013 methodology [ISO, 2013]. Serial dilutions ranging from 10^{-1} to 10^{-6} were prepared employing a sterile physiological saline solution (0.85% NaCl). A 1 mL aliquot from each dilution was inoculated onto the surface of the PCA, followed by incubation at a controlled temperature of 37°C in an IN55 incubator (Memmert), for 24 h post-incubation, the visible colonies were enumerated, and the results were articulated in terms of colony-forming units (cfu) *per* g of honey.

Total coliforms

The quantification of total coliforms was executed on deoxycholate-lactose agar (DCL), which was organized in a dual-layer configuration, in accordance with the NF V08-050 standard methodology [AFNOR, 2009]. Following the inoculation of 1 mL from the serial dilutions (10^{-1} to 10^{-6}), a second layer of molten and cooled agar (maintained at 45°C) was applied. The plates were incubated at 37°C for 24 h in an IN55 incubator (Memmert). Colonies exhibiting a dark red pigmentation, with a diameter measuring greater than or equal to 0.5 mm, were classified as total coliforms and subsequently enumerated.

Fecal coliforms

The quantification of fecal coliforms was carried out using the same methodology as that employed for total coliforms, with the exception of the incubation temperature, which was adjusted to 44°C for a period of 24 h. Colonies displaying analogous morphological characteristics were counted and recorded in terms of cfu/g honey.

Sulfite-reducing anaerobes

The analysis of sulfite-reducing anaerobes was conducted utilizing a sulfite-polymyxine-sulfadiazine (SPS) medium [Gomes *et al*, 2010]. Aliquots of 1 mL from the serial dilutions were inoculated into tubes containing the aforementioned medium. These tubes were subsequently placed within an anaerobic jar equipped with a reducing atmosphere generator and incubated at 46°C for 24 h in an IN55 Memmert incubator. The presence of black colonies, indicative of the reduction of sulfites to sulfides, was quantified.

Salmonella spp. detection

Salmonella spp. detection followed the ISO 6579-1:2017 method [ISO, 2017]. It commenced with a selective enrichment phase. A sample of 25 g was incubated in 225 mL of selenite-cystine broth at 37°C for 24 h. Following the enrichment phase, an inoculation was performed on *Salmonella-Shigella* agar (SS agar) in streak patterns, followed by incubation at 37°C for 24 h in an IN55 Memmert incubator. Suspicious colonies, characterized by either a colorless appearance or a black center, were isolated on Mueller-Hinton agar for the purpose of purification. The biochemical identification of these suspicious colonies was executed utilizing a biochemical gallery specifically designed for enterobacteria, incorporating standardized tests such as glucose fermentation and hydrogen sulfide (H_2S) production.

Yeasts and molds

The yeasts and molds were cultivated on Sabouraud agar enriched with chloramphenicol and on potato dextrose agar (PDA) in accordance with the ISO 21527-2:2008 method [ISO, 2008]. A 1 mL aliquot from the serial dilutions was inoculated onto each distinct type of medium. The Petri dishes were incubated at 30°C in an IN55 Memmert incubator. The yeasts were examined after a duration of 24 to 48 h, whereas the molds were observed for a period extending up to seven days. The enumeration of colonies was performed based on the unique morphological characteristics exhibited by the colonies.

Colorimetric estimation of total phenolic content

Total phenolic content (TPC) was estimated using Folin-Ciocalteu reagent (FCR), following the method of Singleton *et al*. [1999]. An aliquot (0.1 mL) of the diluted honey (0.2 mg/mL) was mixed with 0.5 mL of FCR, diluted 10 times, and the mixture was shaken and left to rest for 5 min at room temperature. After 2 h of incubation in the dark, 0.4 mL of a 7.5% Na_2CO_3 solution was added. The absorbance was measured at 760 nm using a Cary

60 UV-Vis spectrophotometer (Agilent). Gallic acid solutions with concentrations between 20 and 100 mg/L were used to establish the calibration curve (y=0.0138x+0.0352; R^2=0.993). Honey TPC was expressed as mg gallic acid equivalents *per* 100 g of honey (mg GAE/100 g honey).

Colorimetric estimation of total flavonoid content

The colorimetric method reported by Turkoglu *et al*. [2007] was used to estimate the total flavonoid content (TFC) in the honey, with slight modifications. An aliquot (1 mL) of the diluted honey (0.2 mg/mL) was added to a test tube with 4.3 mL of 80% (*v/v*) aqueous methanol solution, containing 0.1 mL of 10% aluminum nitrate, and 0.1 mL of 1 M potassium acetate. The resulting mixture was kept at room temperature for 40 min. The absorbance was measured at 415 nm using an Agilent Cary 60 UV-Vis spectrophotometer. Quercetin solutions with concentrations between 20 and 100 mg/L were used to establish the calibration curve (y=0.0111x+0.0122; R^2=0.994). Honey TFC was expressed as mg quercetin equivalents *per* 100 g of honey (mg QE/100 g honey).

Antioxidant capacity analysis

DPPH assay

The antioxidant capacity of the honey samples was estimated as their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$) using the method developed by Brand-Williams *et al*. [1995], with thorough modifications. A volume of 0.1 mL from each honey sample at various concentrations (6.25––100.00 mg mL) was added to 2.9 mL of a DPPH$^{\bullet}$ methanolic solution (6×10^{-5} M). The mixtures were agitated and incubated for 1 h at 25°C in darkness. The absorbance was measured at 517 nm using an Agilent Cary 60 UV-Vis spectrophotometer. Ascorbic acid (0.01–1.00 mg/mL) was used as a reference standard. The percentage of DPPH$^{\bullet}$ scavenged by the honey samples in each concentration was calculated and, additionally, the concentration of honey or ascorbic acid corresponding to half maximal DPPH$^{\bullet}$ scavenging activity (IC_{50}, mg/mL) was reported.

Reducing power assay

The method of Oyaizu [1986] was followed to evaluate the ability of the honey samples to reduce Fe^{3+}. Butylated hydroxytoluene (BHT) was used as a reference standard. In summary, 1.5 mL of the honey sample solution at different concentrations (3.125 to 50.00 mg/mL) or BHT (0.01–1.00 mg/mL) was added to a test tube containing 2.5 mL of a phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of a potassium ferricyanide solution (1%). The tubes were transferred to a water bath (50°C) for 20 min. Then, 1.25 mL of trichloroacetic acid was added, and the resulting mixture was thoroughly centrifuged (3,000×*g*, 10 min). Finally, 2.5 mL of the resulting upper layer was mixed with 20.5 mL of distilled water and 0.50 mL of a ferric chloride aqueous solution (0.1%), and the absorbance was measured at 700 nm using an Agilent Cary 60 UV-Vis spectrophotometer. The reducing power (RP) was expressed as the concentration of honey or a BHT solution giving an absorbance of 0.5 ($A_{0.5}$, µg/mL).

Determination of anti-inflammatory activity

The anti-inflammatory activity of the honey samples was estimated as their ability to denaturate bovine serum albumin (BSA) according to the method reported by Williams *et al*. [2008]. In summary, 0.5 mL of different concentrations of solutions of the honey samples (31.25 to 500.00 µg/mL) or diclofenac sodium as a reference compound (31.25 to 125.00 µg/mL) was mixed with a BSA solution (0.5 mL of 0.2%, *w/v*) prepared in Tris-HCl buffer (pH 6.8). The resulting solution was allowed to stand at 37°C for 15 min and then heated at 72°C for 5 min. After cooling, the absorbance at 660 nm was measured using an Agilent Cary 60 UV-Vis spectrophotometer. The percentage inhibition (I) of protein denaturation by the honey samples or diclofenac in each concentration was calculated using Equation (3):

$$I\ (\%) = ((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100 \quad (3)$$

where: $Abs_{control}$ is the absorbance of the BSA solution without the honey sample or diclofenac and Abs_{sample} is the absorbance with the honey sample.

The half maximal inhibitory concentration (IC_{50}, mg/mL) was determined using curves of percentage inhibition *vs.* concentration of honey or diclofenac.

Determination of antimicrobial activity

The antimicrobial activity of both honey samples was determined by the agar-well diffusion technique against the strains of clinical origin and pathogenic to humans; they include four Gram-negative bacteria: *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*; two Gram-positive bacteria*: Enterococcus faecium* and *Staphylococcus aureus*; and two yeasts of the Candida genus*: Candida albicans* and *Candida tropicalis*, provided by the University Hospital Center, Resuscitation Services, Annaba, Algeria. Amoxicillin (AK10), amoxicillin-clavulanic acid (AMC30) for bacterial strains, and amphotericin-B (AM-B) for fungal strains were used as positive controls. The microbial suspension was prepared from an 18–24-h pure culture adjusted between 0.08 and 0.10 at 600 nm using an Agilent Cary 60 UV-Vis spectrophotometer, corresponding to a concentration of 1×10^8 cfu/mL following the McFarland scale [Benzaid *et al*., 2021].

The selective culture media used were: Mueller-Hinton agar for bacterial strains and Sabouraud agar for fungal strains in Petri dishes. The inoculation process involved flooding the Petri dishes with the culture and spreading it evenly over the surface. Plates were further incubated for 20 min at 37°C in an IN55 Memmert incubator to allow adequate uptake of the inoculum into the agar medium; wells 6 mm in diameter were aseptically prepared on the agar before being filled with 150 µL of each honey sample. After 24 h of incubation at 37°C, the antimicrobial effect was evaluated by measuring the diameter of the zone of inhibition (DZI) in mm, which was shown by a clear halo around the wells. The mean zone of inhibition was determined by repeating the test three times, with zones categorized as weak (less than

Table 1. Composition and physicochemical properties of arbutus honey and heather honey.

Compound/parameter	Arbutus honey	Heather honey	International standard limit
pH at 20°C	4.37±0.02[a]	4.22±0.02[b]	–
Total acidity (meq/kg)	17.40±0.05[b]	22.37±0.04[a]	≤50*
Water (g/100 g)	19.76±0.06[b]	21.53 ±0.05[a]	≤20*,**
Refractive index	1.49±0.00[a]	1.48±0.00[b]	–
Density (g/mL)	1.41±0.00[a]	1.38±0.001[b]	–
EC (mS/cm)	0.73±0.005[b]	0.78±0.006[a]	≤0.80*
Ash (g/100 g)	0.29±0.005[b]	0.35±0.02[a]	–
HMF (mg/kg)	11.56±0.15[b]	14.90±0.2[a]	≤40*
Total sugars (g/100 g)	78.18±0.02[a]	76.56±0.05[b]	–
Reducing sugars (g/100 g)	74.05±0.03[a]	70.25±0.04[b]	≥60*
Sucrose (g/100 g)	3.90±0.02[b]	6.12±0.03[a]	≤5*

Results are expressed as mean ± standard deviation. Different letters indicate significant differences within rows ($p<0.05$). *Codex Alimentarius [2001]; **Water content of heather honey –not more than 23 g/100 g. EC, electrical conductivity; HMF, hydroxymethylfurfural.

10 mm), moderate (10–13 mm), strong (10–13 mm), or major (greater than 13 mm) [Abdellah *et al*., 2020]. The minimum inhibitory concentration (MIC) was determined using the dilution technique for microbial organisms that showed susceptibility to honey samples with an inhibition zone of 10.0 mm and greater. The MIC represents the minimum concentration at which microorganisms do not multiply.

Statistical analysis

All tests were done in triplicate for three samples of each type of honey, and results are presented as mean and standard deviation (SD). Comparisons between means were analyzed using the Tukey test, and differences are deemed significant at a $p<0.05$. Statistical analyses were performed using GraphPad Prism version 9.0.0 (GraphPad, Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Composition and physicochemical properties of honey samples

Table 1 summarizes the results of analyses of the composition and physicochemical parameters studied in arbutus honey and heather honey. Honey's water content varies depending on a variety of factors, including maturity, climate, botanical source, and the beekeeper's manipulation during collection [Alvarez-Suarez *et al*., 2010]. It is one of the parameters that determine the quality and stability of honey during storage [da Silva *et al*., 2016]. Our study shows that heather honey had the highest water content (21.53 g/100 g), followed by arbutus honey, with 19.76 g/100 g, corresponding to a refractive index of 1.48 and 1.49, respectively (Table 1). The water content of the analyzed honey samples falls within the accepted norms: less than 20 g/100 g for arbutus honey and less than 23 g/100 g for heather honey [Codex Alimentarius, 2001]. These findings were similar to those previously found by other researchers for monofloral honey, including Algerian honey [Homrani *et al*., 2020], Moroccan honey [Bouhlali *et al*., 2019], and Tunisia honey [Boussaid *et al*., 2018]. Regarding heather honey, it is a unique variety known for its high water content, as reported by Waś *et al*. [2011] and Kavanagh *et al*. [2019].

The density of honey varies mainly according to the water content. Therefore, as expected, the density of arbutus honey was higher than that of heather honey – 1.41 and 1.38 g/mL, respectively (Table 1).

The pH and total acidity are important parameters for determining the quality of the honey and provide information about its geographic and botanical origins [Majewska *et al*., 2019]. They can be used to classify and differentiate unifloral honeys [Makhloufi *et al*., 2021]. The pH values of both studied honey samples, arbutus and heather, lean towards acidity, with values of 4.37 and 4.22, respectively (Table 1). These values are in line with the pH range of Algerian honey [Homrani *et al*., 2020; Makhloufi *et al*., 2021] and similar to those reported by previous studies on Algerian monofloral [Mesbahi *et al*., 2019; Otmani *et al*., 2019] and Moroccan [Bouhlali *et al*., 2019] honey samples, confirming the nectar honey status of the studied samples. The total acidity results (Table 1) fall within the international standard of less than 50 meq/kg. Heather honey exhibited a higher total acidity (22.37 meq/kg) than arbutus honey, which was 17.40 meq/kg.

Electrical conductivity of honey is an important physical parameter that helps identify honey's floral source and purity [Makhloufi *et al*., 2021]. As shown in Table 1, the EC values of the honey samples were 0.73 and 0.78 mS/cm for arbutus

Table 2. Microbial profile (cfu/g) of arbutus honey and heather honey.

Honey	Total aerobic mesophilic flora	Molds and yeasts	Total coliforms and fecal coliforms	*Salmonella* spp.	Sulfite-reducing anaerobes
Arbutus	<10	Negative	Negative	Negative	Negative
Heather	<10	Negative	Negative	Negative	Negative

Table 3. Total phenolic content (TFC) and total flavonoid content (TFC) of arbutus honey and heather honey.

Honey	TPC (mg GAE/100 g)	TFC (mg QE/100 g)
Arbutus	108.3±5.4^{a}	6.50±0.11^{a}
Heather	65.2±3.1^{b}	4.72±0.19^{b}

Results are expressed as mean ± standard deviation. Means in a column with different letters are significantly different (p<0.05). GAE, gallic acid equivalents; QE, quercetin equivalents.

and heather honey, respectively. The results meet the international standard for nectar honey, which is less than 0.8 mS/cm [Codex Alimentarius, 2001]. However, the established limiting values are ≤0.8 mS/cm for nectar honey and ≥0.8 mS/cm for honeydew honey. Similar EC values to those found in our study were also previously reported in Algerian honey samples [Khalil *et al*., 2012] and in some monofloral honey from Morocco [El-Haskoury *et al*., 2018] and Malaysia [Moniruzzaman *et al*., 2013].

The investigated honey samples (arbutus and heather) had ash content of 0.29 and 0.35 g/100 g, respectively (**Table 1**). These values are within reported ash content range in honey, which varies from 0.02 to 1.03 g *per* 100 g, according to da Silva *et al*. [2016] for nectar honey, and fall within the range of values reported for Algerian honey [Nabti & Tichati, 2022].

HMF is a critical parameter for assessing honey's freshness and purity. It is found in trace concentrations in honey and is influenced by heat treatment and storage time [da Silva *et al*., 2016]. In our study, the HMF content of both honey samples met the Codex Alimentarius [2001] standards, stipulating that it should not exceed 40 mg/kg. Heather honey showed a higher HMF content (14.90 mg/kg) compared to arbutus honey, which was 11.56 mg/kg (**Table 1**). The HMF content determined in our study aligns with findings from previous studies on some monofloral Algerian honey samples [Mesbahi *et al*., 2019; Nakib *et al*., 2024].

The data obtained for the sugar content of both investigated honey samples showed that sugars were the predominant compounds. Arbutus honey exhibited slightly higher contents of total and reducing sugars at 78.18 and 74.05 g/100 g, compared to heather honey in which these values reached 76.56 and 70.25 g/100 g, respectively (**Table 1**). The results for reducing sugars align with international standards on sugar, which require ≥60 g/100 g for reducing sugars in floral honey [Codex Alimentarius, 2001]. The obtained values were closer to those reported by Achour & Khali [2014] and Mesbahi *et al*. [2019] for some monofloral honey samples from Algeria.

For sucrose, the results revealed that arbutus honey had a sucrose content of 3.90 g/100 g (**Table 1**), which falls within the acceptable range of ≤5 g/100 g for all types of honey. In contrast, heather honey's sucrose content was 6.12 g/100 g, exceeding the above-mentioned limit set by the Codex Alimentarius [2001] standard. Indeed, the cause of this high content could be early honey harvesting, where the sucrose is not fully converted into glucose and fructose, as evidenced in our study by the water content (21.53 g/100 g), or due to overfeeding the bees with sucrose syrup [Achour & Khali, 2014; da Silva *et al*., 2016].

Microbiological quality

Honey is subject to various sources of microbial contamination, including plant-derived substances such as nectar and pollen, as well as endogenous microbial flora in the digestive tract of bees. In addition, it may be affected by environmental factors such as air, dust, soil, post-harvest handling, and processing practices [Valdés-Silverio *et al*., 2018]. The most commonly detected microbial contaminants include molds, yeasts, and bacterial spores, especially those of *Bacillus* spp. and *Clostridium* spp. In this study, the two honey samples were tested for total mesophilic aerobic flora, coliforms and fecal coliforms, *Salmonella* spp., sulfite-reducing anaerobes, molds and yeasts, and the results are illustrated in **Table 2**. Mesophilic aerobic bacteria were detected in both honey samples, with a concentration of less than 10 cfu/g. These levels comply with the current Algerian regulations [AOJ, 2017], which set a maximum permissible limit of 1,000 cfu/g. Detecting mesophilic aerobic bacteria may suggest inadequate hygiene practices during the production and storage [Fernández *et al.*, 2017]. Furthermore, the analysis of all samples did not reveal any presence of fecal coliforms, sulfite-reducing anaerobes, or *Salmonella* spp. Concerning molds or yeasts, no contamination was observed in the samples analyzed in this study.

Total phenolic and flavonoid contents

Phenolics are essential compounds for honey's appearance and functional properties due to their great structure diversity and their properties. **Table 3** shows the contents of these compounds in the two monofloral honey samples. Arbutus honey had a higher total phenolic content (108.3 mg GAE/100 g) compared to heather honey (65.2 mg GAE/100 g). Regarding total flavonoid content, arbutus honey presented a slightly higher content (6.50 mg QE/100 g) than heather honey, which was 4.72 mg QE/100 g. These results are consistent with findings from other Algerian arbutus honey studies [Nakib *et al*., 2024; Otmani *et al*., 2019], especially for TPC. This phenolic richness determines antioxidant capacity and therapeutic values of honey

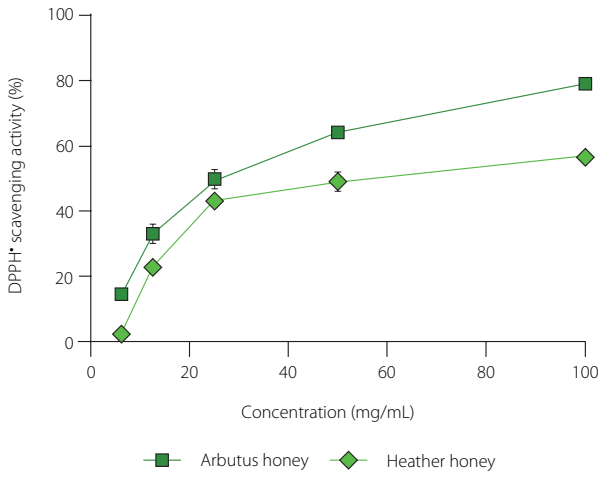

Figure 2. DPPH radical scavenging activity (%) of arbutus honey and heather honey.

[Jurič *et al*., 2022; Lovaković *et al*. 2018]. It can be mentioned that the TFC and TFC in heather honey were similar to those previously reported for Estonian heather honey [Kivima *et al*., 2021], but lower than those found by Homrani *et al*. [2020] in heather honey samples from the El Taref region, Algeria, and by Kavanagh *et al*. [2019] in Irish honey. The observed differences could be attributed to various factors, including botanical origin, honey ripeness, processing techniques, and collection site [Harbane *et al*., 2024; Kavanagh *et al*., 2019].

Antioxidant capacity

The antioxidant capacity of both honey samples was analyzed using two different *in vitro* methods: DPPH and RP assays. The antiradical capacity was assessed using the IC_{50} value, which is the concentration of the honey sample or a reference compound required to scavenge 50% of DPPH•. A lower IC_{50} value indicates higher antioxidant capacity. Arbutus honey exhibited lower IC_{50} value of 25.4 mg/mL compared to heather honey (IC_{50} of 60.28 mg/mL). Additionally, based on the results in **Figure 2**, the DPPH• scavenging activity of the honey samples (arbutus and heather) was dose-dependent, as it increased proportionally with honey concentration. It is worth noting that the reference compound, ascorbic acid, showed higher antioxidant activity than both honey samples, with an IC_{50} of 0.007 mg/mL. The DPPH• inhibition percentages observed in this study for both honey types were within the range reported in previous studies on Algerian honeys [Harbane *et al*., 2024; Zaidi *et al*., 2019]. The antioxidant capacity observed was mainly attributed to the phenolic content. Previous studies have indicated that honey samples with a high phenolic content tend to exhibit strong antioxidant activities, suggesting a causal relationship [Abdellah *et al*., 2020; Otmani *et al*., 2021], which may be linked to the redox properties of phenolic compounds. These compounds act as free radical scavengers through electron donation, and also chelate pro-oxidant metal ions [Becerril-Sánchez *et al*., 2021; Tichati *et al*., 2021].

In the reducing power assay, both honey samples (arbutus and heather) showed significant antioxidant capacity, with $A_{0.5}$ values of 8.17 mg/mL and 13.86 mg/mL, respectively, as reported in **Table 4.** These findings align with previous studies on Algerian honey samples [Khalil *et al*., 2012; Otmani *et al*., 2021]. The observed reducing power can be attributed to the presence of agents, which act as antioxidants due to their electron-donating capabilities [Ruiz-Ruiz *et al*., 2017].

In vitro anti-inflammatory activity

The denaturation of proteins, a significant contributor to inflammation in rheumatic diseases, results in the loss of their configuration due to the disruption of the bonds maintaining their three-dimensional conformation [Alamgeer *et al*., 2017]. This severe consequence includes the loss of biological activities and properties of the proteins, leading to the formation of autoantigens, which are critical in developing autoimmune disorders such as arthritis [Ruiz-Ruiz *et al*., 2017; Zaidi *et al*., 2019]. In our study, the BSA denaturation method was used to examine the *in vitro* anti-inflammatory activity of the honey samples. The results are demonstrated in **Figure 3** and **Table 4**. Both honey samples exhibited concentration-dependent inhibition of protein denaturation induced by high temperature. At a concentration of 0.5 mg/mL, arbutus and heather honey inhibited

Table 4. Antioxidant capacity determined as DPPH• scavenging activity and reducing power as well as anti-inflammatory activity determined as BSA denaturation inhibition of arbutus honey and heather honey.

Honey/standard	DPPH• scavenging activity (IC_{50}, mg/mL)	Reducing power ($A_{0.5}$, mg/mL)	BSA denaturation inhibition (IC_{50}, mg/mL)
Arbutus honey	25.4±2.4^{a}	8.17±0.06^{b}	0.29±0.03^{a}
Heather honey	60.3±4.6^{b}	13.86±0.27^{a}	0.38±0.04^{a}
Ascorbic acid	0.007±0.003^{c}	NT	NT
BHT	NT	0.049±0.002^{c}	NT
Diclofenac	NT	NT	0.031±0.002^{b}

Results are expressed as mean ± standard deviation. Means in a column with different letters are significantly different ($p<0.05$). $A_{0.5}$, honey or standard concentration corresponding to 0.5 absorbance in reducing power assay; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DPPH•, 2,2-diphenyl-picrylhydrazyl radical, IC_{50}, half maximal inhibitory/scavenging concentration; NT, no tested.

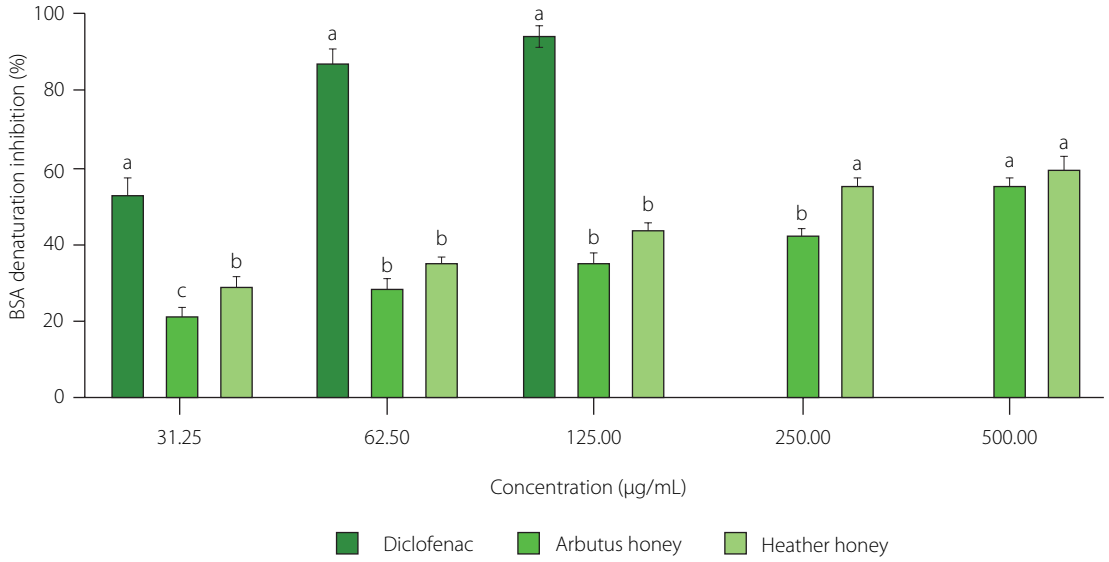

Figure 3. *In vitro* anti-inflammatory activity of arbutus honey, heather honey, and standard (diclofenac). Data are expressed as mean and standard deviation. Values with different superscript letters (a, b, c) indicate significant differences ($p<0.05$) between tested samples at the same concentration. BSA, bovine serum albumin.

Table 5. Antimicrobial activity of arbutus honey and heather honey.

Strain	Arbutus honey		Heather honey		AMC30		AK10		AM-B	
	DZI (mm)	MIC (µg/mL)	DZI (mm)	MIC (µg/mL)	DZI (mm)	MIC (µg/mL)	DZI (mm)	MIC (µg/mL)	DZI (mm)	MIC (µg/mL)
Acinetobacter baumannii	12±0.5	500	10±1.0	500	R	–	R	–	–	–
Klebsiella pneumoniae	14±0.0	250	15±0.0	500	R	–	R	–	–	–
Pseudomonas aeruginosa	28±1.0	500	10±0.0	125	R	–	19±0.5	500	–	–
Escherichia coli	35±0.5	500	R	–	R	–	–	–	–	–
Enterococcus faecium	36±0.5	62.5	38±0.5	62.5	R	–	–	–	–	–
Staphylococcus aureus	R	–	R	–	R	–	–	–	–	–
Candida albicans	R	–	R	–	–	–	–	–	R	–
Candida tropicalis	R	–	R	–	–	–	–	–	R	–

Results are expressed as means ± standard deviation. AK10, amikacin; AM-B, amphotericin-B; AMC30, amoxicillin-clavulanic acid; MIC, minimum inhibition concentration; DZI, diameter of zone inhibition; R, resistant.

BSA denaturation by 60.23% and 55.61%, respectively, with IC_{50} values of 0.29 and 0.38 mg/mL. These values were lower than those for the reference anti-inflammatory drug diclofenac, which inhibited BSA denaturation by 94.65% at a concentration of 0.125 mg/mL, and with an IC_{50} value of 0.031 mg/mL. These results demonstrate that both honey samples inhibit the thermal denaturation of BSA, confirming their anti-inflammatory effects. The bioactive compounds, including phenolic compounds, present in the honey samples may be responsible for this inhibitory activity [Ruiz-Ruiz *et al*., 2017; Zaidi *et al*., 2019].

Antimicrobial potential

Antibiotic-resistant pathogens pose a significant challenge in the clinical environment, necessitating the development of new and more effective therapies. Honey offers a promising alternative due to its proven antimicrobial properties [Bakchiche *et al*., 2020]. In our study, eight clinical isolates, including Gram-positive and Gram-negative bacteria, as well as two fungi with high antibiotic resistance rates, were tested. The inhibition zone diameter and MIC for both honey samples are shown in **Table 5**. The results revealed a wide range of inhibitory effects on *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, and *E. faecium*, with inhibition zones ranging from 12 to 36 mm for arbutus honey and from 10 to 38 mm for heather honey. The antibacterial activity of both honeys was similar, with moderate MICs ranging from 62.5 to 500 µg/mL, proving more effective than the antibiotics AK10 and AMC30 used as controls. Strains of *A. baumannii*, *K. pneumoniae*, *E. coli*, and *E. faecium*, which had shown antibiotic resistance, were sensitive to both honeys. Additionally, we observed that both honey samples had notable contents of total

phenolics and total flavonoids, along with a low pH, low water content, and high reducing sugar content, which may contribute to their antibacterial activities [Chettoum *et al*., 2023; Otmani *et al*., 2021]. These findings are consistent with other results reported for Algerian honey [Bakchiche *et al*., 2020; Chettoum *et al*., 2023]. However, the *S. aureus* strain exhibited resistance to both honey samples, which contrasts with the findings of Bouacha *et al*. [2018] and Chettoum *et al*. [2023] who reported the antimicrobial activity of honey against this bacterium, emphasizing that Gram-positive bacteria are typically more sensitive. This resistance could be attributed to the strain's inherent resistance, as it was also resistant to the tested antibiotics, or it could be related to the honey type and concentration used [Almasaudi, 2021].

Regarding antifungal activity, the results indicated that both honey samples lacked activity against *C. albicans* and *C. tropicalis*. These findings align with those of Latifa *et al*. [2020], who demonstrated that *C. albicans* was resistant to honey samples at all concentrations. This resistance is not directly linked to the floral origin of the honey but may be due to yeasts and fungal strains' higher tolerance to concentrated media, which act primarily as bacteriostatic agents [Latifa *et al*., 2020]. In contrast, other studies on Algerian honeys have reported sensitivity in different *Candida* strains [Ahmed *et al*., 2020; Bakchiche *et al*., 2020].

CONCLUSIONS

This study demonstrates that the physicochemical qualities of arbutus and heather honey meet international quality standards with unique microbiological quality. Arbutus exhibited higher phenolic content, superior antioxidant activity, and anti-inflammatory potential than heather honey. Both honeys displayed moderate antibacterial effects against multi-resistant pathogens, with notable efficacy against *E. faecium*. However, no antifungal activity was detected. Further research is needed to characterize other honey samples from the Edough Peninsula, Algeria.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Nanocarriers and Curcumin in Blueberry Shelf-Life Management: *In Vitro* Preliminary Antibacterial Effect

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Blueberries are very perishable, with fungi and bacteria affecting their spoilage along all the supply chain. No studies considered up to now the application of curcumin-loaded nanobubbles (NBs) or curcumin nanocrystals (NCs) to maintain their freshness. The aim of this preliminary work was to evaluate these two nanoformulations in terms of their *in vitro* antimicrobial effect against blueberry bacterial microbiota and establishing a quick-answer protocol *in vitro* in Petri plates. The effect was tested under three different light conditions (dark environment, blue LED and white LED). Results show that the presence of a lighting step (blue LED or white LED) after the microorganisms have been in contact with NBs and NCs was fundamental to activate the nanostructure and obtain a positive answer as inhibition halo. Notably, in relation to NBs, blue light significantly increased the antimicrobial potential compared to white LED; moreover, a curcumin concentration-dependent effect was highlighted (50 µg/mL with respect to 25 µg/mL). No significant differences were presented applying NCs. The results obtained from this preliminary study pointed out the sensitivity of bacteria from blueberry microbiota to NBs and NCs containing curcumin, nevertheless further research should be carried out to evaluate the *in vivo* applicability of the nanotechnologies.

Keywords: *Curcuma longa* L., inhibition, nanovectors, protocol, quality, *Vaccinium corymbosum* L.

ABBREVIATIONS

BPW, buffered peptone water; LED, light-emitting diode; NB, nanobubble; NC, nanocrystal; PCA, plate count agar

INTRODUCTION

The highbush blueberry (*Vaccinium corymbosum* L.) is one of the fruit whose consumption has been increasing in the last years due the important health properties of the species. The fruit quality is affected by the interaction of abiotic and biotic factors that can occur along all the supply chain (from the orchard to the post-harvest phase). Berries can be consumed fresh or stored frozen mainly and, as a consequence, these two major steps in the supply chains generate different quality requirements. The visual appearance (skin bruising and softening) and the shelf-life are the main attributes for the fresh market while the maintenance of nutritional and health value is required after the processing phase in the food industry. Diseases caused by fungi, bacteria and viral spoilage organism are the greatest cause of post-harvest losses in berries. The high humidity conditions in the storage phase, that are necessary to maintain the freshness of fruits, represent a critical factor in the berries supply chain if there is no adequate air flow to prevent moisture condensation on berries skin [Almenar *et al.*, 2007]. The main post harvest phytopathological disorders caused by fungi and some

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bacteria can in fact compromise the marketability of the berries up to 20% if they are not properly managed along all the supply chain [Bell *et al*., 2021]. The gray mold caused by *Botrytis cinerea*, Rhizopus rot by *Rhizopus stolonifera* and anthracnose fruit rot (black spot) by *Colletotrichum acutatum* and *Colletotrichum gloesosporiodes* are the most common diseases. *Phytophthora cactorum*, Cladosporium sp., Fusarium sp. and Alternaria sp. occasionally occur on berries [Bell *et al*., 2021; Ding *et al*., 2023]. The incidence of Botrytis on fresh fruits (after prolonged cold storage) is not influenced by conidial density on the surfaces, but by the resistance level of the host [Jiang *et al*., 2022]. In the case of Rhizopus instead the development is on overripe berries, but the fungi cannot grow below 5°C [Bell *et al*., 2021].

The main actors involved in the production, transport, handling, display, and sale of berries are responsible for the fruit safety. This is a very important and complex issue for the fresh supply chain. To extend blueberries shelf-life different approaches have been explored in the last years. The most common used were the cold atmospheric pressure plasma [Bovi *et al*., 2019; López *et al*., 2019], the use of coating with bio-films [Maringgal *et al*., 2020; Nain *et al*., 2021; Sempere-Ferre *et al*., 2022], the pulsed light [Pratap-Singh *et al*., 2023] and the UV-B application [Varaldo *et al*., 2024]. Nanotechnology applications could potentially address many of these needs due their large versatility bringing revolution in the agri-food supply chain from production, processing, packaging and transportation to storage. Different nanodelivery systems, such as nanoliposomes, colloidosomes, nanoemulsions, nanofibers, and polymeric nanoparticles, together with their application have been studied by food technologists exploiting proteins, carbohydrates, and lipids among ingredients to provide benefits in the food sensory attributes or to enhance the efficacy of the nanodelivered bioactive compounds [Biswas *et al.,* 2022]. Considering that research on the application of nanovectors in fresh fruits, in particular blueberries, are still limited [Jafarzadeh *et al*., 2021; Lee *et al*., 2024; Stura *et al*., 2023], it seems interesting to evaluate this tool to improve the shelf-life of berries as innovative post-harvest management. The present work describes a preliminary approach to evaluate the antimicrobial effect *in vitro* of two curcumin-based nanostructures, such as curcumin-loaded nanobubbles (NBs) and curcumin nanocrystals (NCs), alone and under photoactivation with a white- and blue light-emitting diode (LED). The encapsulation of curcumin in NB system has been previously optimized [Bessone *et al*., 2019; Munir *et al*., 2023]. This preparatory approach proposed might be the first step towards the study, development, and application of a storage treatment for freshly picked berries with natural compounds (curcumin) with the support of the nanotechnology tool. The development of a new type of photoactivated coating for berries during post-harvest aims to preserve and extend shelf-life and exploit natural compounds without costly and high-impact treatments, such as freezing and high-pressure techniques. This method could address the solutions for sustainability and resource optimization strategy in the agri-food supply chain. Fruit safety is an intrinsic requirement in the supply chain as also the control of phytopatological disorders. Hence, this study aimed at tentative evaluation of the *in vitro* antimicrobial activity of different nanocarriers in the blueberry post-harvest phase, investigating the optimum concentrations, nanostructure and the role of the photoactivation of curcumin from *Curcuma longa* L.

MATERIALS AND METHODS

Fruit material and bacterial extraction

Blueberries imported from Peru were retrieved from a supermarket in Grugliasco (Italy) and directly taken to the Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Grugliasco (Italy) to isolate microorganisms.

Notably, 20 g of blueberries were weighed and placed in a stomacher bag with 180 mL of buffered peptone water (BPW, Scharlab srl, Lodi, Italy). A suspension was prepared by smashing the berries in the stomacher (Seward, Worthing, United Kingdom) for 30 s at 2,300 rpm. Subsequently, 100 µL of the suspension was inoculated into Petri dishes with a plate count agar (PCA, Scharlab srl) and incubated at 30°C for 48 h. The grown colonies were randomly selected and picked, then resuspended in about 40 mL of a Ringer's solution, reaching a concentration of 7 log CFU/mL using a McFarland standard turbidity scale (Biomerieux, Marcy-l'Étoile, France). The microorganisms were directly isolated from commercially available blueberries and not purified. To prepare the suspension, colonies were screened for morphology, and a representative number of morpho-type was selected on PCA plates. This selection was performed to obtain a representative broth culture of the blueberry bacterial microbiota. The suspension was homogenized using a vortex to eliminate cell aggregates. The final broth (750 µL) was dispensed in 2 mL tubes with glycerol (250 µL) to allow a long-term storage at −18°C without affecting the vitality of the cells. The bacteria were revived for 48 h before the experiment (1 mL of bacterial suspension inoculated in 30 mL of brain-heart infusion broth (BHI) and incubated at 30°C), to obtain an initial suspension of microorganisms. A dilution-streak plate was made from the broth culture used to screen the various morphologies growing in it.

Preparation of curcumin nanoformulations

Curcumin from *Curcuma longa* L. (Sigma-Aldrich, Merk Life Science S.r.l., Milan, Italy) was used to prepare two nanoformulations, *i.e.*, curcumin-loaded NBs and curcumin NCs. For the preparation of curcumin-loaded NBs, curcumin was dissolved in Epikuron 200 (Cargill, Minneapolis, MN, USA) (3%, *w/v*) and palmitic acid (0.5%, *w/v*) ethanol solution by using *N*-methyl-2-pyrrolidone as a co-solvent. The mixture was then added to decafluoropentane and distilled water and homogenized using an Ultra-Turrax homogenizer (IKA, Staufen im Breisgau, Germany) for 2 min. Subsequently, an aqueous solution of chitosan (2.7%, *w/w*, pH 4.5) was added under magnetic stirring [Munir *et al*., 2023]. Three different formulations were prepared with curcumin concentrations of 25, 50 and 100 µg/mL, respectively.

Curcumin NCs were prepared by wet media milling method using a planetary ball mill (PM 100, Retsch, Haan, Germany). An aqueous suspension of curcumin (5%, *w/v*) was prepared

and added into the milling chamber containing milling pearls (3 mm stainless-steel grinding balls). The milling process was performed at 400 rpm for 2 h, with a pause of 5 min at every 30 min rotation [Malamatari *et al.,* 2018]. Different dilutions (0.5, 1.0, and 2.0 mg/mL) of the produced curcumin NC nanosuspension were prepared in phosphate buffered saline (PBS, pH 7.4) for the microbiological assays. At least ten different formulation batches for curcumin NBs and NCs were produced.

In vitro characterization of curcumin nanoformulations

The average diameter, polydispersity index and zeta potential of the curcumin formulations (*i.e.*, NBs and NCs) were determined by dynamic light scattering (DLS) using a 90 plus instrument (Brookhaven Instruments Corporation, New York, NY, USA), at a fixed scattering angle of 90° and a temperature of 25°C. The samples were diluted in filtered distilled water prior to measurements.

The dissolution profile of curcumin NCs was evaluated in water by suspended weighed amounts (5 mg) of curcumin NCs in 50 mL of distilled water under magnetic stirring at room temperature. At fixed times (0.5, 1, 2, 3, 4, 5, 6 h), 500 µL samples were withdrawn and replaced with the same amount of fresh dissolution medium. After centrifugation and filtration through a membrane filter (0.22 µm), the content of curcumin in the samples was determined by high-performance liquid chromatography (HPLC) analysis [Argenziano *et al.,* 2022]. A Shimadzu system (Kyoto, Japan) equipped with a UV/Vis detector set at a wavelength of 425 nm was used to this end. The analysis was performed using a mobile phase consisting of acetonitrile and water (70:30, *v/v*) at 1 mL/min flow rate through a reverse phase TC-C18(2) column (250 × 4.6 mm, pore size 5 µm, Agilent, Santa Clara, CA, USA).

The *in vitro* release kinetics of curcumin from curcumin-loaded NBs was determined using a multi-compartment rotating cell system. The curcumin-loaded NBs were placed in the donor chamber separated from the receiving compartment through a dialysis cellulose membrane (Spetra/Por cellulose membrane, cut-off 14 kDa, Spectrum Laboratories, Rancho Dominguez, CA, USA). The receiving phase contained PBS supplemented with 0.1% (*w/v*) sodium dodecyl sulfate (SDS). At fixed times (0.5, 1, 2, 4, 6, 22, 24, 28 h), the receiving phase was withdrawn and replaced with the same amount of fresh PBS [Bessone *et al.,* 2019]. The withdrawn samples were analyzed by HPLC to determine the curcumin content as described above. The experiments were performed in triplicate.

In vitro antibacterial activity determination by well diffusion method

The antimicrobial effect was tested using agar diffusion method as presented by Nadjib *et al.* [2014] with minor modifications. In particular, in all the trials the initial microbial concentration was 6 log CFU/mL, where 1 mL of the initial microbial suspension was added to 9 mL of fluid PCA and poured into a Petri dish. After the media solidified, 1.5 mm wells were made using the edge of a sterile glass pipette. Curcumin suspensions were inoculated without dilutions, using 20 µL into each well. Wells cut in agar were chosen instead of disks for practical reasons – very small quantity of nanocarriers suspensions. A suspension (20 µL) of three NBs (with curcumin concentrations of 25, 50 and 100 µg/mL) or three NCs (with curcumin concentrations of 0.5, 1.0 and 2.0 mg/mL) (separate plates for treatments with NBs and NCs) and a sterilized Ringer's solution (as the negative control) were inoculated into each well. Six plates were prepared for each lighting condition (white LED, blue LED, dark). After preparation and photoactivation, plates were incubated at 30°C to allow microbial growth, and inhibition halos (mm) were determined as follows:

$$\text{Inhibition halo (mm)} = (\emptyset_{halo} - \emptyset_{well})/2 \quad (1)$$

where: $\emptyset$ is the diameter.

Photoactivation and storage conditions

The NBs-inoculated plates (first trial) and NCs-inoculated plates (second trial) were photoactivated with white and blue LEDs at refrigerated temperature (4°C) for 3 h. Thanks to the freezing procedure with glycerol it has been possible to use the same bacterial mixture for both trials. Petri dishes were arranged under the LEDs to be homogeneously lit on the plate surface independently from their relative position. Incubation at 30°C for 48 h followed to allow the bacterial growth. Besides white LED and blue LED, six plates *per* trial were also kept in the dark, as a reference. Six replicates for each curcumin-loaded nanovector, under each LED color (white and blue) were considered.

Statistical analysis

The *in vitro* antimicrobial activity evaluation was performed with six replicates of each concentration of curcumin nanoformulation under each lighting condition. Two-way analysis of variance (ANOVA) was carried out on the results of halos, separately for NBs and NCs, considering light color and curcumin concentration as factors. Statistically significant differences were identified by comparison of mean values through Bonferroni's *post-hoc* test ($p \leq 0.05$). The R software 4.4.2 version (Free Software Foundation, Boston, MA, USA) was used for these elaborations.

RESULTS AND DISCUSSION

Two different curcumin nanoformulations were developed to evaluate the antimicrobial effect. In particular, curcumin NCs, a carrier-free curcumin colloidal nanosystem, were compared to a nanostructure in which curcumin was molecularly dispersed, such as curcumin-loaded NBs. Both formulations were characterized *in vitro*, evaluating the physicochemical parameters. Their average diameter, polydispersity index and zeta potential are shown in **Table 1**. NBs and NCs showed sizes in the nanometer range and good polydispersity indices. Indeed, the polydispersity index values of NB and NC formulations of about 0.2 indicated a rather homogenous nanoparticle size distribution [Danaei *et al.,* 2018]. No significant difference in NB average diameter was observed between the three NB formulations having different curcumin contents. All the NB formulations exhibited a positive

Table 1. Physicochemical characteristics of curcumin nanocrystals (NCs) and curcumin-loaded nanobubbles (NBs).

Formulation	Curcumin concentration	Average diameter (nm)	Polydispersity index	Zeta potential (mV)
Curcumin NCs	50 mg/mL	450.4±5.4	0.196	–15.2±4.6
Curcumin-loaded NBs	25 µg/mL	378.5±9.3	0.202	28.1±6.4
	50 µg/mL	380.2±10.6	0.205	28.7±5.3
	100 µg/mL	381.7±8.5	0.204	27.5±4.8

The results are presented as mean ± standard deviation (n=3).

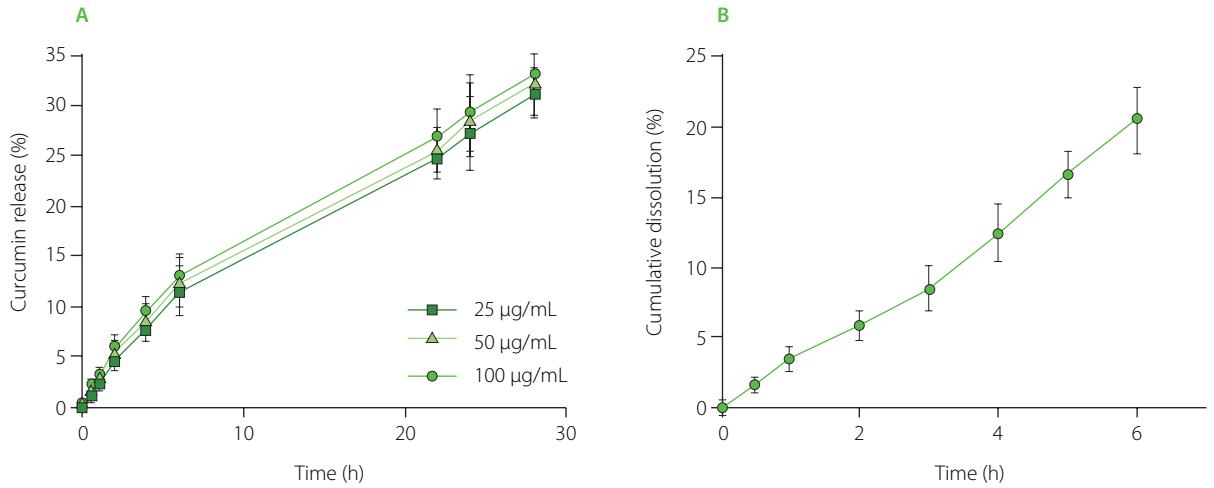

Figure 1. (**A**) *In vitro* release profile of curcumin from curcumin-loaded nanobubbles (NBs) formulations at different curcumin concentrations and (**B**) *in vitro* dissolution profile of curcumin nanocrystals (NCs).

surface charge with zeta potential values of about +28 mV, due to the presence of the chitosan shell, while curcumin NCs showed a negative surface charge. Additionally, the curcumin release from NBs as a function of time and dissolution profile of NCs are presented in **Figure 1**. In particular, NCs (**Figure 1B**) dissolved more percentage of curcumin in less time in respect of NBs (**Figure 1A**), as expected. Indeed, NCs were pure curcumin agglomerates, while NBs had less curcumin, but their structure was able to release it slowly. A prolonged *in vitro* release profile of curcumin from NBs with no initial burst effect was observed, indicating the curcumin incorporation in the NB core (**Figure 1A**). NCs, instead, enhanced the dissolution rate of curcumin due to the increased surface area and saturation solubility.

Curcumin-loaded NBs and curcumin NCs have been evaluated *in vitro*, under photoactivation with white or blue LED, on the microbiota extracted from blueberries and all showed an antimicrobial effect (**Figure 2** and **3**). The antibacterial effect was not perceivable in terms of halo presence for the samples incubated under no light (**Figure 2C** and **3C**), concluding that the presence of light is fundamental to boost the activation. Considering NBs, the results from halos analysis highlighted a significant difference between both white and blue LEDs and for the different curcumin concentrations (**Table 2**). The antibacterial effect of the application of white light was less impactful as the mean halo dimension was smaller compared to blue light ($p \leq 0.05$). Concentration effect was not statistically significant between 50 µg/mL and 100 µg/mL and between 25 µg/mL and 100 µg/mL ($p > 0.05$). These results encourage the fact that a smaller dose with respect to 100 µg/mL may have a more significant and valuable antimicrobial efficacy. Regarding NCs, no significant difference ($p > 0.05$) was evident, for white or blue LEDs (**Table 2**). The lack of differences observed for NC formulations may be attributed to the nanovector structure. NBs are nanometric core-shell structures, in which curcumin is molecularly dissolved in their inner core, made of decafluoropentane, a perfluorocarbon. The NB structure can affect the antimicrobial efficacy of curcumin favoring the delivery of curcumin molecules and their interaction with the fruit matrix. Therefore, this capability has an impact on the availability of the active ingredient (curcumin). Moreover, it has been reported that the encapsulation can have a role in retarding the curcumin degradation [Naksuriya *et al*., 2016]. Indeed, the rapid degradation of curcumin as such limited its possible application. In addition, a synergic antimicrobial effect can be obtained thanks to NB chitosan coating, although this property was not investigated in this experimental work. It is worth noting that the chitosan had antimicrobial activity [Confederat *et al*., 2021]. The results obtained should be considered as preliminary and evaluated as screening. Generally, they disclosed the potential of NBs and NCs coupled to light to inhibit bacterial communities, but

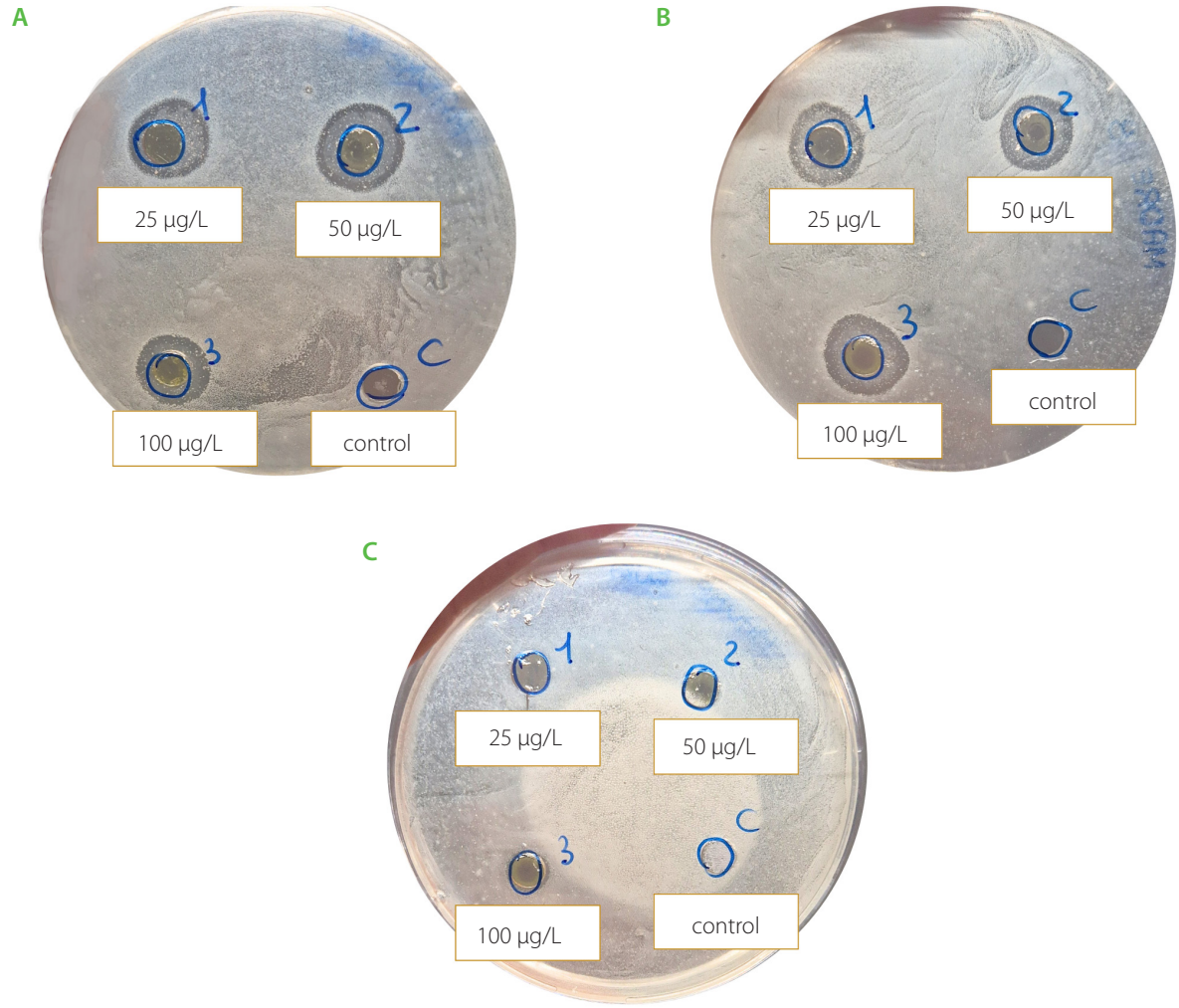

Figure 2. Inhibition halos caused by nanobubbles (NBs) loaded with curcumin at concentrations of 25, 50 and 100 µg/L. Sterile Ringer's solution was used as a negative control. The plates were photoactivated with white LED light (**A**), blue LED light (**B**) and kept in the dark (**C**) at 4°C.

this cannot necessarily be translated into a clear proof of inhibition against spoiling bacteria and improvement of fruit quality. Further assays should investigate the effect on specific pure bacterial cultures considered fruit spoiling agents but also on the main fungal diseases affecting berries, to have a complete assessment of the treatment efficacy.

On the other hand, curcumin NCs are carrier-free pure drug crystals with a size in the nanometer range. Therefore, curcumin is not present at a molecular level but a dissolution step is necessary for its delivery on the fruit matrix. Moreover, no external adjuvant structure and no other ingredients were used to activate or amplify the curcumin effect, possibly justifying the absence of the dose-related effects. Notably, the NC structure was investigated to possibly extend the nanovector technology to food products, as the presence of a perfluorocarbon is not allowed in food due to safety reasons and is in no way included in the list of food additives authorized by the European Parliament and the Council of the European Union [Regulation (EC) No 1333/2008]. The promising results obtained with NBs pave the way for developing nanovesicles without perfluorocarbon for the delivery of curcumin.

This experiment aimed to define a microbiological *in vitro* protocol to screen the efficacy of the NBs and NCs nanosuspensions on the real microbiota present on the fruit reference, in this case blueberries. In fact, the preliminary phase should clarify if the further *in vivo* tests may have scientific significance, as different conditions and the non-homogeneity of the fruit matrix usually lead to uneven results, reduced or altered antimicrobial activity [Lichtemberg *et al.*, 2016]. It is necessary to recognize a limitation of the methodology applied: the microbiological process followed was not previously compared to traditional and acknowledged one, performed on single pathogens or specific mix of relevant species. Thus, the conclusions cannot be generalized but are dependent on the microbiota present on the specific blueberries from which they were extracted.

The refrigeration temperature and the LED activation in the study should simulate the storage conditions of berries at the point of sale of the large-scale organized market, where the packaged berry fruits are kept at least overnight. Moreover, the cold chain may prevent possible damages of the fruit product caused by the heat deriving from light exposure. The possible

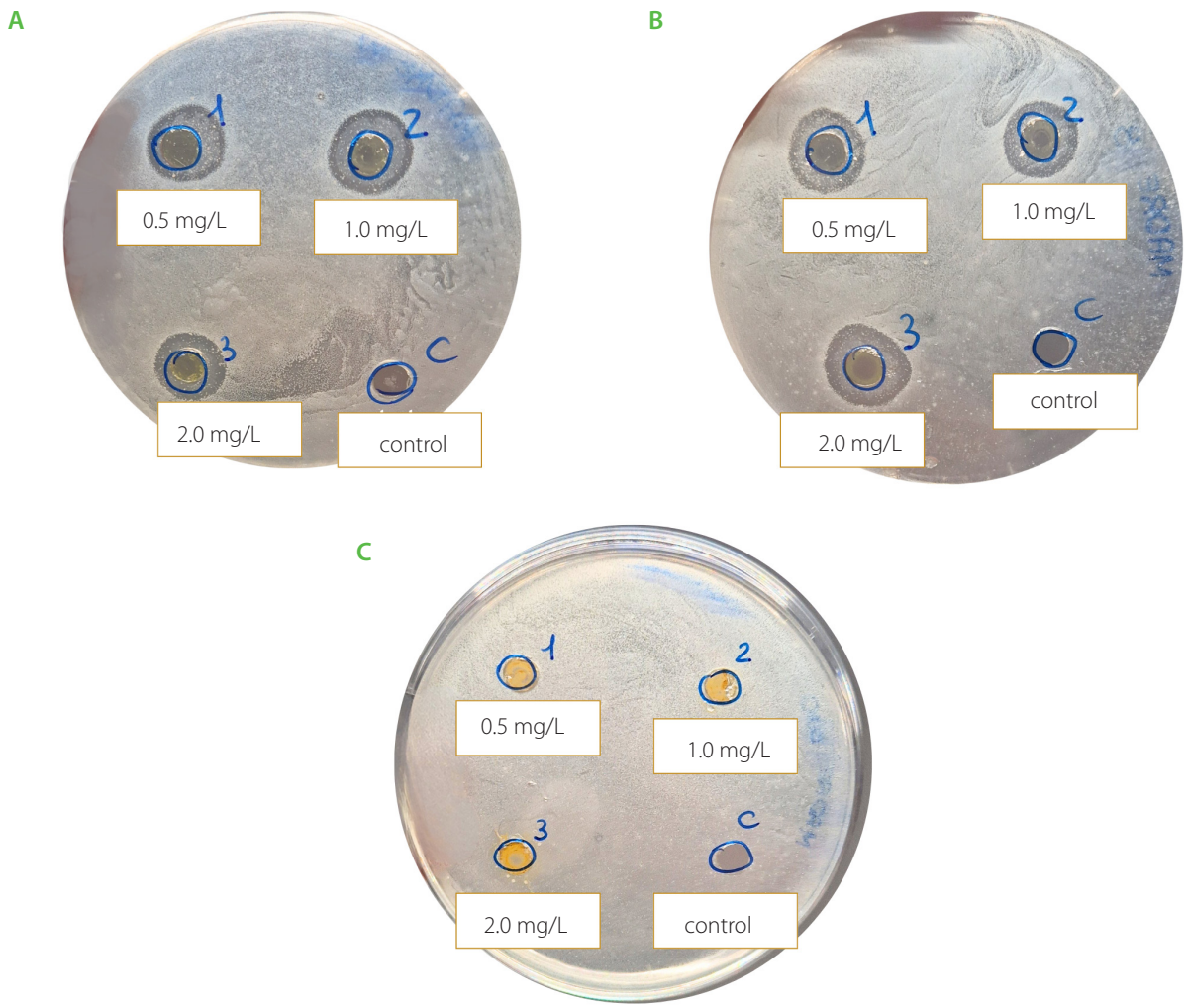

Figure 3. Inhibition halos caused by nanocrystals (NCs) loaded with curcumin at concentrations of 0.5, 1.0 and 2.0 mg/mL. Sterile Ringer's solution was used as control. The plates were photoactivated with white LED light (**A**), blue LED light (**B**) and kept in the dark (**C**) at 4°C.

Table 2. Halo measurement results for curcumin-loaded nanobubbles (NBs) and curcumin nanocrystals (NCs) at different curcumin concentrations and different photoactivation modes.

Formulation	Curcumin concentration	Inhibition halo (mm)	
		Blue LED light	White LED light
Curcumin-loaded NBs	25 µg/mL	4.04±0.39^{b}	3.35±0.20^{b}
	50 µg/mL	5.16±0.92^{a}	4.12±0.54^{a}
	100 µg/mL	4.52±0.30^{ab}	3.91±0.68^{ab}
Curcumin NCs	0.5 mg/mL	2.53±0.98^{a}	2.29±0.24^{a}
	1.0 mg/mL	2.42±0.36^{a}	2.32±0.84^{a}
	2.0 mg/mL	2.18±0.74^{a}	2.45±0.85^{a}

Data are reported as mean ± standard deviation (n=6). Different letters highlight statistically significant differences among the samples' mean ($p \leq 0.05$). Light color and curcumin concentration were considered as factors of the two-way ANOVA. Nanobubbles (NBs) and nanocrystals (NCs) were investigated separately.

effect of light on the fruit should be also considered. Both release patterns and LEDs efficacy are reported to work better at room temperature or even higher (50°C); however, these conditions cannot be applied in the fruit supply chain, as the product would be damaged [Liu *et al*., 2015]. The blue light was demonstrated to exert an antimicrobial effect on pathogenic bacteria, such as *Escherichia coli* [Braatsch & Klug, 2004]. Blue light spectrum should better match with curcumin activation. Nevertheless, also white LEDs were considered as their spectrum, because as reported by Vera-Duarte *et al*. [2021] they overlap to a large extent with the blue light spectrum. Moreover, white LEDs are currently used in the illumination system of refrigerated counters at large retailers.

When assuming the use of nanocarriers and nanosolids in a food context, a complete assessment of their impact on the safety and sensory quality of the product is required. As previously discussed, the presence of perfluorocarbon in NBs is the first issue. After considering this primary aspect, the sensory aspects should not be neglected. The addition of an ingredient and possible structures containing it may cause changes in the traditionally perceived flavor. Many research studies

in literature focused on the possibility of introducing nanoparticles in the pharmaceutical sector to reduce the taste and aftertaste of medicines and drugs, especially for pediatric patients [Krieser *et al*., 2020; Naik *et al*., 2021; Zhang *et al*., 2022]. In agreement with these studies, the presence of a nanovector covering the active ingredient should encapsulate the curcumin flavor, certainly in relation to the structure and composition of the nanostructure itself and proportionally to the applied dose. The evaluation of the possibility of color release onto the berries surface needs to be investigated as appearance is the first and primary interaction point between the fruit product and the consumer during the purchase.

In relation to the analytical methodology adopted, it is meaningful to remark that the overall microbiota present on the blueberry surface may be constituted of strains acting as biocontrol agents with antagonistic effect on the main fungal pathogens [Chacón *et al*., 2022], thus further characterization of the bacteria extracted may help in defining the range of activity of the curcumin-loaded nanostructures.

CONCLUSIONS

Producing and managing fresh berries in a sustainable way meeting market and consumers' high-quality requirements is the key for the future challenge in the post-harvest sector. This study provides a first insight into understanding how curcumin NBs and NCs under photoactivation act against a bacterial mixture isolated from blueberries. Results validate the positive role of these innovative tested treatments in the *in vitro* inhibition of bacteria isolated from the fruit matrix. This may help evaluate the treatment for the further application of the methodology *in vivo*. The approach followed in this study is a mandatory step to understand the feasibility of a post-harvest protocol for blueberries' management treated *in vivo* with nanocarriers. Further studies are needed to consider the feasibility and applicability of the technique and especially the knowledge of the raw material in terms of the picking time and conditions before the management in the laboratory should be considered. In fact, all storage treatments normally used in the picking store (use of modified atmosphere or controlled atmosphere) could directly affect the results of the study. Thus, the interaction between nanocarrier treatment and other post-harvest storage technique should be considered for further investigations.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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Pithellobium dulce (Roxb.) Benth. Fruit Flour Intake Enhances Short-Chain Fatty Acid Production and Glucose Metabolism in BALB/c Mice

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Pithecellobium dulce (Roxb.) Benth. (*P. dulce*) is a legume native to Mexico with a rich profile of bioactive compounds, including dietary fiber (DF) and phenolics, which have shown potential health benefits. This study investigates the effects of *P. dulce* fruit flour (PDF) on glucose metabolism and short-chain fatty acid (SCFAs) production in healthy BALB/c mice. Four groups – unmanipulated control (UC), vehicle control (VC), positive control inulin-treated (PC), and PDF-treated – received oral solutions for 60 days. Variables such as body weight and food intake were monitored, glucose and insulin tolerance tests were conducted, SCFAs in cecal contents were analyzed *via* gas chromatography, besides goblet cells in the descending colon were quantified. PDF in 100 g dry matter contained 57.44 g of carbohydrates and 10.48 g of DF including 7.77 g of insoluble DF and 2.71 g of soluble DF. Total phenolic content of PDF was 526 mg GAE/100 g. PDF consumption significantly improved glycemic regulation, as evidenced by lower blood glucose levels and enhanced glucose clearance in tolerance tests compared to controls. This was accompanied by increased SCFAs production, particularly propionic and butyric acids, associated with improved glucose homeostasis. PDF also promoted intestinal health by significantly increasing goblet cells in the descending colon, indicating enhanced epithelial barrier integrity. These effects were attributed to DF fermentation and phenolic compound activity, which boosted SCFAs production and reduced postprandial glycemia. These findings highlight *P. dulce* as a promising functional ingredient for improving glycemic control and intestinal health, providing a foundation for future research in metabolic disorders and prebiotic therapies.

Keywords: glycemic response, guamuchil, polyphenols, short-chain fatty acids

ABBREVIATIONS

AUC, area under the curve; DF, dietary fiber; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; FFAR2, free fatty acid receptor 2; FFAR3, free fatty acid receptor 3; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GPCR, G protein-coupled receptors; ITT, insulin tolerance test; MeOH, methanol; OGTT, oral glucose tolerance test; PC, positive control; PDF, *Pithecellobium dulce* (Roxb.) Benth. fruit flour; PSS, physiological saline solution; PYY, peptide tyrosine-tyrosine; RS, resistant starch; SCFA, short-chain fatty acids; T2DM, type 2 diabetes mellitus; UC, unmanipulated control; VC, vehicle control.

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INTRODUCTION

The promotion of foods with bioactive properties has become a crucial strategy for maintaining health and reducing the risk of metabolic diseases. Functional foods, particularly those rich in dietary fiber (DF) and phenolic compounds, have garnered significant attention due to their demonstrated benefits on key metabolic processes [Adefegha, 2018]. These bioactive compounds contribute to glycemic control, enhance insulin sensitivity, and support intestinal health through mechanisms such as modulation of gut microbiota, reduction of oxidative stress, and regulation of inflammatory pathways. As diet plays a fundamental role in preventing and managing chronic diseases, encouraging the inclusion of these functional components in daily nutrition represents a promising approach to improving overall metabolic health and quality of life [Mondal *et al.,* 2024].

Within this context, *Pithecellobium dulce* (Roxb.) Benth. stands out as a promising source of these bioactive components. Native to Mexico and also found in South America, Africa, and Southeast Asia, *P. dulce* has a long history of traditional use in food, medicine, and as animal forage [Wall-Medrano *et al.,* 2016]. The fruit of this legume consists of sweet arils surrounding black seeds, and its nutritional profile is particularly notable for its dietary fiber content (~6 g/100 g) and phenolic compounds [Pio-León *et al.,* 2013]. The red arils are rich in anthocyanins, such as pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside, which contribute to its antioxidant and metabolic effects [López-Angulo *et al.,* 2018]. However, despite the fruit's availability in Mexico, its consumption remains limited due to the seasonal nature of its maturation, as it is produced only once a year [Wall-Medrano *et al.,* 2016].

The health benefits of DF and phenolic compounds are supported by well-documented mechanisms. DF improves glycemic control by enhancing viscosity and undergoing fermentation, a process that generates short-chain fatty acids (SCFAs). These SCFAs activate free fatty acid receptors 2 and 3 (FFAR2/FFAR3) and stimulate the secretion of anorexigenic hormones, such as glucagon-like peptide-1 (GLP-1) and peptide tyrosine-tyrosine (PYY), which regulate appetite, glucose homeostasis, and insulin sensitivity [Dalile *et al.,* 2019]. On the other hand, phenolic compounds exert complementary mechanisms that contribute to glycemic regulation and metabolic health. These include the inhibition of key digestive enzymes, such as α-amylase and α-glucosidase, which modulate carbohydrate digestion and postprandial glucose levels. Additionally, phenolic compounds enhance glucose uptake through the activation of glucose transporter type 4 (GLUT-4) and peroxisome proliferator-activated receptor-γ (PPAR-γ), as well as reduce oxidative stress and protect pancreatic beta cells from damage, supporting overall glucose homeostasis [Kang *et al.,* 2020]. Since 90% of phenolics pass into the colon, they influence the gut microbiota, which can transform them into bioactive, low-molecular-weight phenolic metabolites [Gowd *et al.,* 2019]. These metabolites help regulate metabolic networks, favoring the growth of SCFAs-producing bacteria like propionate and butyrate, which enhance insulin sensitivity and reduce inflammation [Wang *et al.,* 2022]. These effects position phenolics as key compounds in dietary strategies to prevent and manage metabolic disorders.

Given its distinctive nutritional and phytochemical profile, *P. dulce* may act as a functional food with the potential to support metabolic health. While its antioxidant and hypoglycemic properties have been observed in previous *in vitro* and *in vivo* studies [López-Angulo *et al.,* 2018; Pradeepa *et al.,* 2013], the impact of *P. dulce* on glucose homeostasis and SCFA production in biological models remains unexplored. This study seeks to fill this gap by evaluating the administration of *P. dulce* flour (PDF) on SCFA production and glucose metabolic responses in BALB/c mice, providing valuable insights into its potential application for the management of metabolic diseases.

MATERIALS AND METHODS

Plant material

Pithecellobium dulce (Roxb.) Benth. fruit was harvested between March and April 2022 in the town of Autlan de Navarro, Jalisco, Mexico (19.7682200° N, 104.3666400° W). The peel was removed from the arils, which were then placed in 500 g hermetic bags and frozen at −18°C until use. To obtain the flour, the arils were thawed, and the seeds were manually removed. They were then washed with distilled water, excess water was drained, and the arils were ground in a food processor until a crushed paste was obtained. This paste was spread on trays and dehydrated in an oven (DESH304/032014, Todumex, Guadalajara City, Mexico) at 50°C for 8 h. The dried arils were ground in the food processor until fine flour and then passed through a No. 200 mesh sieve (<74 µm). The PDF was packaged in airtight, vacuum-sealed bags to preserve its stability and extend shelf life.

Chemicals and reagents

Folin-Ciocalteu reagent, gallic acid, catechin, vanillin, 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$), methanol, ethanol, chicory inulin (I2255), *n*-butanol, total DF assay kit (TDF100A), and citric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Proximate chemical analysis

To further investigate the potential of PDF as a functional food, its Proximate chemical composition (ashes, crude protein, and total lipids) was analyzed using standard AOAC International methods [AOAC, 1995]. Moisture content was determined by Norma Mexicana regulation [NMX-F-083-1986]. The DF content determination was performed with Sigma-Aldrich total DF assay kit, separating the soluble from the insoluble fiber fractions. Total carbohydrates were calculated by difference. Total reducing sugars were determined based on Norma Official Mexicana regulation [NOM-086-SSA1-1994]. The results of the analyses were expressed on a PDF dry matter (dm) basis. The kilocalories expressed *per* 100 g of dry matter were calculated using the conversion factors for fruits (proteins 3.36 kcal/g, carbohydrates

3.60 kcal/g, and fat 8.37 kcal/g) due to their similarity in the nutritional content of fruits and not legumes acc. to *P. dulce* classification [FAO, 2003].

The pectin content of the PDF was determined using the Carré & Haynes [1922] method. PDF (5 g) was added to 100 mL of distilled water and boiled for 15 min on three occasions. After each boiling, the mixture was filtered, and the volume was adjusted to 400 mL by adding 100 mL of water. The filtrate was treated with 100 mL of 0.1 M NaOH and left to rest for 12 h. Then, 50 mL of 1 M acetic acid was added, followed by 50 mL of 1 M calcium chloride, with resting for 1 h. The mixture was boiled for 5 min and filtered. The residue was washed with 500 mL of hot distilled water, re-dissolved in 100 mL of distilled water at 25°C, and boiled for 5 min. Finally, it was filtered through previously weighed open-pore filter paper, washed, and dried until a constant weight was achieved. Pectin content in PDF dm (g/100 g dm) was calculated using Equation (1), based on the weights of the residue (w_1), paper (w_2), and sample (w_3):

$$\text{Pectin content} = \frac{w_1 - w_2}{w_3} \times 100 \quad (1)$$

Analysis of phenolic compounds

Extraction

Based on the method described by Mattila & Kumpulainen [2002], with adaptations, 2 g of PDF were extracted using 7 mL of 80% methanol with 10% acetic acid (85:15, *v/v*). The mixture underwent 30 min of sonication (1510R-MT, Brandsonic, Connecticut, USA) followed by centrifugation at 560×*g* for 10 min (Z326 HERMLE Labortechnik GmbH, Wehingen, Germany). The residue was re-extracted using the same procedure. The pooled extracts were analyzed for total phenolic content, total flavonoid content, tannin content and antioxidant capacity.

Total phenolic content

The total phenolic content was quantified using the Folin-Ciocalteu reagent [Singleton & Rossi, 1965]. A mixture consisting of 3 mL of deionized water, 50 µL of extract, and 250 µL of Folin-Ciocalteu reagent was prepared. Following 8 min, 750 µL of a 20% Na_2CO_3 solution was added, and deionized water was used to bring the volume up to 950 µL. Using a UV-vis spectrophotometer (UNICO S-2150, Dayton, NJ, USA), the absorbance was measured at 765 nm. A calibration curve was constructed using gallic acid as the standard, with 13 points ranging from 0 to 1,000 mg gallic acid/L. The results were expressed as mg of gallic acid equivalents in 100 g of PDF (mg GAE/100 g).

Total flavonoid content

A catechin solution of 25 mL was prepared by dissolving 0.0250 g of catechin in 80% (*v/v*) methanol to obtain a calibration curve. Each catechin dilution or extract (1,000 µL), 4,000 µL of distilled water and 300 µL of 5% $NaNO_2$ were mixed. After 5 min, 300 µL of 10% $AlCl_3$ was added and 1 min later 2 mL of 1 M NaOH and 2.4 mL of distilled water were added to complete the volume to 10 mL. Each tube was shaken for 30 s, and absorbance was measured at 415 nm [Moo-Huchin *et al.,* 2015]. Calibration curves were constructed using catechin with concentrations ranging from 0 to 1,000 µg catechin/mL. The results were expressed in mg catechin equivalents in 100 g of PDF (mg CE/100 g).

Tannin content

The tannin content of PDF was determined with minor modifications based on the methodology outlined by Broadhurst & Jones [1978]. A calibration curve was generated using catechin (0.02655 g) dissolved in 80% (*v/v*) methanol, with concentrations ranging from 0 to 1,000 µg catechin/mL. In test tubes, 50 µL of extract, 3.0 mL of 4% vanillin solution in methanol, and 1.5 mL of HCl were combined. The mixture was thoroughly shaken and allowed to rest for 15 min. Absorbance was measured at 500 nm, and the results were expressed as mg CE/100 g of PDF.

Antioxidant capacity

The antioxidant capacity of PDF was evaluated using the DPPH assay [Brand-Williams *et al.*, 1995], with modifications. Trolox was used for the standard curve (1.97 to 11.75 µg/mL). Absorbance of reaction mixtures was measured at 517 nm, after 30 min of incubation in darkness. The DPPH radical scavenging activity was expressed as µg of Trolox equivalents (TE) *per* g of PDF dm.

In vivo assays

Animal model and experimental design

The Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Centro Universitario de Ciencias Exactas e Ingienerias de la Universidad de Guadalajara (CUCEI-UDG) accepted the animal experimentation protocol (approval opinion: 02/2022). Healthy male mice of the BALB/c strain (7 weeks of age with an approximate weight of 21–26 g) were purchased from the Vivarium of the Universidad Autonoma del Estado de Hidalgo (Pachuca, Hidalgo, Mexico). They were transported to the experimental animal room of Pharmacobiology Department from CUCEI/UDG. Mice were acclimatized for 2 weeks before starting the experimental process, in a controlled environment (12 h light-dark cycle, temperature: 22°C) with a standard pellet diet (Purina brand Nutri-cubos, Mexico City, Mexico) and drinking water *ad libitum*. The nutritional content of the diet is shown in **Table S1** in Supplementary Materials. Animal experimentation was conducted by the protocols outlined in the NOM regulation [NOM-062-ZOO-1999]. Four groups of 10 mice each were randomly assigned after they were weighed: unmanipulated control (UC) group, vehicle control (VC) group, positive control with inulin (PC) group and PDF group.

Dose standardization

A preliminary test was conducted to determine the most effective dosages of inulin and PDF during the first intervention phase. To prevent precipitation and improve sample dissolution, the sample water solutions were ultrasonicated in bath for 10 min (Sonicator Lab-Tech Instrumentation S.A. de C.V., Mexico).

Based on concentrations of 6.25, 12.50, and 25.00 mg/kg for PDF and 6.25, 25.00, and 50.00 mg/kg for chicory inulin, dose-response curves were generated using the oral glucose tolerance test (OGTT). A vortex (G560, Scientific Industries Inc, Bohemia, NY, USA) was used to shake the samples before each administration. Using an orogastric cannula, the dosages were given orally (v. o.). Before OGTT, mice were fasted for 4 h. Using an Accu-Check Active glucometer (ROCHE, Basil, Switzerland) blood was drawn by a tail puncture at the following intervals to test blood glucose (mg/dL): −30, 0, 15, 45, 60, 90 and 120 min. The different doses tested were administered orally at a time of 30 min.

Prolonged administration

The second stage of the experiment began after the animals had been acclimated for two weeks to routine feeding in a controlled setting (22°C, 12-h light/dark cycles). The VC, PC and PDF groups received the appropriate solution orally. The VC group was administered physiological saline solution (SSF), the PC group received reconstituted inulin at a concentration of 50 mg/kg, and the PDF group was given a solution containing 6.25 mg/kg of PDF, dissolved in SSF. The administration volume was standardized to 60 µL daily for all groups. During 60 days, the mice body weight and daily food and water consumption were recorded, along with their weekly weight. Subsequently, the OGTT was performed, and after 3 days of rest for the mice, the insulin tolerance test (ITT) was performed. At the conclusion of the testing period, mice were sacrificed by decapitation in accordance with the guidelines set forth by NOM [NOM-062-ZOO-1999], and intestinal samples were harvested.

Monitoring body weight and food intake

The body weight of the mice was assessed weekly, and the body weight differences (Δ) were analyzed during the 1st, 4th, and 8th weeks, corresponding to the beginning, middle, and end of the experimental period. The mice weight was measured using a digital scale (KD 160, TANITA, Tokyo, Japan). The weight of pellets consumed daily was obtained to assess each group's meal intake. Caloric intake was estimated based on the grams of food consumed *per* day according to the provider's caloric intake reported (**Table S1** in Supplementary Materials).

Acute response evaluation (with preload and without preload)

Each group was divided equally before starting the OGTT to evaluate the acute response to solution administration. Mice were fasted for 4 h, and glucose was measured after 3.5 h. Preloads were administered to the VC, PC, and PDF groups, followed by an oral glucose load (2 g/kg body weight). Glucose levels (mg/dL) were recorded at specific intervals, and the area under the curve (AUC) was calculated using the trapezoidal rule to assess total glucose exposure [Andrikopoulos *et al.,* 2008].

Insulin tolerance test

Insulin-sensitive cells can be observed to see how well insulin stimulates the internalization of circulating glucose using the approved approach known as ITT [Gelding *et al.,* 1994]. Mice were fasted for 4 h and administered intraperitoneally with 0.070 IU/kg insulin. Glucose was subsequently monitored at the same times mentioned above for OGTT.

Serum metabolic hormone analysis

Serum samples were obtained by dividing each study group into two subgroups (n=4 each) to evaluate metabolic hormone levels under fasting (4 h) and postprandial conditions. Sacrifices were conducted over 4 days (one group per day), with animals maintained on a standard diet and without experimental solutions before assignment to fasting or postprandial subgroups. Blood was centrifuged to isolate serum, and metabolic hormone concentrations glucose-dependent insulinotropic polypeptide (GIP), insulin, leptin, and resistin were measured using the MILLIPLEX MAP Mouse Panel Kit (MMHE-44K, Millipore, Burlington, MA, USA).

Short-chain fatty acid analysis

After sacrifice, cecal contents (n=4) were carefully collected in microtubes and stored at −80°C. The quantification of SCFAs was performed using a Shimadzu GC 2010 Plus gas chromatograph equipped with a flame ionization detector (FID) (Shimadzu Scientific Instruments, Kyoto, Japan). Samples (20–35 mg) were homogenized in distilled water and subjected to a liquid-liquid extraction method using a solvent mixture consisting of *n*-butanol, tetrahydrofuran, acetonitrile, 0.1 M HCl, citric acid, and sodium chloride, following the protocol described by Ribeiro *et al.* [2018]. The supernatant was obtained by centrifugation at 13,000×*g* for 10 min and filtered using a Whatman GD/X syringe filter with 0.22 µm polyvinylidene fluoride (PVDF) membrane (MERCK, Billerica, MA, USA). Chromatographic analysis was performed using a high-polarity Mega-Acid column (MEGA, Legnano, Italy). Data were processed with LabSolutions software (Shimadzu), and the contents of SCFAs, specifically acetic acid, butyric acid, and propionic acid, were determined using 6-point standard curves (12.5, 25, 50, 100, 200, and 400 mg/L).

Goblet cell count

Descending colon tissue samples were embedded in paraffin and sectioned to 5 µm thickness. Each sample slide included five tissue sections, stained with Alcian blue (HYCEL, Zapopan, Mexico) and hematoxylin to identify goblet cells. Goblet cells were counted in three randomly chosen sections *per* sample, with 10 crypts assessed *per* section, totaling 30 crypts *per* sample. Using APERIO Image Scope software (Leica, Vista, CA, USA) at 20× magnification (100 µm), results were recorded as goblet cells *per* crypt *per* 100 µm [Rodríguez-Mejía *et al.,* 2022].

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (version 8.0.1, San Diego, CA, USA). Descriptive statistics, including mean and standard deviation, were applied to the proximate chemical and phenolic compound analyses of PDF. These analyses were conducted in triplicate. For evaluations

of data from *in vivo* experiment, one-way analysis of variance (ANOVA), two-way ANOVA or the non-parametric Kruskal-Wallis test was used, depending on the normality of the data (assessed with the Shapiro-Wilk test), to identify significant differences between groups ($p<0.05$). *Post hoc* comparisons were performed using Tukey's test or Dunn's test, as appropriate.

RESULTS AND DISCUSSION

Nutritional and phenolic content in *P. dulce* flour

The proximate composition of PDF, revealing a crude protein content of 9.26 g/100 g dm, is provided in **Table 1**. This protein content was lower than the 12–15 g/100 g dm reported for dehydrated *P. dulce* arils from India [Rao *et al.*, 2011]. The levels of moisture, ash, total lipids, and total carbohydrates were like those reported in the cited study. The proximate composition of fresh *P. dulce* arils has been previously reported on a fresh matter (fm) basis, though the values varied due to methodological differences and environmental factors during plant harvest. For instance, in regions of Mexico such as Sinaloa, the arils exhibited a high moisture content (77.20 g/100 g fm), a low protein content (2.48 g/100 g fm), and significant levels of dietary fiber (5.97 g/100 g fm) [Pio-León *et al.,* 2013].

Proximate analysis of PDF (**Table 1**) revealed a total DF content of 10.48 g/100 g dm, with 7.77 g/100 g dm as insoluble fiber and 2.71 g/100 g dm as soluble fiber, primarily pectin.

Table 1. Nutrient composition, phenolic compound content, and antioxidant capacity of *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

Parameter	Content/capacity
Energy value (kcal/100 g dm)	245.4±1.8
Crude protein (g/100 g dm)	9.26±0.55
Total lipids (g/100 g dm)	0.89±0.01
Total carbohydrates (g/100 g dm)	57.44±0.47
Total reducing sugars (g/100 g dm)	47.5±3.4
Total dietary fiber (g/100 g dm)	10.48±0.02
Insoluble fiber (g/100 g dm)	7.77±0.74
Soluble fiber (g/100 g dm)	2.71±0.92
Pectin (g/100 g dm)	2.68±0.39
Moisture (g/100 g dm)	19.76±0.46
Ash (g/100 g dm)	2.15±0.02
Total phenolics (mg GAE/100 g)	526±32
Total flavonoids (mg CE /100 g)	242±33
Tannins (mg CE/100 g)	75.3±1.8
Antioxidant capacity (µg TE/g dm)	656±34

Each value represents the mean of three repetitions ± the standard deviation. GAE, gallic acid equivalent; CE, catechin equivalent; TE, Trolox equivalent; dm, dry matter.

The predominance of insoluble fiber suggested the presence of hemicelluloses previously identified in *P. dulce* arils [Preethi & Mary Saral, 2016].

Like DF, phenolic compounds play a critical role in modulating glycemic response and are integral to the food matrix. *P. dulce* contains a variety of phytochemicals, including flavonoids, tannins, glycosides, saponins, phytosterols, triterpenes, and alkaloids [Pradeepa *et al.,* 2013]. To evaluate the potential functional benefits of PDF, its total phenolic content, total flavonoid content, tannin content and antioxidant capacity were assessed (**Table 1**). The total phenolic content was notably high as consistent with previously reported values for dehydrated *P. dulce* arils, which range between 462.40 and 829.80 mg GAE/100 g depending on the aril color (white or red) [Rao *et al.,* 2011].

Interestingly, the total phenolic content in fresh materials was not significantly different (392.2 and 517.8 mg GAE/100 g fm in white and red arils, respectively) [Pio-León *et al.,* 2013]. While dried fruit flour might be expected to have higher phenolic content, dehydration can also cause a reduction in phenolic compounds. Compared to other commonly consumed fruits in their fresh state, such as raspberries (383–400 mg GAE/100 g fm) [Mihailović *et al.,* 2019] and blueberries (275–645 mg GAE/100 g fm) [Hernández-Carrión & Caicedo Narvaez, 2022], the PDF contained a respectable amount of phenolics. The current study reports a higher total flavonoid content in the PDF compared to fresh arils, with 50.0 and 86.6 mg quercetin equivalents/100 g in white and red arils, respectively [Pio-León *et al.,* 2013]. These levels were comparable to those in pomegranates (84–214 mg CE/100 g) [Shams Ardekani *et al.,* 2011] and colored grapes (210.6–758.2 mg CE/100 g) [Liang *et al.,* 2014], known for their high antioxidant capacity. Consistently, the antioxidant capacity of the PDF (656 µg TE/g dm) underscored the significant contribution of the bioactive compounds present in the sample. Additionally, tannin content in the PDF was lower than in fresh arils (148.2 and 309.2 mg/100 g in white and red arils, respectively) [Pio-León *et al.,* 2013]. Tannins, common in many plants, are known for their astringency and antinutritional properties, and their content may be reduced during food processing steps like soaking [Ojo, 2022]. The preparation of the fruit before dehydration has likely contributed to the reduced tannin levels in the flour.

Dose standardization

Dose-response evaluation of *P. dulce* flour

Glycemic curves obtained to establish the effective dose of PDF are shown in **Figure 1A**. The highest glucose peak was observed at 15 min in all groups. The VC group showed a decrease in their glucose levels from the 30th minute with a slow reduction to 120 min. The group administered the 25.0 mg/kg dose showed higher glucose levels, observing a marked decrease up to the 60th minute. The dose group of 12.50 mg/kg showed a shortened and delayed curve over all measured times to reach their baseline values. The lowest dose group (6.25 mg/kg) showed a lower glucose concentration in the 15th minute (below 20 mg/dL), shortening the time to reach their baseline blood glucose

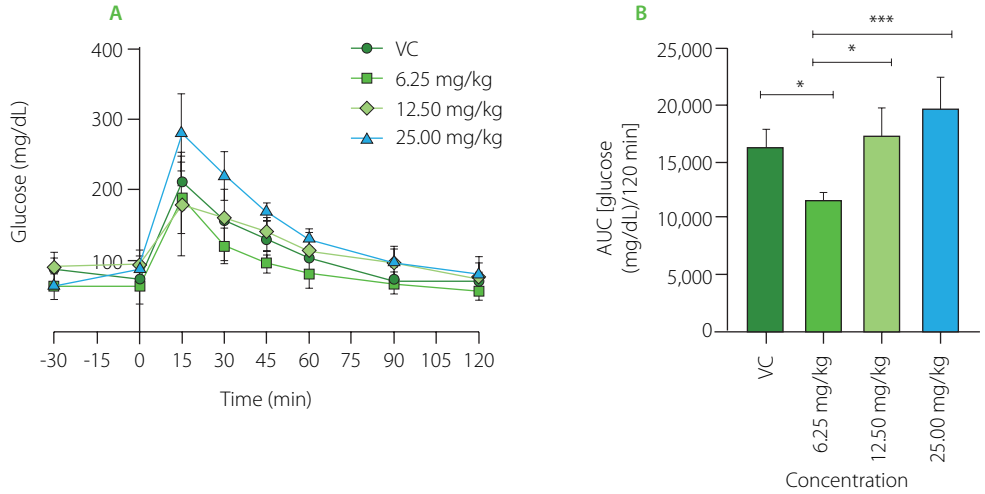

Figure 1. Glucose dose-response evaluation of *Pithecellobium dulce* (Roxb.) Benth. fruit flour (PDF) at 6.25, 12.50, and 25.00 mg/kg. (**A**) Oral glucose tolerance test (OGTT). (**B**) Area under the curve (AUC). Statistical analysis: one-way analysis of variance and Tukey's *post hoc* test. Significant differences: ***$p<0.001$, *$p<0.05$. VC, vehicle control.

values. When compared to doses of 25.00 mg/kg ($p<0.0001$), 12.50 mg/kg ($p<0.001$), and the VC group ($p<0.05$), the dose of 6.25 mg/kg resulted in lower glucose concentrations, according to the AUC (**Figure 1B**) which displays the glucose concentration present in the groups with different doses during the 120 min test.

The sugar content in the dry matter of *P. dulce* fruit has been reported to vary depending on its ripeness. These sugars include fructose (12–14 g/100 g), glucose (11–14 g/100 g), and sucrose (2–4 g/100 g) [Wall-Medrano *et al.,* 2016]; in addition, monosaccharides such as xylose, rhamnose, ribose, galactose and mannose have been extracted from the fruit [Preethi & Mary, 2016].

PDF has been obtained and developed from the whole fruit, which is why the establishment of the most effective dose was required to begin prolonged administration. As indicated in the dose-response evaluations, the lowest dose (6.15 mg/kg/day) demonstrated superior efficacy in reaching basal glucose levels by obtaining lower blood glucose levels throughout the OGTT. Findings demonstrated that PDF did not provide a larger reaction at higher dosages, which might happen if its mechanism of action to enhance glycemic control was caused by DF viscosity in the chyme, which functions in a dose-dependent manner, particularly in soluble fiber. DF properties are related to the cell wall matrix of its polysaccharides (porosity, cell rupture and viscosity), which can affect the bioaccessibility of nutrients, gastric emptying and gastrointestinal transit rates, as well as the degree of digestion and absorption of macronutrients [Giuntini *et al.,* 2022]. DF is involved in nutrient encapsulation, remaining structurally intact in plant tissues, which slows down and impairs digestion, thus reducing postprandial glycemic increase [Holland *et al.,* 2020]. However, it has been suggested that xylose could be a potent sucrase inhibitor, mitigating postprandial glucose increase [Bae *et al.,* 2011]. In this context, xylose has been found able to immediately decrease postprandial peaks in blood glucose and insulin in healthy individuals after ingesting this monosaccharide together with sucrose. The xylose efficacy combined with a high-protein and high-fat food matrix (muffins) was also investigated by the authors. They observed a significant decrease in postprandial glycemia responses, however, not as much as when xylose was consumed alone with a sucrose solution. These findings imply that the glycemic response in OGTTs at an acute dosage observed in our study might have been influenced by the xylose present in PDF. *In vitro* digestion models have demonstrated the efficacy of phenolic compounds found in certain fruits in inhibiting enzymes involved in the metabolism of carbohydrates, particularly in anthocyanin-rich fractions [Prpa *et al.,* 2021].

In addition to DF, the white and red arils of *P. dulce* are rich in phytochemicals, including high levels of vitamin C and anthocyanins, especially in red arils [Pio-León *et al.,* 2013]. *In vitro*, the methanolic extract from the red arils demonstrated superior α-glucosidase inhibition compared to the reference drug, acarbose. This effect is primarily due to anthocyanins such as cyanidin 3-*O*-glucoside and pelargonidin 3-*O*-glucoside, which interact with α-glucosidase in both competitive and non-competitive ways. This enzyme, located in the small intestine, plays a key role in carbohydrate breakdown and its inhibition may help delay or reduce postprandial blood glucose spikes [López-Angulo *et al.,* 2018]. The observed dose-response effect in this study is likely linked to the high phenolic content in the PDF. Additionally, the glycemic response is often influenced by the viscosity of soluble DF, which delays gastric emptying and forms a physical barrier that reduces nutrient absorption in the small intestine [Alexander *et al.,* 2019].

Dose-response evaluation of inulin

Inulin was used as a positive control due to its prebiotic properties and its widespread use as a dietary supplement to enhance insulin sensitivity and metabolic syndrome markers

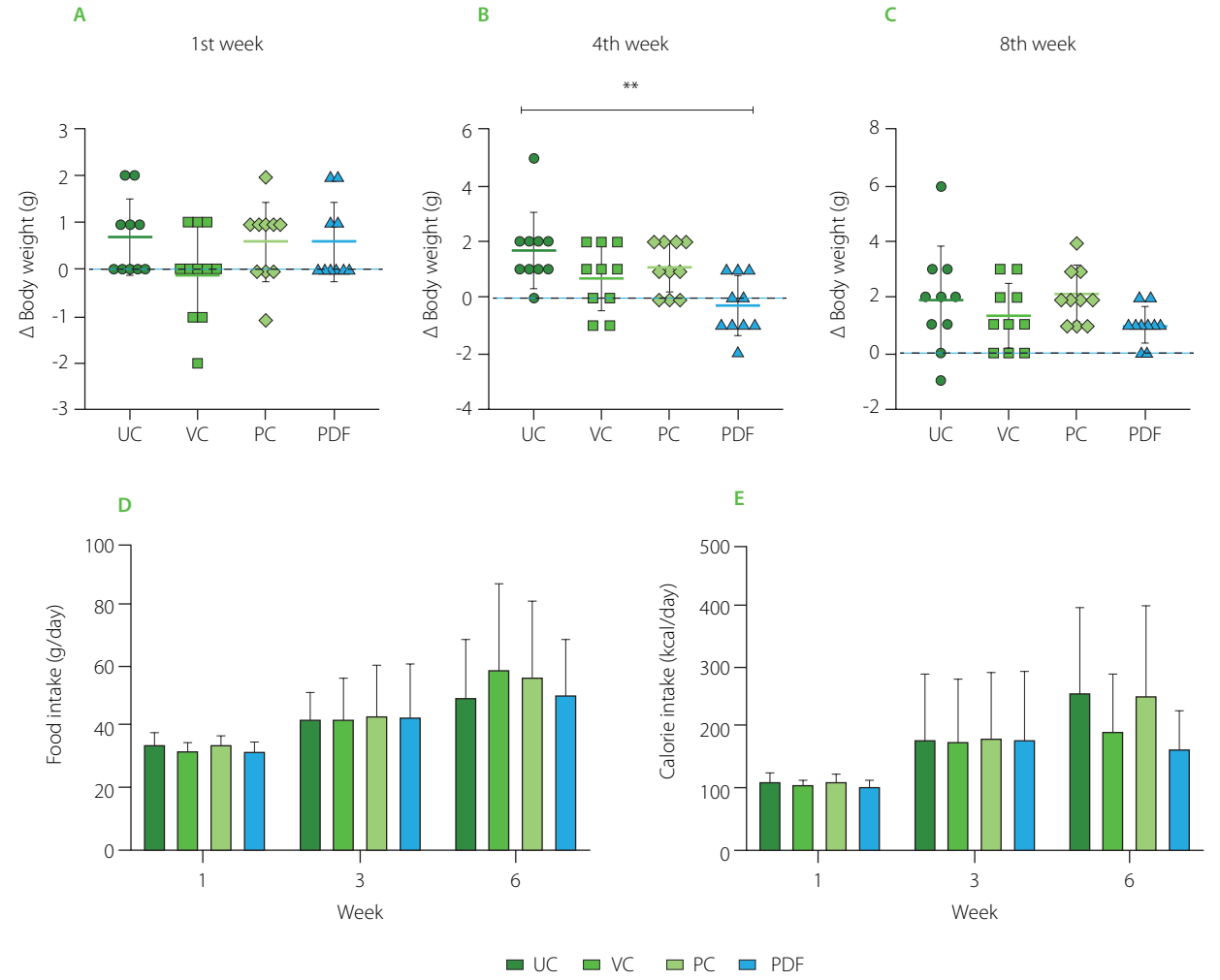

Figure 2. Changes in body weight during the study period: (**A**) first week, (**B**) fourth week, (**C**) eighth week. (**D**) Food intake at weeks 1, 3, and 6. (**E**) Caloric intake across the same time points. Statistical analysis: non-parametric Kruskal-Wallis analysis of variance (ANOVA) and Dunn's *post hoc* test (**A**, **B**, **C**); one-way ANOVA and Tukey's *post hoc* test (**D**, **E**). Significant differences: **$p<0.01$. Groups: UC, unmanipulated control; VC, vehicle control; PC, positive control (inulin-treated); PDF, treated with *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

[Li *et al.,* 2021]. As a non-digestible compound in the small intestine, it is fermented in the colon, selectively promoting the growth of beneficial bacteria that produce SCFAs [Nakajima *et al.,* 2022]. This prebiotic effect plays a key role in metabolic regulation. Additionally, inulin has been associated with reduced postprandial glycemic and insulinemic responses, as it does not contribute to blood glucose upon ingestion [Lightowler *et al.,* 2018].

In the present study, different doses of inulin were evaluated to identify the best glycemic response during the OGTT (**Figure S1A** in Supplementary Materials). The results showed that the glycemic concentration in AUC for the maximum dose (50 mg/kg) was considerably lower than for the lower doses, though it remained quite close to the glycemic concentration reported for the VC group (**Figure S1B**).

Changes in body weight and food intake

The mice in the four groups had comparable average weight during the first week of the trial (**Figure 2A-C**). During the first month, there were significant differences between the UC and PDF groups' Δ body weight values, showing that the UC group had gained weight while the PDF group had lost weight. By the eighth week, there had been no discernible variations in weight among the four research groups.

In the food intake and their respective caloric intake *per* week, no significant differences ($p\geq0.05$) were observed between groups (**Figure 2D** and **2F**). But there was a significant difference in food intake when comparing the first week to the sixth week of the study ($p<0.001$) across all groups, with a variation of 23.78%. Similarly, calorie intake showed a significant increase over time, with a notable difference between the first and sixth weeks ($p<0.001$), resulting in an 18.5% variation during this period. Animal studies on body weight and nutritional intake have demonstrated that eating different forms of fiber had a stronger impact on obesity [Isken *et al.,* 2010]. In contrast, in the present study, mice did not have obesity, so the expected approach was to maintain body weight, as observed in all groups. Likewise, there were no differences in food intake between these. The addition of DF to diet may have an impact on the fluctuation in weight and dietary intake,

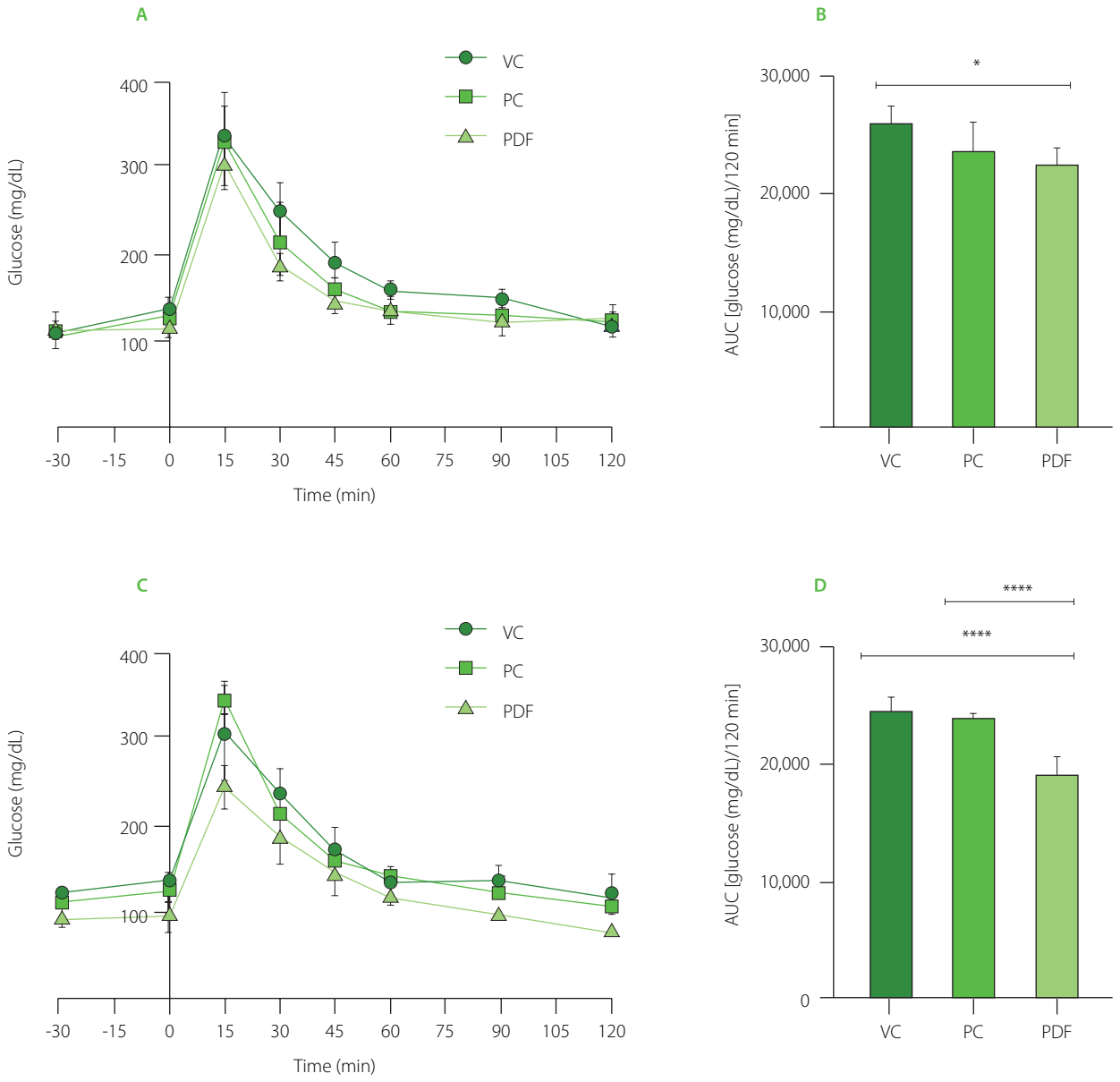

Figure 3. Oral glucose tolerance test (OGTT) results after 60 days of administration. (**A**) OGTT in preloading groups. (**B**) Area under the curve (AUC) of preloading groups. (**C**) OGTT in non-preloading groups. (**D**) AUC of non-preloading groups. Statistical analysis: one-way analysis of variance and Tukey's *post hoc* test. Significant differences: ****$p<0.0001$, *$p<0.05$. Groups: VC, vehicle control; PC, positive control (inulin-treated); PDF, treated with *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

even if the mice do not start the trial with altered weight. Mao *et al*. [2018] reported that large doses of fructo-oligosaccharides (FOS) (about 25% of diet) supplemented in non-obese mice resulted in dose-dependent body weight control. The animals also tended to reduce their food intake and exhibited soft, sticky feces. These effects have been related to intestinal microbiota modification promoted by the FOS prebiotic effect. Maintenance weight observed in all groups, including the PC, to whom inulin was administered, shows an effect like that reported by Zhang *et al.* [2018] regarding their control group made up of healthy rats administered with inulin (3 g/kg), after 12 weeks, the weight remained the same as in the group that was not administered with this DF.

Oral glucose tolerance test after 60 days of administration

Acute preloading of the solutions was done during OGTT at the end of the prolonged administration (**Figure 3A** and **3B**). The AUC showed that the PDF group's glucose levels were lower than the VC group's ($p<0.05$). In the preload-free curves, the PDF group had lower glucose levels than the VC group ($p<0.0001$) and the inulin-administered group ($p<0.0001$) (**Figure 3C** and **3D**). To determine the effects of the solution given before a high glucose load, the differences were evaluated between each experimental group's performance on the OGTT with and without preloading solutions. AUC glucose did not change substantially between the VC and PC groups, but when

a preload was not given, the level in the PDF groups was significantly lower (p<0.001) (244.60 mg/dL/15 min) *vs.* with preload (305.20 mg/dL/15 min) (**Figure 3A** and **3C**, respectively).

These results indicate that administering PDF for a prolonged period presented greater benefits in glycemic control after high sugar ingestion. According to these results, a meta-analysis has been reported on the benefits of glycemic control of chronic treatment with DF in patients with type 2 diabetes mellitus (T2DM), it reports a decrease in blood sugar, serum insulin, and fasting glycosylated hemoglobin [Mao *et al.,* 2021]. However, the evidence regarding the type of fiber's effectiveness in enhancing postprandial insulinemia and glucose response is inconsistent [Tsitsou *et al.,* 2023].

The antidiabetic potential of *P. dulce* fruit is understudied, with existing research highlighting the hypoglycemic effects of its ethanolic aril extracts. In diabetic rats, 30 days of treatment (300 mg/kg/day) improved glucose tolerance and reduced blood glucose, glycated hemoglobin, urea, and creatinine levels [Pradeepa *et al.,* 2013]. These effects were linked to inhibited glucose absorption, enhanced glycogen storage, and increased insulin secretion. In contrast, in this study, flour derived from dried *P. dulce* arils was used, obtained through dehydration without the addition or removal of any components. The resulting PDF primarily contains carbohydrates (57.44 g/100 g dm), dietary fiber (10.48 g/100 g dm), and protein (9.26 g/100 g dm), while also containing a notable amount of phenolics (**Table 1**).

The effects of this flour on mice may depend on the various compounds it contains. The metabolic state of the rodents plays a crucial role in how DF affects glycemic regulation. A meta-analysis found that FOS supplementation reduced fasting glycemia more significantly in conditions like obesity, T2DM, or a high-fat diet compared to healthy animals [Le Bourgot *et al.,* 2018]. In this study, despite using healthy mice, prolonged PDF consumption improved glycemic regulation, suggesting that these effects could be stronger under impaired glucose homeostasis. The response to the OGTT in the PC group was like that of the VC group, highlighting that inulin's beneficial effects on glucose regulation are most evident in altered metabolic states. Zhang *et al*. [2018] observed that inulin supplementation in diabetic rats enhanced the abundance of beneficial SCFAs-producing bacteria. However, in healthy rats, inulin had no effect on the diversity of the intestinal microbiota.

Furthermore, the greater effects observed in the PDF group compared to the PC group are attributed to the integrity of the food matrix in the PDF, which in addition to containing fermentable fiber fractions, was a rich source of phenolics. In contrast, the chicory inulin used in the PC group is a purified source. These results contrast with the findings reported by Fotschki *et al.* [2019], who compared the effects of chicory root flour and pure FOS in rats with necrotic colitis. In their study, greater benefits were found with the use of chicory root flour, due to its greater complexity in terms of fibers and bioactive compounds, which were associated with an increase in SCFAs production and improved intestinal integrity regeneration. These results highlight the importance of using whole dietary ingredients rather than purified supplements to regulate the intestinal microbiota and improve inflammatory conditions.

Insulin tolerance test

The ITT curves were performed during the last week of prolonged solution administration. As shown in **Figure 4A**, both the PC and PDF groups exhibited a progressive decrease in glucose at 30 min, while the VC group continued to decrease until 45 min. All three groups showed variable glycemic responses until their basal levels were restored at 120 min. In terms of the AUC (**Figure 4B**), no significant differences (p≥0.05) in glucose concentrations were observed. The ITT reflects insulin action by measuring the extent of glucose reduction after insulin administration. Since the half-life of insulin in mice is approximately 10 min, glucose reduction after 30 min may not be a direct effect of insulin, but rather a counterregulatory response involving hormones such as glucagon, catecholamines, and cortisol when glucose levels fall below 80 mg/dL [Ayala *et al.,* 2010]. Under normal conditions, increased blood glucose stimulates insulin secretion, which promotes glucose uptake by peripheral tissues and inhibits hepatic gluconeogenesis [Li *et al.,* 2022]. Despite differences in glycemic regulation observed in the PDF group, the homeostatic response to insulin was similar across all groups, likely due to the BALB/c strain's metabolic phenotype. This strain has been shown to maintain glucose tolerance and insulin sensitivity even under high-fat diet conditions [Benedé-Ubieto *et al.,* 2020].

Serum concentration of metabolic hormones

To determine the concentration of hormones that play an important role in glucose metabolism, serum samples were analyzed under fasting and postprandial conditions. In fasting, no significant differences (p≥0.05) were reported between the PDF group and the other groups (**Figure 5A**). GIP levels in the PDF group were lower in the postprandial state compared to the other groups, although the differences did not reach statistical significance. This trend suggests that *P. dulce* may attenuate the GIP response following food intake. This effect could be associated with the presence of phenolics and fermentable fiber in the fruit, which modulate hormonal responses through mechanisms such as delayed gastric emptying and interaction with the gut microbiota, impacting the release of incretins like GIP [Guccio *et al.,* 2022; Williamson, 2022]. However, a larger sample size is required to confirm these findings.

The insulin response in the postprandial state was significantly higher (p<0.05) compared to fasting conditions in the PC and PDF groups (**Figure 5B**), indicating greater insulin sensitivity. This effect was not observed in the control groups (VC and UC). As the solutions were not administered during the three days preceding sacrifice (in the PC and PDF groups), the serum samples collected for metabolic hormone analysis reflect cumulative effects from prolonged administration rather than acute responses to the solutions. These cumulative effects were likely attributable to the fiber and phenolics within the PDF matrix. The phenolic compounds present in PDF may have accumulated in tissues, exerting sustained antioxidant and anti-inflammatory

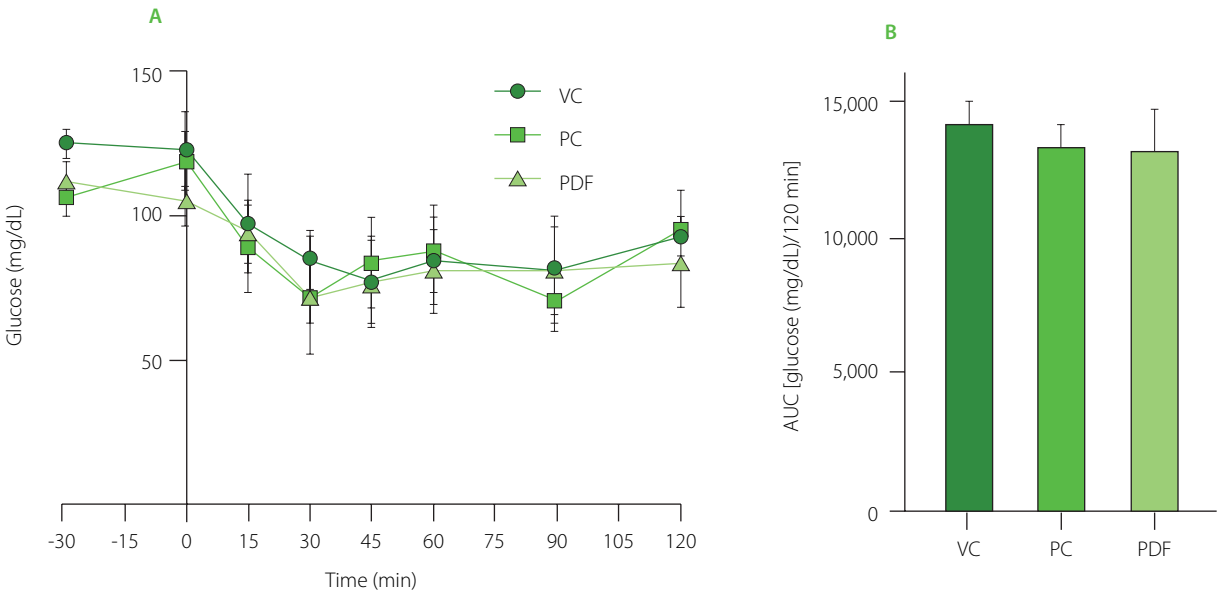

Figure 4. (**A**) Insulin tolerance test (ITT) results after 60 days of administration. (**B**) Area under the curve (AUC) of glucose concentrations during the 120-min ITT. Statistical analysis: one-way analysis of variance and Tukey's *post hoc* test. No significant differences ($p \geq 0.05$) in AUC between groups. Groups ($n=5$): VC, vehicle control; PC, positive control (inulin-treated); PDF, treated with *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

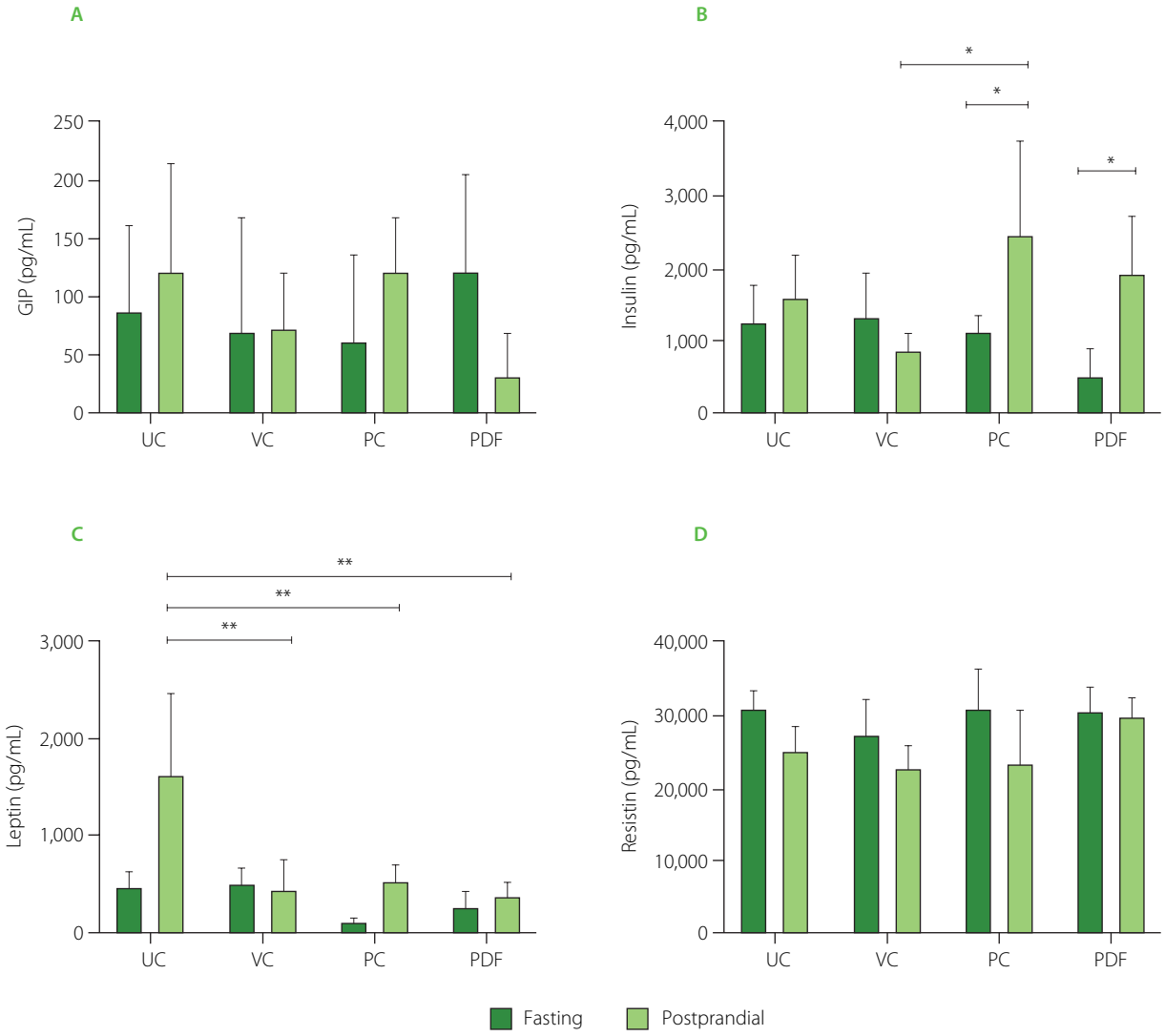

Figure 5. Serum concentrations of metabolic hormones: (**A**) Glucose-dependent insulinotropic polypeptide, GIP, (**B**) insulin, (**C**) leptin, (**D**) resistin. Groups ($n=4$) evaluated under fasting and postprandial conditions. Statistical analysis: one-way analysis of variance (ANOVA) and Tukey's *post hoc* test; resistin was analyzed using Kruskal-Wallis ANOVA and Dunn's *post hoc* test. Significant differences: $^{**}p<0.01$, $^{*}p<0.05$. Groups: UC, unmanipulated control; VC, vehicle control; PC, positive control (inulin-treated); PDF, treated with *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

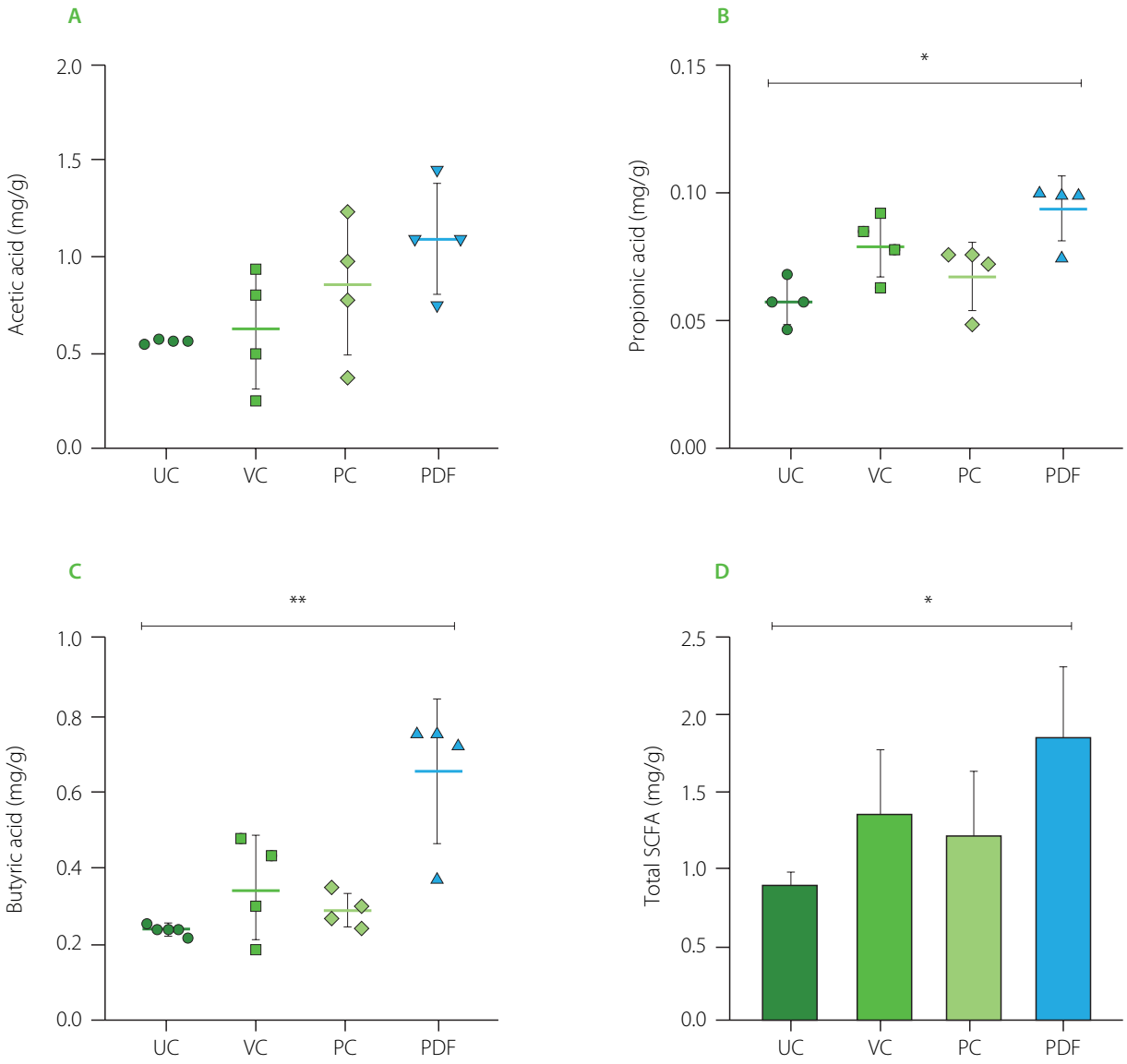

Figure 6. Short-chain fatty acid (SCFA) content in the cecum: (**A**) propionic acid, (**B**) acetic acid, (**C**) butyric acid. (**D**) total SCFA content by group. Statistical analysis: Kruskal-Wallis analysis of variance (ANOVA) and Dunn's *post hoc* test (**A–C**); one-way ANOVA and Tukey's *post hoc* test (**D**). Significant differences: $^{**}p<0.01$, $^{*}p<0.05$. Groups (n=4): UC, unmanipulated control; VC, vehicle control; PC, positive control (inulin-treated); PDF, treated with *Pithecellobium dulce* (Roxb.) Benth. fruit flour.

effects [Yahfoufi *et al.,* 2018]. Furthermore, phenolic compounds can influence the modulation of the expression of genes related to glucose metabolism, improving insulin sensitivity [Kang *et al.,* 2020]. It is possible that, despite the discontinuation of PDF consumption for four days, these compounds continue to have a modulatory effect on the metabolic pathways that regulate insulin and GIP levels.

Anthocyanins have demonstrated positive effects on glucose metabolism, including the neutralization of oxidative stress, prevention of apoptosis in pancreatic β cells, and regulation of genes involved in glucose transport, insulin sensitivity, and hepatic glucose production [Kang *et al.,* 2020]. Studies on mice and human 3T3-L1 adipocytes have shown that anthocyanins, such as cyanidin-3-*O*-glucoside, and their metabolites, such as protocatechuic acid, improve insulin sensitivity and promote glucose uptake by facilitating the translocation of GLUT-4 to the cell membrane. Furthermore, they positively regulate PPAR-γ and the adiponectin gene [Scazzocchio *et al.,* 2011]. PPAR-γ controls the expression of key proteins involved in glucose and fatty acid uptake, while adiponectin, produced by adipocytes, enhances insulin sensitivity. Together, these mechanisms play crucial roles in glucose and lipid metabolism [Den Besten *et al.*, 2015].

Regarding leptin levels, only the control group (UC) showed significantly higher ($p<0.01$) serum concentrations in postprandial conditions (**Figure 5C**), consistent with the body weight fluctuations observed in this group (**Figure 2C**). Leptin levels are influenced by adipose tissue reserves and food availability. In the postprandial state, gastric leptin secretion responds to food intake and is modulated by energy reserves [Mendoza-Herrera *et al.,* 2021]. The UC group's characteristics may reflect

stress from minimal experimental manipulation, causing maladjustment to standard conditions [Marin *et al.,* 2023]. Additionally, stress in mice can induce leptin resistance, leading to uncontrolled eating and weight gain [Patterson & Abizaid, 2013]. The reduced serum leptin levels in the postprandial condition observed in the PC and PDF groups are consistent with the findings of Den Besten *et al*. [2015] who also reported a decrease in serum leptin levels in animals directly treated with SCFAs. This reduction was attributed to the lower white adipose tissue mass in these mice. Despite the lower levels, which indicate reduced satiety signaling, food intake in SCFAs-treated animals did not increase, a pattern that aligns with the feeding behavior observed in the present study.

Serum resistin concentrations represent a relevant marker to evaluate the health status of the mice in the study, since the levels recorded in all groups remained within the physiological range of 40 to 80 ng/mL [Rajala *et al.,* 2003], without significant differences ($p \geq 0.05$) between them (**Figure 5D**). In murine models, resistin has been shown to function as an antagonist in insulin signaling, and its elevated levels are implicated in the development of insulin resistance [Steppan *et al.,* 2001]. Although the relevance of this relationship in humans remains a matter of debate, a recent meta-analysis indicated that serum resistin concentrations significantly correlate with insulin resistance in the contexts of obesity and T2DM [Su *et al.,* 2019].

Changes in gut microbial metabolites

Administration of PDF significantly altered SCFAs production, underscoring its prebiotic potential. Analysis of cecal contents revealed a notable increase in propionic acid levels ($p<0.05$) and a trend toward higher acetic acid levels in the PDF group compared to the unmanipulated control (**Figure 6A** and **6B**). Furthermore, butyric acid levels were significantly elevated in the PDF group compared to the UC group ($p<0.01$).

Fermentation occurs in the colon, particularly in regions with lower pH, such as the cecum, leading to higher SCFA contents. In the colon and stool, the typical molar ratio of acetic acid to, propionic acid, and butyric acid is 3:1:1, depending on microbiota composition, diet, and intestinal transit time [Nogal *et al.,* 2021].

Overall, total SCFA contents were significantly higher in the PDF group, showing notable differences compared to the UC group ($p<0.05$) (**Figures 6D**). This suggests enhanced fermentation of dietary fibers in PDF, likely driven by its composition rich in fermentable polysaccharides such as hemicelluloses [Preethi & Mary, 2016].

The increase in acetic acid in the intestine reflects the activity of bacteria such as *Bifidobacterium spp*., with this SCFA acting as a functional mediator in various systemic metabolic processes. Its increased availability in the body has been linked to improved insulin sensitivity through the activation of the FFAR2 receptor on pancreatic β-cells. Furthermore, acetic acid exerts an anti-inflammatory effect by inhibiting pro-inflammatory cytokines and enhancing the function of the intestinal barrier, thereby supporting metabolic homeostasis and protecting against inflammatory disorders [Fusco *et al.,* 2023].

The increase in propionic acid, associated with *Bacteroidetes*, further supports the observed metabolic benefits, as this SCFAs serves as a substrate for hepatic gluconeogenesis and stimulates enteroendocrine L cells to secrete GLP-1 and PYY, hormones essential for glucose regulation and appetite control [Psichas *et al.,* 2015].

Butyric acid was the metabolite that stood out with significantly higher concentrations compared to the UC group, suggesting modifications in the microbiota and improvements in intestinal integrity. Given that butyric acid is a key energy source for colonocytes and a modulator of epithelial inflammation, it plays a crucial role in maintaining intestinal integrity and promoting glucose homeostasis through FFAR3-mediated gluconeogenesis. This SCFA is primarily produced by *Faecalibacterium prausnitzii* and *Ruminococcus spp.* [Salvi & Cowles, 2021], whose activity may have been stimulated by the fermentable fractions of PDF.

These findings underscore the potential of PDF to modulate gut microbiota and enhance SCFAs production, offering promising health benefits, particularly in metabolic regulation. However, future research employing microbiome sequencing is crucial to precisely identify the bacterial shifts underlying these effects.

Goblet cells count in descending colon

PDF significantly increased goblet cells *per* crypt in the descending colon (**Figure 7**), suggesting a possible improvement in the epithelial barrier. This finding is consistent with that reported by Yang *et al*. [2024] who demonstrated that fermentable fibers such as arabinoxylan and resistant starch increase both the number of goblet cells and the thickness of the mucosa, favoring the integrity of the intestinal barrier. Similarly, the inulin-treated group (PC) showed a significant increase in these cells compared to UC and VC (**Figure 7A**). This aligns with the results reported by Corrêa *et al*. [2023] who associated inulin intake with remodeling of the colonic epithelium, promoting the proliferation and differentiation of intestinal stem cells, as well as the improvement of the intestinal barrier and mucin secretion.

The intestinal mucosal barrier is essential for preventing pathogen translocation, with goblet cells, which secrete mucus containing antimicrobial peptides, playing a key role in microbiota regulation and intestinal transit [Gustafsson & Johansson, 2022]. In this study, mice treated with PDF exhibited a significant increase in the number of goblet cells in the colonic crypts, possibly associated with the fermentation of dietary fibers and the production of metabolites such as butyric acid, which was found at higher concentrations in this group. These findings are consistent with previous studies highlighting the role of fermentable fiber as an energy substrate for colonocytes, promoting intestinal homeostasis [Fusco *et al.,* 2023]. Similarly, Hunt *et al.* [2021] reported that fiber consumption over 112 days in healthy mice increased intestinal weight, stimulated epithelial cell proliferation, and maintained intestinal integrity, in contrast to mice fed a fiber-deficient diet, underscoring its preventive potential in supporting intestinal health.

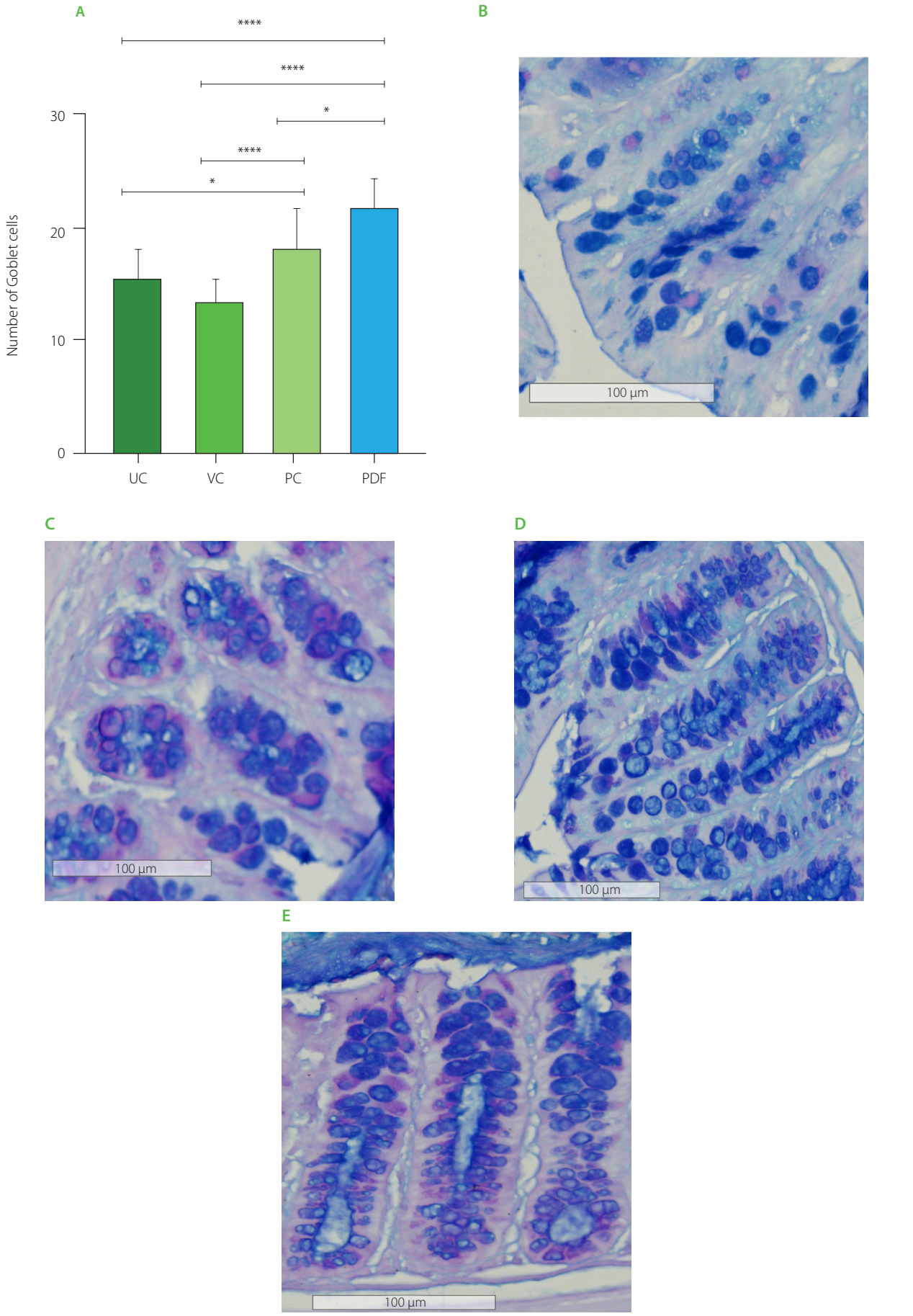

Figure 7. (**A**) Goblet cells count in the ascending colon. Histological staining with Alcian blue and hematoxylin in (**B**) unmanipulated control (UC), (**C**) vehicle control (VC), (**D**) positive control (PC), and (**E**) *Pithecellobium dulce* (Roxb.) Benth. fruit flour (PDF) groups (n=4). Statistical analysis: two-way analysis of variance and Tukey's *post hoc* test. Significant differences: ****p<0.0001, *p<0.05.

While promising, these results do not establish a direct link between PDF consumption and improved intestinal barrier integrity, as functional markers of barrier function were not assessed. Additionally, the absence of a taxonomic analysis of the microbiome limits the ability to pinpoint specific changes in its composition that might explain these effects. Nevertheless, the observed increase in SCFAs in the PDF group may be associated with certain bacterial phyla involved in fiber fermentation, hinting at potential interactions between PDF fiber and the intestinal microbiota.

Overall, the effects observed after prolonged PDF administration in healthy mice highlight the potential of its bioactive compounds to modulate SCFA production and potentially influence metabolic processes associated with intestinal health. However, these findings should be interpreted cautiously and serve as a foundation for further research to elucidate the underlying mechanisms and evaluate relevant functional markers.

The extrapolation of these results to humans is complex due to interindividual variability in the microbiota, influenced by factors such as diet, lifestyle, genetics, and medications [Nogal *et al.,* 2021]. Although SCFAs, such as butyrate and propionate, have shown benefits in preclinical models [De Vadder *et al.,* 2014; Psichas *et al.,* 2015], the evidence in humans is limited and sometimes contradictory [Shortt *et al.,* 2018]. Their effects, such as improving insulin sensitivity and intestinal integrity, largely depend on the interaction between diet, microbiota, and metabolic status [Ahrén, 2022]. Therefore, clinical trials are crucial to establish optimal dosages and assess their efficacy in humans, considering metabolic and digestive differences between species. These complexities underscore the need for broader research to understand the translational potential of PDF in human health.

CONCLUSIONS

This study highlights the preliminary functional potential of PDF as a dietary intervention to support glucose homeostasis, opening the door to future research on metabolic disorders. The observed metabolic effects suggest the need for further exploration of the pathophysiological mechanisms involved in glucose dysregulation, such as those seen in obesity, T2DM, and metabolic syndrome. However, the translational application of these findings to humans is complex due to the physiological and metabolic differences between murine models and humans. This underscores the need for more comprehensive research to adequately evaluate the potential of PDF in human health. By addressing these gaps, it may be possible to integrate *P. dulce* fruit consumption into clinical practice and develop functional products derived from this promising food source, but this requires further studies to validate its clinical applicability.

SUPPLEMENTARY MATERIAL

The following are available online at **https://journal.pan.olsztyn.pl/-Pithellobium-dulce-Roxb-Benth-Fruit-Flour-Intake-Enhances-Short-Chain-Fatty-Production,202003,0,2.html**; **Table S1**. Nutrient content of the standard diet Nutri-cubos brand Purina, Mexico. **Figure S1**. Glucose dose-response evaluation of the positive control (inulin). (A) Oral glucose tolerance test (OGTT) at low (6.25 mg/kg), medium (25.00 mg/kg), and high (50.00 mg/kg) doses. (B) Area under the curve (AUC). Statistical analysis: one-way analysis of variance and Tukey's *post hoc* test. Significant differences: ***$p<0.001$, **$p<0.01$, *$p<0.05$. VC, vehicle control.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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