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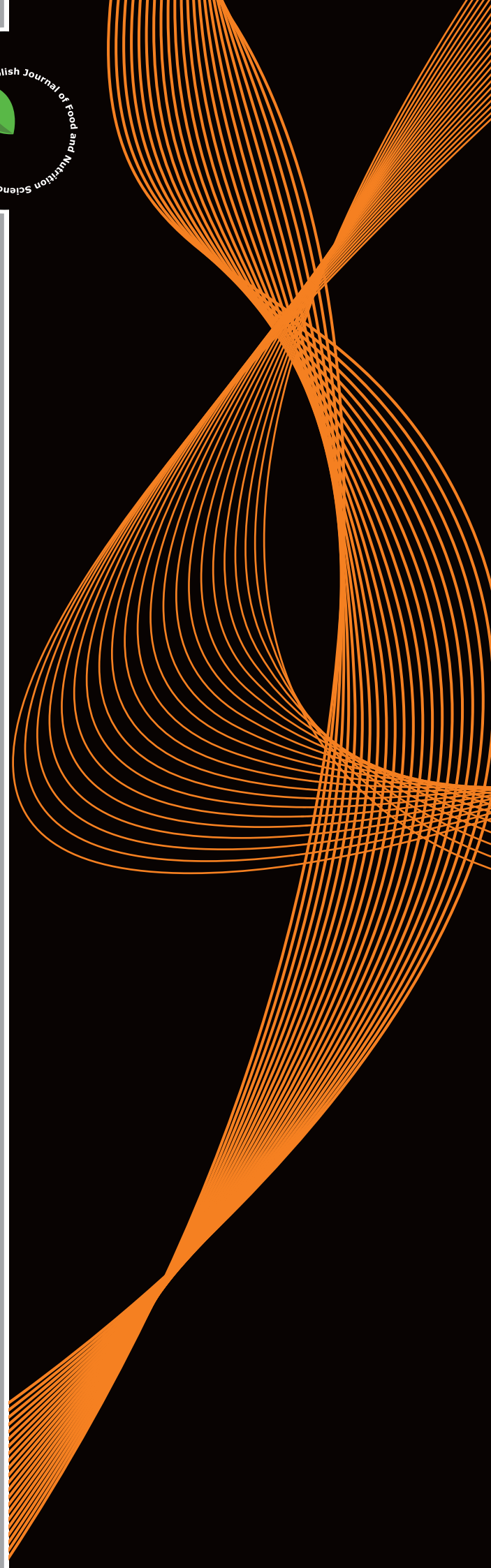

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CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
InLife Institute of Animal Reproduction and Food Research,
Polish Academy of Sciences,
ul. Trylińskiego 18, 10-683 Olsztyn, Poland
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Consequences of the Addition of Bread Making Improvers to Strong Flour-Based Formulations

Antonietta Baiano, Anna Fiore

Dipartimento di Scienze Agrarie, Alimenti, Risorse Naturali e Ingegneria (DAFNE), University of Foggia, 71122 Foggia, Italy

Bread improvers are used to enhance key attributes of bread quality that, in turn, affect consumer preference and acceptability. They are used to counterbalance the deficiencies of weak soft flours, while the effects of their addition to strength flours are controversial. For this reason, this study investigated the effects of adding gluten (replacing 2% of flour, *w/w*), lecithin (1% of flour, *w/w*), xylanase (0.01% of flour, *w/w*), ascorbic acid (0.02% of flour, *w/w*), and combinations of lecithin (1% of flour, *w/w*) with gluten (1% of flour, *w/w*) or ascorbic acid (0.02% of flour, *w/w*) on the quality of Manitoba flour breads. Xylanase gave bread with the darkest colour (*L** values of 52.5 and 59.3 for crust and crumb, respectively), the highest total phenolic content (TPC, 140.5 mg gallic acid/100 g dm) and quantity of crust (41.4%), as well as the lowest specific volume (1.98 mL/g) and overall sensory quality (6.0). The crumb pores of bread produced with xylanase had a shape closer to a perfect circle than the other types. Ascorbic acid allowed obtaining breads with the highest volume (2.78 mL/g), crumb cohesiveness (8.5), stickiness (1.5), and similar TPC (137.9 mg gallic acid/100 g dm) as the bred with the addition of xylanase. The control breads and those produced with combinations of lecithin and ascorbic acid exhibited the highest antioxidant capacity. The use of improver combinations almost never exerted synergistic effects on bread quality. Only the antioxidant capacity of these breads was higher than that of the samples in which the improvers were used alone. The overall sensory quality was significantly, positively correlated with specific volume, malty and freshly baked bread aroma with correlation coefficients above 0.8. According to the experimental data, the best improvers that can be conveniently added to a strong flour are those that influence the bread structural characteristics (increasing its volume and alveolation). Due to the positive relationship between the overall sensory quality and structural properties, the choice of an improver to be added to a strong flour in baking should fall on those additives that improve variables such as volume and alveolation.

Keywords: ascorbic acid, bread quality, gluten, lecithin, Manitoba flour, xylanase

INTRODUCTION

Historically, bread is one of the most widespread products, being consumed by 80% of the world's population under different formulations, processing, and shapes. In the 27 EU member states, the bread market is around 32 million tons. Despite of these considerable numbers, the consumption of the common bread is declining in the last years due to changes in eating patterns and the availability of bread enriched with functional ingredients [Mencin *et al*., 2023; Uriho *et al.,* 2024] and several bread substitutes [Angelino *et al*., 2020]. The key to bringing this product back into vogue is to improve its quality and/or consumer quality perception to effectively counteract the competition from its substitutes. The suggestion that improving bread quality is key to reviving its popularity aligns with the actual consumption trends, which show a growing preference for quality ingredients and products over quantity. Among the factors influencing

***Corresponding Author:**
e-mail: **antonietta.baiano@unifg.it** (Prof. A. Baiano)

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quality, the choice of ingredients directly affects product's texture and flavour.

The key attributes of bread include appearance, flavour, and texture that, in turn, affect acceptability, pleasantness, preference and willingness to consume. The crumb colour mainly depends on the ingredients used while that of crust is influenced by Maillard reactions and caramelisation, although the latter can be partially masked by the colour imparted by bread formulation [Martins *et al*., 2017]. These reactions are also responsible for the crust flavour compounds originating during baking, while fermentation and enzymatic reaction mostly affect intensity and quality of crumb flavour. In the end, loaf volume, crumb density, and crust and crumb structure determine biting properties and chewiness. All these attributes are very critical for the overall quality evaluation of bread. The enrichment of bread with functional ingredients must be carefully investigated since such compounds can determine undesired changes of textural, mechanical, and sensory properties. Uriho *et al.* [2024] studied the combined effects of encapsulated vitamin C and ω-3-rich sablefish oil on both the quality of bread and the stability of the bioactive ingredients. The combination of such compounds was able to both increase the retention of encapsulated vitamin C and decrease the lipid oxidation with respect to the non-combined forms while maintaining the textural properties and masking the fishy flavour.

The strategies implemented to enhance bread quality include the use of integrated bread baking improvers, *i.e.*, of ingredients such as oxidants, gluten-reducing agents, enzymes, emulsifiers, hydrocolloids, organic acids, and other food additives, selected based on their activity and synergistic effect within a wide range of compounds, and added to the bread formulation. The advantage of using integrated bread-making improvers is that they act during the entire bread-making process. Their use is widely spread to: standardize the technological quality of wheat flour; compensate for the deficiencies of weak flours and mitigate their negative effects on dough development and properties of the finished products. On the other hand, the effects deriving from the addition of improvers to strong flours have been little investigated. Improvers could negatively change the viscoelastic properties of strong flours with detrimental effects on loaf shape and crumb porosity, as a consequence of an excessive crosslinking that makes the dough too strong [Mohammadi *et al.*, 2015]. The strengthening effects of improvers, such as transglutaminase added to flours with strong gluten, may result in an undesirable bread hardening [Boukid *et al.*, 2018]. According to Boukid *et al.* [2018], the effects of improvers depend on the interaction between the type and level of improvers and flour strength level. As an example, the specific volume of strong flour based-bread increased with the addition of low concentrations of transglutaminase (0.1 g/100 g) and decreased at higher added levels due to an excessive crosslinking [Mohammadi *et al*., 2015]. Instead, vital gluten added to astrong flour induces a slight increase in springiness (due to better elasticity of the gluten network) and cohesiveness. The effects of other kinds of improvers have not been investigated.

In view of the above findings, this study aimed at investigating the effect of the addition of bread improvers on the quality of loaves produced with a strong Manitoba flour. Manitoba is a high-protein, high-gluten soft wheat flour originating from the province of Manitoba, Canada, from which it takes its name. It is known for its exceptional strength, water-absorbing capacity, and a strong gluten network, making dough elastic, stable, and suitable for long-leavening time. The choice fell on the following improvers: gluten because, by forming a network that envelops starch, it limits α-amylase access to starch, thus reducing the glycaemic index of the corresponding bread [Zeng *et al*., 2023]; lecithin, because it not only improves bread quality characteristics but also preserves product freshness [Codinã & Mironeasa, 2016]; xylanase, because it is able to release and transfer free water from pentoses to protein, increasing gluten hydration and, moreover, because the water-soluble arabinoxylans can stabilize gas cells [Mohammadi *et al*., 2022]; ascorbic acid, because of its documented ability to improve oxidative action in bread-making [Kiyashko & Sideltsev, 2022]; a combination of lecithin and gluten, selected because Mohamed *et al*. [2006] documented its ability to reduce degradation in maize starch but not in rice starch; and an unusual combination of lecithin with ascorbic acid (previous research used additives containing lecithin and ascorbic acid not alone but together with other improvers [Lambert-Meretei *et al*., 2010]). The comparisons among improvers were performed by investigating their effects on physiochemical, sensory, and structural parameters but also on the bread antioxidant content. This latter approach is less common, having been implemented by few researchers to date. For example, Hemalatha *et al*. [2012] investigated changes in nutraceutical and antioxidant properties of chapatis obtained by doughs supplemented with amylases and xylanase, observing an increase of soluble dietary fibre and phenolic compounds when the first enzyme was used. The possibility to homogenously group the bread samples according to the type of improvers added to the formulation was also explored.

MATERIALS AND METHODS

Bread formulation

The ingredients used in bread production included: Manitoba soft wheat flour type 0 free from additives (strength 350±10; tenacity/extensibility ratio equal to 0.6±0.1; COOP, Casalecchio di Reno, Italy), water, extra-virgin olive oil (Pazienza, Foggia, Italy), sodium chloride (Compagnia Europea Sali, Margherita di Savoia, Italy), and dehydrated *S. cerevisiae* yeast (Cameo, Desenzano del Garda, Italy). The bread improvers used were as follows: gluten (Elgranero, Madrid, Spain), soy lecithin (Céréal, Lainate, Italy), food-grade xylanase (10,000 U/g), Vland Biotech Group, Qingdao, China), and ascorbic acid (Balducci, Faenza, Italy).

A control (without improvers) and six bread types obtained by adding one or two improvers were produced according to the formulations described in **Table 1** and appeared as in **Figure 1**. As can be inferred, the volume of water requested by the various formulations was the same, giving doughs of comparable consistency. The quantity of each improver has been calculated as

Table 1. Bread formulations (amount of ingredients in g).

Ingredient	B_control	B_gluten	B_lecithin	B_xylanase	B_ascorbic acid	B_asc_lec	B_glu_lec
Manitoba flour	500	490	495	499.95	499.90	494.90	485
Water	350	350	350	350	350	350	350
Extra virgin olive oil	40	40	40	40	40	40	40
Sodium chloride	12	12	12	12	12	12	12
Dehydrated *S. cerevisiae*	7	7	7	7	7	7	7
Gluten	–	10	–	–	–	–	10
Soy lecithin	–	–	5	–	–	5	5
Xylanase	–	–	–	0.05	–	–	–
Ascorbic acid	–	–	–	–	0.10	0.10	–

B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread soy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic acid, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively).

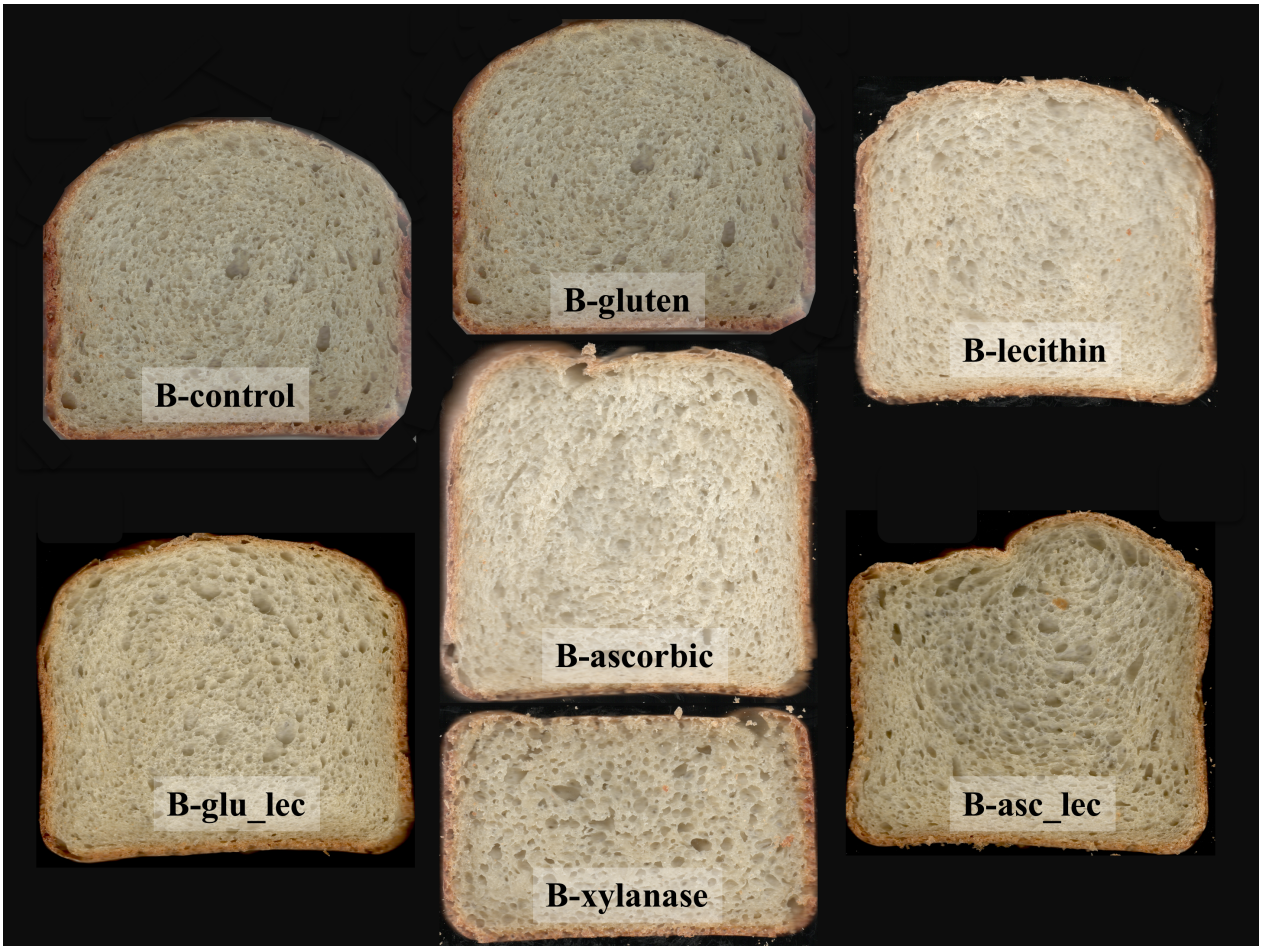

Figure 1. Appearance of bread slices. B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread soy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively).

the percentage of the flour weight of the control bread and used to replace the same amount of flour: gluten, 2%; soy lecithin, 1%; xylanase, 0.01%; ascorbic acid, 0.02%; ascorbic acid plus soy lecithin, 0.02% and 1%, respectively; gluten plus soy lecithin, 2% and 1%, respectively. The percentages represent the average values indicated by the improver suppliers. Three technological replicates were produced in a randomized order for each bread type. Loaves of regular shape were produced as described by

Baiano *et al*. [2023] using a Zero-Glu Pro bread-making machine (Imetec, Azzano S. Paolo, Italy) through the following steps: mixing the powdered ingredients (except salt); adding water and, after 2 min, adding the salt; kneading for 22 min; leavening for 40 min; stirring for 5 s; leavening for 73 min; stirring for 5 s; leavening for 50 min; and baking for 47 min at a temperature of 180°C.

Analyses of breads

Physical and chemical analyses

To evaluate the bread chromatic characteristics, the loaves were cut using an electric slicer into 1 cm-thick slices, and slice images (resolution 1,200 dpi) were acquired using an Epson scanner (mod. XP-3100, Cinisello Balsamo, Italy) and saved in the tiff format. The free ImageJ software ver. 1.52a (Bethesda, MD, USA) was used to process the acquired images according to Baiano *et al*. [2023]. The crust and crumb colours were expressed as follows: *L** (lightness/brightness), ranging from black to white on a 0–100 scale; *a**, with negative and positive values corresponding to green and red colours, respectively; *b**, with negative and positive values corresponding to blue and yellow colours, respectively. An image-based colour calibration was performed using a standard colour calibration chart [Sunoj *et al*., 2018].

Moisture and ash quantification were performed following the AACC International methods 44-15.02 and 08-01.01, respectively, and expressed as g/100 g of bread [AACC, 2012].

The bread phenolic extracts were obtained according to method described by Baiano *et al*. [2023]. More precisely, for each type of bread, the percentage of crust in relation to the total weight of the loaf was quantified. That percentage ranged from 28 to 43%. Then, the bread samples to be submitted to the phenolic extraction were prepared by mixing the crust and crumb in the correct proportions. One g of each bread sample prepared in that manner was added to 30 mL of a hydroalcoholic solution (58% ethanol, *v/v*). The obtained suspension was first sonicated (37 kHz, 30°C, 30 min), then centrifuged (2,000×*g*, 25 min, 20°C), and the supernatant was recovered and filtered through a nylon filter (0.45 µm).

The total phenolic content (TPC) was quantified using the Folin–Ciocalteu reagent [Almeida da Rosa *et al*., 2017]. Briefly, 150 µL of diluted phenolic extracts or 58% (*v/v*) ethanol (blank) were mixed with 7,500 µL of distilled water and 750 µL of Folin–Ciocalteau reagent. After 3 min, 2,250 µL of 15% sodium carbonate and 4,350 µL of distilled water were added, and the resulting mixture was incubated for 2 h, at 25°C, in the dark. The absorbance was read at 765 nm. A calibration curve for gallic acid was prepared in parallel. The results were expressed as mg of gallic acid equivalents *per* 100 g of bread dry matter (dm).

The phenolic profile of the extracts was analysed by high-performance liquid chromatography with diode array detector (HPLC-DAD) system (Agilent 1100 Liquid Chromatograph, Santa Clara, CA, USA) using a 100×4.6 mm, 3 µm particle size, RP-C18 Gemini column (Phenomenex, Aschaffenburg, Germany) and the following separation conditions: column temperature at 30°C; injection volume 100 µL; flow rate 1 mL/min; mobile phase solvent A (1.0% acetic acid in water, *v/v*) and solvent B (50% methanol, 50% acetonitrile, *v/v*) applied in a linear gradient from 5% to 30% B in 25 min, from 30% to 40% B in 10 min, from 40% to 48% B in 5 min, from 48% to 70% B in 10 min, from 70% to 100% B in 5 min, 100% B for 5 min, return to the initial conditions in 10 min, and column equilibration for 12 min [Baiano *et al*., 2023]. Retention times and spectra of extract phenolic compounds were compared to those of pure standards. Quantification (mg/100 g bread dm) was performed by comparing the peak areas of extract phenolic compounds calculated at 280 or 320 nm with those of the standard curves.

The antioxidant capacity of breads was measured through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [Brand-Williams *et al*., 1995]. The diluted phenolic extracts (0.1 mL) were added to 3.9 mL of a 6×10^{-5} M methanol DPPH radical solutions. The absorbance at 515 nm was measured at 0 min, 1 min and every 15 min until the reaction reached a plateau. The results were expressed as mmol of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents *per* 100 g of bread dm.

Determination of structural properties

The following structural characteristics were determined starting from the slice images acquired and processed as already described: minimum and maximum slice height (cm); pore density (number of pores/mm^2); average pore size (mm^2); porosity %, *i.e.*, the surface of the slice occupied by pores; pore circularity, calculated as 4π*area/perimeter^2 (it ranges between 1.0 and 0.0, with a value of 1.0 indicating a perfect circle and value near to 0.0 indicating increasingly elongated shapes). The specific volume (mL/g) was determined as the ratio between volume and weight of whole (crust+crumb) loaves. The loaf volume was measured according to rapeseed displacement using AACC method 10-05.01 [AACC, 2010]. The crust % was also evaluated multiplying by 100 the ratio between the weight of the crust withdrawn from a loaf and the total weight of the same bread loaf.

Sensory analysis

A quantitative descriptive analysis (QDA) was carried out according to Baiano *et al*. [2023] by a trained panel made of 10 judges (5 women and 5 men) between 20 and 65 years of age, with long experience in the sensory evaluation of baked foods. The profile sheet included the following attributes: visual (colour and thickness of crust; colour, pore size, and development of crumb); olfactory (overall, freshly baked bread, wheat, and malty aroma on crust and crumb together; toasty aroma on crust; yeast aroma on crumb); gustatory (sweetness, saltiness, and sourness of crumb), and tactile (hardness and crispiness of crust; resistance to chewing, cohesiveness, graininess, stickiness of crumb). Panellists were also asked to evaluate the overall sensory quality of each bread, *i.e.*, the comprehensive quality of the sample evaluated by considering all the sensory attributes. The intensity of each parameter was evaluated on a 0–9 scale.

Statistical analysis

Each analysis was replicated three times, except for the image analysis, with five acquisitions for each sample. The means

and the standard deviations were calculated. Analysis of variance (ANOVA) followed by least square difference (LSD) test was applied at $p<0.01$ to highlight any statistically significant differences induced by the addition of the improvers on each variable. Principal component analysis (PCA) was used to evaluate if the experimental data allowed the homogeneous grouping of breads according to the improver used. Pearson correlation coefficients at $p<0.01$ were applied to highlight any significant correlations among pairs of bread sample variables. The statistical analyses were performed using Statistica for Windows ver. 7.0. (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Physiochemical properties

The physiochemical characteristics of the breads produced from formulations including improvers are reported in **Table 2** and compared to those of the control bread. The type of improver influenced the colour coordinates of bread, with xylanase that made the colour of crust and crumb darker compared to the colour of the control bread and to the colour imparted by the other additives. This may be a consequence of the hydrolysis of the β-1,4 glycosidic bonds of arabinoxylan and the release of reducing sugars, which increases the extent of Maillard reaction [Wang *et al*., 2018]. However, it was opposite to what was highlighted in the whole-wheat bread by Ghoshal *et al*. [2013], who observed a brighter colour compared to that of bread prepared without enzyme despite of the high quantities of enzyme added (from 0.3 to 0.6 g/100 g of flour). The addition of xylanase also increased the intensity of crumb red and yellow hues, probably due to the changes induced in the structural characteristics as described subsequently. The highest values of crust a^* and b^* detected in lecithin-supplemented bread were due to the thermal transformation of lecithin during baking with production of four pyridinium compounds whose formation mechanism involves a pseudo-Maillard re-arrangement reaction [Fujimoto *et al*., 2021]. In agreement with Yeşil & Levent [2022], the highest crumb L^* values were measured on bread supplemented with lecithin alone or combined with gluten, depending on the ability of lecithin to act as a natural antioxidant. Crust a^* values and crumb a^* and b^* values detected on bread supplemented with ascorbic acid were indicative of its ability to act as a colour stabilizer.

After baking, the breads supplemented with lecithin or ascorbic acid showed the lowest crust moisture values (**Table 2**), but this common result can be explained by different mechanisms of action of these improvers. In the case of bread supplemented with lecithin, the binding of emulsifiers with starch granules prevented moisture migration from crumb to crust during baking [Tebben *et al*., 2022]. Regarding the effect of ascorbic acid, its addition was responsible for the decrease in vaporization temperature because of the reduced interaction of the tightly bound water with crust bread components [Kerch *et al*., 2012]. Instead, when the two improvers were applied together, crust was able to retain the highest moisture percentage, which is in agreement with findings reported by Latif *et al*. [2005]. Regarding crumb, it is well known that the greater the loss of water during baking, the quicker the bread ages and stales [Kotoki & Deka, 2010]. Bread supplemented with ascorbic acid alone or together with lecithin had the lowest crumb moisture since, during baking, the interaction of lecithin with starch delayed water absorption and granule swelling while the inclusion of ascorbic acid resulted in the already cited reduction in vaporization temperature [Codină & Mironeasa, 2016; Kerch *et al*., 2012]. The control bread showed the highest crumb moisture (24.5 g/100 g). Crumb moisture content was not affected by gluten as highlighted by the absence of significant difference ($p\geq0.01$) with respect to the control, which is in agreement with the findings of Curti *et al*. [2014]. The ash contents ranged from 2.98 g/100 g of xylanase-supplemented bread to 3.13–3.14 g/100 g of breads supplemented with combinations of ascorbic acid with lecithin or gluten, whose greater ash content depended on the contribution of minerals from the last two ingredients.

The effect of the addition of bread improvers on the content of antioxidants is of particular interest as it is a poorly investigated subject. The control bread, together with that supplemented with the mixture of gluten and lecithin showed the lowest TPC (**Table 3**). The total phenolic content was increased by

Table 2. Physiochemical characteristics of breads with and without improvers.

Bread type	Crust			Crumb			Crust moisture (g/100 g)	Crumb moisture (g/100 g)	Ash (g/100 g)
	L^*	a^*	b^*	L^*	a^*	b^*			
B_control	57.0±1.4^{a}	16.6±1.0^{bc}	47.0±0.4^{b}	69.2±1.0^{ab}	2.6±0.4^{bc}	29.4±0.8^{d}	10.5±0.9^{b}	24.5±0.1^{a}	3.08±0.00^{b}
B_gluten	56.7±1.0^{ab}	18.1±0.7^{b}	47.7±1.5^{ab}	69.8±1.9^{ab}	2.9±0.1^{b}	32.9±0.9^{c}	10.4±0.8^{b}	24.2±0.2^{a}	3.06±0.03^{b}
B_lecithin	57.4±1.6^{a}	20.3±1.0^{a}	49.7±0.3^{a}	71.9±0.5^{a}	2.4±0.6^{bc}	28.8±1.7^{cd}	7.7±1.1^{c}	19.0±1.0^{cd}	3.04±0.03^{b}
B_xylanase	52.5±1.3^{b}	17.0±0.9^{bc}	47.8±1.8^{ab}	59.3±0.7^{d}	4.8±0.3^{a}	37.3±0.1^{a}	10.7±0.5^{b}	23.2±0.3^{b}	2.98±0.00^{d}
B_ascorbic acid	57.9±0.8^{a}	15.8±0.5^{c}	46.5±0.1^{b}	67.6±1.0^{bc}	2.0±0.5^{c}	28.6±0.2^{d}	7.1±1.0^{c}	18.0±0.5^{d}	3.01±0.00^{c}
B_asc_lec	58.9±0.7^{a}	19.8±1.0^{a}	46.0±0.8^{b}	65.5±1.3^{c}	2.5±0.5^{bc}	32.5±1.5^{c}	12.3±0.2^{a}	17.3±0.6^{d}	3.13±0.01^{a}
B_glu_lec	57.2±0.5^{a}	17.8±0.8^{b}	45.5±1.0^{b}	72.6±0.5^{a}	2.5±0.6^{bc}	35.1±0.5^{b}	10.8±0.0^{b}	20.8±0.4^{c}	3.14±0.02^{a}

Results are shown as men ± standard deviation. In column, different letters indicate significant differences at $p<0.01$. B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread soy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic acid, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively).

Table 3. Total phenolic content (TPC), content of individual phenolic compounds, and antioxidant capacity (AC) of breads with and without improvers.

Bread type	TPC (mg gallic acid/100 g dm)	Individual phenolic compound content (mg/100 g dm)					AC (mmol Trolox/100 g dm)
		Gallic acid	4-Hydroxybenzoic acid	Ferulic acid	*p*-Coumaric acid	Caffeic acid	
B_control	90.6±1.0^{d}	2.8±0.1^{bc}	nd	nd	2.7±0.0^{a}	nd	0.628±0.020^{a}
B_gluten	103.8±2.0^{c}	3.1±0.1^{b}	nd	nd	2.7±0.0^{a}	nd	0.568±0.010^{b}
B_lecithin	114.4±4.1^{b}	2.8±0.1^{bc}	nd	nd	2.6±0.1^{ab}	nd	0.460±0.057^{c}
B_xylanase	140.5±2.5^{a}	3.1±0.2^{b}	nd	nd	2.5±0.0^{b}	nd	0.599±0.050^{ab}
B_ascorbic acid	137.9±1.3^{a}	7.5±0.6^{a}	2.5±0.7	nd	nd	nd	0.159±0.040^{d}
B_asc_lec	104.6±2.1^{c}	2.7±0.0^{c}	nd	2.4±0.6	2.6±0.0^{ab}	0.1±0.0	0.617±0.031^{a}
B_glu_lec	87.5±1.6^{d}	2.6±0.2^{c}	nd	nd	2.6±0.0^{ab}	nd	0.463±0.085^{c}

Results are shown as mean ± standard deviation. In column, different letters indicate significant differences at p<0.01. B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread soy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic acid, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively); dm, dry matter; nd, not detected.

the treatment with xylanase, due to the hydrolysis of arabinoxylans and the higher release of reducing sugars (able to react with the Folin-Ciocalteu reagents) and bound phenolic compounds, and by the addition of ascorbic acid, able to protect oxidizable molecules such as phenolic and flavour compounds and to reduce the *o*-quinones generated through the reaction catalysed by polyphenol oxidase [Chen *et al*., 2019; Landi *et al.*, 2013]. **Table 3** also shows the phenolic compounds detected in control and supplemented breads. Gallic acid was the major phenolic compound in all bread samples. As observed by Meral & Köse [2019], its content increased during fermentation and baking. Bread with ascorbic acid showed the highest content of gallic acid and was the only bread type containing 4-hydroxybenzoic acid. Probably, the overall phenolic content of that bread was preserved by ascorbic acid because this compound acts as a synergistic antioxidant, regenerating oxidized phenolic antioxidants [Nahas, 2012]. However, it was the only bread whose *p*-coumaric acid content was under the detection limit. The lowest gallic acid contents (2.6–2.8 mg/100 g) were detected in breads produced with lecithin alone or in combinations with ascorbic acid or gluten, probably as a consequence of the ability of phenolic compounds to protect lecithin against oxidation [Mazaletskaya *et al*., 2024; Saleh *et al*., 2022]. Bread supplemented with the combination of ascorbic acid and lecithin was the only one that contained ferulic and caffeic acids, probably because they were regenerated by ascorbic acid [Alemán *et al*., 2015; Vijayalakshmi *et al*., 2014]. At the same time, bread with ascorbic acid and soy lecithin showed a low total phenolic content as a consequence of the lower gallic acid content. Antioxidant capacity ranged from 0.159 mmol Trolox/100 g dm in bread supplemented with ascorbic acid to 0.628 mmol Trolox/100 g dm in the control bread. Antioxidant capacity values were positively correlated with the content of *p*-coumaric acid (correlation coefficient, r=0.913) and negatively correlated with gallic acid (r=–0.883) content.

Structural properties

Bread structure was significantly influenced by the type of improver added to the formulation. The first interesting parameter is the incidence of the crust weight on the total weight, whose percentages were in the following increasing order: 29.6%, control bread; 31.4%, bread with ascorbic acid and lecithin; 32.5%, bread with gluten; 33.5%, bread with ascorbic acid; 34.6%, bread with gluten and lecithin; 35.5%, bread with lecithin; 41.4%, bread with xylanase. These data must be read together with those relating to the development of the loaves during leavening, since the incidence of the crust increased as the specific volume (**Figure 2**) decreased (r=–0.802). The importance of measuring the bread specific volume relies on the evidence that it can be considered a good predictor of bread firmness [Eduardo *et al*., 2014]. In other words, the greater the specific volume, the softer the crumb. The lowest specific volume detected for xylanase-supplemented bread was opposite to the findings of previous research. Jaekel *et al*. [2012] stated the absence of significant differences among the white breads supplemented with that enzyme in concentrations between 0 and 12 g/100 kg flour. Ahmad *et al.* [2014] described a larger specific volume of bread with xylanase. They observed that brans absorbed large amount of water, making it less available for gluten proteins and resulting in a reduced gluten network development. Xylanases are able to hydrolyse soluble and insoluble pentosans, facilitating the release of free water that becomes available for a proper gluten development, thus resulting in a higher loaf volume. However, according to Ahmad *et al*. [2014], the excessive breakdown of starch may have negative effects, as excessively leavened doughs collapse during baking leading to a decreased loaf volume. Another reason that could explain the unusual behaviour of xylanase-added bread could be a low amount of arabinoxylans in the Manitoba flour so that, although the correct dose of enzyme was used, it resulted in an overdosage. Bread supplemented with ascorbic acid had the highest specific volume, with an increase of almost 6%

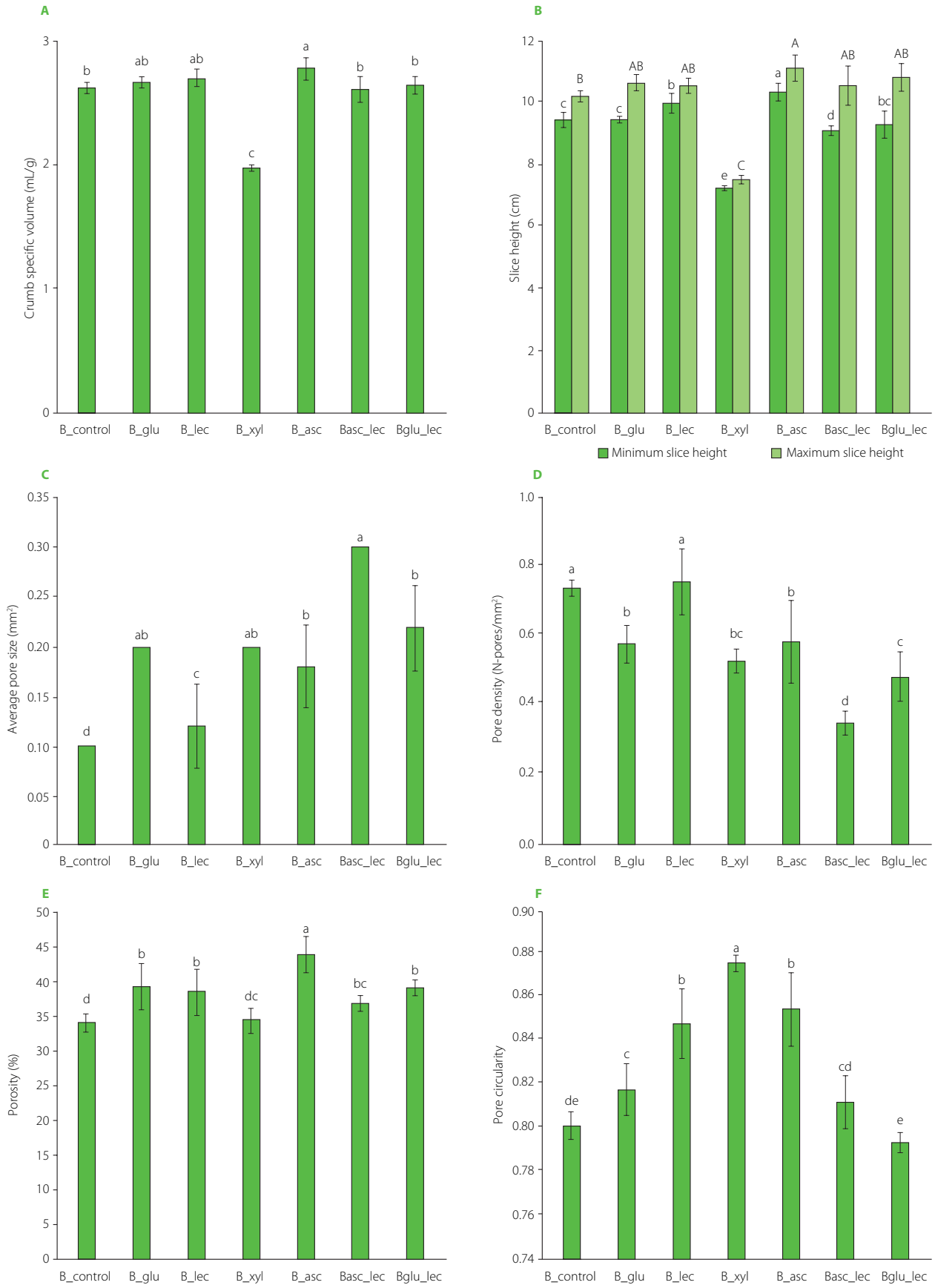

Figure 2. Structural characteristics of breads with and without improvers: (**A**) crumb specific volume, (**B**) slice height (minimum and maximum), (**C**) average pore size, (**D**) pore density, (**E**) porosity, and (**F**) pore circularity. Different letters above bars indicate significant differences at $p<0.01$. B_control, bread without improvers; B_glu, bread with gluten (replacing 2% of flour, *w/w*); B_lec, bread soy lecithin (1% of flour, *w/w*); B_xyl, bread with xylanase (0.01% of flour, *w/w*); B_asc, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively).

compared to the control bread. This effect is due to the ability of dehydroascorbic acid formed during mixing to oxidize gluten sulfhydryl groups producing the strong disulphide bonds that further stabilize the dough obtained from Manitoba flour [Koehler, 2003]. The supplementation with gluten or lecithin or their combinations did not increase the specific volume with respect to the control bread, contrary to literature reports [Tebben *et al*., 2022]. However, the improving effect of the two substances was generally observed in breads obtained from low- or medium-strength flours.

Consistently with the specific volume, breads supplemented with xylanase and ascorbic acid had the lowest and highest loaf height, respectively (**Figure 2**); the correlation coefficient of the specific volume was equal to 0.971 with the minimum loaf height and equal to 0.988 with the maximum loaf height. These data should be considered together with the loaf shape. As can be inferred from **Figure 1**, the control bread and those supplemented with gluten, lecithin, and gluten+lecithin had a regular shape with the highest point corresponding to the central part of the loaf. The shape of bread with ascorbic acid was also regular but slightly flatter than the others and, therefore, with a minimal difference between the highest and lowest points. The formulation with xylanase gave breads poorly developed and slightly concave in shape. The bread produced with ascorbic acid and lecithin showed an irregular shape with a partially collapsed portion to highlight the absence of synergistic effects of the two improvers when added to a strong flour contrary to what was reported by El Halal *et al*. [2018] in the case of using a common soft wheat flour.

The results concerning pore density and average pore size (**Figure 2**) must be discussed together since they highlighted a significantly high negative correlation (r=–0.983). Pore size is generally considered as an index of structural damage, with large size related to a weak gluten network or to an extended damage of the gluten skeletal framework of pore walls [Polaki *et al*., 2010]. Based on this statement, it would be expected to find larger pore sizes in poorly developed loaves. Instead, in our research, the correlation between pore size and specific volume at a r=–0.134 was insignificant (p<0.01). The control bread showed the most compact crumb, having the lowest pore size paired with the highest number of pores *per* mm^2. Compared to the control, lecithin did not improve the bread structure probably because of its addition to a strong flour. When added to a common wheat flour, lecithin determines the formation of a higher number of smaller pores [Garzón *et al*., 2018]. The bread with ascorbic acid+lecithin showed a lower number of larger pores because ascorbic acid strengthens the gluten network increasing the gas structure-retaining ability, while lecithin increases dough extensibility, thus allowing the expansion of carbon dioxide.

Porosity represents a measure of the incidence of the void space (pores) on the total loaf volume and was, therefore, quite well correlated with the specific volume (r=0.623). Its values ranged from 34.93% for the control bread to 45.09% for the bread supplemented with ascorbic acid (**Figure 2**).

In general, pore circularity is considered another attribute able to highlight structural damages. It is generally accepted that fresh breads have ellipsoidal pore shapes while the pores of damaged structures appear rounder because of the destruction of the gluten skeletal framework of pore walls [Polaki *et al*., 2010]. Our research partially confirmed this finding, since the formulations including xylanase gave breads with the roundest pores, but the more elongated pores were observed in bread with gluten+lecithin and in the control bread but not in breads supplemented with ascorbic acid or lecithin, as expected (**Figure 2**). The value of the correlation coefficient (r=–0.523) objectively quantified this behaviour.

Sensorial properties

Although a high specific volume and a uniform pore distribution are among the priority requirements in a bread loaf due to their close relationship with crumb firmness, the choice of the improver must rely not only on structural features but also on visual, olfactory and gustatory characteristics since they determine the overall sensorial quality and, finally, improvers can enhance some quality parameters but make others worse [Tebben *et al*., 2022]. The results of the sensorial evaluation are listed in **Table 4**.

The colour of crust was affected only by the addition of xylanase that made it darker (**Table 2**). Moreover, the visual colour evaluation (**Table 4**) was highly and negatively correlated with the instrumental measurement of lightness (r=–0.796). Similarly, only the addition of xylanase affected the sensorial evaluation of crust thickness by increasing it, consistently with the crust % (r=0.609). Except for the breads supplemented with gluten or lecithin, the addition of improvers significantly changed crumb colour with respect to the control, showing the following increasing colour intensity: ascorbic acid < xylanase < ascorbic acid+lecithin < gluten+lecithin. The visual crumb colour was positively correlated with the instrumental b^* values (r=0.649).

The human perception of crumb pore size (**Table 4**) was not correlated with its objective evaluation performed through the image analysis technique, but it was highly correlated with the specific volume (r=–0.953), with the smallest and the largest pores visually detected in bread supplemented with gluten and xylanase, respectively. Crumb development, whose lowest score was attributed to the bread supplemented with xylanase, was negatively correlated with crust % (r=–0.680) and positively correlated with the specific volume (r=0.724).

There are few studies, generally rather dated, focused on the influence of the interactions between flour components and baking improvers on bread flavour, which is known for its remarkable influence on consumer choices. Umelo *et al*. [2014] evaluated the effects of different improvers/techniques (ascorbic acid, ethylene dough conditioner, egg, azodicarbonamide, and screw thread kneading) on the bread sensory properties and observed that the highest score was assigned to the flavour of bread supplemented with an egg improver as a consequence of the greater extent of Maillard reaction. A study on the effects of five bread improvers (four lipase enzymes and diacetyl tartaric esters of mono-glycerides (DATEM) emulsifier) highlighted

Table 4. Sensorial characteristics of breads with and without improvers.

Sensory characteristics			B_control	B_gluten	B_lecithin	B_xylanase	B_ascorbic acid	B_asc_lec	B_glu_lec
Visual	Crust	Colour	1.0±0.0^{c}	2.0±0.5^{b}	2.0±0.3^{b}	4.0±0.0^{a}	2.0±0.5^{b}	2.0±0.0^{b}	2.3±0.8^{b}
		Thickness	3.5±1.0^{b}	3.0±1.0^{b}	2.5±1.0^{b}	5.0±0.0^{a}	3.5±0.5^{b}	2.5±1.0^{b}	3.5±0.5^{b}
	Crumb	Colour	1.0±0.0^{e}	1.0±0.1^{e}	1.0±0.2^{e}	2.0±0.0^{c}	1.5±0.0^{d}	2.5±0.1^{b}	3.0±0.2^{a}
		Pore size	3.0±0.5^{bc}	2.0±0.2^{d}	2.5±0.5^{cd}	6.0±1.0^{a}	2.5±0.4^{cd}	3.5±0.0^{b}	2.5±0.6^{cd}
		Development	8.5±0.4^{a}	6.0±1.0^{bc}	8.0±0.5^{a}	5.0±0.3^{c}	7.5±0.2^{ab}	8.0±0.5^{a}	8.3±0.5^{a}
Aroma	Crust and Crumb	Overall intensity	4.5±0.5^{ab}	5.0±0.6^{ab}	5.5±0.4^{a}	5.0±0.2^{ab}	4.5±0.3^{ab}	5.0±0.4^{ab}	4.0±0.1^{b}
		Freshly baked bread	5.0±0.0^{a}	5.0±0.0^{a}	4.0±0.2^{b}	1.0±0.0^{d}	3.5±0.4^{b}	3.5±0.2^{b}	2.8±0.1^{c}
		Wheat	1.5±0.7^{c}	2.0±0.4^{bc}	2.5±0.2^{bc}	3.0±0.1^{a}	2.5±0.2^{ab}	3.0±0.2^{a}	2.5±0.3^{ab}
		Malty	1.0±0.0^{a}	1.0±0.1^{a}	0.0^{b}	0.0^{b}	0.0^{b}	1.0±0.0^{a}	0.5±0.3^{ab}
	Crust	Toasty	1.0±0.0^{a}	1.5±0.2^{a}	1.5±0.2^{a}	1.0±0.2^{a}	1.0±0.0^{a}	1.5±0.2^{a}	1.5±0.3^{a}
	Crumb	Yeast	1.0±0.0^{c}	1.0±0.0^{c}	1.0±0.0^{c}	2.0±0.1^{b}	1.5±0.5^{bc}	4.5±1.0^{a}	2.3±0.2^{b}
Taste	Crumb	Sweetness	6.0±0.4^{ab}	5.0±1.4^{ac}	4.0±0.4^{bc}	7.0±0.0^{a}	3.5±0.5^{bc}	3.0±0.3^{c}	5.5±1.5^{ac}
		Saltiness	6.5±0.5^{ab}	5.0±0.1^{c}	5.0±0.2^{c}	7.0±08^{a}	5.5±0.0^{bc}	5.0±0.3^{c}	5.5±0.0^{bc}
		Sourness	2.0±0.0^{a}	1.5±0.5^{a}	1.5±0.4^{a}	2.0±0.2^{a}	1.5±0.3^{a}	2.0±0.0^{a}	1.8±0.0^{a}
Tactile characteristics /Texture	Crust	Hardness	4.0±0.2^{b}	3.5±0.7^{b}	2.0±0.0^{c}	5.0±0.4^{a}	2.0±0.0^{c}	5.0±0.0^{a}	5.5±0.5^{a}
		Crispiness	4.5±0.5^{b}	6.0±0.2^{a}	1.5±0.0^{d}	3.0±0.1^{c}	4.5±0.3^{b}	3.0±0.1^{c}	2.8±0.5^{c}
	Crumb	Resistance to chewing	0.5±0.4^{b}	1.0±0.2^{b}	1.5±0.5^{b}	5.0±1.0^{a}	1.5±0.5^{b}	4.5±0.5^{a}	6.3±0.5^{a}
		Cohesiveness	7.5±0.3^{bc}	7.5±0.4^{bc}	7.5±0.1^{bc}	7.0±0.5^{c}	8.5±0.5^{a}	7.5±0.0^{bc}	8.0±0.5^{ab}
		Graininess	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.2^{a}	0.5±0.2^{a}	0.5±0.0^{a}
		Stickiness	0.5±0.2^{bc}	1.0±0.5^{ab}	1.0±0.3^{ab}	0.0^{c}	1.5±0.5^{a}	0.5±0.2^{bc}	0.3±0.1^{bc}
Overall sensory score			8.0±0.5^{a}	8.0±0.0^{a}	7.5±0.7^{a}	6.0±0.4^{b}	7.5±0.8^{a}	7.5±0.5^{a}	7.8±0.3^{a}

Results are shown as mean ± standard deviation. In line, different letters indicate significant differences at $p<0.01$. B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread soy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic acid, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively).

the absence of significant differences among the samples for soapy flavour intensities, overall flavour desirability and undesirable aromas [Moayedallaie *et al.*, 2010]. In our study, lecithin was able to maximize the overall flavour intensity when used alone (**Table 4**), probably because the binding of flavours to emulsifier molecules facilitated the aroma retention, while it minimized the score when combined with gluten [Li *et al*., 2016]. The highest score for the freshly baked bread flavour was assigned to the control bread and to that supplemented with gluten while the formulation containing xylanase gave a less fragrant bread. Concerning the consumer choice, the freshly baked bread flavour is just one of the most preferred [Oručević Žuljević & Spaho, 2024]. All the improvers were able to increase the wheat flavour but with higher intensity in bread supplemented with xylanase or with ascorbic acid+lecithin. The yeast flavour intensity was greatly enhanced by the combination of ascorbic acid and lecithin. The ability of ascorbic acid to enhance the bread flavour profiles thanks to its oxidation preventing potential. Finally, malty and toasty flavours were weakly perceived in all breads.

With reference to the bread taste, the supplementation with xylanase was able to maximize sweetness and saltiness of bread (**Table 4**), because of the increased content of the corresponding compounds in a lower volume as highlighted by the negative correlation coefficients between specific volume and sweetness (r=–0.703) or saltiness (r=–0.754). The sourness taste was weakly perceived in all the samples without statistically significant differences (p≥0.01).

Concerning bread texture, the supplementation with lecithin or with ascorbic acid reduced the crust hardness, while the addition of xylanase or ascorbic acid+lecithin or gluten+lecithin increased it with respect to the control bread (**Table 4**). A close relationship was highlighted between hardness and moisture content (r=0.897), probably due to the formation of a glassy state. Always taking the control bread as reference, the crust crispiness was increased only by the addition of gluten to the formulation. All the other improvers exerted a detrimental effect on this feature, with the bread supplemented with lecithin showing the softest crust. Analogously to the crust hardness, the addition of xylanase or ascorbic acid+lecithin or gluten+lecithin increased the crumb resistance to chewing with respect to the control bread. The resistance to chewing was negatively correlated with pore density (r=–0.730) and positively correlated with pore size (r=0.667), indicating the influence of the crumb alveolation on its chewing behaviour, *i.e.*, showing that the greater pore size could be an indicator of its hardness. All breads showed high scores for cohesiveness (7.0–8.0) but within this narrow range, the lowest and the highest values were attributed to xylanase and ascorbic acid-supplemented breads, which is consistent with findings reported by Sarabhai *et al*. [2021] and Gujral *et al*. [2003]. At the same time, the crumb stickiness was low for all samples (0.0–1.5), and the highest values were attributed to xylanase and ascorbic acid-supplemented breads. Moreover, cohesiveness and stickiness increased with the increase of the specific volume (r=0.705 and r=0.724, respectively) and of porosity % (r=0.861 and r=0.806, respectively). None of the samples showed a grainy consistency (scores between 0.0 and 0.5).

Finally, all breads obtained high scores (from 6.0 to 8.00) for the overall sensory quality, with the lowest value assigned to the bread supplemented with xylanase (**Table 4**). The differences among the other samples were not statistically significant (p≥0.01). The overall sensory score positively correlated with ash content (r=0.630), brightness of crust and crumb (r=0.793 and r=0.845, respectively), loaf development evaluated instrumentally with the specific volume (0.893) and sensorially as crumb development (r=0.677), freshly baked and malty aroma (r=0.891 and r=0.609, respectively). Instead, it was negatively correlated with pore circularity (r=–0.785), crust thickness (r=–0.725), crumb pore size (r=–0.922), wheat aroma (r=–0.692), and crumb saltiness (r=–0.597). The negative correlation between the overall sensory quality and pore circularity was not in agreement with the findings of Naumenko *et al*. [2017], who stated that round-shaped pores enhance the customer appeal. It should be considered that elongated pores evoke the irregular porous structure of homemade breads and their typicality and authenticity.

Principal component analysis

The results of the multivariate analysis (**Figure 3A** and **B**) summarize the analytical description of the supplemented breads. The first two principal components (PC) explained 62.64% of the total variance. The bread with xylanase differed from all the others since it is located in the quadrant characterised by positive scores of PC1 (~9) and PC2 (~2). It was characterised by high values of crumb a^*, crust thickness, average pore size, saltiness, sweetness, and wheat aroma. The other supplemented breads and the control bread are placed in the part of the factorial plane characterised by values of PC1 between 0 and –3 and values of PC2 depending on the bread type. More in depth, the breads made with combinations of lecithin and gluten, or lecithin and ascorbic acid are close to each other and were characterised by high values of crust a^*, crumb development, and toasty aroma. The control bread and the bread supplemented with gluten are close to each other and have intermediate specific volume and high freshly baked aroma. Bread with lecithin, characterised by low crust crispiness, and bread with ascorbic acid, having a high total phenolic content, high crumb stickiness and cohesiveness, are in isolated positions in the plane. The bread supplemented with xylanase clearly stands out from all the others for its already lowest loaf development, intensity of the freshly baked bread flavour, and cohesiveness, and its highest saltiness, crust hardness, and crumb resistance to chewing which, in turn, significantly reduced its overall sensory score.

CONCLUSIONS

Improvers used in our study exerted unexpected effects on the characteristics of bread produced with a strong flour. Ascorbic acid allowed reaching the greatest specific volume, together with the highest scores for cohesiveness and stickiness.

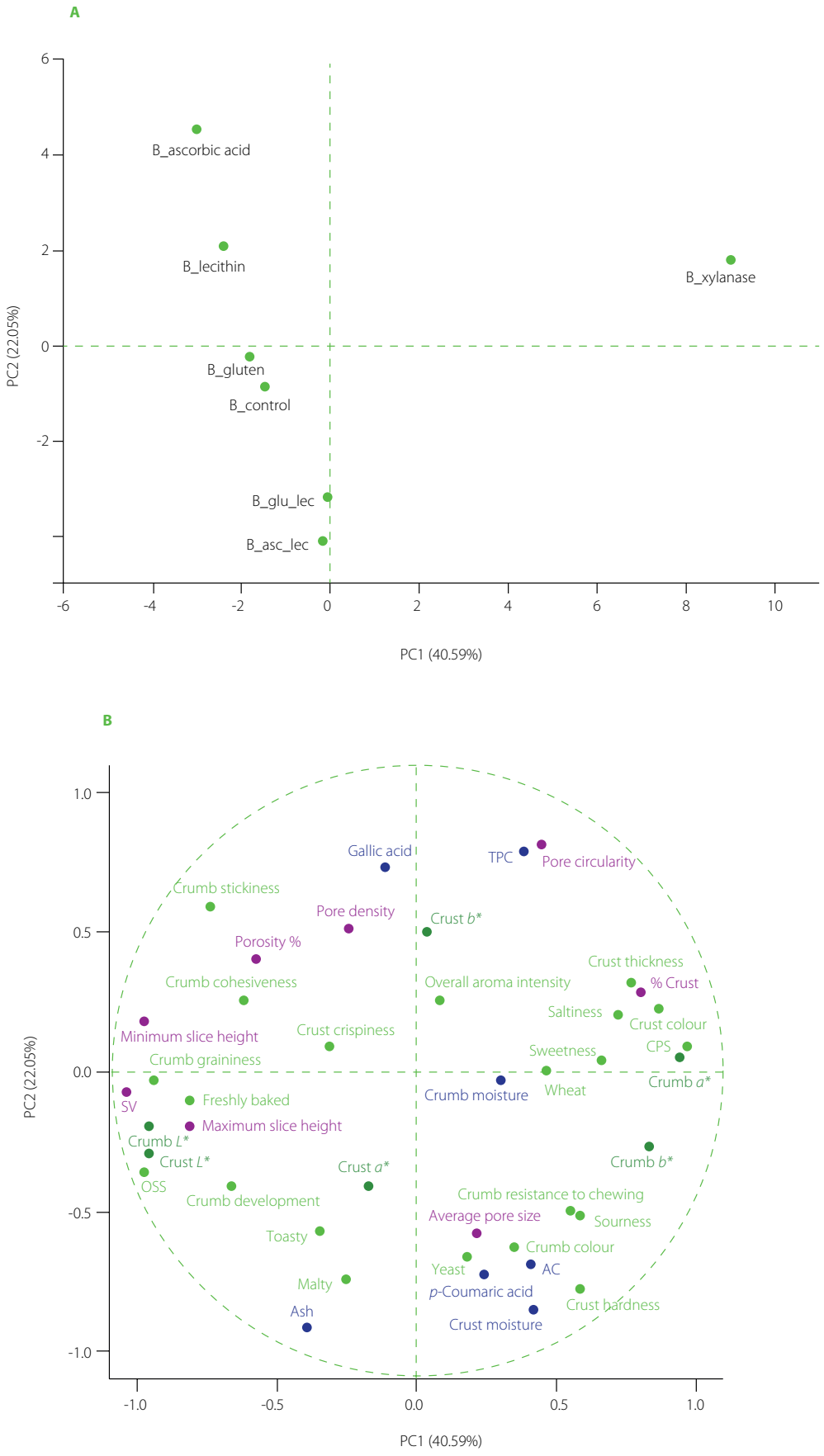

Figure 3. Principal component analysis (PCA) plots of breads: projection of samples (**A**) and variables (**B**). B_control, bread without improvers; B_gluten, bread with gluten (replacing 2% of flour, *w/w*); B_lecithin, bread s oy lecithin (1% of flour, *w/w*); B_xylanase, bread with xylanase (0.01% of flour, *w/w*); B_ascorbic acid, bread with ascorbic acid (0.02% of flour, *w/w*); B_asc_lec bread with ascorbic acid and soy lecithin (0.02% and 1% of flour, *w/w*, respectively); B_glu_lec bread with gluten and soy lecithin (2% and 1% of flour, *w/w*, respectively). Physical data are in dark green, structural data are in purple, sensorial data are in light green, and chemical data are in navy blue font. AC, antioxidant capacity; CPS, crumb pore size; OSS, overall sensory score; SP, specific volume; TPC, total phenolic content.

The highest overall sensory scores were assigned to the control bread and to that supplemented with gluten, probably as a consequence of their high specific volume and the highest intensity of freshly baked bread flavour. The total phenolic content (higher in the breads whose formulations contained xylanase or ascorbic acid) was not correlated with the antioxidant capacity (higher in the control bread and in that supplemented with ascorbic acid+lecithin). Except for antioxidant capacity, the use of two improvers together (ascorbic acid+lecithin, or gluten+lecithin) almost never exerted synergistic effects on bread quality, since they did not determine an improvement in quality parameters compared to the samples in which they were used alone. The sensory evaluation of texture strongly depended on specific volume and crumb characteristics (number and size of pores, porosity, and pore circularity).

According to the experimental data, the best improvers that can be conveniently added to a strong flour are those that influence the bread structural characteristics (increasing its volume and alveolation) because of their significant correlation with positive sensory aspects.

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The study received no external funding.

CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL APPROVAL

Ethical commission's approval was not necessary. The sensory study was performed using human volunteers that were previously asked to sign an informed consent form.

ORCID IDs

A. Baiano https://orcid.org/0000-0002-8191-8248
A. Fiore https://orcid.org/0000-0003-3912-4441

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Development and Quality Assessment of Fat-Reduced Turkish Fermented Sausage (Sucuk) Formulated with Functional Lemon Fiber

Zeki Gürler[1], Teslime Ekiz Ünsal[2]*

[1]Department of Food Hygiene and Technology, Veterinary Faculty, Afyon Kocatepe University, Afyonkarahisar, Turkey
[2]Department of Food Engineering, Faculty of Engineering, Afyon Kocatepe University, Afyonkarahisar, Turkey

This study aimed to produce Turkish fermented sausage (sucuk) with a reduced fat content and to minimize the quality defects typically associated with fat reduction by incorporating lemon fiber into the formulation. For sucuk production, six different dough formulations were prepared, comprising two fat levels (24 and 28 g/100 g of dough) and three lemon fiber levels (addition at 0%, 1%, and 2% of dough, *w/w*). After production, physical, chemical, and textural analyses were performed on the sucuk samples, and lactic acid bacteria (LAB) counts were determined microbiologically. The results indicated that there were no significant differences among the formulations in terms of protein and moisture content, water activity (a_w), pH, color, thiobarbituric acid reactive substances (TBARS), weight loss, lactic acid bacteria count, cholesterol content, residual nitrite and residual nitrate levels. However, lemon fiber addition had a significant effect on cooking loss, with the lowest cooking loss (16.8%) observed in the formulations containing 2% (*w/w*) lemon fiber compared to those without the fiber. Differences among the samples were observed in certain texture parameters, particularly hardness, springiness, and chewiness, depending on the interaction between fat level and lemon fiber addition. As a result, the formulation containing 24 g fat/100 g and 2% (*w/w*) lemon fiber was determined to be a suitable alternative for the production of low-fat sucuk in terms of technological properties.

Keywords: fat reduction strategy, fermented meat product, functional ingredient application, product reformulation, technological quality

INTRODUCTION

Meat and meat products are valuable sources of high-quality protein and essential nutrients; however, concerns regarding high fat and salt contents and their association with chronic diseases have increased consumer demand for healthier meat products [Grasso *et al*., 2014; Kausar *et al*., 2019; Toldrá & Reig, 2011]. There is a growing global demand for healthier meat products with reduced levels of fat, cholesterol, salt, and nitrites, as well as improved nutritional profiles [Kausar, 2019]. Accordingly, current research and industrial practices focus on reformulating meat products by reducing detrimental components and incorporating functional ingredients such as fibers, proteins, antioxidants, and polyunsaturated fatty acids [Biswas, 2011; Grasso *et al*., 2014; Yadav *et al*., 2016].

Fat plays a crucial role in determining the sensory quality of meat products, including flavor, juiciness, tenderness, and texture, and its reduction often results in increased firmness, reduced juiciness, and lower consumer acceptance [Hoffman & Wiklund, 2006; Keeton, 1994; Zhang *et al*., 2010]. For these reasons, fat level reduction cannot be achieved simply by using

*Corresponding Author:
e-mail: tekiz@aku.edu.tr (T. Ekiz Ünsal)

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less fat in the formulation. Water, which is added from non-meat ingredients used for fat reduction, has several advantages, such as being safe and inexpensive [Hughes *et al*., 1998]. However, incorporating water alone into meat products may lead to undesirable effects such as discoloration and increased cooking losses [Claus *et al*., 1990]. To mitigate these issues, water is typically combined with fat, carbohydrate, or protein-based ingredients [Hughes *et al*., 1998]. Carbohydrate-based fat replacers include fibers, gums, dextrins, maltodextrins, hydrocolloids, pectin, cellulose, and starches [Nikolić *et al*., 2024]. There are various studies in the literature on the use of fat replacers derived from different sources, including plant-based fibers, animal-derived ingredients, and carbohydrate-based compounds, in the production of different types of fat-reduced sausages [dos Santos *et al*., 2021; Guo *et al*., 2024; Kim *et al*., 2019; Tomaschunas *et al*., 2013]. Sucuk, a dry fermented sausage, holds a prominent place among Turkey's most cherished and widely consumed traditional meat products. Due to its high contents of fat (~35–40 g/100 g), saturated fatty acids and cholesterol, its healthier versions are developed [Ercoşkun & Demirci-Erçoşkun, 2010].

Dietary fibers are widely recognized for their health-promoting effects and functional properties, which have led to their increasing use as functional ingredients in meat products [Grasso *et al*., 2014; Pintado & Delgado-Pando, 2020]. Plant-derived dietary fibers exhibit diverse technological characteristics, such as water-holding and fat-binding capacities, that can influence the physicochemical and quality attributes of food products [Nieto *et al*., 2021; Zinina *et al*., 2019]. Lemon-derived fiber, which contains phenolic compounds, carotenoids, and pectin, has gained attention as a promising functional ingredient due to its antioxidant potential [Fu *et al*., 2015; Nieto *et al*., 2021].

Recent studies have shown that the incorporation of citrus fibers can improve technological properties, such as cooking yield and texture, while reducing weight loss in meat products [Chappalwar *et al*., 2020; Sarıcoban & Unal, 2022]. However, most studies have focused on the use of orange or mixed citrus fibers in cooked or emulsified sausages, with few studies on their application in low-fat formulations. Information on the use of lemon fiber, rich in antioxidants and pectin, in fermented or dry-cured meat products (particularly sucuk) is limited. Therefore, this study was designed to fill this knowledge gap by evaluating the effects of lemon fiber addition on the quality characteristics of low-fat sucuk, with the aim of developing a healthier and more functional traditional meat product. Accordingly, sucuk samples were produced using two different fat levels and three different lemon fiber levels. Various physicochemical analyses, including determinations of fat, protein, and moisture contents, pH, water activity (a_w), residual nitrite, residual nitrate, cholesterol content, thiobarbituric acid reactive substances (TBARS) level, cooking loss, and weight loss, as well as color measurements and texture profile analyses, were performed.

The main hypothesis of this study was that reducing the amount of animal fat would improve the nutritional profile of sucuk by decreasing total fat and cholesterol levels, while the addition of lemon fiber would compensate for potential adverse effects of fat reduction on the physicochemical and textural properties of the product. Results obtained enabled identifying the most suitable formulation.

MATERIAL AND METHODS

Materials

The sucuk samples used in this study were produced in a commercial meat products processing plant located in Afyonkarahisar, Turkey. The meat and tallow used in the formulations of sucuk were obtained from cattle slaughtered in the plant's own abattoir. Beef from the brisket region was used as the meat source. To facilitate temperature control during mincing and mixing, the meat was used as a mixture of fresh and frozen portions. All spices and additives used in sucuk production were supplied by the same production plant and from the same batch to ensure standardization. Lemon fiber (containing 5 g of protein, 1 g of fat, 90 g of total dietary fiber, and 10 g of moisture *per* 100 g) was supplied by Herbafood Ingredients GmbH (Werder (Havel), Germany).

Production of Turkish fermented sausage

The meat-to-fat ratio for the sucuk dough formulation was determined in collaboration with the industrial manufacturer where the trials were conducted. These ratios were selected as the lowest technologically feasible and sensorially acceptable fat levels, since further reduction in fat content would result in products that lost the characteristic properties of traditional sucuk and exhibited textural and sensory attributes similar to cooked meat. Six sucuk doughs were prepared for each experiment based on the fat level (fat level 1: 5.5 kg meat + 1.5 kg tallow, fat level 2: 5.25 kg meat + 1.75 kg tallow) and lemon fiber (0%, 1% and 2%, *w/w*). The following ingredients were added to sucuk batters: NaCl (15 g/kg), garlic (7.5 g/kg), red pepper (7 g/kg), hot red pepper (7 g/kg), black pepper (6 g/kg), cumin (10 g/kg), pimento (1 g/kg), cinnamon (0.01 g/kg), ascorbic acid (0.9 g/kg), ascorbate (0.06 g/kg), phosphate (0.6 g/kg), $NaNO_2$ (0.15 g/kg), and starter culture (0.2 g/kg). The amount of lemon fiber added to doughs was calculated over the total mixture weight. After the sucuk doughs was prepared, the fat content was measured using the Foss FoodScan meat analyzer (FOSS, Hillerød, Denmark); the doughs with fat levels of 1 had 24 g fat/100 g, and the doughs with fat levels of 2 had 28 g fat/100 g.

For sucuk production, meat and fat were minced. Spices, salt, garlic, additives, and starter culture were then added and thoroughly mixed. The mixture was filled into natural beef casings (46 mm diameter) using a filling machine. After filling, fermentation was carried out at 28°C and 95% relative humidity until the pH reached approximately 5.35 (~20 h). Following fermentation, the products were placed in an oven at 72°C for approximately 4.5–5.0 h, until the internal temperature reached 68°C. They were subsequently cooled with a water shower at ~20°C for 15 min. Finally, the products were transferred to a drying room maintained at 12–15°C, where they were stored until the relative humidity decreased below 40% (~12 h).

Sucuk production and analyses were conducted in three independent batches (experimental replicates) at different time points. From each batch, two coils of sucuk were sampled, and each sample was analyzed in duplicate (technical replicates).

Lactic acid bacteria count determination

For lactic acid bacteria (LAB) enumeration, 1 g of each sucuk sample was homogenized in 9 mL of sterile peptone water, and decimal serial dilutions were prepared [ISO 15214:1998]. From each dilution, 0.1 mL was plated in duplicate onto De Man, Rogosa and Sharpe (MRS) agar and spread on the surface using a sterile spreader. Plates were incubated at 37°C for 48 h under anaerobic conditions. Colonies were counted and expressed as CFU/g.

Physical and chemical parameter analysis

Measurement of pH value and water activity

The pH value of the sucuk samples was measured with a Testo 205 pH meter (Testo SE & Co. KGaA, Lenzkirch, Germany) at room temperature (~20–25°C) by direct immersion of the electrode into the samples. The a_w of the sucuk samples was measured with a Novasina LabMaster-aw device (Novasina AG, Lachen, Switzerland) following the manufacturer's guidelines, after equilibrating the samples at room temperature.

Determination of fat, protein, and moisture contents

The fat, protein, and moisture contents of the sucuk samples were determined using a Foss FoodScan meat analyzer (FOSS, Hillerød, Denmark) based on the near-infrared (NIR) spectroscopy principle. Prior to analysis, the sucuk samples were cut into small pieces and blended until a homogeneous consistency was achieved, and then placed into the sample chamber of the device. The instrument was operated according to the calibration sets and standard protocols provided by the manufacturer. During the measurements, the ambient temperature and humidity were maintained within the ranges recommended by the device. Protein, fat, and moisture contents were obtained directly through the device software, and results were expressed as g/100 g sucuk.

Determination of residual nitrite and nitrate contents

Nitrite and nitrate contents of the sucuk samples were determined at the TÜBİTAK Marmara Research Center (Turkey) using a spectrophotometric method based on extraction following protein precipitation, according to the method described by Schormüller [1968]. Results were expressed as mg/kg sucuk.

Determination of the level of thiobarbituric acid reactive substances

To 10 g of sucuk, 30 mL of a 7.5% trichloroacetic acid solution weres added, and the sample was homogenized using a digital homogenizer (HG-15D, Daihan Scientific Co., Ltd., Wonju, Gangwon-do, South Korea) for 15–20 s, followed by filtration through filter paper [Mielnik *et al.*, 2006]. The filtrate was mixed with a 0.02 M water solution of thiobarbituric acid at the ratio of 1:1 (*v/v*) in test tubes, which were kept in a water bath at 100°C for 35 min. Then, after cooling in cold water for 5 min, the absorbance was measured at 532 nm against a reagent blank containing reagent and water instead of the sample, using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Subsequently, the level of TBARS was calculated using the standard curve for 1,1,3,3, tetraethoxy propane (TEP), and the result was given as µmol malonaldehyde (MDA)/g sucuk.

Cholesterol content determination

The cholesterol content of the sucuk samples was determined at the TÜBİTAK Marmara Research Center (Turkey) using a chromatographic method based on lipid extraction followed by gas chromatographic analysis, in accordance with the method described by Fenton & Sim [1991]. Results were expressed as mg/100 g sucuk.

Weight loss evaluation

The sucuk samples were first weighed immediately after being filled into casings using an analytical balance with a precision of 0.01 g. Following the drying stage, the samples were weighed again under the same conditions. The weight loss of each sample was calculated as the difference between the initial and final weights and expressed as a percentage of the initial weight.

Cooking loss evaluation

After drying, 6 slices of 3 mm thickness were cut from the sucuk samples and weighed. Each slice was grilled for 1 min on each side for a total of 2 min. The slices were weighed after they cooled. The initial and final weights of the slices were taken into account, and the percent cooking loss was calculated.

Color analysis

The color of the sucuk samples was determined using a Minolta chroma meter (CR-400, Konica Minolta, Inc., Tokyo, Japan), following the CIE Lab system. Prior to measurements, the instrument was calibrated using a white standard according to the manufacturer's instructions. Measurements were taken at three different points on both the inner and outer surfaces of each sample to account for surface heterogeneity. The arithmetic mean of these readings was recorded as the final L^* (lightness), a^* (redness), and b^* (yellowness) values. Ambient light and temperature were maintained constant during the measurements to minimize variability.

Texture profile analysis

After drying, samples from each sucuk group were cut into 1.5-cm long pieces using a sharp knife for texture analysis. Cylindrical samples were analyzed using a TA.XT Plus texture analyzer (Stable Micro Systems Ltd, Godalming, Surrey, United Kingdom) equipped with a 36 mm cylindrical probe. They were compressed to 40% of their original height at a test speed of 2 mm/s, with a 1 s interval between the first and second compression. Textural parameters were derived from the force–time curves as follows: hardness was determined from the maximum force recorded

during the first compression, adhesiveness was calculated as the negative area under the curve, and cohesiveness was obtained as the ratio of the area under the second compression to that of the first. Springiness was assessed as the ratio of the recovered height during the second compression to the original height. Chewiness was calculated as the product of hardness, cohesiveness, and springiness, and resilience was evaluated as the ratio of the force recovered during the first compression [Herrero *et al*., 2007].

Statistical analysis

Descriptive statistics (mean and standard deviation) were calculated for all quantitative data. Two-way analysis of variance (ANOVA) was performed to examine the effects of fat and fiber levels and their interaction (fat×fiber) on the measured parameters. Significant differences among means were further evaluated using Duncan's multiple range test at a 5% significance level. All statistical analyses were conducted using SPSS ver. 26 (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Fat, protein, and moisture contents

The fat, protein and moisture contents of sucuk samples are presented in **Table 1**. Obviously, fat level in the sausage recipe had statistically significant effects ($p<0.01$) on their fat content. In contrast, their fat level was not significantly ($p\geq0.05$) affected by the level of lemon fiber in the dough formulation, as another primary variable. This result may be attributed to the fact that lemon fiber mainly affects water-holding capacity and textural properties, while having only a limited influence on fat retention or fat release during processing. Similar findings were reported by Yuca *et al*. [2019], who observed significant differences in fat content among treatments due to variations in fat levels in their study using β-glucan in sausages.

Fat level and lemon fiber level had no statistically significant effect ($p\geq0.05$) on the protein content of sucuk samples (**Table 1**). Although minor numerical variations were observed among formulations, these differences were not statistically significant and therefore cannot be attributed to the effects of fat reduction or fiber addition. Similar findings have been reported in previous studies, where low or moderate levels of dietary fiber incorporation resulted in no significant changes or only minor variations in protein content [Akoğlu *et al*., 2015; Bis-Souza *et al*., 2020; Sarıçoban & Unal, 2022].

The moisture content of sucuk is a critical parameter influencing texture, microbial stability, and overall product quality. In this study, fat level had a significant effect ($p<0.05$) on moisture content, with higher fat levels resulting in lower moisture values (**Table 1**). This effect can be attributed to the partial replacement of lean meat by fat in the formulation, which reduces the proportion of muscle proteins responsible for water binding. Similar effects of fat level on moisture content in fermented meat products have been reported in a recent study [Simunovic *et al*., 2022]. In contrast, the addition of lemon fiber did not have a significant effect ($p\geq0.05$) on the moisture content of sucuk (**Table 1**). This may be attributed to the relatively low inclusion levels used in the present study, which might not have been sufficient to enhance water retention. Moreover, the water-holding capacity of dietary fibers is influenced by their physicochemical properties, such as pectin content, porosity, and surface area [Elleuch *et al*., 2011]. Therefore, the lack of a significant effect of lemon fiber on the moisture content observed in this study could be attributed both to the low inclusion rate and to the specific hydration characteristics of the fiber used.

pH values and water activity

No significant differences in water activity of sucuk were observed as a result of changes in fat and fiber levels in product recipe ($p\geq0.05$) (**Table 1**). Similar findings were reported by Campagnol *et al*. [2012], García *et al*. [2002], and Yalınkılıç *et al*. [2012], who also found that variations in fat or fiber content did not markedly affect a_w values of fermented sausages. The absence of a significant effect in the present study may be

Table 1. Effect of fat and lemon fiber levels in the formulation on the pH value, water activity (a_w), and contents of protein, fat, and moisture of sucuk.

Variable	Level	pH	a_w	Protein (g/100 g)	Fat (g/100 g)	Moisture (g/100 g)
Lemon fiber (LF)	0%†	5.28±0.12^a	0.66±0.04^a	15.22±0.69^a	29.3±2.0^a	48.8±1.6^a
	1%	5.26±0.17^a	0.66±0.05^a	15.13±0.66^a	28.7±2.3^a	48.9±1.6^a
	2%	5.24±0.17^a	0.66±0.05^a	15.00±0.56^a	28.6±2.2^a	48.7±1.8^a
	Significance	NS	NS	NS	NS	NS
Fat (F)	F1	5.23±0.17^a	0.65±0.05^a	15.30±0.63^a	27.9±1.9^b	49.5±1.7^a
	F2	5.28±0.12^a	0.66±0.04^a	14.93±0.58^a	29.8±1.7^a	48.1±1.3^b
	Significance	NS	NS	NS	**	*
Interaction F×LF	–	NS	NS	NS	NS	NS

Results are shown as means ± standard deviation. Means in the same column, separately for LF and F, with different letters are significantly different (*$p<0.05$, **$p<0.01$), NS, not significant; F1, 24 g fat/100 g of dough; F2, 28 g fat/100 g of dough. †g/100 g of dough.

attributed to the limited amount of fiber added and the low water-binding potential of the matrix during drying and fermentation. On the other hand, several researchers have reported a reduction in a_w values with the inclusion of dietary fibers [Aminzare *et al*., 2024; Eim *et al*., 2008; dos Santos *et al*., 2021; Yuca *et al*., 2019]. These discrepancies among studies could be explained by differences in fiber type and addition level, as well as variations in processing conditions (*e.g.*, temperature, relative humidity, or drying duration), which strongly influence the rate of moisture migration and binding capacity of the added fiber.

In the present study, the addition of lemon fiber did not lead to a statistically significant change ($p \geq 0.05$) in pH values (**Table 1**). This finding is consistent with previous studies reporting that the incorporation of citrus-derived fibers at low to moderate levels did not significantly affect pH [Aleson-Carbonell *et al*., 2003; Fernández-Ginés *et al*., 2004]. The absence of a marked pH change has been attributed to the buffering capacity of the meat system [Fernández-Ginés *et al*., 2004]. In particular, Aleson-Carbonell *et al*. [2003] observed no significant pH change with raw albedo addition up to 5%, whereas its higher incorporation levels resulted in a significant decrease, indicating that pH modification depends primarily on the fiber addition level. Therefore, the absence of a significant pH change in the present study can be attributed to the relatively low level of lemon fiber used and the buffering effect of meat proteins. No statistically significant differences were observed in pH values across different fat levels ($p \geq 0.05$) (**Table 1**), which is consistent with the observations made by Yalınkılıç *et al*. [2012] for sucuk. This finding can be explained by the fact that fat acts as a neutral component in the mixture, not participating in fermentation or acid production, and therefore does not directly influence product pH.

Residual nitrite and nitrate

As shown in **Table 2**, lemon fiber and fat level had no significant effects ($p \geq 0.05$) on the residual nitrite and nitrate of sucuk samples. Similarly, Ruiz-Capillas *et al*. [2012] reported that the addition of a fat replacer at the end of the ripening period did not significantly affect the residual nitrite content of fermented sausages. In contrast, Yalınkılıç *et al*. [2012] found that the addition of orange fiber significantly influenced nitrite levels in low-fat sucuk formulations, which was attributed to the interaction between the active bio-compounds in the fiber matrix and nitrite. The lowest residual nitrite levels were detected in the samples containing 4% fiber, likely due to the ability of phenolic compounds to bind or reduce nitrite. The absence of a significant change in our study may be attributed to the lower level of lemon fiber used and the differences in its chemical composition compared with orange fiber. Furthermore, the relatively stable pH values of the samples may have limited the rate of nitrite decomposition during fermentation and drying. Aleson-Carbonell *et al*. [2003] also reported that the addition of lemon albedo reduced residual nitrite levels in sausage samples. However, the lower inclusion level of lemon fiber used in the present study likely restricted such an effect.

Thiobarbituric acid reactive substances and cholesterol content

The effects of fat level, lemon fiber level, and their interaction on the TBARS content of sucuk samples was not significant ($p \geq 0.05$) (**Table 3**). In a previous study, a fat replacer was added to the formulation of dry fermented sausages with a reduced fat content and it was concluded that the TBARS values of the products were not affected by the fat content [Ruiz-Capillas *et al*., 2012]. Fernández-López *et al.* [2007] reported that TBARS values were lower in sausage samples formulated with orange fiber compared to control sausages produced without fiber addition. According to Yuca *et al*. [2019], lower TBARS values were determined as a result of the addition of a fat replacer and fat reduction upon β-glucan addition as a fat replacer to fermented sausage formulations. Dos Santos *et al*. [2021] reported that the incorporation of fat replacers

Table 2. Effect of fat and lemon fiber levels in the formulation on the residual nitrite, the residual nitrate, and total nitrite and nitrate values of sucuk.

Variable	Level	Residual nitrite (mg/kg)	Residual nitrate (mg/kg)	Total residual nitrite and nitrate (mg/kg)
Lemon fiber (LF)	0%†	3.7±1.5^a	37.4±7.7^a	41.0±6.6^a
	1%	3.9±0.8^a	44.4±6.0^a	48.3±5.7^a
	2%	3.3±1.3^a	44.4±3.0^a	47.7±2.5^a
	Significance	NS	NS	NS
Fat (F)	F1	3.9±1.5^a	40.3±6.1^a	44.3±5.4^a
	F2	3.3±0.9^a	43.8±5.4^a	47.1±4.8^a
	Significance	NS	NS	NS
Interaction F×LF	–	NS	NS	NS

Results are shown as means ± standard deviation. Means in the same column, separately for LF and F, with different letters are significantly different (*p<0.05, **p<0.01), NS, not significant; F1, 24 g fat/100 g of dough; F2, 28 g fat/100 g of dough. †g/100 g of dough.

Table 3. Effect of fat and lemon fiber levels in the formulation on the thiobarbituric acid reactive substances (TBARS), cholesterol content, weight loss, cooking loss, and lactic acid bacteria (LAB) count of sucuk.

Variable	Level	TBARS (μmol MDA/kg)	Cholesterol (mg/100 g)	Weight loss (%)	Cooking loss (%)	LAB count (log cfu/g)
Lemon fiber (LF)	0%†	9.02±0.10^{a}	104.3±6.0^{a}	9.03±0.68^{a}	20.1±2.5^{a}	3.64±0.61^{a}
	1%	8.92±0.08^{a}	102.6±5.3^{a}	8.75±0.68^{a}	19.9±2.0^{a}	4.08±1.25^{a}
	2%	8.97±0.14^{a}	103.4±3.1^{a}	8.58±0.68^{a}	16.8±2.2^{b}	3.37±1.38^{a}
	Significance	NS	NS	NS	**	NS
Fat (F)	F1	8.78±0.09^{a}	101.7±4.4^{b}	9.09±0.56^{a}	18.4±2.2^{a}	3.56±0.77^{a}
	F2	9.15±0.12^{a}	105.3±4.7^{a}	8.49±0.56^{a}	19.4±2.3^{a}	3.83±1.42^{a}
	Significance	NS	*	NS	NS	NS
Interaction F×LF	–	NS	NS	NS	*	NS

Results are shown as means ± standard deviation. Means in the same column, separately for LF and F, with different letters are significantly different (*p<0.05, **p<0.01), NS, not significant; MDA, malondialdehyde; F1, 24 g fat/100 g of dough; F2, 28 g fat/100 g of dough. †g/100 g of dough.

in reduced-fat fermented sausages led to an increase in TBARS values. The discrepancies between the results of the present study and those reported in the literature may be attributed to differences in the type and inclusion level of fat replacers or dietary fibers used, as well as variations in formulation, processing conditions, and product type. In the present study, the relatively low level of lemon fiber addition and the absence of statistically significant changes in fat content may have limited the potential effect of the fiber on lipid oxidation, resulting in no significant differences in TBARS values.

A significant reduction in sucuk cholesterol content was observed with a decreased fat level in the formulation (p<0.05), whereas the addition of fiber did not result in a statistically significant change (p≥0.05) (**Table 3**). The reduction in cholesterol level with a lower fat content can be explained by the decreased proportion of animal fat in the formulation, as cholesterol is predominantly associated with lipid fractions in meat products [Jiménez-Colmenero, 2007]. Although previous studies reported that the addition of dietary fiber can also reduce cholesterol content in sausages [Campagnol *et al*., 2012; Candogan & Kolsarici, 2003; Cengiz & Gokoglu, 2005], the lack of a significant effect in our study may be due to the type and level of fiber used. Lemon fiber primarily affects water retention and textural properties rather than lipid content, and its content in our formulations may have been insufficient to produce measurable changes in cholesterol content [Aleson-Carbonell *et al*., 2003; Fernández-Ginés *et al*., 2004].

Weight and cooking losses

The weight and cooking loss of sucuk samples are presented in **Table 3**. None of the main variables (fat level, lemon fiber level, and their interaction) had significant effects on weight losses (p≥0.05). Although the addition of fiber slightly reduced weight loss in sucuk samples, this effect was not statistically significant (p≥0.05). In a study by Sarıçoban & Unal [2022], the incorporation of citrus albedo in sucuk formulations reduced weight loss, which was attributed to the high water-holding capacity of the fiber. Similarly, dos Santos *et al*. [2012] reported that dietary fiber addition reduced weight loss in meat products. The lack of a significant reduction in weight loss in the present study may be explained by the relatively low lemon fiber inclusion levels used, which may not have been sufficient to induce a pronounced water-binding or gel-forming effect within the meat matrix. In addition, differences in fiber source, processing conditions, and product formulation may influence the extent to which dietary fibers contribute to water retention.

Cooking loss is a critical parameter for assessing the physicochemical properties of meat products during thermal processing and is mainly influenced by the water- and lipid-binding capacities of proteins. A low fat content can reduce the ability of the meat matrix to retain moisture, since fat contributes to the structural stability of the protein–lipid network and helps entrap water and melted fat within the matrix [Han *et al*., 2018; Oz *et al*., 2016]. In our study, a significant interaction between fat and fiber levels affecting cooking loss was detected (p< 0.05) (**Table 3**). The distribution of cooking loss values for different fiber levels within each formulation is illustrated in **Figure 1**. The F×LF interaction suggests that the water-holding effect of fiber depends on the fat level of the formulation. At the lower fat level (F1), samples with 2% (*w/w*) fiber exhibited the lowest cooking loss among all treatments, whereas at the higher fat level (F2), the same fiber level resulted in comparatively higher cooking loss. This tendency may be explained by the water- and fat-binding properties of citrus fiber, which have been shown to improve matrix stability and moisture retention, particularly in reduced-fat meat systems [Elleuch *et al*., 2011; Fernández-Ginés *et al*., 2004; Saha & Bhattacharya, 2010].

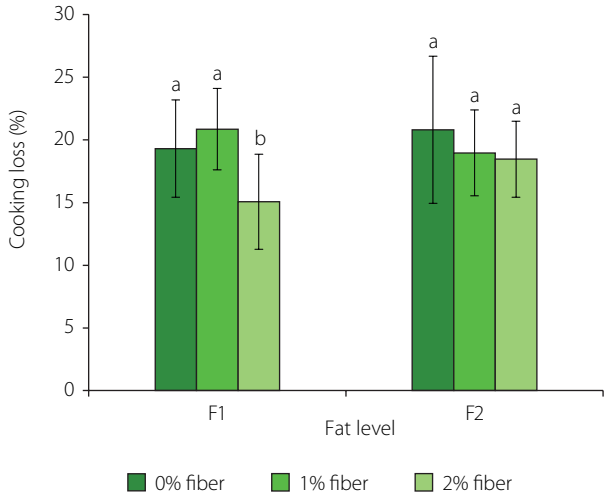

Figure 1. Cooking loss of sucuk samples prepared with two fat levels, F1 (24 g fat/100 g dough) and F2 (28 g fat/100 g dough), at fiber contents of 0%, 1%, and 2% (*w/w*, based on dough weight). Bars represent mean values and error bars indicate standard deviation (SD). Different letters above the bars indicate statistically significant differences ($p<0.05$).

Lactic acid bacteria count

The results showed that neither fat level, lemon fiber level, nor their interaction had a significant effect ($p\geq0.05$) on lactic acid bacteria (LAB) counts (**Table 3**). Similarly, several studies have reported that the incorporation of dietary fibers into fermented meat products had no significant impact on LAB populations [García *et al*., 2002; Mendoza *et al*., 2001; Ruiz-Capillas *et al*., 2012]. Most plant-derived fibers are insoluble, which may curb their functional impact in meat matrices [Elleuch *et al*., 2011]. This, combined with the limited fermentable carbohydrate content, may explain why their addition did not significantly affect LAB counts.

In contrast, Yalınkılıç *et al.* [2012] reported that increasing levels of orange fiber in low-fat sucuk formulations significantly affected LAB counts, with the highest values observed in the samples containing 4% fiber. This effect was attributed to the slight pH reduction caused by the addition of orange fiber, which created more favorable conditions for LAB activity. Therefore, the lack of a significant change in LAB counts in the present study may be related to the lower inclusion level of lemon fiber and the differences in contents of fermentable carbohydrates and phenolic compounds between lemon and orange fibers [Gorinstein *et al*., 2001; Fernández-Ginés *et al*., 2004].

Color parameters

Color parameters of sucuk samples are given in **Table 4**. The effect of fat level, lemon fiber level, and their interaction on the outer and inner surface *L** values of sucuk samples was not significant. Similarly, Araujo-Chapa *et al*. [2023] reported that the incorporation of soybean husk as a plant-derived dietary fiber had no significant effect on the *L** values of sausage products. The outer and inner surface *a** and *b** values were also not affected by the variables (**Table 4**). Previous studies reported that fat content and dietary fiber addition did not significantly affect *a** values in dry-ripened sausages, fermented sausages, and fermented cooked sausages formulated with different dietary fibers, including β-glucan and fructooligosaccharides [Bis-Souza *et al*., 2020; dos Santos *et al*., 2012; Yuca *et al*., 2019]. Fernández-Ginés *et al*. [2004] found that adding different amounts of lemon albedo to sausage samples was expected to increase *b** values, but it did not cause a change. The observed effect was attributed to the potential masking of yellow pigments in the albedo by the meat emulsion matrix. Supporting this, Aleson-Carbonell *et al*. [2003] reported that the incorporation of lemon albedo led to significant variations in *b** values, particularly at the 2.5% inclusion level. In a study, it was concluded that the effect of dietary fiber addition on *b** values of sucuk samples was not significant [Akoğlu *et al*., 2015].

Table 4. Effect of fat and lemon fiber levels in the formulation on the *L**, *a** and *b** values of the outer surfaces and the inner cross-sectional surfaces of sucuk.

Variable	Level	Outer surfaces			Inner surfaces		
		*L**	*a**	*b**	*L**	*a**	*b**
Lemon fiber (LF)	0%†	40.4±3.8^a	25.0±1.9^a	10.9±1.4^a	49.6±3.0^a	26.3±1.5^a	15.4±1.4^a
	1%	40.3±4.3^a	23.9±2.5^a	9.9±1.7^a	49.7±3.2^a	26.7±1.5^a	16.3±1.7^a
	2%	40.4±4.5^a	24.2±2.5^a	10.3±2.0^a	49.7±2.8^a	26.2±1.6^a	15.9±1.6^a
	Significance	NS	NS	NS	NS	NS	NS
Fat (F)	F1	40.2±4.1^a	24.1±2.4^a	9.9±1.7^a	49.0±2.9^a	26.8±1.5^a	15.8±1.6^a
	F2	40.5±4.2^a	24.6±2.2^a	10.9±1.7^a	50.1±2.9^a	26.0±1.4^a	16.0±1.6^a
	Significance	NS	NS	NS	NS	NS	NS
Interaction F×LF	–	NS	NS	NS	NS	NS	NS

Results are shown as means ± standard deviation. Means in the same column, separately for LF and F, with different letters are significantly different (*$p<0.05$, **$p<0.01$), NS, not significant; F1, 24 g fat/100 g of dough; F2, 28 g fat/100 g of dough. †g/100 g of dough.

Table 5. Effect of fat and lemon fiber levels in the formulation on the texture properties of sucuk.

Variable	Level	Hardness (N)	Adhesiveness (N×s)	Springiness (mm)	Cohesiveness	Chewiness (N)	Resilience
Lemon fiber (LF)	0%†	267.31±37.20^a	0.11±0.08^a	0.83±0.05^a	0.66±0.03^a	146.75±26.53^a	0.32±0.02^a
	1%	269.65±30.59^a	0.15±0.13^a	0.81±0.06^a	0.66±0.03^a	145.36±23.93^a	0.32±0.02^a
	2%	283.05±37.49^a	0.16±0.12^a	0.81±0.05^a	0.67±0.03^a	153.11±26.31^a	0.32±0.02^a
	Significance	NS	NS	NS	NS	NS	NS
Fat (F)	F1	282.64±33.71^a	0.11±0.08^b	0.82±0.05^a	0.67±0.03^a	153.43±25.2^a	0.33±0.02^a
	F2	264.04±35.15^b	0.17±0.13^a	0.82±0.05^a	0.66±0.03^a	143.38±22.91^a	0.32±0.02^a
	Significance	*	**	NS	NS	NS	NS
Interaction F×LF	–	**	NS	*	NS	**	NS

Results are shown as means ± standard deviation. Means in the same column, separately for LF and F, with different letters are significantly different (*p<0.05, **p<0.01), NS, not significant; F1, 24 g fat/100 g of dough; F2, 28 g fat/100 g of dough. †g/100 g of dough.

Overall, these findings suggest that the limited impact of lemon fiber on color parameters may be explained by its relatively low inclusion level, the composition of the fiber, and interactions with the meat matrix. The water- and fat-binding capacities of dietary fibers can influence pigment visibility, which may account for the minimal changes observed in L^*, a^*, and b^* values [Aleson-Carbonell *et al*., 2003; Fernández-Ginés *et al*., 2004].

Texture properties

A highly significant interaction between fat and fiber levels affecting sucuk hardness was detected (p<0.01) (**Table 5**). This finding indicates that the impact of fiber inclusion on hardness is dependent on the fat content of the samples. Hardness values at different fiber levels within each formulation are illustrated in **Figure 2**. At fat level F1, the addition of 2% (*w/w*) fiber produced the highest hardness values, whereas at fat level F2 the same fiber level resulted in lower hardness. Conversely, the samples with 1% fiber showed an opposite trend, with hardness increasing from F1 to F2. Among all combinations, the highest hardness was observed at F1×2% (*w/w*) fiber. Ruiz-Capillas *et al*. [2012] found an increase in hardness due to the decrease in fat content. In turn, Aleson-Carbonell *et al*. [2003] stated that the hardness of the samples to which albedo was added increased in dry-cured sausages. In a study examining the effect of oat fiber on different types of sausages (Bologna and Frankfurter types), it was reported that the hardness of Bologna type sausages increased with the addition of oat fiber, while in Frankfurter type sausages, the added oat fiber did not affect the hardness of the samples much [Steenblock *et al*., 2001], while Yuca *et al*. [2019] reported that the incorporation of dietary fiber, such as β-glucan, increased the hardness values of fermented sausage products [Yuca *et al*., 2019].

This effect of fat level and lemon fiber level interaction on texture (hardness) may be attributed to the opposite structural

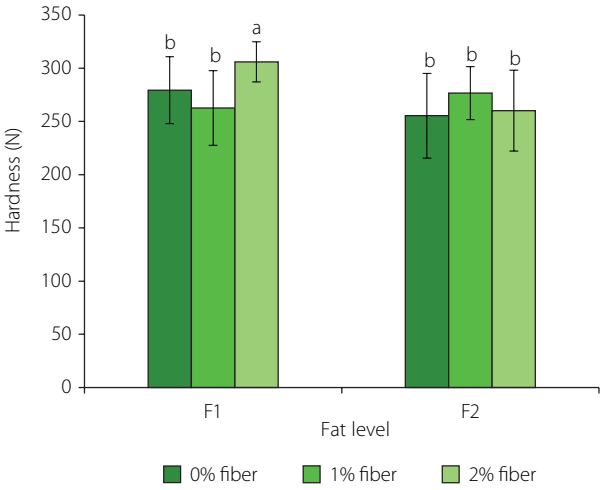

Figure 2. Hardness of sucuk samples prepared with two fat levels, F1 (24 g fat/100 g dough) and F2 (28 g fat/100 g dough), at fiber contents of 0%, 1%, and 2% (*w/w*, based on dough weight). Bars represent mean values and error bars indicate standard deviation (SD). Different letters above the bars indicate statistically significant differences (p<0.05).

roles of fat and fiber within the meat matrix. Fat generally acts as a plasticizer and lubricant, interrupting protein–protein interactions and resulting in a softer texture [Ruiz-Capillas *et al*., 2012]. In contrast, dietary fibers can increase matrix compactness by binding water and reinforcing the protein network, leading to a firmer texture [Aleson-Carbonell *et al*., 2003; Steenblock *et al*., 2001]. The combined effect suggests that at low-fat levels, fiber addition enhances protein–fiber crosslinking and increases hardness, whereas at higher fat contents, fat globules may disrupt this network, thereby diminishing the fiber's strengthening effect.

Among the variables, only the fat level had a highly significant effect on the adhesiveness of sucuk samples (p<0.01), while the effect of fiber level was not significant (p≥0.05) (**Table 5**). The observed differences in adhesiveness may be attributed to

the structural roles of fat and fiber within the meat matrix. Fat likely acts as a lubricant and filler, weakening protein–protein interactions and thereby reducing surface stickiness. At lower fat levels, a higher amount of denatured proteins may become exposed on the surface, which could increase adhesiveness. In contrast, dietary fibers may retain water and interact with proteins, possibly leading to a more cohesive and integrated gel network. Such interactions can influence moisture distribution and surface properties depending on the formulation [Ruiz-Capillas *et al*., 2012; Saha & Bhattacharya, 2010; Totosaus *et al*., 2002].

A significant interaction was detected between fat and fiber levels affecting sucuk springiness ($p<0.05$) (**Table 5**). In turn. springiness values at different fiber levels within each formulation are illustrated in **Figure 3**. In this case, springiness decreased with an increasing fat level when fiber was at 0% or 2% (*w/w*), whereas at 1% (*w/w*) fiber an opposite response was noted, with values increasing at F2. These findings clearly show that the effect of fiber on springiness is determined by fat content. In the study conducted by Aleson-Carbonell *et al*. [2003], lemon albedo was added to dry-cured sausages, reducing their springiness compared to the control sample. No difference was observed in terms of springiness among the samples with different albedo concentrations. Ruiz-Capillas *et al*. [2012] concluded that the use of a fat replacer in dry fermented sausages with a reduced fat content had no significant effect on the springiness of the samples.

The significant impact of the interaction between fat level and lemon fiber level on springiness may be explained by the contrasting structural effects of fat and dietary fiber within the meat matrix. Fat tends to act as a plasticizer, weakening the protein network and thereby reducing the elastic recovery of the product. In contrast, dietary fibers can promote the formation of a denser gel matrix through water-binding and protein interaction properties, enhancing elasticity depending on their content and compatibility with the protein structure [Aleson-Carbonell *et al*., 2003; Ruiz-Capillas *et al*., 2012; Saha & Bhattacharya, 2010]. The observed pattern suggests that moderate fiber levels may optimize matrix elasticity in formulations with reduced fat, whereas excessive fiber or high fat levels may disrupt the uniformity of the protein–fiber network, leading to decreased springiness.

The interaction between fat and fiber levels had a highly statistically significant ($p<0.01$) effect on sucuk chewiness (**Table 5**). In turn, chewiness values at different fiber levels within each formulation are illustrated in **Figure 4**. Specifically, chewiness decreased from F1 to F2 when fiber was at 0% or 2% (*w/w*), whereas in the presence of 1% (*w/w*) fiber, chewiness increased under the same fat level change. The interaction yielded the highest chewiness at the F1×2% (*w/w*) fiber combination, whereas the same fiber level produced substantially lower chewiness at F2. Once again, the observed crossing trends emphasize that chewiness was determined by the joint influence of fat and fiber levels, and not by their main effects alone. This interaction may be attributed to the structural roles of fat and fiber within the meat matrix. The extent of this effect likely depends on the balance between fat and fiber levels, which influences water distribution and protein network density within the meat matrix [Fernández-Ginés *et al*., 2004; Ruiz-Capillas *et al*., 2012].

The effect of fat level, lemon fiber level, and their interaction on the cohesiveness and resilience of sucuk samples was not statistically significant ($p\geq0.05$).

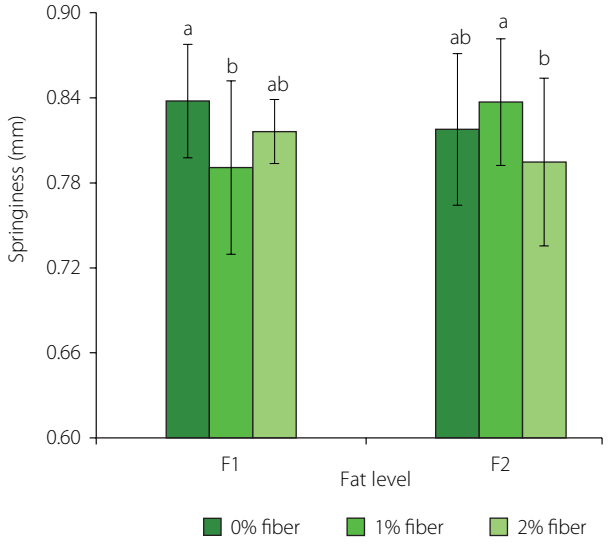

Figure 3. Springiness of sucuk samples prepared with two fat levels, F1 (24 g fat/100 g dough) and F2 (28 g fat/100 g dough), at fiber contents of 0%, 1%, and 2% (*w/w*, based on dough weight). Bars represent mean values and error bars indicate standard deviation (SD). Different letters above the bars indicate statistically significant differences ($p<0.05$).

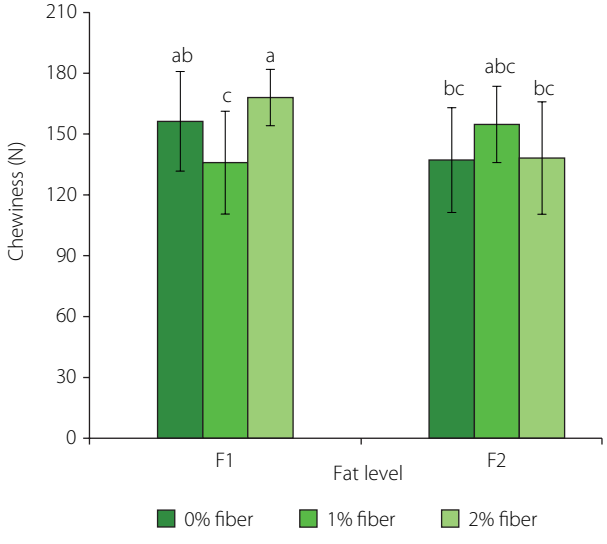

Figure 4. Chewiness of sucuk samples prepared with two fat levels, F1 (24 g fat/100 g dough) and F2 (28 g fat/100 g dough), at fiber contents of 0%, 1%, and 2% (*w/w*, based on dough weight). Bars represent mean values and error bars indicate standard deviation (SD). Different letters above the bars indicate statistically significant differences ($p<0.05$).

CONCLUSIONS

The study demonstrated that lemon fiber can be effectively used as a natural fat replacer in sucuk formulations. The sucuk samples with a lower fat content in the formulation (24 g fat/100 g dough) and higher lemon fiber (2%, *w/w*) showed the lowest cooking loss and no significant differences in physical and chemical properties or lactic acid bacteria counts compared to the other experimental formulations with different fat and fiber levels. These findings indicate that lemon fiber contributes to the development of healthier sucuk products without compromising their technological quality.

Future studies should focus on evaluating the sensory acceptability of lemon fiber–enriched sucuk using consumer panels, optimizing the level and particle size of lemon fiber for different fat levels, and investigating the synergistic effects of combining lemon fiber with other natural antioxidants or plant-based fibers. Additionally, assessing the shelf-life stability and lipid oxidation behavior under various storage conditions would provide further insights into the industrial applicability of lemon fiber in low-fat meat products.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

ADDITIONAL INFORMATION

This study is part of the doctoral thesis of Teslime Ekiz Ünsal.

ORCID IDs

T. Ekiz Ünsal https://orcid.org/0000-0001-7708-3420
Z. Gürler https://orcid.org/0000-0002-9037-2945

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Optimizing the Hydrogen Peroxide Concentration During Soaking and the Germination Time: A Simple Strategy to Modify Phenolic Content and Enhance Antioxidant Capacity in Runner Bean Sprouts

Luis M. Sánchez-Magaña[1], Luisa F. Uriarte-Franco[2], Liliana León-López[1,2*], Saraid Mora-Rochín[1,2], Edith O. Cuevas-Rodríguez[1,2], Sarita Montaño[1], Israel Benítez-García[3], Cuauhtémoc Reyes-Moreno[1,2]

[1]Programa de Posgrado Integral en Biotecnología, Facultad de Ciencias Químico Biológicas (FCQB), Universidad Autónoma de Sinaloa (UAS), Ciudad Universitaria, C.P. 80030, Culiacán, Sinaloa, México
[2]Maestría en Ciencia y Tecnología de Alimentos, FCQB-UAS, Ciudad Universitaria, C.P. 80030, Culiacán, Sinaloa, México
[3]Programa educativo Ingeniería en Biotecnología, Programa de Maestría en Ciencias Aplicadas, Universidad Politécnica de Sinaloa, C.P. 82199, Mazatlán, Sinaloa, México

Runner bean (*Phaseolus coccineus* L.) is a crop of significant economic importance in Mexico, but its inclusion in diets remains limited to certain regions. The bioprocessing of legumes through germination expands their consumption possibilities. Applying oxidative stress during germination has been suggested to improve the functional qualities of legume seeds. In this study, response surface methodology (RSM) with a central composite rotatable design (13 treatments) was used to identify the optimal hydrogen peroxide soaking concentration ([H_2O_2]; 0–35 mM) and germination time (Gt; 0–96 h) to enhance germination percentage (GP), free phenolic content (FPC), free flavonoid content (FFC), and antioxidant capacity in black runner bean sprouts. Regression analysis generated predictive models for each response. Optimal conditions were identified as [H_2O_2] of 30 mM and Gt of 92 h, achieving a GP of 95.7%. Under these conditions, sprouts exhibited increases in FPC from 67.6 to 72.7 mg GAE/100 g dry weight (DW), FFC from 26.4 to 28.6 mg CE/100 g DW, $ABTS^{\bullet+}$ scavenging activity from 3,028 to 3,782 µmol TE/100 g DW, and oxygen radical absorbance capacity (ORAC) from 5,793 to 6,573 µmol TE/100 g DW compared to those germinated without H_2O_2 stress. Soaking with 30 mM H_2O_2 enhanced the content of ferulic and *p*-coumaric acids in free and bound phenolic fractions, whereas catechin and quercetin showed notable reductions in both fractions as a result of H_2O_2 treatment. These findings reveal that H_2O_2 treatment can modify the phenolic profile of runner bean sprouts, thereby boosting their nutraceutical value.

Keywords: ayocote bean, H_2O_2 soaking, *Phaseolus coccineus*, phenolic profile, sprouted beans

ABBREVIATIONS

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA, analysis of variance; AOC, antioxidant capacity; C3GE, cyanidin 3-glucoside equivalents; CCRD, central composite rotatable design; CE, catechin equivalents; CV, coefficient of variation; DW, dry weight; FFC, free flavonoid content; FPC, free phenolic content; GAE, gallic acid equivalents; GP, germination percentage; Gt, germination time; [H_2O_2], hydrogen peroxide concentration; ORAC, oxygen radical absorbance capacity; R^2, coefficient of determination; ROS, reactive oxygen species; RSM, response

***Corresponding Author:**
e-mail: **lili.leon@uas.edu.mx** (Dr. L. León-López)

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surface methodology; TAC, total anthocyanin content; TE, Trolox equivalents.

INTRODUCTION

Runner bean (*Phaseolus coccineus* L.), also known as ayocote bean, is indigenous to Mesoamerica and is adaptable to various climatic conditions, particularly in the tropical high-humidity zones of Mexico [Vargas Vázquez *et al.*, 2012]. Although it is a valuable nutritional resource, runner bean remains underutilized as a legume [Alvarado-López *et al.*, 2019]. Traditionally, beans are consumed after cooking [Osuna-Gallardo *et al.*, 2023]. Nevertheless, heightened awareness of health and sustainability issues has spurred interest in alternative methods of consuming legumes and in novel plant-based foods [Aloo *et al.*, 2021].

Sprout production constitutes an effective strategy for diversifying legume consumption [Wojdyło *et al.*, 2020]. Germination can increase the bioactive compound content, such as phenolic acids and flavonoids. These compounds are crucial in treating and preventing chronic degenerative diseases linked to oxidative stress, such as diabetes, hypertension, and cancers [Hernández-Miranda *et al.*, 2025]. Recent research suggests that applying stress during sprout production, particularly during pre-germination stages such as soaking, may enhance the levels of bioactive compounds in germinated legumes [León-López *et al.*, 2020; Yang *et al.*, 2019; Yu *et al.*, 2023]. Nevertheless, the extent of these effects depends on the specific germination conditions [da Silva *et al.*, 2021].

The response surface methodology (RSM) provides a powerful statistical framework for optimizing complex processes such as germination. Within RSM, the central composite rotatable design (CCRD) – which incorporates axial points at lower and upper extremes beyond the factorial range – facilitates accurate response prediction, nonlinear model development, curvature detection, and enhanced model robustness while optimizing multiple parameters with fewer experimental runs and efficient quadratic polynomial fitting [Andres *et al.*, 2020; Mahapatra *et al.*, 2025]. Consequently, this study aimed to evaluate the effects of 24-h soaking with hydrogen peroxide (H_2O_2) at different concentrations, as well as germination time, on the nutraceutical properties of black runner bean sprouts. The findings provide a basis for developing high-value ingredients for functional foods.

MATERIALS AND METHODS

Plant material

Black runner bean seeds (*P. coccineus*) used in this study were purchased at the local market in Tecpatán, Chiapas, Mexico, in 2019. The seeds were cleaned and stored in plastic bags at 4°C for subsequent germination assays and chemical determinations.

Runner bean germination process

The germination protocol was adapted from León-López *et al.* [2020]. Black runner bean seeds were sanitized by immersion in a 0.5% sodium hypochlorite solution (1:5 *w/v*, ratio) for 10 min. The sanitizing solution was discarded and the seeds were then rinsed three times with purified water. Batches of 30 g of seeds were soaked for 24 h at room temperature with different H_2O_2 concentrations, as detailed in **Table 1**. After soaking, the seeds were rinsed with distilled water. The soaked seeds were sown uniformly on filter paper folds placed in plastic containers (20×30 cm). Germination was conducted in an incubator chamber (I-36VL Model, Percival Scientific Inc. Perry, IA, USA) at 25°C and 80–90% of relative humidity until germination time established in the experimental design was reached (**Table 1**). Germination percentage was calculated at the end of each incubation period. Each assay was performed in quadruplicate. Two replicates were used for physical characterization, while the remaining samples were dried and stored for subsequent chemical analysis.

Table 1. Central composite rotatable design (CCRD) consisting of 13 experiments produced by different combinations of two process variables and five levels: X_1 – soaking hydrogen peroxide concentration ($[H_2O_2]$) and X_2 – germination time (Gt).

No.[1]	$[H_2O_2]$ (mM) (X_1)	Gt (h) (X_2)
1	5 (–1)	14 (–1)
2	30 (+1)	14 (–1)
3	5 (–1)	82 (+1)
4	30 (+1)	82 (+1)
5	0 (–1.414)	48 (0)
6	35 (+1.414)	48 (0)
7	17.5 (0)	0 (–1.414)
8	17.5 (0)	96 (+1.414)
9	17.5 (0)	48 (0)
10	17.5 (0)	48 (0)
11	17.5 (0)	48 (0)
12	17.5 (0)	48 (0)
13	17.5 (0)	48 (0)

[1]Does not correspond to order of experiments. Values in parentheses correspond to coded levels.

Experimental design

The independent variables were: X_1 – soaking hydrogen peroxide concentration ($[H_2O_2]$, 0–35 mM) and X_2 – germination time (Gt, 0–96 h). The experimental design employed a CCRD comprising 13 randomized experiments, as detailed in **Table 1**. These experiments were conducted in random order, and performed in triplicate.

Evaluation of germination percentage

Germination percentage (GP) was evaluated in seeds treated with different H_2O_2 concentrations and germinated for different times. GP was calculated as the number of successfully germinated seeds, identified by the presence of visible radicles (>1 mm), divided by the total number of seeds sown *per* treatment, and then multiplied by 100 [León-López *et al.*, 2020].

Estimation of sprout growth

After each germination period, the physical characteristics of the runner bean sprouts were recorded. Radicle length (mm) and radicle diameter (mm) were measured using a digital caliper (CALDI-6MP, Truper. S.A. de C.V., Mexico City, Mexico). The number of seeds that developed secondary roots was recorded to calculate the percentage of secondary root appearance.

Flour obtaining process

The sprouted seeds obtained from each treatment were dried at 55°C for 24 h in a food dehydrator (Hamilton Beach, 32100a, 500 W, Glen Allen, VA, USA). After drying, the sprouts were ground in a coffee grinder (Hamilton Beach, 80350R, Glen Allen, VA, USA). The resulting flours were sifted through an 80-mesh sieve (sieve aperture: 0.180 mm).

Preparation of flour extracts

For optimization purposes, a 0.5 g portion of each flour was extracted by orbital agitation in a horizontal rotary shaker (RKVSD, ART Inc., Laurel, MD, USA) (200 rpm, 25°C) with 4 mL of 80% (*v/v*) aqueous ethanol during 10 min [Osuna-Gallardo *et al.*, 2023]. The mixture was then centrifuged (Eppendorf 5810R, AG, Hamburg, Germany) at 4,000×*g* for 10 min, and the supernatant was collected and stored at −20°C for subsequent analysis.

Determination of free phenolic content and free flavonoid content

The free phenolic content (FPC) was determined with the Folin-Ciocalteu colorimetric method of Singleton *et al.* [1999]. Twenty µL of appropriately diluted extracts obtained as described above were reacted by adding 180 µL of the Folin-Ciocalteu reagent. Following a 20-min incubation, the absorbance of the blue complex was measured at 750 nm using a microplate reader (Synergy HT Multi-Detection BioTek Instruments, Inc., Winooski, VT, USA). Results were calculated using a calibration curve of gallic acid (0–300 mg/L) and presented in mg of gallic acid equivalents (GAE) *per* 100 g of dry weight (DW) of sprouted seeds (mg GAE/100 g DW).

The free flavonoid content (FFC) was determined using the methodology proposed by Heimler *et al.* [2005]. In a 96-well plate, 20 µL of the extract were mixed with 100 µL of distilled water and 6 µL of 5% $NaNO_2$, rested for 5 min, then 12 µL of 10% $AlCl_3$ were added. After another 5 min, 40 µL of 1 M NaOH and 22 µL of water were incorporated, followed by a 30-min dark incubation at room temperature. Absorbance was read at 510 nm using a microplate reader (Synergy HT Multi-Detection BioTek Instruments, Inc., Winooski, VT, USA). Results were calculated using a catechin calibration curve (0–300 mg/L) and expressed in mg of catechin equivalents (CE) *per* 100 g of sprouted seed dry weight (mg CE/100 g DW).

Determination of antioxidant capacity

The antioxidant capacity (AOC) of the sprouted seed flours was evaluated using the 2,2′-azinoazino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [Re *et al.*, 1999]. Each extract (7.5 µL) was added to a 96-well plate and brought to a total volume of 300 µL *per* well with 292.5 µL of the $ABTS^{\bullet+}$ solution. The plate was incubated at room temperature for 10 min, followed by absorbance measurement at 735 nm (Synergy HT Multi-Detection BioTek Instruments, Inc., Winooski, VT, USA). Trolox served as the reference standard, with a calibration curve from 0 to 800 µg/mL. Results were expressed as µmol of Trolox equivalents (TE) *per* 100 g of seed flour on a dry weight basis (µmol TE/100 g DW).

Additionally, the AOC of unprocessed black runner beans and sprouted beans under optimized ($[H_2O_2]$ of 30 mM, Gt of 92 h) and control ($[H_2O_2]$ of 0 mM, Gt of 92 h) conditions was determined using the oxygen radical absorbance capacity (ORAC) assay [Ou *et al.*, 2001]. Peroxyl radicals were generated with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and free radical-induced fluorescence loss was measured in a microplate reader (Synergy HT Multi-Detection, BioTek Instruments, Inc., Winooski, VT, USA) at excitation/emission wavelengths of 485/538 nm. Results were expressed as µmol of Trolox equivalents (TE) *per* 100 g of seed flour on a dry weight basis (µmol TE/100 g DW).

Regression analysis and optimization

The optimal soaking H_2O_2 concentration and Gt to maximize GP, FPC, FFC, and AOC in the ABTS assay of sprouted black runner beans were established using the response surface methodology (RSM). The experimental design (CCRD) comprised 13 randomized runs (**Table 1**), varying $[H_2O_2]$ (0–35 mM) and Gt (0–96 h). PG, FPC, FFC, and AOC were modelled as quadratic responses according to Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{2}\beta_i X_i + \sum_{i=2}^{2}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{i=j+1}^{2}\beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where: Y is the predicted response variable (Y_1 = PG, Y_2 = FPC, Y_3 = FFC, Y_4 = AOC); β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and interaction terms, respectively; X_i and X_j represent the independent variables ($X_1 = [H_2O_2]$, X_2 = Gt); and ε denotes the experimental error.

Sprouts characterization

Proximate chemical composition

The moisture (method 925.09), protein (method 920.87), ash (method 923.03), and lipid (method 922.06) contents of black

runner beans and sprouted beans under optimized and control conditions were determined according to the official AOAC International methods [AOAC, 2005]. The carbohydrate content was calculated by subtracting the sum of moisture, protein, fat, and ash from 100 g of sample. All results were expressed in g/100 g DW.

Total anthocyanin content

The total anthocyanin content (TAC) was determined after extraction of a weighed 0.1 g portion of flours of unsprouted, control sprouted, and optimal sprouted beans into a 2 mL vial with 1 mL of acidified methanol (95% methanol and 1 M HCl, 85:15, *v/v*). The samples were vortexed for 10 min (Genie 2 mixer, model G560, Scientific Industries, Inc., Bohemia, NY, USA) and then centrifuged (Eppendorf 5810R, AG, Hamburg, Germany) at 3,000×*g* for 10 min, and the supernatants were collected. Sample absorbance was immediately recorded at 535 nm (A_{535}) and 700 nm (A_{700}) using a microplate reader (Synergy HT Multi-Detection BioTek Instruments, Inc., Winooski, VT, USA). TAC was quantified using the following Equation (2) [Abdel-Aal & Huel, 1999]:

$$TAC = \frac{A_{535} - A_{700}}{\varepsilon} \times V_{extract} \times MW \times \frac{1}{w_{sample}} \quad (2)$$

where: ε is cyanidin 3-glucoside molar absorption coefficient (23,900 L/(cm×mol), $V_{extract}$ is total extract volume (L), MW is cyanidin 3-glucoside molecular weight (449.2 g/mol), and w_{sample} is weight of the sample (g).

Results were expressed as mg of cyanidin 3-glucoside equivalents (C3GE) *per* 100 g of sprouted bean dry weight (C3GE/100 g DW).

Composition of free and bound phenolic fractions

The free phenolic fraction was obtained as described in "Preparation of flour extracts" section. The bound phenolics were extracted from the precipitate remaining after free phenolic extraction [Mora-Rochín *et al.*, 2010]. The precipitate was treated with 10 mL of 2 M NaOH, heated at 95°C for 30 min, and stirred for 1 h at room temperature. The mixture was neutralized with 2 mL concentrated HCl, vortexed for 2 min, and defatted by adding 5 mL of hexane. The defatted fraction was then extracted four times with 5 mL of ethyl acetate each time (vortexed for 10 min and centrifuged at 3,000×*g* for 10 min *per* extraction). The combined ethyl acetate fractions were evaporated to dryness under reduced pressure in a Speed Vac Concentrator SC 250 EXP (Thermo Scientific Inc., Sunnyvale, CA, USA), and reconstituted in 1 mL of 50% methanol.

The phenolic compound profile of the free and bound phenolic fractions was determined using a Dionex UltiMate 3000 high-performance liquid chromatography (HPLC) system with a photodiode array detector (DAD3000) (Thermo Fisher Scientific, New York, NY, USA) according to the procedure previously used by Valdez-Morales *et al.* [2014]. The injection volume was 10 µL. Separation was performed on a C18 Acclaim 120 Å analytical column (C18, 5 µm, 120 Å, 4.6×250 mm) from Dionex (Thermo Fisher Scientific, New York, NY, USA), at room temperature using a gradient elution of acetic acid–acidified water (pH 2.8) (A) and acetonitrile (B). The gradient program varied the proportion of solvents over 45 min as follows: 95% A (0–8 min); 6–12% B (8–14 min); 12–20% B (14–18 min); 20–35% B (18–24 min); 35–95% B (24–27 min); 95–60% B (27–31 min); 60–40% B (31–34 min); 40–20% B (34–38 min); and 20–5% B (38–45 min). The flow rate was 0.5 mL/min. Detection was set at wavelengths of 280, 320, and 360 nm. Chromatographic peaks were identified by comparing their retention times and UV-Vis spectra with those of authentic standards. Samples were injected in triplicate, and data were analyzed using Chromeleon 7.0.200 software (Thermo Fisher Scientific, Sunnyvale, CA, USA). Results were expressed as µg/g seed flour DW.

Statistical analysis

The effect of H_2O_2 treatment on seedling growth, chemical composition, AOC, free phenolic content, free flavonoid content, and phenolic profile was evaluated using a one-way analysis of variance (ANOVA). Mean comparisons were performed with Tukey's test at the 95% confidence level ($p<0.05$).

RESULTS AND DISCUSSION

Effect of the soaking with H_2O_2 and germination time on the growth performance of runner bean sprouts

Radicle length, diameter, and the number of secondary roots were measured in sprouts as outlined in the experimental design (**Table 1**). Elevated H_2O_2 concentrations consistently promoted radicle elongation at both 48 h and 82 h of germination (**Table 2**, **Figure 1**). At 48 h, radicle length increased from 17.3 mm (0 mM H_2O_2) to 23.9 mm (35 mM H_2O_2). At 82 h, values ranged from 41.0 mm (5 mM H_2O_2) to 46.3 mm (30 mM H_2O_2). The effect was more pronounced at 48 h, highlighting that H_2O_2 substantially enhances early root development in runner bean sprouts. No root growth was detected with germination times shorter than 14 h, regardless of H_2O_2 treatment. Secondary roots were absent at short Gt. At 48 h, secondary root formation was not induced irrespective of the H_2O_2 concentration applied, and at 82 h, 30 mM H_2O_2 increased secondary roots by 36 percentage points compared to 5 mM. The presence of secondary roots was primarily determined by Gt and further influenced by H_2O_2 treatment at high concentrations. These results emphasize the importance of optimizing both Gt and H_2O_2 concentration to maximize root development.

Previous studies have shown that exogenous H_2O_2, applied at optimal concentrations, promotes adventitious root formation and elongation across various plant species [Li & Jia, 2013; Roussos, 2023]. In accordance with these findings, Barba-Espin *et al.* [2010] reported that pea seeds imbibed with H_2O_2 at concentrations of 0, 5, 10, and 20 mM exhibited a clear stimulation of seedling length, with the highest concentration showing the most pronounced effect. Exogenous H_2O_2 at suitable doses acts as a priming agent, boosting seedling vigor while limiting oxidative damage [Wojtyla *et al.*, 2016].

Table 2. Length and diameter of radicles, and percentage of seeds that developed secondary roots after soaking runner bean in an H_2O_2 solution in different concentrations ([H_2O_2]) and germinating for different times (Gt).

No.[1]	[H_2O_2] (mM)	Gt (h)	Length (mm)	Diameter (mm)	Secondary roots (%)
1	17.5	0	0.0	0.0	NP
2	5	14	0.0	0.0	NP
3	30	14	0.0	0.0	NP
4	0	48	17.3	2.1	NP
5*	17.5	48	18.9	2.3	NP
6	35	48	23.9	2.5	NP
7	5	82	41.0	3.0	14.5
8	30	82	46.3	3.4	50.5
9	17.5	96	45.8	3.1	56.9

[1]Does not correspond to order of the experiments. *Central point repeated by 5. NP, secondary roots were not present.

Reactive oxygen species (ROS), particularly H_2O_2, are key signaling molecules in seed physiology, shifting the view from harmful by-products of aerobic respiration to regulators of developmental transitions. During imbibition, H_2O_2 accumulates through mitochondrial, peroxisomal, and nicotinamide adenine dinucleotide phosphate oxidase activity, facilitating endosperm weakening and hormonal modulation by enhancing gibberellin biosynthesis while reducing abscisic acid and ethylene levels [Černý *et al.*, 2018]. In cereals, H_2O_2 also promotes α-amylase activation and programmed cell death in the aleurone layer *via* DELLA protein interactions, processes essential for nutrient mobilization [Nazir *et al.*, 2020]. In turn, antioxidant systems, both enzymatic and non-enzymatic, balance oxidative stress while permitting H_2O_2 to act as a signaling molecule [Chu *et al.*, 2022]. Moreover, nuclear accumulation of H_2O_2 at radicle protrusion may regulate redox-sensitive transcription factors, leading to gene expression changes linked to phytohormone signaling [Wang *et al.*, 2025]. Collectively, these findings highlight the multifaceted role of H_2O_2 in coordinating germination success and phytochemical outcomes.

Mathematical models of response variables

As shown in **Table 3**, the process variables, *i.e.*, [H_2O_2] and Gt, significantly influenced the response variables, including GP, FPC, FFC, and AOC. Using multiple regression analysis, quadratic polynomial equations for each response variable were accurately fitted to the experimental data of germination conditions. The response surfaces and the obtained contour plots were then analyzed to visualize these effects (**Figure 2**). The validity and adequacy of the predictive models were confirmed by considering the statistical parameters, including a high coefficient of determination (R^2) and adjusted R^2 (>0.80 for both), a very low *p*-value (<0.05), a coefficient of variation (CV) below 10%, and a non-significant lack-of-fit test (p>0.05) (**Table 4**).

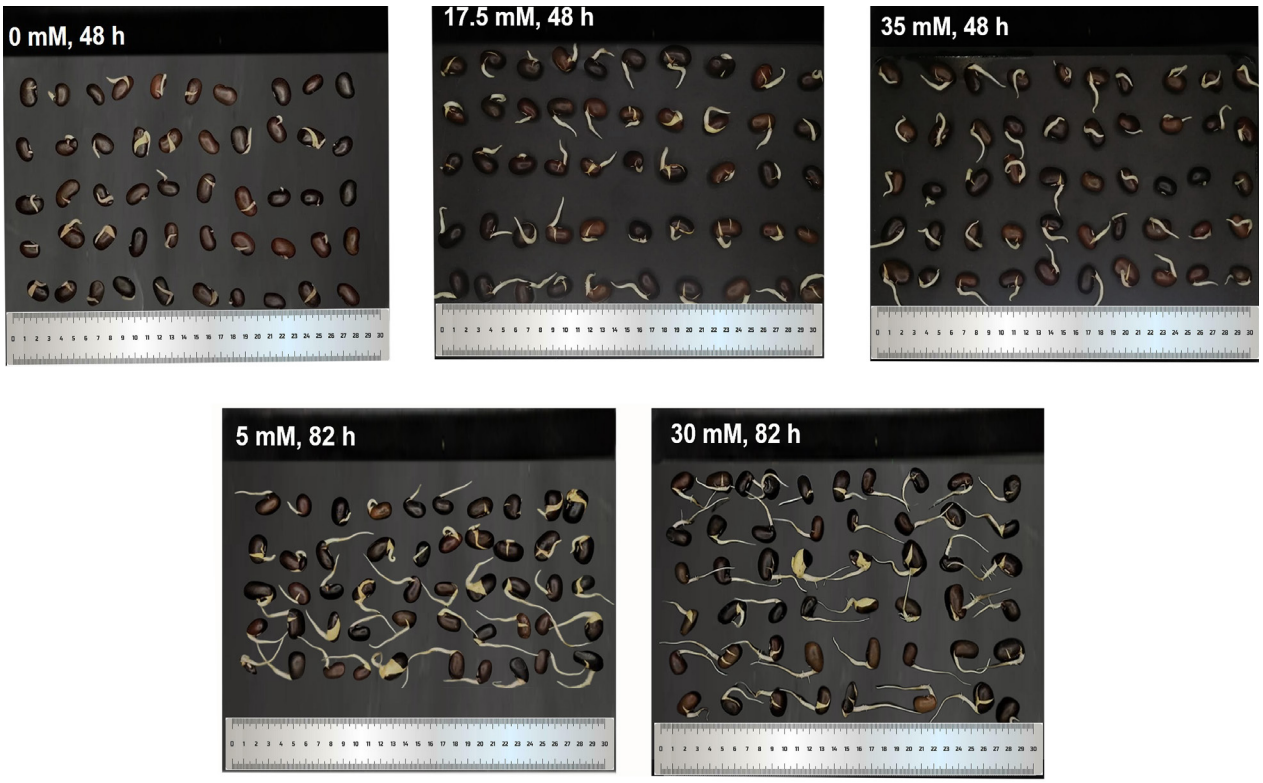

Figure 1. Appearance of runner bean sprouts at different germination times (48–82 h) after soaking in H_2O_2 solutions of varying concentrations (0–35 mM).

Germination percentage

The process variables, [H_2O_2] and Gt, affected the germination performance in black runner bean sprouts, with GP ranging from 14.0 to 98.0%, depending on the experimental conditions (**Table 3**). The analysis of variance yielded a significant quadratic model for GP (p<0.0001), which includes the linear terms of [H_2O_2] and Gt, the quadratic term Gt (Gt^2), and the interaction between both variables ([H_2O_2]×Gt) (**Table 4**).

The contour plot (**Figure 2A**) shows a clear GP increase with longer Gt. The treatment with 35 mM H_2O_2 for 48 h resulted in GP values similar to those achieved after more than 82 h at lower H_2O_2 concentrations, suggesting that oxidative elicitation expedited germination. The highest GP was observed at H_2O_2 concentrations above 30 mM combined with Gt exceeding 48 h. Comparable responses have been documented in other legumes treated with H_2O_2 during imbibition [Barba-Espín *et al.*, 2012; León-López *et al.*, 2020; Santhy *et al.*, 2014]. This behavior is explained by the inductive effect of H_2O_2 in breaking seed dormancy and increasing germination. The oxidative stress caused by the natural accumulation of H_2O_2 enhances the production of ROS that diffuse from the seed surface to its interior and, by interacting with other molecules, inhibit the action of abscisic acid cytokinins, and indole-3-acetic acid, while simultaneously increasing the biosynthesis and inhibiting the catabolism of gibberellic acid, thereby promoting the germination process [Delis-Hechavarría *et al.*, 2021; Wojtyla *et al.*, 2016].

Free phenolic content

The FPC in black runner bean sprouts was affected by the process variables, ([H_2O_2] and Gt), showing values ranging from 46.9 to 71.8 mg GAE/100 g DW across the 13 treatments (**Table 3**). The analysis of variance yielded a significant quadratic model for FPC (p<0.0001), which includes the linear and quadratic terms of [H_2O_2] and Gt, and the interaction between both variables ([H_2O_2]×Gt) (**Table 4**).

The contour plot (**Figure 2B**) shows the interaction of the process variables [H_2O_2] and Gt with FPC in runner bean sprouts, where increasing [H_2O_2] resulted in the highest FPC. A similar trend was observed with increasing Gt; however, the stimuli caused by the chemical stress induced by different H_2O_2 concentrations (0, 17.5, and 35 mM) at the same Gt (48 h) showed significant increases in FPC. The highest FPC (71.8 mg GAE/100 g DW) was obtained at Gt of 96 h and [H_2O_2] of 17.5 mM (**Table 3**), demonstrating that the synergy between high peroxide concentrations and prolonged germination times maximized FPC in black runner bean sprouts. Previous studies have reported similar positive effects of germination time and H_2O_2 stress on total phenolic content in wheat sprouts, *Ficus deltoidea* Jack plant,

Table 3. Experimental rotatable central composite design used to obtain treatment combinations of different concentrations of H_2O_2 ([H_2O_2]) and germination time (Gt) for producing runner bean sprouts, along with the experimental values obtained for the selected response variables.

No.[1]	Process variables		Response variables			
	[H_2O_2] (mM)	Gt (h)	GP (%)	FPC (mg GAE/100 g DW)	FFC (mg CE/100 g DW)	AOC (μmol TE/100 g DW)
1	5	14	14.0	51.0	16.3	2,117
2	30	14	45.3	55.7	19.7	2,106
3	5	82	94.3	69.0	23.5	2,889
4	30	82	**98.0**	70.0	**28.3**	**3,526**
5	0	48	57.6	59.9	19.7	2,416
6	35	48	90.2	65.9	25.9	2,948
7	17.5	0	0.0	46.9	15.7	1,980
8	17.5	96	95.9	**71.8**	26.6	3,426
9	17.5	48	81.7	60.9	21.0	2,579
10	17.5	48	75.3	61.3	21.2	2,670
11	17.5	48	74.3	61.7	21.1	2,713
12	17.5	48	80.8	61.5	21.2	2,652
13	17.5	48	79.6	60.6	21.2	2,661

[1]Does not correspond to order of the experiments. GP, germination percentage; FPC, free phenolic content; FFC, free flavonoid content; AOC, antioxidant capacity in ABTS assay; GAE, gallic acid equivalent; CE, catechin equivalent; TE, Trolox equivalent, DW, dry weight. Values in bold mean maximum values.

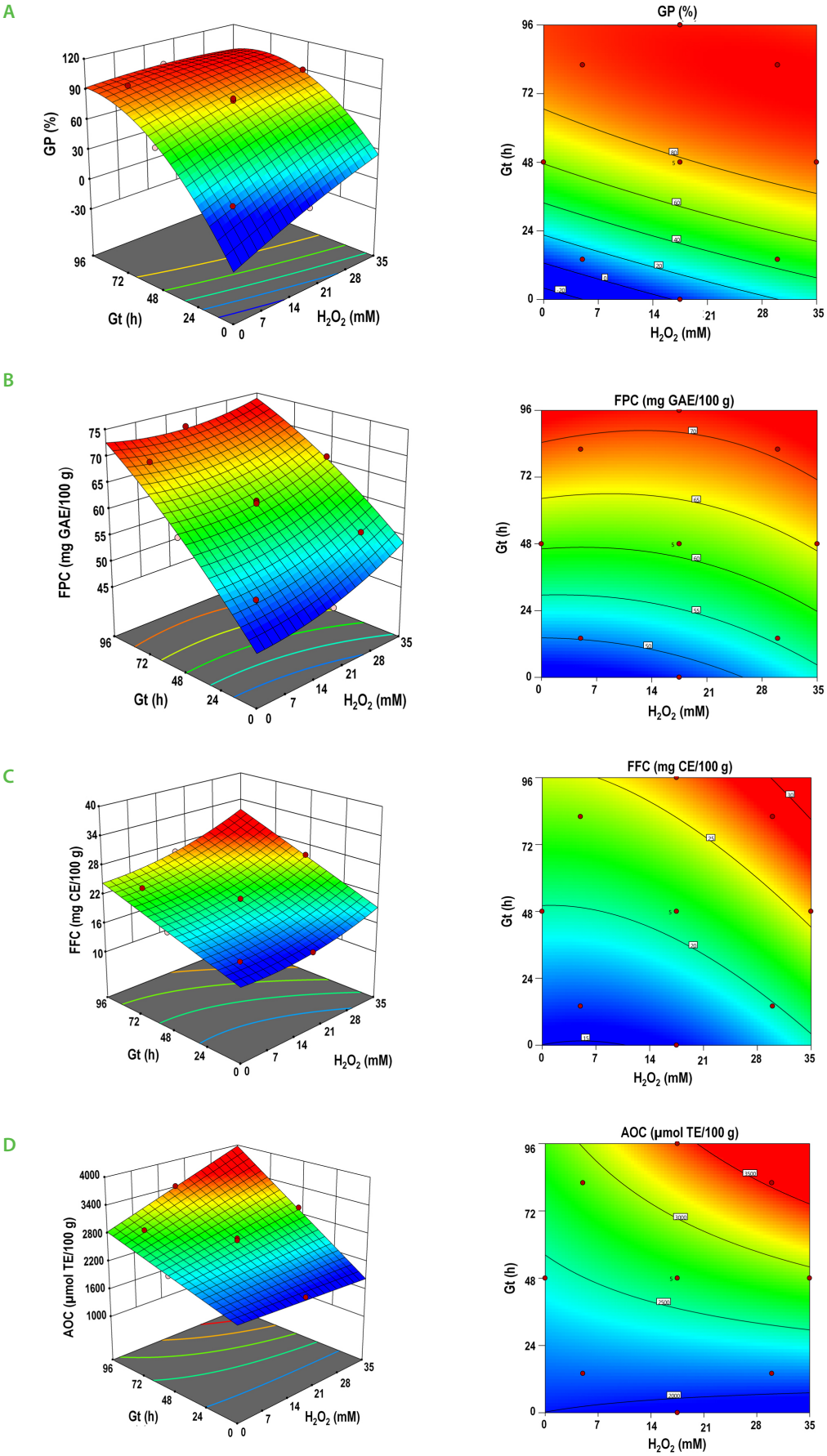

Figure 2. Response surface and contour plots for the effect of the soaking hydrogen peroxide concentration (H_2O_2), and germination time (Gt) on the response variables: (**A**) germination percentage (GP), (**B**) free phenolic content (FPC), (**C**) free flavonoid content (FFC), and (**D**) antioxidant capacity (AOC) of black runner bean sprouts.

Table 4. Parameters of predicted quadratic polynomial model of regression analysis for the response variables including germination percentage (GP), free phenolic content (FPC), free flavonoid content (FFP), and antioxidant capacity (AOC) after adjusting the experimental data.

Parameter	GP	FPC	FFC	AOC
β_0	77.15	61.20	21.14	2,667.97
β_1, $[H_2O_2]$ (X_1)	10.08***	1.78***	2.10***	171.15***
β_2, Gt (X_2)	33.54***	8.43***	3.89***	529.30***
β_{11}, $[H_2O_2]$ (X_{12})	NS	0.91***	0.80***	NS
β_{22}, Gt (X_{22})	14.46***	–0.84**	NS	NS
β_{12}, $[H_2O_2]$×Gt (X_1X_2)	–6.84***	–0.92**	0.37***	160.14***
P-value for model	<0.0001	<0.0001	<0.0001	<0.0001
R^2	0.992	0.995	0.999	0.993
$R^2_{ajusted}$	0.988	0.992	0.999	0.991
P-value for lack of fit	0.476^{NS}	0.483^{NS}	0.331^{NS}	0.628^{NS}
CV (%)	4.93	1.08	0.55	1.69

**Significant at $p<0.01$; ***significant at $p<0.001$; NS, not significant at $p\geq0.05$; $[H_2O_2]$, H_2O_2 concentration; Gt, germination time; R, coefficient of determination; CV, coefficient of variation.

and chia sprouts, supporting the present findings [Dziki *et al.* 2015; Nurnaeimah *et al.*, 2020; Gómez-Velázquez *et al.*, 2021]. Dziki *et al.* [2015] evaluated the influence of germination time (2, 4, 6, and 8 days) on the total phenolic compound content in wheat sprouts, finding that the highest values were obtained at 8 days of germination. On the other hand, several authors observed the same positive effect when stressing seeds or plants with H_2O_2, finding that concentrations of 16 and 30 mM improved the total phenolic content in ethanolic extracts of *F. deltoidea* [Nurnaeimah *et al.*, 2020], and concentrations of 10 and 20 mM significantly increased phenolic content in chia sprouts (*Salvia hispanica* L.) [Gómez-Velázquez *et al.*, 2021].

The observed increase in FPC in H_2O_2-treated sprouts may result from *de novo* synthesis and metabolic transformation. Enzymes such as L-tyrosine ammonialyase and L-phenylalanine ammonialyase are highly responsive to both germination duration and H_2O_2 elicitation [Świeca, 2016]. Additionally, seeds can upregulate defense enzymes like peroxidase and polyphenol oxidase to manage the rapid increase in ROS under stress conditions [Nurnaeimah *et al.*, 2020].

Free flavonoid content

The free flavonoid content in black runner bean sprouts was affected by the H_2O_2 soaking concentration and Gt showing values ranging from 15.7 to 28.3 mg CE/100 g DW (**Table 3**). The analysis of variance yielded a significant quadratic model for FFC, which includes the linear terms of $[H_2O_2]$ and Gt, the quadratic terms of $[H_2O_2]$, and the interaction term of both variables ($[H_2O_2]$×Gt) (**Table 4**).

The response surface (**Figure 2C**) shows a progressive increase in FFC with increasing Gt, reaching a maximum at the longest durations tested. H_2O_2 concentration exerted a secondary but meaningful effect, enhancing flavonoid accumulation at intermediate doses, particularly in combination with extended Gt. These findings indicate that flavonoid biosynthesis is primarily regulated by developmental processes associated with germination and is further amplified by oxidative signals. H_2O_2 functions as a signaling molecule that modulates the expression of genes involved in flavonoid biosynthetic pathways, while prolonged germination supports sustained metabolic activity and substrate availability. Comparable responses have been reported in Dalia bean [Mendoza-Sánchez *et al.*, 2016], quinoa [Świeca, 2016], *F. deltoidea* [Nurnaeimah *et al.*, 2020], and chia seeds [Gómez-Velázquez *et al.*, 2021], demonstrating the robustness of H_2O_2 elicitation across various plants.

Antioxidant capacity

The AOC in black runner bean sprouts ranged from 1,980 to 3,526 µmol TE/100 g DW and was affected by both process variables (**Table 3**). The analysis of variance yielded a significant two-factor interaction model for AOC, which includes the linear terms of $[H_2O_2]$ and Gt, as well as the interaction of both variables ($[H_2O_2]$×Gt) (**Table 4**).

The contour plot (**Figure 2D**) shows that AOC increased as both H_2O_2 concentration and Gt rose, mirroring the trends observed for FPC and FFC. Notably, the highest AOC value (3,526 µmol TE/100 g DW) was achieved by soaking seeds in 30 mM H_2O_2 and germinating for 82 h, indicating that high

peroxide concentrations can reduce the time required to reach near-maximum AOC, whereas at shorter germination times (*e.g.*, 14 h) peroxide concentration had little effect on AOC. The observed increase in AOC during germination is primarily attributed to elevated levels of phenolic compounds, as the FPC and FFC significantly correlated with AOC.

In line with previous studies on amaranth, lentil, Dalia bean, and chia sprouts [Gómez-Velázquez *et al.*, 2021; Mendoza-Sánchez *et al.*, 2016; Perales-Sánchez *et al.*, 2014; Świeca, 2015], these findings suggest that flavonoids and other phenolics enhanced by H_2O_2 treatment are key contributors to the improved antioxidant potential of black runner bean sprouts.

Optimization of hydrogen peroxide soaking concentration and germination time

For optimizing process conditions, the RSM was used through the graphical method to determine the optimal combination of the [H_2O_2] and Gt that maximize the values of the GP, FPC, FFC, and AOC in the sprouts. The overlaid contour plot (**Figure 3**) was used to determine the best combinations of the process variables. The dark green area shows the region with the best combination of process variables to achieve the highest levels of the four response variables, from which the optimal conditions of [H_2O_2] (30 mM) and Gt (92 h) were selected.

The predicted values at the optimal point under the aforementioned conditions were: GP=96.7%, FPC=72.3 mg GAE/100 g DW, FFC=29.6 mg CE/100 g DW, and AOC=3,737 µmol TE/100 g DW. To validate the accuracy of the prediction model, runner bean seeds were germinated under the optimal conditions, and the response variables GP, FPC, FFC, and AOC were determined. Results are shown in **Table 5**. Growth performance and proximate chemical composition of sprouts obtained under optimal conditions were also analyzed.

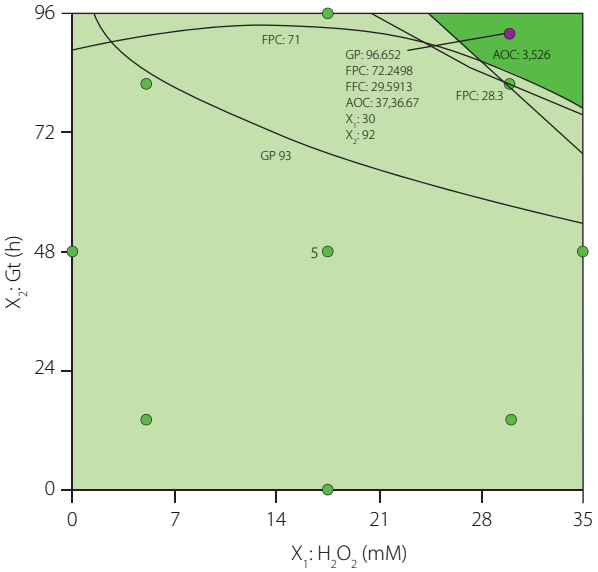

Figure 3. Optimization graph for the response variables: germination percentage (GP), free phenolic content (FPC), free flavonoid content (FFC), and antioxidant capacity (AOC) of black runner bean sprouts. X_1, soaking hydrogen peroxide concentration (H_2O_2); X_2, Germination time (Gt).

Effect of the optimal treatment on growth performance and proximate chemical composition of runner bean sprouts

To evaluate the effect of soaking treatment with H_2O_2 on the growth of runner bean sprouts, radicle length and diameter, as well as the percentage of secondary roots, were analyzed. Sprouts obtained under optimal conditions were compared with those obtained by soaking without H_2O_2 for 92 h (control sprouts). The radicle length in the optimal and control treatments differed significantly ($p<0.05$), with the former having a 32.0% higher value (**Table 5**). This indicates a positive trend in the development of structures in runner bean sprouts due to the inductive effect of the H_2O_2 treatment. However, the radicle's diameter did not show significant differences ($p\geq0.05$) between the two treatments. Regarding the percentage of seeds with secondary roots, the optimal treatment registered a higher percentage compared to the treatment without the stressor, with 76.7% and 43.0%, respectively, showing a difference, which suggests that H_2O_2 accelerates the emergence of lateral structures in the seed's root system. The root is a fundamental organ whose main function is to absorb water and minerals; therefore, a greater length implies a higher probability of success for the establishment, development, and survival of the seedling. Interestingly, Saleh *et al.* [2019] found a correlation between the increase in their length and the rise in antioxidant capacity as well as the content of phenolic compounds and flavonoids in different legumes. Other authors have observed the same inductive trend of H_2O_2 on root growth in various seeds [Barba-Espín *et al.*, 2012; Chaplygina *et al.*, 2020]. These results could be explained by the interaction between the redox state and plant hormones orchestrated by H_2O_2 in the induction of proteins related to signaling and plant development during the early growth of seedlings [Barba-Espin *et al.*, 2010]. In addition, the natural accumulation of H_2O_2 promotes the energy metabolism required for the growth of the radicle and plumule rather than promoting water absorption in the early stage of germination through an increase in osmotic regulators; it mobilizes sugar reserves derived from stored starch, inhibits catabolism, and promotes the biosynthesis of gibberellic acid, a growth and development regulatory substance in seedlings [Delis-Hechavarría *et al.*, 2021; Song *et al.*, 2023].

The unsprouted runner black beans contained 18.96 g of protein, 2.25 g of lipids, 5.50 g of ash, and 73.29 g of total carbohydrates in 100 g DW (**Table 5**). This proximate composition largely coincides with those reported by various authors for different bean varieties [Alvarado-López *et al.*, 2019; Corzo-Ríos *et al.*, 2020; Osuna-Gallardo *et al.*, 2023], with minimal variations that could be explained by environmental conditions during cultivation and harvest, the grain variety, and the methodology used. The protein and lipid content in the sprouts obtained under optimal conditions and control sprouts showed significant increases ($p<0.05$) compared to the unsprouted seeds (**Table 5**). This increase can be attributed to the loss of dry weight due to the oxidation of carbohydrates during seed respiration and the activation of certain enzymes during

Table 5. Proximate composition, phenolic content, and antioxidant capacity of unsprouted runner bean and sprouted under control and optimal conditions, as well as growth performance of sprouts.

Parameter	Unsprouted bean	Sprouted bean	
		Control conditions (0 mM H_2O_2, 92 h)	Optimal conditions (30 mM H_2O_2, 92 h)
Germination percentage (%)	–	82.5±5.8^b	95.7±1.2^a
Radicle length (mm)	–	53.5±9.0^b	70.7±11.0^a
Radicle diameter (mm)	–	3.2±0.5^a	3.3±0.5^a
Secondary roots (%)	–	43.0±1.1^b	76.7±6.6^a
Protein (g/100 g DW)	18.96±0.26^b	21.73±0.56^a	22.76±0.50^a
Lipids (g/100 g DW)	2.25±0.09^b	2.75±0.08^a	2.76±0.03^a
Ash (g/100 g DW)	5.50±0.21^a	5.76±0.17^a	5.91±0.08^a
Total carbohydrates (g/100 g DW)	73.29±0.56^a	69.60± 0.81^b	68.72±0.61^b
Free phenolic content (mg GAE/100 g DW)	58.8±1.6^c	67.6±0.9^b	72.7±1.8^a
Free flavonoid content (mg CE/100 g DW)	22.3±1.5^c	26.4±1.4^b	28.6±1.5^a
Total anthocyanin content (mg C3GE/100 g DW)	9.5±0.2^a	3.8±0.2^b	3.0±0.3^c
ABTS assay (µmol TE/100 g DW)	2,472±56^c	3,028±38^b	3,782±58^a
ORAC (µmol TE /100 g DW)	4,982±303^c	5,793±280^b	6,573±283^a

Different lowercase letter superscripts in the same row show significant difference (p<0.05). ABTS assay, assay with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalent; CE, catechin equivalent; TE, C3GE, cyanidin 3-glucoside equivalent; Trolox equivalent, DW, dry weight.

the germination process [Nazih *et al.*, 2025]. No significant difference (p≥0.05) was found between the proximate chemical composition of the control sprouted seeds and the seeds sprouted under optimized conditions, contrary to what was reported by León-López *et al.* [2020], who reported a significant increase in protein content in germinated chickpea grains under chemical stress with H_2O_2.

Effect of the optimal treatment on phytochemicals and antioxidant properties of runner bean sprouts

The free phenolic content measured in unsprouted runner bean (58.8 mg GAE/100 g DW) was consistent with values reported by Osuna-Gallardo *et al.* [2023], but lower than those observed in other *P. coccineus* varieties [Alvarado-López *et al.*, 2019]. Germination resulted in significant (p<0.05) increases in FPC for both the optimal (to 123.7%) and control (to 115.0%) treatments compared to the unsprouted seeds (**Table 5**). Additionally, the optimal H_2O_2 treatment yielded significantly (p<0.05) higher FPC than the control, supporting findings in other seeds or grains, including chickpea, chia, and barley, where H_2O_2 elicitation promoted phenolic accumulation [Delis-Hechavarría *et al.*, 2021; Gómez-Velázquez *et al.*, 2021; León-López *et al.*, 2020]. A higher phenolic content is noteworthy, as these compounds contribute substantially to the antioxidant activity of sprouts.

The free flavonoid content also increased significantly (p<0.05) in both optimal (to 128.1%) and control (to 118.1%) treatments relative to the unsprouted seeds (**Table 5**). The increase in FFC under the optimal H_2O_2 treatment is consistent with previous studies in other species exposed to hydroxyl peroxide elicitation [Gómez-Velázquez *et al.*, 2021; Uchegbu & Amulu, 2015], highlighting the role of H_2O_2 as an effective stressor to enhance flavonoid biosynthesis.

For total anthocyanin content, the value obtained in this study (9.5 mg C3GE/100 g DW) was lower than those previously reported for bean of some *P. coccineus* varieties [Alvarado-López *et al.*, 2019], but higher than those observed by Osuna-Gallardo *et al.* [2023], who reported values close to 4 mg C3GE/100 g for unprocessed runner bean. In our study, germination led to a significant (p<0.05) reduction in TAC in both optimal and control treatments compared to the unsprouted seeds (**Table 5**). James *et al.* [2020] reported a decrease in anthocyanin content as germination time increased in different legumes, probably because various enzyme systems are mobilized and activated during germination, leading to anthocyanin loss through oxidation and leaching. Meanwhile, Dueñas *et al.* [2006] evaluated the effect of germination and elicitation on the phenolic profile of bean seeds (*Phaseolus vulgaris* L.) germinated for 8 days and observed a reduction in total and some specific anthocyanins, reporting

that germination in the presence of inducers or stressors caused a more extensive decrease in anthocyanin content even below the quantification limit. The behavior recorded in the present study for the anthocyanin content in black runner bean soaking under the induction of chemical stress with H_2O_2 is in agreement with these findings, since a greater reduction was observed in the optimal germination treatment than in the control one (**Table 5**).

The antioxidant capacity (AOC) of unsprouted seeds and sprouts germinated under both optimal and control conditions was evaluated as $ABTS^{\bullet+}$ scavenging activity and ORAC. According to Munteanu & Apetrei [2021], the ABTS assay operates *via* a mixed mechanism (involving both electron and hydrogen atom transfer), whereas the ORAC assay relies primarily on hydrogen-atom-transfer mechanisms. The use of both methods provided a more comprehensive assessment of antioxidant properties. The value obtained by the ABTS assay for the unsprouted seed (2,472 µmol TE/100 g DW) was consistent with that reported by Osuna-Gallardo *et al*. [2023], who reported 2,657.94 µmol TE/100 g of unprocessed runner bean. However, it was higher than the values reported by Orak *et al*. [2016] for ten white bean (*P. vulgaris*) varieties (350–517 µmol TE/100 g), and also higher than those reported by Weidner *et al*. [2018] for four *P. vulgaris* varieties (421–640 µmol TE/100 g DW). Optimal germination treatment and the control germination treatment showed significant increases ($p<0.05$) of 53.0% and 22.5%, respectively, compared with the unsprouted bean (**Table 5**). Likewise, a significant increase ($p<0.05$) of 24.9% was observed as a result of the soaking treatment with H_2O_2 when comparing the optimal sprouts to the controls. This increase in the AOC in the sample germinated under optimal conditions could be associated with the stimulation of the synthesis of compounds with high antioxidant activity, such as phenolic compounds, due to the chemical stress produced by the application of H_2O_2 during germination [León-López *et al*., 2020].

Several authors have emphasized the increase in AOC during the germination process using H_2O_2 as an inducing stress agent. For instance, Gómez-Velázquez *et al*. [2021] recorded an increase in the AOC of chia seeds germinated with 10, 20, and 30 mM H_2O_2, obtaining increases in the range of 29–37% compared to seeds germinated without the stressor. Similarly, León-López *et al*. [2020] reported that in white chickpea germination, chemical H_2O_2 elicitation produced an increase of 14.8% when comparing their optimal treatment ([H_2O_2] of 30 mM, Gt of 72 h) with control seeds without elicitor ([H_2O_2] of 0 mM, Gt of 72 h). Conclusions from these studies agree with the present findings regarding the increase in AOC measured by the ABTS assay in runner bean sprouts treated with H_2O_2.

The ORAC showed the same trend as that determined by ABTS assay (**Table 5**). Optimal and control germination treatments showed significant increases ($p< 0.05$) of 31.9% and 16.3%, respectively, compared with ungerminated runner beans, moreover, a significant increase ($p<0.05$) of 13.5% was observed between runner beans germinated under optimal conditions and those germinated under control conditions. The ORAC of the ungerminated seed (4,982 µmol TE/100 g DW) was close to that reported by Alvarado-López *et al.* [2019], who reported values of 5,162; 3,694; 2,557; and 2,031 µmol TE/100 g, for four varieties of *P. coccineus* bean: purple, black, brown, and white, respectively. However, Osuna-Gallardo *et al.* [2023] recorded an ORAC value of 3,866 µmol TE/100 g of runner bean flour, which was lower than those reported in this study. These variations may be associated with differences in the phenolic profiles among bean varieties, as well as with the extraction and quantification techniques used.

In summary, these results demonstrate that H_2O_2 treatment during soaking significantly enhanced the antioxidant capacity of black runner bean sprouts, supporting their potential use as nutraceutical ingredients with improved health-promoting properties.

A variety of phenolic acids, flavonoids, and anthocyanins have been reported in runner bean varieties [Baeza-Jiménez & López-Martínez, 2024; López-Martínez, 2020]. Particularly, gallic acid, sinapic acid, ferulic acid, chlorogenic acid, *p*-coumaric acid, protocatechuic acid, and 4-hydroxybenzoic acid have been identified in unprocessed black runner beans [Baeza-Jiménez & López-Martínez, 2024]. Flavonoids, such as kaempferol 3-glucoside, catechin, and epicatechin, were also reported in unprocessed black runner beans. In this study, gallic, syringic, ferulic, chlorogenic, and *p*-coumaric acids were identified in unsprouted runner beans (**Table 6**). Regarding flavonoids, consistent with Baeza-Jiménez & López-Martínez [2024], catechin and quercetin were identified; in addition, rutin was detected in the free phenolic fraction of the unsprouted seeds. Germination processes can modify the phenolic profile [Domínguez-Arispuro *et al.,* 2018; Yu *et al.,* 2023], and even soaking stress can elicit an increase in some phenolic contents [León-López *et al*., 2020].

Among the main findings, the significant ($p<0.05$) increase in the content of gallic acid and syringic acid, and the presence of caffeic acid, stand out as a result of germination in the free phenolic fraction (**Table 6**). The content of catechin and quercetin also significantly increased ($p<0.05$) in the free phenolic fraction as a result of the control germination treatment. The stress treatment with H_2O_2 during soaking induced specific changes in the free phenolic fraction compared to the unprocessed seed and the control sprouts; notably, a significant increase in ferulic acid and *p*-coumaric acid was observed. In contrast, the content of catechin and quercetin decreased significantly ($p<0.05$) compared to the control sprouts.

Although the content of bound phenolic compounds was not used as a response variable for the RSM optimization, nor were their antioxidant properties individually measured, it was considered important to analyze the phenolic profile of this fraction to fully discuss the metabolic changes that occurred. Ferulic and *p*-coumaric acids, which increased in both free and bound phenolic fractions in runner bean sprouts obtained under optimal conditions, serve as potent antioxidants with applications across food, nutraceuticals, and pharmaceuticals. Ferulic acid supports metabolic health by enhancing glucose and lipid metabolism and mitigating oxidative stress, while

Table 6. Phenolic compound profile of unsprouted runner bean and sprouted under control and optimal conditions (μg/g dry weight).

Phenolic compound	Unsprouted bean		Sprouted bean			
			Control conditions (0 mM H_2O_2, 92 h)		Optimal conditions (30 mM H_2O_2, 92 h)	
	Free	Bound	Free	Bound	Free	Bound
Gallic acid	158.5±3.6^{c}	72.7±1.3^{f}	218.0±6.8^{a}	120.6±2.3^{d}	197.2±3.3^{b}	97.3±2.4^{e}
Syringic acid	7.8±0.5^{d}	10.4±0.7^{d}	52.9±2.4^{c}	137.4±3.7^{a}	57.0±4.0^{c}	76.3±1.8^{b}
Ferulic acid	89.2±1.7^{c}	184.2±4.8^{b}	43.9±2.5^{d}	86.8±2.7^{c}	190.1±3.9^{b}	356.2±7.2^{a}
Chlorogenic acid	26.7±0.1^{b}	ND	25.5±0.7^{b}	15.3±1.5^{c}	27.3±2.1^{b}	42.8±0.7^{a}
Caffeic acid	ND	ND	14.0±0.4^{a}	14.2±0.3^{a}	14.04±0.1^{a}	7.1±0.0^{b}
p-Coumaric acid	25.4±0.2^{e}	81.7±2.4^{b}	24.2±2.7^{e}	39.1±0.14^{d}	67.6±2.7^{c}	205.9±4.1^{a}
Catechin	53.3±3.6^{d}	72.5±1.9^{c}	85.7±3.7^{b}	178.6±4.7^{a}	76.8±1.4^{bc}	82.4±4.6^{b}
Quercetin	14.8±0.9^{d}	41.4±1.5^{b}	38.2±2.4^{b}	103.4±4.1^{a}	20.1±0.9^{cd}	25.3±2.3^{c}
Rutin	128.4±3.1^{b}	ND	125.1±4.7^{b}	189.8±5.9^{a}	120.0±6.3^{b}	13.3±0.8^{c}
Total phenolics	504.1±13.0^{d}	462.9±12.6^{d}	613.4±26.1^{c}	871.0±26.2^{a}	756.1±24.8^{b}	899.4±24.0^{a}

Different lowercase letter superscripts show significant differences across rows (p<0.05). ND, not detected.

also exerting anti-inflammatory, antimicrobial, neuroprotective, and cardiovascular effects [Jacobo-Velázquez, 2025; Kumar *et al.*, 2025]. Meanwhile, *p*-coumaric acid provides anti-inflammatory, antidiabetic, anticancer, cardioprotective, and hepatoprotective benefits, positioning both phenolic acids as promising candidates for drug formulations and chronic disease prevention [Kaur & Kaur, 2022; Kumar *et al.*, 2025].

CONCLUSIONS

The present study demonstrates that H_2O_2 treatment before black runner bean seed germination is an effective strategy to enhance sprout quality. H_2O_2 elicitation improved germination performance, stimulated radicle growth, increased the accumulation of total phenolic compounds, and enhanced the antioxidant capacity of sprouts. Optimal conditions, 30 mM H_2O_2 combined with prolonged germination (around 92 h), resulted in higher levels of certain free and bound phenolic acids and flavonoids, as well as improved $ABTS^{\bullet+}$ scavenging activity and ORAC of the sprouts. The use of response surface methodology (RSM) was critical for identifying these optimal elicitation conditions, offering a robust statistical framework that facilitates reproducibility and potential scale-up. These findings highlight the promise of H_2O_2-assisted sprouting for the development of functional flours with enhanced nutritional and potential health benefits.

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CONFLICT OF INTERESTS

Authors declare no conflicts of interest.

ORCID IDs

I. Benítez-García https://orcid.org/0000-0002-9047-9355
E.O. Cuevas-Rodríguez https://orcid.org/0000-0001-6648-7323
L. León-López https://orcid.org/0000-0003-4973-2096
S. Montaño https://orcid.org/0000-0002-0420-4268
S. Mora-Rochín https://orcid.org/0000-0002-9630-2518
C. Reyes-Moreno https://orcid.org/0000-0002-4384-1286
L.M. Sánchez-Magaña https://orcid.org/0000-0003-1268-5986
L.F. Uriarte-Franco https://orcid.org/0009-0007-2131-3347

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Modification of Tartary Buckwheat Bran and Its Application in Steamed Bread Processing

Xinru Guo[1]†, Xuan Zhang[2]†, Yuan Liu[1], Jian Wang[1]*, Lin Yuan[1], Yunlong Li[3], Peng Dong[1], Dongjie Wang[1], Chunheng Dai[1], Haitao Zhou[4]

[1]Hebei Key Laboratory of Quality & Safety Analysis-Testing for Agro-Products and Food, Hebei North University, Zhangjiakou 075000, China
[2]College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China
[3]Institute of Functional Food of Shanxi, Shanxi Agricultural University, Taiyuan 030031, China
[4]Zhangjiakou Academy of Agricultural Sciences, Zhangjiakou 075000, China

This study investigated the modification of Tartary buckwheat bran (Tbb) and its application in enhancing the quality and functional properties of steamed bread. The effects of three modification methods, including extrusion, fermentation, and superfine grinding, on soluble dietary fiber (SDF) content, total phenolic content (TPC), total flavonoid content (TFC) and functional properties of Tbb were evaluated. The SDF content increased from 1.87 g/100 g in unmodified Tbb to 3.87, 2.98, and 2.69 g/100 g after extrusion, fermentation, and superfine grinding, respectively. The modified Tbb also showed higher TPC and TFC compared to the unmodified bran, with the greatest increase found for fermented Tbb to 19.07 mg GAE/g and 2.06 mg RE/g, respectively. Among the three modification methods, fermentation resulted in the most pronounced improvement in the water-holding capacity, oil-holding capacity, and swelling capacity of Tbb. The optimal fermentation conditions, determined through response surface methodology, were a temperature of 32°C, a fermentation time of 6 h, and a yeast to lactic acid bacteria ratio of 2:1 (*w/w*). The fermented Tbb was then mixed with wheat flour at various substitution levels to produce steamed bread. Results showed that the steamed bread produced with a 10% (*w/w*) substitution of wheat flour by fermented Tbb exhibited the highest sensory score (84 points), indicating superior consumer acceptability while maintaining enhanced functional properties. This research offers valuable insights into the utilization of Tbb as a functional ingredient in the development of healthier food products.

Keywords: extrusion, *Fagopyrum tataricum*, fermentation, soluble dietary fiber, steamed bread quality

INTRODUCTION

Buckwheat, a dicotyledonous angiosperm of *Fagopyrum* in the Polygonaceae family, is a traditional crop widely planted in the world. The main buckwheat species are common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) [Kim *et al*., 2023]. Tartary buckwheat has strong ecological adaptability and can still grow well in harsh environments. It has a balanced amino acid composition, with contents of protein, fat, minerals, vitamins, and trace elements being generally higher than in common buckwheat, wheat, rice, and corn grains [Zhu, 2016]. In addition, it is rich in flavonoids, such as rutin, which are lacking in many cereal crops [Xue *et al*., 2022]. Tartary buckwheat has been reported to prevent and control diabetes [Wu *et al*., 2018], lower blood lipids [Sun *et al*., 2019], lower blood pressure [Hou *et al*., 2017], as well as elicit anti-cancer [Zhou *et al*., 2019] and anti-fatigue [Miao *et al*., 2016] effects. Due to these health-promoting characteristics, Tartary buckwheat is considered a promising raw material for functional

***Corresponding Author:**
e-mail: lywjbfxy@163.com (Dr. J. Wang)
†These authors had equal contribution to this work

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food development with both nutritional and bioactive value. At present, it is commonly used in the production of various foods, such as tea, noodles, biscuits, and beverages [Zhu, 2016].

Tartary buckwheat bran (Tbb) is the outermost layer of Tartary buckwheat seeds and is usually a by-product of Tartary buckwheat flour processing. Due to its coarse texture and low digestibility, it is mostly used for feed production, as a fermentation medium or directly discarded, thus not only polluting the environment but also greatly increasing waste resources. However, Tbb is also rich in protein, minerals, and other nutrients and functional compounds [Sinkovič *et al*., 2022]. Studies have shown that Tbb contains more bioactive compounds than Tartary buckwheat flour [Noda *et al*., 2023; Xue *et al*., 2022]. The dietary fiber in Tbb confers important physiological benefits, including the prevention of constipation, reduction of serum cholesterol, and regulation of blood glucose levels [Guo *et al*., 2012]. As such, Tbb holds considerable potential as a functional food ingredient with broader nutritional and application potential than the flour itself.

Tbb is broadly utilized, particularly in the processing of traditional staple foods like steamed bread, which is a traditional Chinese food with a long history, primarily produced through wheat flour fermentation, dough mixing, and steaming. While steamed bread is known for retaining more nutrients than Western-style baked bread, its widespread consumption has contributed to nutritional imbalances, such as excessive macronutrient intake and insufficient dietary fiber, leading to health issues like coronary heart disease, type 2 diabetes, and obesity [Liu *et al*., 2011; Rose *et al*., 2010]. Adding Tbb to wheat flour formulations could help develop bran-enriched steamed bread, offering a nutritionally-enhanced alternative to conventional steamed bread. However, incorporating Tbb into steamed bread presents challenges, including poor taste, altered texture, and darkened color [Ma *et al*., 2021]. These issues are largely attributed to Tbb's physicochemical properties, which interfere with the gluten network, slowing fermentation, reducing gas retention, and decreasing loaf volume [Zhao *et al*., 2025].

To address these challenges, various modification techniques have been explored to improve the sensory and nutritional qualities of Tbb-enriched products. The main methods for modifying bran include fermentation [Coda *et al*., 2014], superfine grinding [Xiao *et al*., 2022], extrusion expansion [Li *et al*., 2023], microwave heating [Jiang *et al*., 2016], steam explosion [W. Li *et al*., 2022], ultra-high pressure [Xia & Li, 2018], *etc*. Despite the widespread application of these techniques to other brans, such as wheat [Guo *et al*., 2025; Saroj *et al*., 2025] and barley [Xi *et al*., 2023; Y. Zhang *et al*., 2024], research on Tbb modification, especially in the context of steamed bread production, remains limited.

This study aimed to investigate the effects of three modification techniques – fermentation, superfine grinding, and extrusion expansion – on the content of soluble dietary fiber (SDF), total phenolics, and flavonoids in Tbb. The optimal modification method was identified, and fermentation parameters were optimized using single-factor experiments combined with response surface methodology (RSM). Modified Tbb was then incorporated into wheat flour at varying substitution levels (0%, 5%, 10%, 15%, 20%, and 25%, *w/w*) to produce steamed bread. The resulting products were evaluated in terms of specific volume, textural profile, soluble dietary fiber content, total flavonoid content, and *in vitro* digestibility. A comprehensive sensory evaluation was also conducted to assess the quality of the bran-enriched steamed bread. This study provides experimental evidence and technological support for the integrated utilization of Tbb and its value-added applications, particularly in improving the quality of traditional steamed bread products.

MATERIALS AND METHODS

Materials

Tartary buckwheat bran powder was from Shanxi Yanmen Qinggao Shiye Co., Ltd. (Shanxi, China), instant active dry yeast was from Angel Yeast Co., Ltd. (Hubei, China), and plant-derived *Lactobacillus spp.* and *Saccharomyces cerevisiae* strains were purchased as dried powders from Kunshan Bisour Biotechnology Co., Ltd. (Jiangsu, China) for use in fermentation processes. Standard gallic acid and sodium carbonate were from Sunny Biotech Co., Ltd. (Shanghai, China), Folin-Ciocalteu-phenol reagent and standard rutin were from Hefei Bomei Biotechnology Co., Ltd. (Anhui, China), sodium hydroxide and anhydrous ethanol were from Tianjin Huihang Technology Co., Ltd. (Tianjin, China), sodium nitrite and sodium sulfite were from Tianjin chemical reagent supply and marketing company (Tianjin, China). Potassium sodium tartrate was obtained from Tianjin FengChuan Chemical Reagent Technology Co., Ltd. (Tianjin, China). Aluminum nitrate was obtained from Xilong Scientific Co., Ltd. (Guangdong, China). Golden embryo corn oil was from Shandong Sanxing Corn Industry Technology Co., Ltd. (Shandong, China). Wheat flour was purchased from China Oil and Foodstuffs Corporation (Beijing, China). Saccharifying enzyme (50,000 U/g) and 3,5-dinitrosalicylic acid (DNS) were bought from Shanghai Ruiyong Biotechnology Co., Ltd. (Shanghai, China). Amylase (3,000 U/mL) was from Nanning Pangbo Biological Engineering Co., Ltd. (Guangxi, China). All reagents used were of analytical reagent grade, and the water was ultrapure.

Tartary buckwheat bran modification procedures

Extrusion expansion

The Tbb was processed by using a twin-screw extruder (TSE70, Jinan Sunward Machinery Co., Ltd., Shandong, China). Material moisture content was 18%. The processing parameters were as follows: feeding rate was 15 Hz; screw speed was 200 rpm; and temperature in zone 1, zone 2, and zone 3 was 60℃, 170℃, and 190℃, respectively. After processing, the extrudates were oven-dried at 50℃ overnight and then treated in a high-speed multifunctional grinder (Beijing Rayleigh Analytical Instrument Corp., Beijing, China), and sifted through a 100-mesh sieve.

Fermentation

The Tbb powder was mixed with distilled water at a ratio of 1:2 (*w/v*), after which the mixed culture of *S. cerevisiae* and *Lactobacillus* (2:1, *w/w*) was inoculated into the resulting mixture at an inoculation rate of 3% (*w/v*). The entire mixture was then incubated at 33°C for 4 h in a constant temperature incubator (GNP-9160, Ningbo Southeast Instrument Co., Ltd., Zhejiang, China). Afterwards, it was dried in a hot air-drying oven (FX101-3, Shanghai Shuli Instrumentation Co., Ltd., Shanghai, China) at 40°C for 48 h, crushed, and sifted through a 100-mesh sieve.

Superfine grinding

The Tbb was put into a superfine grinder (RT-UF26W, Rong Tsong Precision Technology Co., Ltd., Taiwan, China), and the superfine Tbb powder of more than 100 mesh was obtained by using the high-speed airflow generated by the high-speed impact between Tbb and the cutting tool. It was collected according to the centrifugal principle of the cyclone. Processing parameters were as follows: power 1.94 kW, speed 25,000 rpm, and feed size 2 mm.

Determination of contents of functional compounds in Tartary buckwheat bran

Soluble dietary fiber content

The content of soluble dietary fiber (SDF) in Tbb was determined according to the AOAC International method no. 991.43 [AOAC, 2023], and the results were expressed as g of SDF *per* 100 g of Tbb.

Total phenolic content and total flavonoid content

The extraction of unmodified and modified Tbb was conducted in order to determine the contents of total phenolics and total flavonoids. The powdered samples were treated as follows: 25 mL of ethanol (95%) were added to 1 g of each sample, which was then sonicated at 20°C and 100 W for 60 min, and filtered.

The total phenolic content (TPC) and the total flavonoid content (TFC) were determined by Folin-Ciocalteu colorimetric and aluminum nitrate-sodium nitrite colorimetric methods with some modifications. The TPC of Tbb was determined using the procedure reported by Pirca-Palomino *et al*. [2024]. To this end, 0.1 mL of the above extract was mixed with 1.0 mL of the Folin–Ciocalteu reagent. After 2 min, 1.5 mL of a sodium carbonate solution was added, the mixture was thoroughly mixed and incubated in the dark for 1 h, and the absorbance was measured at 765 nm using a UV-1800B ultraviolet spectrophotometer (Beijing Rayleigh Analytical Instrument Corp. Beijing, China). Gallic acid was used as the reference substance, the results were expressed as mg gallic acid equivalents (GAE) *per* g of Tbb. The TFC was determined using the procedure reported by Ge & Wang [2020]. In brief, 1.0 mL of the Tbb extract was mixed with 0.5 mL of a 5% $NaNO_2$ solution and allowed to stand at room temperature for 5 min. Then, 0.5 mL of a 10% $Al(NO_3)_3$ solution was added, mixed thoroughly, and the mixture was left to stand for another 5 min. Subsequently, 2 mL of a 4% NaOH solution were added, followed by 60% (*v/v*) ethanol to bring the final volume to 20 mL. After thorough mixing, the absorbance was measured at 510 nm. The results were expressed as mg rutin equivalents (RE) *per* g of Tbb.

Determination of processing properties of Tartary buckwheat bran

Determination of water-holding capacity

The water-holding capacity (WHC) was determined based on the procedures described by Sangokunle *et al*. [2020] and Nguyen *et al*. [2023], with slight modifications. An accurately weighed modified and unmodified Tbb samples of 1 g (m_0) were immersed in 50 mL of distilled water. The mixture was magnetically stirred for 30 min and then allowed to stand at room temperature for additional 30 min. Subsequently, it was centrifuged at 1,800×*g* for 20 min to remove the supernatant. The remaining residue was collected and weighed as m_1. The WHC of Tbb was calculated according to Equation (1):

$$WHC\ (g/g) = (m_1 - m_0)/m_0 \quad (1)$$

Determination of oil-holding capacity

The oil-holding capacity (OHC) was evaluated using the procedures described by Sangokunle *et al*. [2020] and Nguyen *et al*. [2023], with slight modifications. Both modified and unmodified Tbb samples of 1 g (M_0) were immersed in 20 mL of corn oil and magnetically stirred for 30 min. The mixture was then allowed to stand at room temperature for 30 min, followed by centrifugation at 1,800×*g* for 20 min. After removing the supernatant, any oil adhering to the inner wall of the centrifuge tube was carefully wiped off, and the remaining mass was weighed as M_1. The OHC was calculated using Equation (2):

$$OHC\ (g/g) = (M_1 - M_0)/M_0 \quad (2)$$

Determination of swelling capacity

The procedures described by Sangokunle *et al*. [2020] and Nguyen *et al*. [2023] were used to measure swelling capacity (SC). The samples of modified and unmodified Tbb (m) were immersed in 25-mL graduated cylinder and mixed thoroughly. The initial volume of the sample (V_0) was recorded. Subsequently, 10 mL of distilled water were added, and the mixture was shaken well. After standing at room temperature for 24 h, the final volume of the sample (V_1) was recorded. The swelling capacity (SC) was calculated using Equation (3):

$$SC\ (mL/g) = (V_1 - V_0)/m \quad (3)$$

Optimization of Tartary buckwheat bran fermentation

According to the modification method described above, Tbb was thoroughly mixed with distilled water at a ratio of 1:2 (*w/v*) to obtain a homogeneous mixture. With the inoculation level of the mixed starter fixed at 3% (*w/v*), the effects of different ratios of *S. cerevisiae* to *Lactobacillus* (1:1, 1:2, 1:3, 2:1, 3:1, *w/w*), fermentation temperature (23°C, 28°C, 33°C, 37°C, 42°C), and fermentation

Table 1. Factors and levels of the Box–Behnken design for optimizing Tartary buckwheat bran fermentation conditions.

Factor	Level		
	–1	0	1
Fermentation temperature (°C)	28	33	37
Fermentation time (h)	4	6	8
Ratio of *Saccharomyces cerevisiae* to *Lactobacillus* as inoculation cultures (*w/w*)	1:1	2:1	3:1

time (2, 4, 6, 8, 10 h) on the SDF content of Tbb were investigated using a single-factor experimental design in which one variable was changed at a time while the others were kept constant. Specifically, during analyses of the ratio of inoculation cultures, the fermentation temperature and time were maintained at 33°C and 4 h, respectively; during temperature tests, the ratio of inoculation cultures and fermentation time were maintained at 1:1 (*w/w*) and 4 h, respectively; and during time tests, the ratio of inoculation cultures and fermentation temperature were maintained at 1:1 (*w/w*) and 33°C, respectively.

According to the design principle of the Box-Behnken central combination experiment, a response surface analysis experiment with three factors and three levels was conducted (**Table 1**) to optimize the conditions of Tbb fermentation.

Production of Tartary buckwheat bran steamed bread

The mixed flour was prepared by blending fermented Tbb powder and wheat flour. The composite flours were formulated by replacing wheat flour with fermented Tbb at 0, 5, 10, 15, 20, and 25% (*w/w*, based on total flour). The mixtures were stored in a cool, dry place for later use and the steamed bread was prepared based on the method of S. Zhang *et al.* [2024] with slight modifications. Specifically, 110 g of the prepared flour was weighed, 1% (*w/w*) active dry yeast was added, and subsequently 50 g of water were added gradually in multiple portions. All ingredients were mixed in a dough mixer (CE6001C, Guangdong Weishida Electric Technology Co., Ltd., Guangdong, China), first at a low speed for 2 min, then at a medium-high speed for 8 min, to form a smooth dough. The dough was evenly divided into 50-g portions and kneaded into small dough pieces. The dough pieces were placed in a fermentation chamber and proofed for 1 h at 35°C and 60% relative humidity. Subsequently, the samples were steamed for 20 min. After steaming, the heat was turned off, and the samples were allowed to cool naturally at room temperature.

Determination of steamed bread quality

Specific volume

The specific volume measurement was conducted using the rapeseed displacement method outlined by Ouyang *et al.* [2024]. The cooled steamed bread was placed into a container filled with rapeseed, then the volume of rapeseed displaced was measured with a graduated cylinder. The specific volume was calculated as the ratio of this volume (mL) to the mass of the bread sample (g).

Sensory evaluation

This study was approved by the College of Agronomy and Forestry Science of Hebei North University (Zhangjiakou, China), and informed consent was obtained from all volunteers prior to their participation. Throughout the sensory evaluation, the principles set out in the Declaration of Helsinki were strictly followed. Before the formal assessment, all volunteers attended a brief training session to familiarize themselves with the specific attributes to be evaluated, including appearance, internal structure, color, chewiness, elasticity, odor, and other relevant sensory characteristics. After the steamed bread had been placed at room temperature 25±2°C for 1 h, it was cut into several pieces. Fifty evaluators who met the standards of sensory evaluators both psychologically and physiologically were selected to form a sensory evaluation panel to taste steamed bread. The sensory evaluation included the assessment of several attributes, such as appearance and shape, internal structure, color, chewiness, elasticity, palatability, fragrance, and bitterness. The full score was 100, and the scoring criteria for steamed bread are outlined in **Table 2**.

Texture characteristics

The texture characteristics of the steamed bread were studied by texture profile analysis (TPA) with a TA.XT Plus physical property analyzer (Beijing Lotun Science Co., Ltd., Beijing, China). The parameters measured included hardness (N), chewiness (N), springiness (%), cohesiveness, and resilience (%). Springiness was expressed as the percentage recovery of sample height after compression, while resilience was calculated as the percentage of energy recovered during the first compression cycle. After having been left at room temperature for 1 h, the steamed bread was cut into uniform pieces of 15 mm thickness, and the central two pieces were taken for determination. The measurement parameters were set as follows: pretest speed 1.00 mm/s, test speed 5.00 mm/s, post-test speed 5.00 mm/s, compression degree 60.00%, and trigger force 5.0 g.

Determination of contents of functional compounds in steamed bread

The content of SDF in steamed bread was determined according to the AOAC International method no. 991.43 [AOAC, 2023], and the results were expressed as g of SDF *per* 100 g of dry matter (DM) basis.

Based on the procedures described by Ma *et al.* [2013], the steamed bread samples were freeze-dried, ground into powder, and passed through a 100-mesh sieve. The powder was extracted with 70% (*v/v*) ethanol at a solid-to-liquid ratio of 1:20 (*w/v*) under ultrasonic assistance for 40 min at room temperature. The extract was centrifuged, and the supernatant was collected for TFC determination, which was performed using the above-described method. Rutin was used as the standard,

Table 2. The scoring standard of steamed bread.

Attribute	Scoring standard	Score
Appearance shape (15)	Erect and plump appearance, smooth surface	11–15
	Slight collapse, slight surface wrinkle, slight contraction	6–10
	Shrunken, flat, with a hard surface	0–5
Internal structure (15)	The pores in the longitudinal section are fine and uniform, and are spongy	11–15
	The size of pores in the longitudinal section is uneven, with a small amount of steamed bread crumbs	6–10
	The size of pores in the longitudinal section is uneven, with large holes and a rough texture	0–5
Color (15)	Good	8–15
	Slightly dark	5–7
	Gray and dark	0–4
Chewiness (10)	Palatable, soft, easy to swallow	8–10
	Moderately soft and hard	5–7
	Dry and hard, difficult to swallow	0–4
Elasticity (10)	Good rebound when pressed with fingers	8–10
	Weak resilience	5–7
	No rebound or difficulty in pressing	0–4
Palatability (10)	Refreshing and not sticky to teeth	8–10
	Slightly sticky or refreshing	5–7
	Sticky and not refreshing	0–4
Fragrance (15)	Steamed bread unique fragrance	9–15
	The aroma is not strong or basically odorless	5–8
	Unacceptable odor	0–4
Bitterness (10)	The taste is light and fragrant, with a fermented aroma and moderate bitterness	8–10
	Slightly scented, with a strong bitterness	5–7
	Unscented and unacceptably bitter	0–4

Numbers in parentheses indicate the full score of the sensory evaluation.

and the results were expressed as mg rutin equivalents *per* g of bread DM.

Digestibility of steamed bread

To weighed 0.5-g steamed bread crumbs, 10 mL of sodium acetate buffer (0.2 M) were added, and the mixture was placed in a boiling water bath for 30 min. After cooling to room temperature, it was heated in a 37℃ water bath (single row of two-hole constant temperature water bath pot, Shanghai Shuli Instrumentation Co., Ltd.) for 5 min, and then 1 mL of α-amylase (3,000 U/mL) and 0.1 mL of a saccharifying enzyme (10,000 U/mL) were added. Subsequently, 0.5-mL samples were taken at 0, 20, 60, 90, and 120 min respectively, and 4.5 mL of anhydrous ethanol were added, then the samples were shaken in a constant temperature oscillation incubator several times and centrifuged at 1,800×*g* for 15 min [Englyst *et al*., 2018]. The supernatant was collected, and the glucose content in the sample was determined by the DNS method [Miller, 1959]. The digestibility of starch was characterized by the reducing sugar produced in the digestion process.

Statistical analysis

Design-Expert 11 software (Stat-Ease, Inc, Minneapolis, MN, USA) was used for response surface analysis. Microsoft Office Excel 2019 software (Microsoft Corporation, Redmond, WA, USA) was used to calculate means and standard deviations of quality indicators. Data analysis and image processing of the texture analysis were conducted by Texture Exponent 32 software (Beijing Lotun Science Co., Ltd., Beijing, China). Statistical analysis, such as one-way analysis of variance (ANOVA) and Duncan's multiple comparisons, was performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Differences between means were considered statistically significant at $p<0.05$. The statistical plots were produced using Origin 2019 software (OriginLab Corporation, Northampton, MA, USA). Each experiment was repeated three times ($n=3$).

RESULTS AND DISCUSSION

Tartary buckwheat bran modifications

Effect of different modifications on the appearance of Tartary buckwheat bran

Appearance is one of the key indicators directly reflecting product quality. The Tbb appearance before and after modifications is shown in **Figure 1**. The unmodified Tartary buckwheat bran (**Figure 1A**) appeared coarse in texture, yellowish in color, and tended to clump into large aggregates. After modification, the extruded bran became dark brown due to exposure to high temperature and pressure, with larger particles and a more coarse, fluffy texture (**Figure 1B**). In contrast, Tbb modified by fermentation appeared lighter in color and more loosely structured compared to the unmodified version (**Figure 1C**). The superfine ground Tbb had a brighter and more uniform color, and also finer and more delicate particles (**Figure 1D**).

Effects of different modifications on the content of functional compounds in Tartary buckwheat bran

The SDF content before and after modifications of Tbb is shown in **Figure 2A**. The SDF content in all treated Tbb was significantly ($p<0.05$) higher than in the unmodified one. The SDF content of the unmodified Tartary buckwheat bran was 1.87 g/100 g, and it increased by 106.95%, 59.36%, and 43.85% after extrusion expansion, fermentation, and superfine grinding, respectively. Cao *et al*. [2021] treated rice bran with a twin-screw extruder, and the results showed that the SDF content of rice bran increased after extrusion, which was consistent with the results of our study. In the extrusion process, the conditions of high temperature, high pressure, and high shear can destroy the dietary fiber molecules and convert insoluble dietary fiber (IDF) into SDF. Zhao *et al*. [2017] found that the SDF content of wheat bran fermented by yeast and lactic acid bacteria increased. It

Figure 1. Appearance of Tartary buckwheat bran powders in unmodified form (**A**) and after extrusion expansion (**B**), fermentation (**C**), and superfine grinding (**D**).

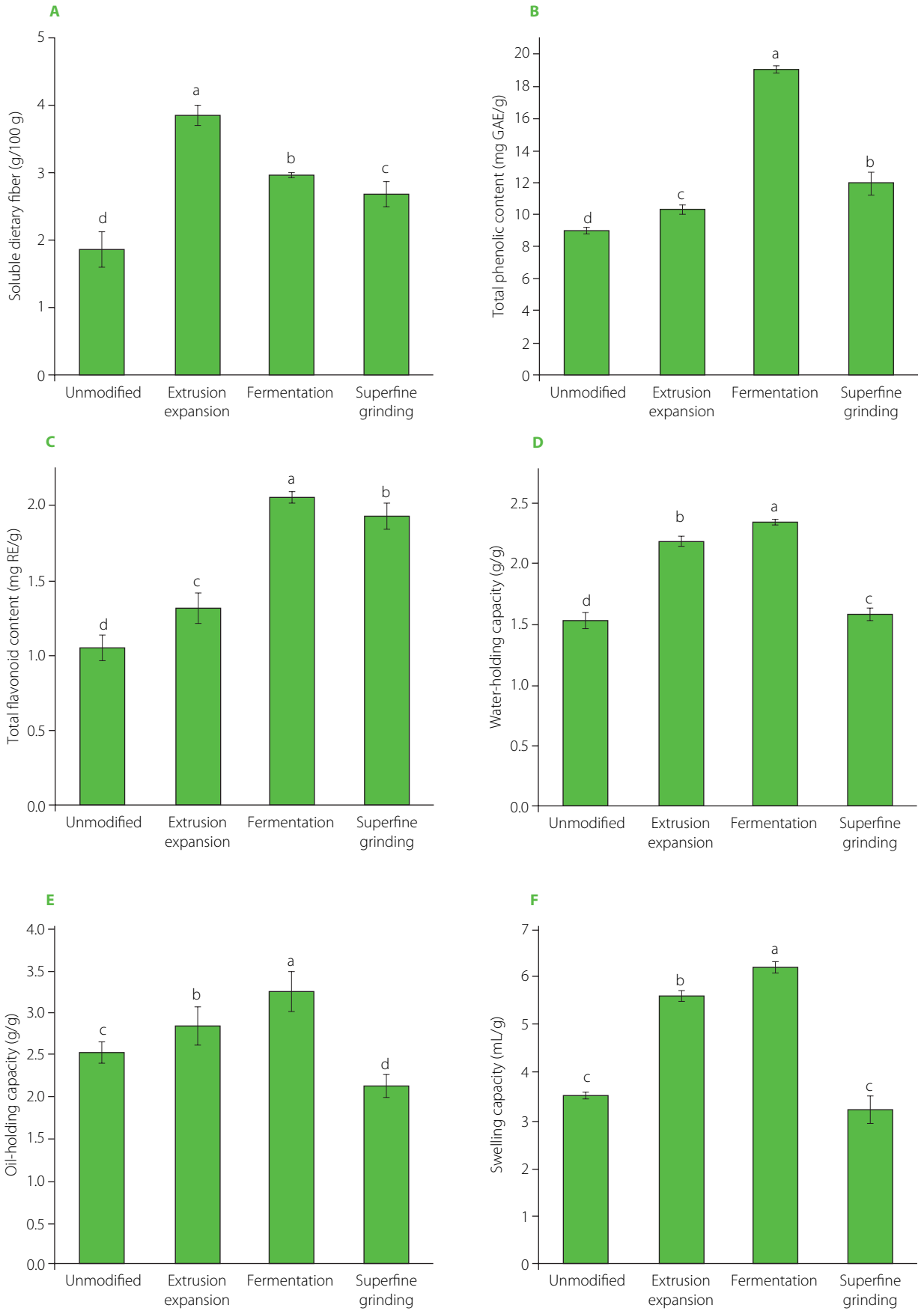

Figure 2. Soluble dietary fiber content (**A**), total phenolic content (**B**), total flavonoid content (**C**), water-holding capacity (**D**), oil-holding capacity (**E**), and swelling capacity (**F**) of unmodified and modified Tartary buckwheat bran. Different letters above the bars indicate significant differences ($p<0.05$). GAE, gallic acid equivalent; RE, rutin equivalent.

might be due to the secretion of various enzymes by microorganisms during the growth and reproduction process, which hydrolyzed the protein and starch in the bran. At the same time, some cellulose was hydrolyzed under the action of microorganisms, and IDF was converted into SDF. The reason for the increase in SDF content in the bran after superfine grinding might be that strong friction and impact reduced the particle size of dietary fiber, exposed more functional groups, enhanced the hydrophilicity of dietary fiber, and ultimately increased the content of the soluble components [Zheng *et al*., 2022].

As shown in **Figure 2B**, the total phenolic content of Tbb increased significantly ($p<0.05$) after extrusion, fermentation, and superfine grinding treatments. Compared with the unmodified Tbb with a TPC of 8.97 mg GAE/g, the total phenolic content increased by 15.62% after extrusion, by 112.62% after fermentation, and by 32.95% after superfine grinding. The TPC in the fermented Tbb reached 19.07 mg GAE/g.

The total flavonoid content of Tbb is shown in **Figure 2C**. After extrusion, fermentation, and superfine grinding treatments, it increased significantly ($p<0.05$), following the trend observed for TPC. Compared with the unmodified Tbb with a TFC of 1.05 mg RE/g, the total flavonoid content increased by 25.26% after extrusion, by 95.25% after fermentation, and by 83.19% after superfine grinding. Fermentation allowed obtaining a Tbb with the total flavonoid content of 2.06 mg RE/g. These results indicate that different modifications effectively promoted the release of soluble dietary fiber and phenolic compounds in Tbb. After extrusion, the TPC of Tbb increased, which was consistent with previous research results; Zhang *et al*. [2018] found that extrusion increased the free phenolic content of rice bran by 27.1% and decreased the bound phenolics by 27.2%, thereby increasing the total phenolic content of rice bran up to 7.3%. This enhancement may be attributed to involving high temperature and pressure during extrusion, which disrupts the cell wall structure and promotes the release and subsequent extraction of bound phenolic compounds. In the process of fermentation, the TPC and TFC in Tbb increased, this may be attributed to the loosening of the originally dense, cross-linked structure of bran, disruption of cell walls, and release of bound phenolic compounds from the cell wall matrix. This has been demonstrated in studies on wheat bran and Tartary buckwheat bran, where fermentation with lactic acid bacteria alone or in combination with yeasts significantly increased the contents of free phenolic acids and other bioactive components, together with enhanced antioxidant activity [Aung *et al*., 2022; Tomassi *et al*., 2025]. In parallel, several reports [Liu *et al*., 2024; Z.Q. Zhang *et al*., 2023] have shown that when the raw material was subjected to superfine grinding, the content of extractable phenolics increased markedly. For Tartary buckwheat bran, superfine grinding combined with endogenous enzyme activity substantially modified the flavonoid profile and improved antioxidant and α-glucosidase inhibitory activities [Xiao *et al*., 2022]. This may be because superfine grinding improves powder uniformity, decreases the average particle size, expands the contact area between phenolics and the extraction solvent, and accelerates their dissolution, thereby increasing the measured phenolic content [Y. Zhang *et al*., 2023].

■ Effect of different modifications on the functional properties of Tartary buckwheat bran

As shown in **Figure 2D**, all used treatments significantly ($p<0.05$) improved WHC of Tbb. Compared with the unmodified buckwheat bran with a WHC of 1.52 g/g, the corresponding data for the extruded, fermented, and superfine-ground buckwheat bran increased by 42.91%, 52.69%, and 3.67%, respectively. Among them, the fermented bran had the highest WHC of 2.33 g/g.

OHC is an important processing characteristic that can represent the ability of food to absorb and retain fat, improving food flavor and palatability. Compared with the unmodified Tbb, the OHC of Tbb after extrusion and fermentation increased significantly ($p<0.05$) by 12.40% and 28.19%, respectively (**Figure 2E**). In contrast, the OHC of Tbb decreased after superfine grinding.

SC of bran affects the texture and processing performance of products. As shown in **Figure 2F**, compared to the unmodified Tbb, the SC values of Tbb after extrusion and fermentation increased significantly ($p<0.05$) by 58.28% and 75.24%, respectively. For the superfine-ground Tbb, the SC was 3.38 g/g, being similar ($p\geq0.05$) to that determined for the unmodified Tbb (3.54 g/g).

The results showed that both the WHC, OHC, and SC of Tbb increased after extrusion expansion. This might be due to the relatively increased amount of cellulose and hemicellulose in bran under the action of high temperature, high pressure, and high shear force, with some connection bonds breaking, resulting in more low-molecular-weight compounds and soluble polymers, increasing the specific surface area of the material [Deroover *et al*., 2020]. The significant improvements in the WHC, OHC, and SC of Tbb after fermentation may be attributed to the degradation of macromolecules, such as IDF, into smaller molecules. Fermentation also increases the specific surface area of SDF, resulting in a loose and porous structure that exposes more polar functional groups [Gu *et al*., 2020; Y. Li *et al*., 2022]. These groups can form additional hydrogen bonds or dipole interactions with water, enhancing hydration and water retention. At the same time, more non-polar groups are exposed, which contributes to an increase in OHC. The increase in both polar and non-polar groups facilitates the penetration and tight binding of water and oil into the dietary fiber, thus reducing their loss. After superfine grinding, the WHC increased by 3.67%, whereas the OHC and SC presented an opposite trend compared to WHC, probably because Tbb exposed many hydrophilic groups after superfine grinding, which easily combined with water [Zhao *et al*., 2009]. At the same time, under the influence of strong mechanical force, macromolecular compounds broke down into smaller particles, and the fiber matrix was destroyed, resulting in a decrease in OHC and SC, which was consistent with the research results reported by Zhu *et al*. [2010].

In conclusion, extrusion expansion, fermentation, and superfine grinding significantly modified the functional properties of Tbb. Fermentation elicited the most pronounced increase in total phenolic and total flavonoid contents, which are essential

for enhancing Tbb's antioxidant properties – critical attribute for health-focused food development. Furthermore, fermentation offers distinct environmental advantages over extrusion expansion and superfine grinding, which typically demand higher energy inputs and pose greater risk of degradation of heat-sensitive nutrients. Although the SDF content post-fermentation was surpassed by extrusion expansion (**Figure 2A**), and fermentation yielded superior functional properties of Tbb (**Figure 2D–F**). Specifically, it achieved the highest WHC, OHC, and SC among the treatments. This combination of enhanced functional properties and superior bioactive compound contents renders fermented Tbb particularly suitable for diverse food applications. Based on these compelling results, fermentation was selected as the preferred modification method for Tbb, and its process parameters were subsequently optimized.

Optimization of Tartary buckwheat bran fermentation process

Single factor experiment

The effect of different ratios of *S. cerevisiae* to *Lactobacillus* on SDF content was investigated under the condition of 3% (*w/v*) addition of inoculation cultures to the mixture of Tbb in water (1:2, *w/v*) (**Figure 3A**). In general, *Lactobacillus* dominate the microbial community during fermentation, creating an acidic environment that severely affects the growth and metabolic activity of *S. cerevisiae*. On the other hand, an excessive amount of *S. cerevisiae* can result in over-fermentation, causing the accumulation of metabolic products that inhibit the normal activities of both yeast and lactic acid bacteria. When the ratio of *S. cerevisiae* to *Lactobacillus* was 2:1 (*w/w*), the SDF content of Tbb was the highest, reaching 2.36 g/100 g. Therefore, this ratio of yeast to lactic acid bacteria was used in the next experiment.

The effect of different fermentation temperatures on the SDF content of Tbb is shown in **Figure 3B**. The SDF content of the bran continuously increased with the increase of fermentation temperature until it peaked at the temperature of 33°C. However, the SDF content decreased significantly after the temperature continued to increase. The results revealed that both high and low temperatures were not conducive to the vital activities of composite microorganisms, affecting the fermentation speed. This might be because when the temperature is too low, the microbial growth is suppressed, making it difficult for yeast and lactic acid bacteria to play a role together. On the contrary, when the temperature is too high, the physiological metabolism of microorganisms is affected. Therefore, the fermentation temperature of 33°C was found optimal.

As shown in **Figure 3C**, the SDF content presented a trend of first increasing and then decreasing with the prolongation of fermentation time. When the fermentation time was 6 h, the SDF content in the Tbb reached the maximum value of 1.45 g/100 g. This might be because yeast and lactic acid bacteria were still in the growth stage in the initial fermentation phase. As the fermentation time prolonged, the growth of yeast and lactic acid bacteria became more and more stable. When

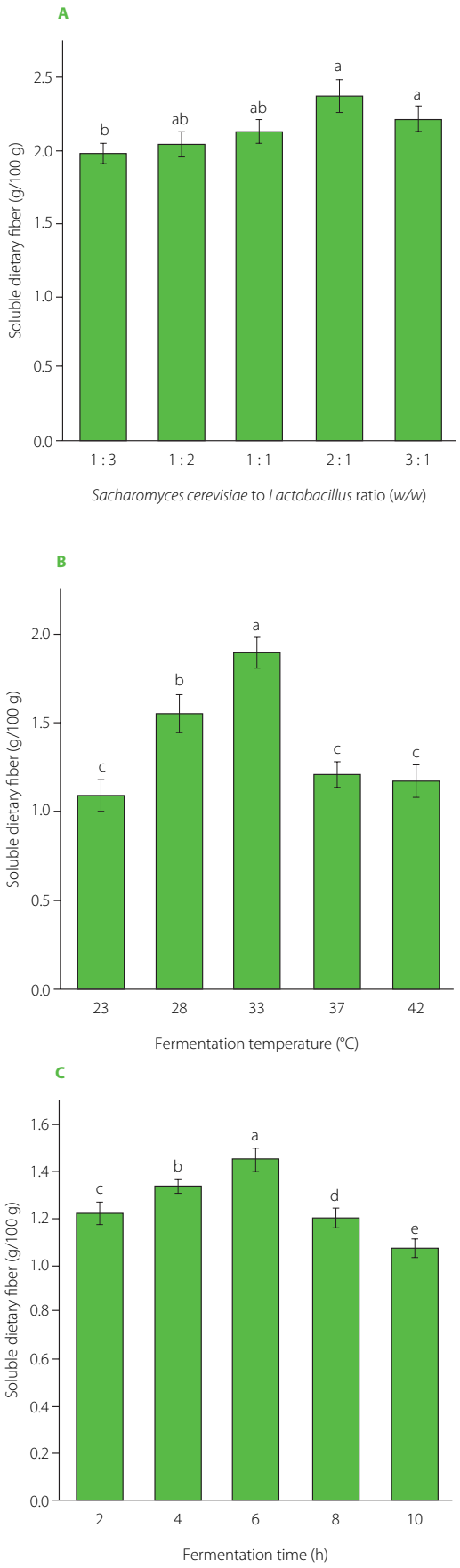

Figure 3. Soluble dietary fiber content of Tartary buckwheat bran powders fermented with different ratios of *Saccharomyces cerevisiae* to *Lactobacillus* as inoculation cultures (**A**), at different temperatures (**B**) and for different times (**C**). Different letters above the bars indicate significant differences ($p<0.05$).

Table 3. The response surface experiment design scheme and results of optimizing Tartary buckwheat bran fermentation conditions.

No.	Factor level			SDF content (g/100 g)
	Fermentation temperature	Fermentation time	Ratio of inoculation cultures (*w/w*)	
1	–1	–1	0	2.42
2	0	1	1	2.41
3	0	–1	–1	2.03
4	–1	1	0	2.17
5	0	0	0	2.98
6	0	–1	1	2.76
7	1	0	–1	2.58
8	–1	0	1	2.54
9	0	0	0	2.93
10	–1	0	–1	2.51
11	1	0	1	2.37
12	0	1	–1	2.57
13	0	0	0	2.83
14	1	1	0	2.52
15	1	–1	0	1.87

the stable period had been reached, the bacterial yield peaked. As the fermentation time was further prolonged, nutrients were depleted, and SDF was also decomposed and utilized by microorganisms, resulting in a decrease in its content. Therefore, the optimum fermentation time was found at 6 h.

Response surface method optimization experiment

To further optimize the conditions of fermentation, a response surface optimization experiment was designed, as shown in **Table 3**. Using the SDF content of Tbb as the response, multiple linear regression analysis and quadratic equation fitting were performed. The regression mathematical model for SDF content (Y), fermentation temperature (A), fermentation time (B), and ratio of inoculation cultures (C) was describe by Equation (4):

$$Y=2.91-0.055A+0.0913B+0.0488C+0.26AB-0.06AC-0.2225BC-0.3229A^2-0.3804B^2-0.0904C^2 \quad (4)$$

The regression model was visualized using the response surface and contour plots (**Figure 4**). With the increase of fermentation temperature and time, the SDF content showed a trend of first increasing and then decreasing (**Figure 4A** and **B**). The slope of the curved surface corresponding to fermentation time was higher than that of the fermentation temperature, indicating that the influence of fermentation time on SDF content was greater than that of fermentation temperature. The contour line was elliptical, indicating a significant interaction between the two factors. **Figure 4C** and D showed that the SDF content first increased and then decreased with the increase of fermentation temperature and the ratio of inoculation cultures The slope of the curved surface corresponding to the ratio of inoculation cultures was higher than that of the fermentation temperature, indicating that the ratio of inoculation cultures had a stronger effect on the SDF content than fermentation temperature. The contour line was also elliptical, which revealed the significant interaction between the two factors. **Figure 4E** and **4F** showed that with the prolonging fermentation time and the increasing ratio of inoculation cultures the SDF content showed a trend of first increasing and then decreasing. The slope of the curved surface corresponding to the ratio of inoculation cultures was higher than that of fermentation time, indicating that the ratio of inoculation cultures had a stronger effect on the SDF content than the fermentation time. The contour line was also elliptical, and the interaction between these two factors was significant.

According to the model prediction, the optimal conditions for fermentation were: fermentation temperature of 31.93℃, fermentation time of 5.96 h, ratio of yeast to lactic acid bacteria of 53:25 (*w/w*), and predicted SDF content of 2.97 g/100 g. Based on the operability of the experiment, the final process conditions were set as follows: fermentation temperature of 32℃, fermentation time of 6 h, and the ratio of yeast to lactic acid bacteria of 2:1 (*w/w*). Three parallel experiments were conducted using these process conditions, and the actual SDF content was determined to be 2.93 g/100 g, which was close to the predicted value, indicating that the process parameters fitted by this model were reliable and had certain practical value.

Effect of different wheat flour substitution levels by fermented Tartary buckwheat bran on steamed bread quality

Sensory evaluation

The sensory scores of steamed breads with different substitution levels of wheat flour by fermented Tbb are shown in **Table 4**. The sensory score of steamed bread with Tbb increased first and then decreased with an increasing wheat flour substitution level. The highest total score had steamed bread at 10% (*w/w*) wheat flour substitution by fermented Tbb (84.7). However, when the bran substitution exceeded 10% (*w/w*), the sensory score decreased significantly ($p<0.05$), with notable declines in appearance shape, internal structure, color, elasticity, and fragrance. This trend can be attributed to the darkening color caused by flavonoid-rich Tbb. Moreover, Tbb disrupts the gluten network at high substitution levels, reduces dough extensibility, impairs the stability of the gluten network structure, and ultimately results in poor texture [Zhao *et al*., 2025]. This is similar to the results

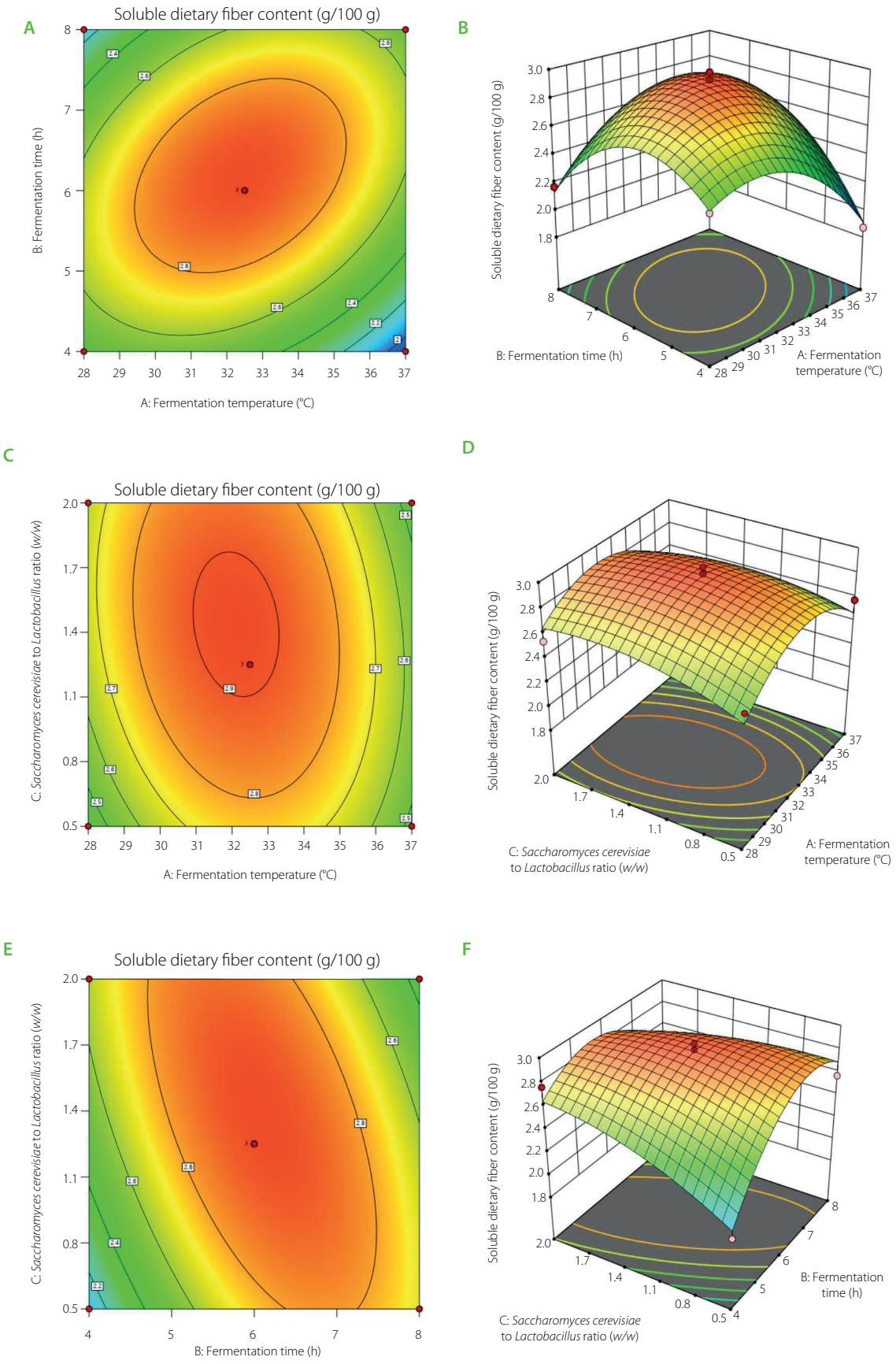

Figure 4. Contour (**A**, **C**, and **E**) and response surface (**B**, **D**, and **F**) plots showing the effect of interaction between fermentation temperature and fermentation time (**A–B**), fermentation temperature and *Saccharomyces cerevisiae* to *Lactobacillus* ratio (*w/w*) (**C–D**), and fermentation time and *Saccharomyces cerevisiae* to *Lactobacillus* ratio (*w/w*) (**E–F**) on the soluble dietary fiber content of Tartary buckwheat bran.

Table 4. Sensory scores of steamed bread produced with different substitution levels of wheat flour with fermented Tartary buckwheat bran powder.

Substitution level (*w/w*)	Appearance shape	Internal structure	Color	Chewiness	Elasticity	Palatability	Fragrance	Bitterness	Total score
0%	11.0±0.8^{c}	12.0±0.7^{b}	12.9±0.6^{a}	9.0±0.6^{a}	9.0±0.5^{a}	7.0±0.6^{c}	13.1±0.6^{a}	8.3±0.6^{b}	82.4±1.8^{c}
5%	12.2±0.5^{b}	12.9±0.6^{a}	12.0±0.6^{b}	8.1±0.5^{b}	8.2±0.5^{b}	8.1±0.5^{b}	13.1±0.4^{a}	8.9±0.5^{a}	83.5±1.6^{b}
10%	12.9±0.6^{a}	12.9±0.5^{a}	12.0±0.5^{b}	8.9±0.4^{a}	8.1±0.4^{b}	9.0±0.5^{a}	12.0±0.6^{b}	8.9±0.4^{a}	84.7±1.7^{a}
15%	11.1±0.4^{c}	10.9±0.5^{c}	9.9±0.8^{c}	6.1±0.7^{c}	6.9±0.5^{c}	8.1±0.4^{b}	11.0±0.5^{c}	7.1±0.4^{c}	71.1±1.5^{d}
20%	9.0±0.7^{d}	8.1±0.8^{d}	7.1±0.6^{d}	6.1±0.5^{c}	5.9±0.5^{d}	8.2±0.4^{b}	10.0±0.7^{d}	6.1±0.5^{d}	60.4±1.8^{e}
25%	8.0±0.6^{e}	6.0±0.5^{e}	6.0±0.6^{e}	5.1±0.4^{d}	5.1±0.5^{e}	8.9±0.5^{a}	9.0±0.5^{e}	5.1±0.5^{e}	53.2±1.6^{f}

Mean ± standard deviation values (n=50) followed by the same column with different letters are significantly different (p<0.05).

Table 5. The texture parameters of steamed bread produced with different substitution levels of wheat flour with fermented Tartary buckwheat bran powder.

Substitution level (*w/w*)	Hardness (N)	Stickiness	Chewiness (N)	Springiness (%)	Cohesiveness	Resilience (%)
0%	1,845±37^{f}	1,783±45^{c}	1,606±64^{c}	0.97±0.02^{a}	0.90±0.01^{a}	0.51±0.02^{a}
5%	2,038±71^{e}	1,962±96^{c}	1,762±98^{c}	0.96±0.01^{a}	0.90±0.01^{a}	0.51±0.01^{a}
10%	2,556±80^{d}	2,448±60^{b}	2,164±64^{b}	0.96±0.01^{ab}	0.88±0.00^{a}	0.49±0.01^{ab}
15%	2,808±30^{c}	2,645±39^{b}	2,276±40^{b}	0.94±0.01^{ab}	0.86±0.00^{b}	0.47±0.00^{bc}
20%	3,274±134^{b}	3,083±197^{a}	2,587±224^{a}	0.94±0.02^{ab}	0.88±0.01^{a}	0.45±0.02^{c}
25%	3,511±41^{a}	3,245±97^{a}	2,596±82^{a}	0.92±0.02^{b}	0.80±0.02^{b}	0.44±0.03^{c}

Mean ± standard deviation values followed by the same column with different letters are significantly different (p<0.05).

of the previous study by Zhang *et al*. [2022], who added different amounts of heat-moisture treated Tbb flour to wheat flour to prepare steamed bread and found that the sensory score first increased and then decreased with the increase of the Tbb flour used.

Texture characteristics

Substituting wheat flour with modified Tbb flour significantly affected the textural parameters of the steamed bread (**Table 5**). Hardness is an important index to evaluate the quality of steamed bread. As can be seen from **Table 5**, with increasing wheat flour substitution level, hardness increased steadily from 1,844 N in the control to 3,511 N in the bread with 25% (*w/w*) Tbb, representing a 90.3% rise (p<0.05). Similarly, stickiness and chewiness increased by 82.0% and 61.6%, respectively, at the highest substitution level. This is primarily due to the high dietary fiber content of Tbb, which competes with gluten proteins for water and interrupts gluten network formation, resulting in a denser and less aerated crumb structure.

Springiness remained relatively stable up to 20% (*w/w*) Tbb but decreased significantly at 25% (*w/w*) Tbb (p<0.05). Cohesiveness decreased from 0.90 at 0% to 0.80 at 25% (*w/w*) Tbb (p<0.05), suggesting a reduction in the bonding strength between gluten proteins. Resilience also decreased significantly (p<0.05), dropping from 0.51 to 0.44 with higher Tbb levels. These reductions indicate that excessive bran incorporation disrupts the continuity of the gluten network, limiting the dough's ability to recover from deformation and retain gas during proofing [Zhang *et al*., 2021].

Therefore, when the substitution level was ≤10%, the steamed bread samples exhibited moderate increases in hardness and chewiness while retaining desirable levels of elasticity, cohesiveness, and resilience. Furthermore, they maintained a soft texture, pleasant taste, and appealing appearance. In contrast, higher substitution levels (>15%) led to excessive gluten dilution by bran fibers, resulting in poor gas retention, a denser and drier crumb structure, and significantly reduced sensory acceptance attributable to elevated hardness, loss of elasticity, and a pronounced bitter aftertaste. Thus, 10% wheat flour substitution by fermented Tbb was found optimal for balancing nutritional enhancement with consumer acceptability.

Specific volume

Specific volume is used to reflect the degree of dough volume expansion and retention ability, and is one of the important indicators of steamed bread quality evaluation. As shown in **Figure 5A**, the specific volume of steamed bread showed an overall

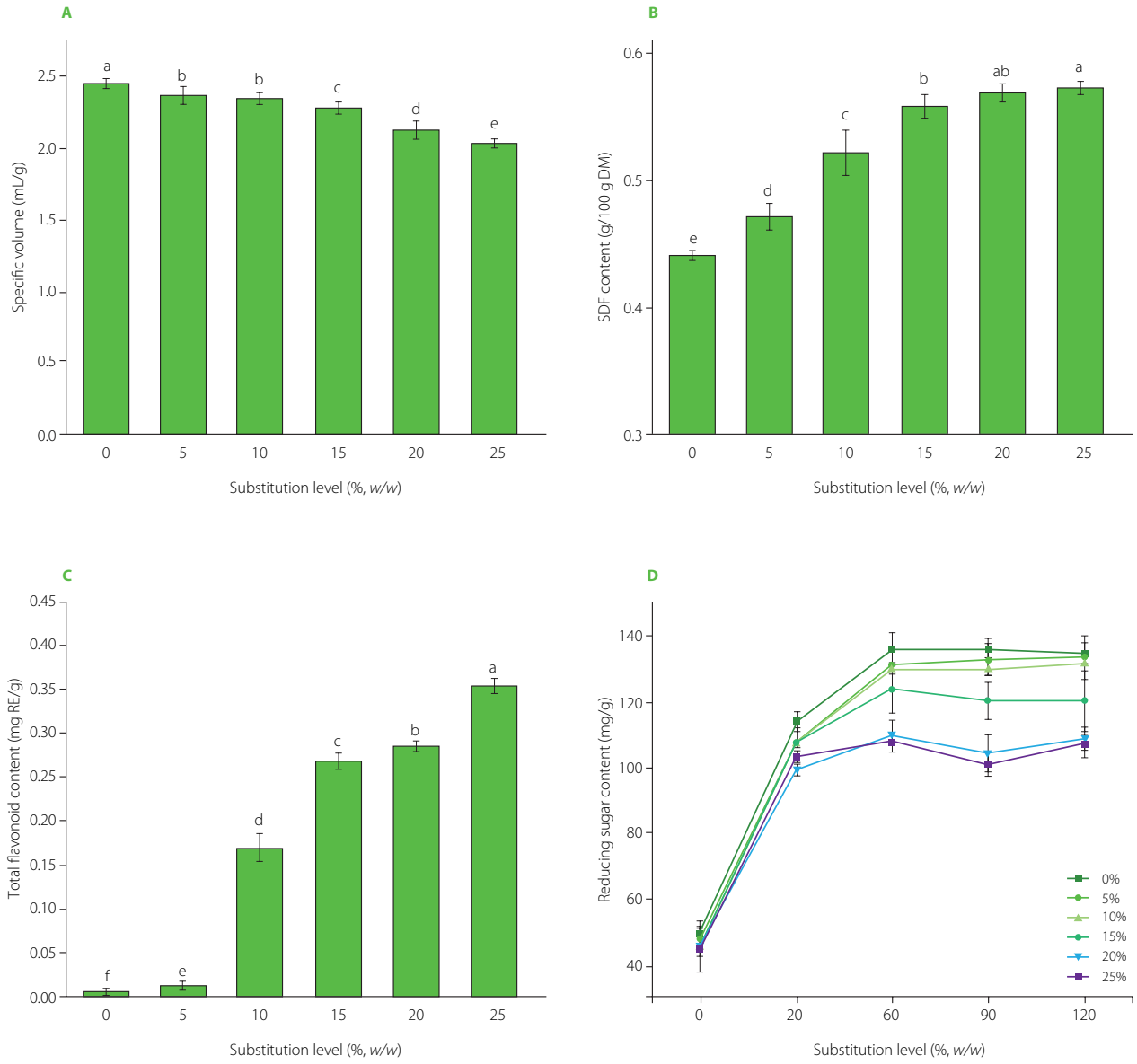

Figure 5. The specific volume (**A**), soluble dietary fiber (SDF) content (**B**), total flavonoid content (**C**), and digestive characteristic (**D**) of steamed bread produced with different substitution levels of wheat flour with fermented Tartary buckwheat bran powder. Different letters above the bars indicate significant differences ($p<0.05$). RE, rutin equivalent; DM, dry matter.

decreasing trend with an increasing substitution level. This is consistent with the results of Zhang *et al*. [2021]. The observed changes might be caused by the fact that the addition of Tbb destroyed the stable gluten network structure in the wheat flour, and the expansion of the steamed bread embryo was blocked, which led to the reduction of steamed bread volume [Li *et al*., 2023].

Internal structure

As shown in **Figure 6**, the internal pores of the steamed bread without Tbb were uniform but too tight. The pores of the steamed breads with 5% (*w/w*) and 10% (*w/w*) substitution levels were even and delicate. When the substitution level exceeded 15% (*w/w*), with the increase of the Tbb amount, the pores of steamed bread began to vary in size, their number increased, and their internal structure became rougher and rougher.

SDF content

As shown in **Figure 5B**, compared with the steamed bread without Tbb, the SDF content in the steamed bread increased gradually with an increasing substitution level, and the increase in SDF slowed down when the bran addition amount exceeded 15%.

Total flavonoid content

The wheat flour substitution level with fermented Tbb had significant ($p<0.05$) effects on the TFC of the steamed bread, which gradually increased with Tbb level increase (**Figure 5C**). This might be due to the wheat flour substitution with fermented

Figure 6. The internal structure of steamed bread produced with substitution by weight of 0% (**A**), 5% (**B**), 10% (**C**), 15% (**D**), 20% (**E**), and 25% (**F**) of wheat flour with fermented Tartary buckwheat bran powder.

Tbb, which underwent further microbial metabolic activities in the dough, leading to the production of enzymes, such as esterases, glucosidases, and proteases. These enzymes enhanced the breakdown of complex plant compounds, facilitating the release of flavonoids [Huynh *et al.*, 2014]. As the Tbb substitution level increased, the TFC in the steamed bread showed a significant rise. Specifically, at the 5% (*w/w*) substitution level, the TFC increased 9.38 times compared to the control samples ($p<0.05$). The increase was particularly pronounced between 5% and 10% (*w/w*), which represented the most significant interval. Beyond 15% (*w/w*), the increase in TPC slowed down, indicating a diminishing return with higher substitution levels.

Digestive characteristics

It can be seen from **Figure 5D** that the reducing sugar content of the steamed bread samples increased first and then stabilized during the whole digestion process. The increase occurred rapidly after the addition of α-amylase within 20 min. Then, the reducing sugars were slowly released and their content slightly increased, reaching a maximum at 60 min. Among all samples, the steamed bread without Tbb exhibited the highest reducing sugar content after digestion, whereas the reducing sugar release decreased progressively with increasing levels of Tbb substitution. This indicates that partial substitution of wheat flour with Tbb significantly reduced starch digestibility. Similar results have been reported in fiber-fortified starch-based foods. Krishnan *et al.* [2012] demonstrated that the incorporation of wheat bran, oat bran, or rice bran into sweet potato–based pasta significantly slowed starch digestion compared with the control samples. In the present study, the reduced reducing sugar release could be attributed to the higher dietary fiber content in Tbb, which diluted the starch fraction in steamed bread and consequently lowered the amount of digestible substrate available for α-amylase during *in vitro* digestion [Xue *et al.*, 2022].

CONCLUSIONS

Evaluation of the functional characteristics of Tbb modified by three different techniques showed that extrusion expansion most effectively increased soluble dietary fiber content, while fermentation produced the greatest improvements in total phenolic and total flavonoid contents. Fermentation also significantly improved the WHC, OHC, and SC of Tbb, compared to unmodified Tbb. Collectively, these results indicate that fermentation offers the strongest enhancement of the functional properties of Tbb. These improvements make fermented Tbb a promising ingredient for functional food development. The optimum conditions of fermentation for Tbb were established as: fermentation temperature 32°C, fermentation time 6 h, and the ratio of *S. cerevisiae* to *Lactobacillus* as inoculation cultures 2:1 (*w/w*). The 10% (*w/v*) substitution of wheat flour by fermented Tbb

allowed the production of steam bread with the highest sensory scores. In conclusion, fermentation significantly improved both the functional and processing properties of Tbb, making it a promising ingredient for the development of healthier food products, particularly in the production of traditional steamed bread.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

INFORMED CONSENT

This study was approved by the College of Agronomy and Forestry Science of Hebei North University (Zhangjiakou, China), and informed consent was obtained from all volunteers prior to their participation.

ORCID IDs

C. Dai https://orcid.org/0009-0003-6435-8706
P. Dong https://orcid.org/0009-0005-3206-9656
X. Guo https://orcid.org/0009-0007-4406-2624
Y. Liu https://orcid.org/0000-0003-0949-8629
D. Wang https://orcid.org/0009-0000-8685-3335
L. Yuan https://orcid.org/0009-0009-0185-3084
X. Zhang https://orcid.org/0000-0001-8804-4144
H. Zhou https://orcid.org/0009-0008-5545-4831

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Nutritional and Preservative Potential of Tunisian *Nigella sativa* L. Seeds: Insights into Lipid Composition, Antioxidant Activity, and Antimicrobial Effects

Faouzi Sakouhi[1]*, Chaima Saadi[1], Sadok Boukhchina[1], Raquel Rodriguez Solana[2,3]

[1]Laboratory of Neurophysiology, Cellular Physiopathology and Biomelcules Valorization (LR18ES03), Department of Biology, Faculty of Sciences, University of Tunis El Manar, El Manar I., Tunisia
[2]Department of Agroindustry and Food Quality, Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), Rancho de la Merced Center, Carretera Cañada de la Loba (CA-3102) Km 3.1., SN, 11471 Jerez de la Frontera, Cádiz, Spain
[3]MED—Mediterranean Institute for Agriculture, Environment and Development & CHANGE—Global Change and Sustainability Institute, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Research on new natural resources with nutritional and preservative properties has gained increasing interest among modern consumers and the contemporary food industry. In this context, this study aimed to explore the bioactive potential of *Nigella sativa* L. seeds collected from various locations in Tunisia. The results highlight the considerable potential of *N. sativa* seeds for oil production, due to high oil yield (23–28%). These findings also reveal the abundance of bioactive lipids, including linoleic acid (C18:2, 49.66 to 58.47% of total fatty acids), β-sitosterol (88.19 to 100.46 mg/100 g oil), triacontanol (24.13 to 30.48 mg/kg oil), and α-tocopherol (10.38 to 11.51 mg/100 g oil). The hydroethanolic seed extracts exhibited the highest antioxidant activity compared to the ethanol and aqueous extracts, due to their higher contents of phenolic compounds, with total phenolic content ranging from 72.50 to 74.35 mg GAE/g, total flavonoid content from 23.48 to 29.47 mg QE/100 g, and total tannin content from 7.18 to 8.07 mg CE/g. Furthermore, ethanolic extracts showed antibacterial activity against bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *Staphylococcus aureus* frequently found in rotten food products. These findings underscore the value of *N. sativa* seeds as a rich source of natural compounds with good potential for use in food preservation and nutritional enhancement.

Keywords: black seeds, phytosterols, tocopherols, policosanols, biological activities

INTRODUCTION

Nigella (*Nigella sativa* L.) is an annual plant native to regions including Western Asia, the Middle East, Central Europe, and North Africa. The plant produces aromatic seeds of an intense black color, commonly known as black cumin. Since antiquity, *N. sativa* seeds have gained centuries-old recognition as a high-quality spice for their distinct aroma and have also been used for therapeutic purposes in several countries around the world [Ahmad *et al.,* 2021]. Lipids and essential oils derived from these seeds have long been valued as natural remedies for treating a wide range of illnesses, including asthma, hypertension, diabetes, and influenza [Majeed *et al.,* 2021]. *N. sativa* seeds contain various natural compounds, including lipids, polysaccharides, proteins, flavonoids, alkaloids, and saponins [Ahmad *et al.,* 2021]. The lipid fraction represents the major group among these organic constituents, accounting for 30–40% of the total seed weight [Ahmad *et al.,* 2021]. This designation qualifies *N. sativa* seeds as an excellent oilseed when compared to other vegetable

***Corresponding Author:**
tel.: +216 22142610; fax: +216 71885480, e-mail: **Faouzi.Sakouhi@fst.rnu.tn** (Dr. F. Sakouhi)

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matrices such as olive, soybean, and sunflower. Vegetable matrices, especially oleaginous fruit and seeds, contain a complex mixture of lipids consisting of glycerides (approximately 98%) and unsaponifiable matter (approximately 2%) [Sakouhi *et al.,* 2011]. The glyceridic fraction of vegetable oils comprises mainly triacylglycerols esterified with saturated, monounsaturated, and polyunsaturated fatty acids [Dubois *et al.,* 2007]. The lipid fraction of vegetable matrices equally contains numerous unsaponifiable compounds, such as sterols, triterpenic alcohols, vitamins, and policosanols, all of which exhibit notable biological activities. Sterols and triterpenic alcohols constitute the major portion of the unsaponifiable fraction in vegetable oils [Goriainov *et al.,* 2021]. The term policosanols refers to a mixture of long-chain (C20–C36) aliphatic primary alcohols. Weerawatanakorn *et al.* [2019] reported that tetracosanol (C24), hexacosanol (C26), octacosanol (C28), and triacontanol (C30) were the main policosanols in oleaginous matrices. Policosanols have been shown to be associated with numerous physiological benefits, such as platelet aggregation inhibition, endothelial damage mitigation, and a cholesterol-lowering effect [Weerawatanakorn *et al.,* 2019]. The unsaponifiable fraction also contains tocopherols, which are well known for their effective antioxidant properties [Patterson, 1981]. These natural compounds protect lipids from peroxidation by neutralizing lipid peroxyl radicals or interacting with singlet oxygen and other reactive oxygen species. They have a crucial role in preventing lipid oxidation, a major form of degradation that can occur during food processing, distribution, storage, and preparation.

In many food industries, protection against spoilage and rancidity is commonly achieved using synthetic antioxidants, including ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG). Nonetheless, concerns have increasingly emerged regarding the potential toxic and carcinogenic effects associated with these synthetic additives [Wang *et al.,* 2021]. As a result, there is growing demand within these industries for natural antioxidants (tocopherols and phenolic compounds) extracted from vegetable matrices. Such additives can be incorporated directly into food products to preserve them against alteration and rancidity. Beyond their antioxidant activity, many plant extracts also contain antimicrobial active compounds that could enhance the storage stability of food products [Karnwal & Malik, 2024]. As far as microbial damage is concerned, most food industries typically rely on synthetic antimicrobial agents, such as benzoates, sorbates, propionates, and nitriles for food preservation from microbial damage. However, several studies have highlighted that these chemical preservatives may exhibit potential toxic, mutagenic, or genotoxic effects, raising growing safety concerns [Piper & Piper, 2017]. Recent reviews have emphasized the need to replace such synthetic compounds with safer, naturally occurring antimicrobial alternatives [Karnwal & Malik, 2024]. These natural additives do not only have the advantage of preserving food products against alterations, rancidity, and microbial infections but also offering nutritional benefits to consumers.

Recently, food industry has started exploring new oil plant matrices with both nutritional and preservative potential. Most of these bioactive compounds are primarily extracted from plant sources, such as chia, flax, pumpkin, sunflower, and sesame seeds [Alasalvar *et al.,* 2021; Sumara *et al.,* 2023]. However, research on the bioactive lipid compounds of *N. sativa* seeds, particularly the unsaponifiable fraction, remains scarce [Albakry *et al.,* 2022]. To the best of our knowledge, this is the first study to provide a comprehensive profile of both unsaponifiable matter and phenolic compounds from *N. sativa* seeds and to discuss their potential applications in nutrition and food preservation. The objective of this research is twofold: first, to identify and characterize the bioactive lipid compounds in *N. sativa* seeds, such as unsaturated fatty acids, phytosterols, policosanol, and tocopherols; and second, to assess their antioxidant and antimicrobial activities of their seed extracts, thereby supporting the potential future applications of *N. sativa* seeds.

MATERIALS AND METHODS

Reagents and standards

Chloroform, diethyl ether, ethanol, methanol, *n*-hexane, and isopropyl alcohol (high-performance liquid chromatography, HPLC, grade) were obtained from Panreac Applichem ITW Reagents (Darmstadt, Germany). Anhydrous sodium carbonate (Na_2CO_3), Folin–Ciocalteu (FC) reagent, aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), anhydrous sodium sulfate (Na_2SO_4), and potassium hydroxide (KOH) were purchased from VWR (Leuven, Belgium). A standard mixture of fatty acid methyl esters, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 5α-cholestanol and 1-eicosanol standards, α-, β-, γ-, and δ-tocopherol isoform standards, gallic acid (GA), quercetin, hydrochloric acid, (+)-catechin standard, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and butylated hydroxytoluene (BHT) standard were supplied by Sigma-Aldrich (Madrid, Spain). 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) was purchased from Fluka (Buchs, Switzerland). Mueller–Hinton agar was obtained from BIOKAR Diagnostics (Allonne, France).

Material

Samples of *Nigella sativa* L. seeds were collected in June 2023 from four distinct geographic regions of Tunisia: Nabeul, Kairouan, Sfax, and Gabès. The cultivation of *N. sativa* in Tunisia was managed and supervised by the Ministry of Agriculture. These regions display a variety of climatic conditions and geographic coordinates: (1) Nabeul: longitude: 36°26′N; latitude: 10°43′E; altitude: 14 m; annual rainfall: 404 mm/year; (2) Kairouan: longitude: 10°05′N; latitude: 10°05′E; altitude: 68 m; annual rainfall: 300 mm/year; (3) Sfax: longitude: 34°45′N; latitude: 10°25′E; altitude: 13 m; annual rainfall: 233.1 mm/year; and (4) Gabès: longitude: 33°49′N; latitude: 9°45′E; altitude: 1 m; annual rainfall: 152 mm/year.

The *N. sativa* seeds were hand-harvested randomly, and 2 kg of seeds approximately were collected from each region. The seeds collected from each site were ground separately into a fine powder and preserved at 4°C for further analysis.

Extraction of fixed oil from *N. sativa* seeds

Oil extraction from *N. sativa* seeds was performed using the Folch method, following the procedure described in our previous publication [Sakouhi *et al.*, 2023]. The extraction of oils was performed using a chloroform/methanol mixture (1:1, *v/v*), with 5 mL *per* 1 g of ground seeds. After extraction, the solvent was removed under reduced pressure using a rotary evaporation. The obtained *N. sativa* seed oil was weighed and stored in dark bottles at 4°C for analysis of fatty acids, phytosterols, policosanol, and tocopherols.

Determination of fatty acid composition of *Nigella sativa* L. seed oil

The fatty acid profile of *N. sativa* seed oil was determined using the method described by Sakouhi *et al.* [2023]. The fatty acid methyl esters from each sample were extracted by vigorously shaking a mixture of *N. sativa* oil in *n*-hexane (0.2 g in 3 mL) with 0.4 mL of 2 M methanolic solution of potassium hydroxide. A 1 µL aliquot of the solution was then injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID) (Hewlett-Packard, Avondale, PA, USA). The analysis was achieved using a fused silica column (50 m length × 0.25 mm i.d.) coated with SGL-1000 phase (0.25 µm thickness; Sugerlabor, Madrid, Spain). Helium served as the carrier gas at a constant flow rate of 1 mL/min. The injector and detector temperatures were set at 250°C, while the oven temperature was maintained at 210°C. Fatty acids were identified by comparing their retention times to those of a standard fatty acid methyl ester mixture. The fatty acid (FA) relative content (expressed as % of total fatty acids) was calculated using Equation (1):

$$\text{FA relative content (\%)} = PA_i/TPA \times 100 \quad (1)$$

where: PA_i is FA's peak area and TPA is total FA peak area. The mean value of three injections was reported.

Saponification of oil and thin layer chromatography separation of phytosterols and policosanols

The unsaponifiable fraction was obtained by saponifying 5 g of *N. sativa* seed oil from each location using 50 mL of a 12% (*w/v*) ethanolic KOH solution following the procedure we used previously [Sakouhi *et al.*, 2023]. The mixture was heated at 60°C for 1.5 h, and the unsaponifiable compounds were then extracted four times with 50 mL of petroleum ether. After extraction, the obtained unsaponifiable matter was separated into subfractions using preparative thin-layer chromatography (TLC) on silica gel 60 G F254 plates (Merck, Darmstadt, Germany) with hexane and diethyl ether mixture (6:4, *v/v*) as a developing solvent [Sakouhi *et al.*, 2023]. To accurately identify the sterol and policosanol bands among the other unsaponifiable subfractions, reference solutions of 5α-cholestanol and 1-eicosanol (external standards) were applied to the TLC plates. After chromatographic development, the plate was sprayed with 2,7-dichlorofluorescein and examined under UV light. Subsequently, the phytosterol and policosanol bands were scraped off, extracted three times with a chloroform and diethyl ether mixture (1:1, *v/v*), filtered to eliminate residual silica, and finally dried with a rotary evaporator. The final extracts were derivatized separately with BSTFA and then stored at –4°C until further analysis by gas chromatography–mass spectrometry (GC-MS).

Gas chromatography-mass spectrometry analysis of phytosterols and policosanols

The analysis of phytosterols and policosanols of *N. sativa* seed oil was conducted according to the GC-MS method [Sakouhi *et al.*, 2023], on a Varian SAR 3400CX gas chromatograph connected directly to a Varian SATURN mass detector (Varian, Palo Alto, CA, USA). A DB-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) was used for the separation. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and detector temperatures were maintained at 250°C. The oven temperature was programmed to increase from 150°C to 300°C at a rate of 4 °C/min, with the final temperature held for 10 min. The transfer line temperature was also set at 250°C. Mass spectra were recorded using electron impact ionization at 70 eV. A 1 µL aliquot of each sample was manually injected in the split mode with a split ratio of 60:1. Phytosterols and policosanols were identified by comparing their retention times and mass spectra with those of authentic standards, and further confirmed using the NIST/EPA/NIH Mass Spectral Library, NIST 2020 version (accessed November 2024). The quantification of phytosterols and policosanols was performed through internal standard methods using 5α-cholestanol and 1-heneicosanol, respectively, and the results for individual phytosterols were expressed in mg/100 g of oil and those for policosanols in mg/kg of oil. The analysis was performed in triplicate.

High-performance liquid chromatography analysis of tocopherols from *N. sativa* seed oil

Tocopherol analysis of *N. sativa* seed oil was conducted following the American Oil Chemists' Society (AOCS) method Ce-8-89 [AOCS, 1989]. In brief, 0.5 g of oil was dissolved in *n*-hexane in a 5-mL volumetric flask. The resulting solution was then filtered through a 0.45-µm polytetrafluoroethylene membrane filter, and 20 µL of the filtrate was injected into an Agilent 1200 Series HPLC system (Agilent Corp., Santa Clara, CA, USA), equipped with a Phenomenex Luna Sil column (250 × 4.6 mm i.d., 5 µm particle size; Phenomenex, Inc., Torrance, CA, USA). The column was maintained at 40°C throughout the analysis. The mobile phase consisted of a mixture of *n*-hexane and isopropyl alcohol (99:1, *v/v*). It was used at a flow rate of 1.0 mL/min. Prior to use, the mobile phase was degassed by sonication for 10 min. Fluorescence detection was performed at an excitation wavelength

of 290 nm and at an emission wavelength of 330 nm. Tocopherol peaks were identified by comparing their retention times to those of external standards of pure α-, β-, γ-, and δ-tocopherol isoforms. The quantification of tocopherols in *N. sativa* oil samples was carried out using the external standard calibration method. Standard solutions of each tocopherol isomer (0.1 mg/mL) were prepared in *n*-hexane. Calibration curves were obtained by plotting the peak area against the corresponding standard concentrations at four calibration levels, and quantification was based on the resulting linear regression equations. The analysis was performed in triplicate, and results were expressed as mg/100 g oil.

Preparation of different solvent extracts of *N. sativa* seeds

N. sativa seeds were extracted using three different solvents: ethanol, a water and ethanol mixture (20/80, *v/v*), and water, using the maceration method. For each solvent, 5 g of seed powder from each location was macerated in 50 mL of the solvent for 24 h at room temperature, with continuous agitation at 150 rpm. The resulting filtrates were dried and then stored at 4°C until further analysis of their antioxidant and antibacterial activities.

Determination of total phenolic content of the extracts

The total phenolic (TP) content of the different *N. sativa* seed extracts (aqueous, hydroethanolic, and ethanolic) was determined using the method of Singleton & Rossi [1965]. Briefly, 63 µL of FC reagent were added to 63 µL of each extract at a concentration of 0.01 g/mL. After a 5-min incubation period, 625 µL of a 7% (*w/v*) Na_2CO_3 solution were added, and the mixture was incubated in the dark for 90 min. After incubation, 200 µL of each mixture were transferred to a microplate and analyzed with a Synergy HTX MultiMode Microplate Reader (Biotek Instruments, Winooski, VT, USA) at a wavelength of 765 nm. The total phenolic content was calculated using a standard calibration curve for gallic acid with a concentration range of 10–100 mg/mL. The linear regression equation obtained was: $y = 8.1722x + 0.0308$, with an R^2 of 0.9974. The TP content was expressed as mg of gallic acid equivalents (GAE) *per* g of dry extract. Measurements for each extract were performed in triplicate.

Determination of total flavonoid content of the extracts

The total flavonoid (TF) content of the *N. sativa* seed extracts (aqueous, hydroethanolic, and ethanolic) was determined using the method with $AlCl_3$, as defined by Zhishen *et al.* [1999]. In short, 38 µL of 5% (*w/v*) $NaNO_2$ were added to 125 µL of each extract (0.01 g/mL). The mixture was allowed to stand for 6 min, after which 75 µL of freshly prepared 10% (*w/v*) $AlCl_3$ solution were added. After incubation, 200 µL of each mixture were transferred to a microplate and analyzed with a Synergy HTX MultiMode Microplate Reader (Biotek Instruments) at a wavelength of 510 nm. TF content was calculated relying on a standard calibration curve for quercetin with concentrations of 10–75 mg/mL. The linear regression equation was: $y = 0.0103x + 0.0943$, with an R^2 of 0.9996. The TF content was expressed as mg of quercetin equivalents (QE) *per* 100 g of dry extract. Measurements were carried out in three experimental replicates for each extract.

Determination of total tannin content of the extracts

The total tannin (TT) content of the *N. sativa* seed extracts (aqueous, hydroethanolic, and ethanolic) was determined using the method described by Julkunen-Tiitto [1985]. Briefly, 50 µL of each extract (0.01 g/mL) were mixed with 1.5 mL of 4% (*v/v*) vanillin in methanol and 750 µL of a 12 M hydrochloric acid solution. The mixture was thoroughly blended and incubated in the dark at room temperature for 20 min. After incubation, 200 µL of each mixture was transferred to a microplate and analyzed with a Synergy HTX MultiMode Microplate Reader (BioTek Instruments) at a wavelength of 500 nm. The TT content was calculated using a (+)-catechin standard calibration curve with concentrations of 0.10–1 mg/mL. The linear regression equation was: $y = 0.8962x + 0.0014$, with an R^2 of 0.9995. The TT content was expressed as mg of (+)-catechin equivalents (CE) *per* g of dry extract. Measurements were taken in triplicate for each extract.

Evaluation of the antioxidant activity of *N. sativa* seed extracts

DPPH assay

The antioxidant activity of each *N. sativa* seed extract was evaluated using the DPPH assay by Brand-Williams *et al.* [1995]. A DPPH radical methanol solution (0.35 g/L), previously adjusted to an absorbance of 0.95 at 515 nm with methanol, was placed into the wells of a microplate at a volume of 190 µL. Stock solutions (0.1 mg/mL) of the extracts were prepared in their respective extraction solvents (ethanol, water, and ethanol-water (20:80, *v/v*)), while the BHT stock solution (0.1 mg/mL) was prepared in methanol. These solutions were then diluted to obtain five different concentrations in a range of 10–100 µg/mL for extracts and 5–25 µg/mL for BHT. Subsequently, 10 µL of each diluted extract and BHT solution or each solvent alone was added to the wells with DPPH radical solution, and the mixtures were incubated in the dark for 50 min at room temperature. After incubation, the absorbance was read. The inhibition percentage (I%) was calculated using Equation (2):

$$I\% = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (2)$$

where: $A_{control}$ is the absorbance of the DPPH radical solution without the antioxidant and A_{sample} is the absorbance of the DPPH radical solution with the extract or BHT.

The antioxidant activity was quantified by the IC_{50} value (expressed as mg/mL), which represents the concentration of an extract or BHT required to reduce 50% of the DPPH radicals. Measurements were performed in triplicate for each sample.

Ferric-reducing antioxidant power assay

The ferric-reducing antioxidant power (FRAP) of the extracts was determined according to the method of Benzie & Strain [1996],

with slight modifications. The FRAP reagent was freshly prepared before each analysis by mixing 10 mL of acetate buffer (300 mM, pH 3.6), 1 mL of a TPTZ solution (10 mM in 40 mM HCl), and 1 mL of an $FeCl_3 \times 6H_2O$ solution (20 mM). The mixture was maintained at 37°C until use. In a 96-well microplate, 25 µL of extract solutions (1 mg/mL) or Trolox standard solutions (20–200 mg/L) were mixed with 175 µL of the pre-warmed FRAP reagent. The absorbance was recorded at 593 nm and the antioxidant power was expressed as Trolox equivalents *per* g of dry extract weight (mg TE/g dry extract) based on the linear standard curve with R^2=0.9976. Measurements were performed in triplicate for each sample.

Determination of the antibacterial activity of *N. sativa* seed extracts

In this part of the study, only the ethanolic extracts of *N. sativa* seeds from various locations were evaluated for antibacterial activity, as the aqueous and hydroethanolic extracts showed no significant activity against the tested bacterial strains. The ethanolic extracts were tested against four bacterial strains commonly found in food products: three Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella enteritidis* ATCC 13076) and one Gram-positive bacterium (*Staphylococcus aureus* ATCC 6538). These bacterial strains were sourced from the American Type Culture Collection (ATCC, Manassas, VA, USA), and the analyses were conducted at the Laboratory for Microbiological Analysis of Food Products, part of the National Laboratory for Analysis and Testing in Tunis, Tunisia. Antibacterial activity was assessed using the agar well diffusion method with sterile Mueller–Hinton agar, as described by Weerakkody *et al.* [2010]. Specifically, a 100 µL aliquot of a freshly prepared bacterial suspension (adjusted to 10^7 CFU/mL) was inoculated onto the surface of the agar plates. Sterile filter paper discs (6 mm in diameter) were impregnated with 20 µL of *N. sativa* seed extract (10 mg/mL) and 20 µL of gentamycin (10 mg/mL), a synthetic antibiotic used as a positive control, and placed on the agar plates. The plates were incubated at 37°C for 24 h. Subsequently, the microbial inhibition was assessed by measuring the diameter (mm) of the inhibition zone (DIZ, clear zone around each disc). Measurements were carried out in triplicate.

Statistical analysis

All data were analyzed using the XLSTAT software package (Addinsoft, NY, USA) for Microsoft Excel. Significant differences in the parameters analyzed among the samples of *N. sativa* seeds from four different Tunisian regions were evaluated using one-way analysis of variance (ANOVA) with a post-hoc Tukey test, with a significance level set at $p<0.05$.

RESULTS AND DISCUSSION

Fatty acid composition of *N. sativa* seed oil

The lipid content of the studied N. sativa seeds was 23.41, 25.32, 27.15, and 28.64 g/100 g, respectively, for seeds collected from the Nabeul, Kairouan, Sfax, and Gabès regions. Fatty acids are the main constituents of the lipid fraction and contribute specific physicochemical characteristics to fats and oils. The fatty acid profile of each *N. sativa* seed oil was determined using GC-FID, with the results summarized in **Table 1**. Nine compounds were identified. Among these, linoleic acid (C18:2) was the most abundant, accounting for 49.66% to 58.47% of the total fatty acids in all samples. The high linoleic acid content, an essential ω-6 fatty acid, suggests that *N. sativa* oil could serve as a valuable source of this vital fatty acid, which plays an important role in regulating cholesterol levels [Azemi *et al.,* 2023]. Additionally, oleic acid (C18:1), the second most prevalent fatty acid, accounted for 19.63% to 29.25% of the total fatty acids in the samples. Thus, it contributed a substantial proportion of monounsaturated fatty acids in *N. sativa* seed oil. This acid is well known for its cardioprotective benefits and positive impact on lipid metabolism [Petersen *et al.,* 2024]. Nevertheless, palmitic (C16:0), stearic (C18:0), eicosadienoic (C20:2), linolenic (C18:3), eicosanoic (C20:0), and eicosenoic (C20:1) acids were found in lower contents across all samples. While these fatty acids are present in smaller amounts, they play an essential role in maintaining the structural integrity of cell membranes [Pashkovskaya *et al.,* 2018]. The results also revealed significant differences ($p<0.05$) in the contents of the major fatty acids across the four regions, with linoleic acid being the highest in Gabès, followed by oleic acid in Nabeul and palmitic acid in Gabès. These variations are likely to be due to the distinct agro-climatic conditions in the studied regions, as they differ considerably in terms of annual rainfall, which ranges from 404 mm in Nabeul to 152 mm in Gabès. Similar results regarding the fatty acid composition of *N. sativa* seed oil have been reported by Kiani *et al.* [2020], who identified the same fatty acids, albeit with slight differences in their relative percentages.

The results of the present study demonstrate that *N. sativa* seeds share a fatty acid composition comparable to that of chia, flax, pumpkin, sesame, and sunflower seeds, which are widely marketed for their health benefits [Ahmed *et al.,* 2026; Sumara *et al.,* 2023]. Our findings highlight *N. sativa* seeds as an equally valuable source of bioactive fatty acids, with linoleic and oleic acids as the major components.

Phytosterol composition of *N. sativa* seed oil

Phytosterols represent the primary constituents of the unsaponifiable fraction found in vegetable oils and are recognized for their nutritional significance in the human diet. In this study, the sterol fraction of *N. sativa* oil was separated from the total unsaponifiable matter using TLC (**Figure 1**) and subsequently characterized through GC-MS. The identified phytosterols and their respective contents are summarized in **Table 2**. A total of eight sterol compounds were detected in *N. sativa* seed oils, which were classified into two structural categories. The first category, Δ^5-sterols, includes cholesterol, campesterol, stigmasterol, β-sitosterol, Δ^5-avenasterol, and $\Delta^{5,24}$-stigmastadienol. The second category comprises Δ^7-sterols, specifically Δ^7-stigmastenol and Δ^7-avenasterol. Quantitative assessment showed that β-sitosterol was the most

Table 1. Fatty acid composition (expressed as % of total fatty acids) in *Nigella sativa* L. seed oils from four Tunisian regions (Nabeul, Kairouan, Sfax, and Gabès).

Fatty acid	Nabeul	Kairouan	Sfax	Gabès
Myristic acid (C14:0)	0.14±0.02^{a}	0.14±0.01^{a}	0.14±0.01^{a}	0.12±0.01^{a}
Palmitic acid (C16:0)	10.51±0.09^{d}	11.14±0.06^{c}	12.43±0.08^{b}	13.74±0.08^{a}
Palmitoleic acid (C16:1)	0.28±0.01^{b}	0.11±0.01^{d}	0.33±0.01^{a}	0.23±0.01^{c}
Stearic acid (C18:0)	2.89±0.05^{c}	2.95±0.07^{c}	3.11±0.02^{b}	3.41±0.02^{a}
Oleic acid (C18:1)	29.25±0.09^{a}	26.23±0.07^{b}	23.54±0.07^{c}	19.63±0.08^{d}
Linoleic acid (C18:2)	49.66±0.25^{d}	53.50±0.02^{c}	56.72±0.09^{b}	58.47±0.49^{a}
Linolenic acid (C18:3)	0.37±0.05^{a}	0.38±0.01^{a}	0.40±0.04^{a}	0.44±0.02^{a}
Arachidic acid (C20:0)	0.19±0.02^{a}	0.19±0.01^{a}	0.21±0.03^{a}	0.19±0.02^{a}
Gondoic acid (C20:1)	0.56±0.02^{a}	0.11±0.02^{c}	0.41±0.10^{b}	0.41±0.02^{b}
Eicosadienoic acid (C20:2)	4.78±0.03^{a}	3.95±0.01^{b}	2.65±0.11^{d}	2.97±0.05^{c}
SFA	13.73±0.11^{d}	14.41±0.02^{c}	15.89±0.06^{b}	17.46±0.07^{a}
MUFA	30.10±0.10^{a}	26.79±0.52^{b}	24.28±0.04^{c}	20.26±0.08^{d}
PUFA	54.81±0.24^{d}	57.83±0.03^{c}	59.77±0.07^{b}	61.88±0.44^{a}

Values are mean ± standard deviation, n=3. Values in row without a common letter are significantly different (p<0.05). SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids.

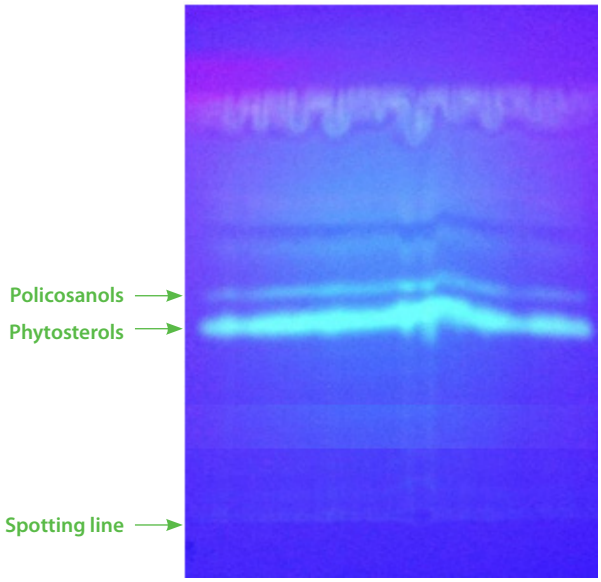

Figure 1. Thin-layer chromatography (TLC) separation of phytosterols and policosanols of the unsaponifiable fraction of *Nigella sativa* L. seed oil.

abundant phytosterol across all oil samples. The highest content of β-sitosterol was found in the seeds collected from Nabeul in northern Tunisia (100.46 mg/100 g oil), while the lowest was observed in the samples from Gabès in the southern region (88.19 mg/100 g oil). Additionally, other sterols such as stigmasterol (ranging from 40.23 to 54.21 mg/100 g oil), campesterol (27.52 to 33.76 mg/100 g oil), and Δ^5-avenasterol (16.60 to 22.42 mg/100 g oil), were consistently present at relatively high levels in seed oils across all regions. The content of the main phytosterols in seeds from different regions exhibited significant variation. This difference can primarily be attributed to agro-climatic factors, as the four studied regions (Nabeul, Kairouan, Sfax, and Gabès) exhibit significantly different climatic conditions. Comparable profiles of phytosterols in *N. sativa* oil have been documented by Albakry *et al.* [2022], although slight variations in their contents were noted.

Phytosterols have attracted growing scientific attention due to their beneficial effects on human health. Evidence from clinical research highlights their potential in reducing cholesterol levels, as well as their anti-inflammatory and anticancer properties [Jiménez *et al.,* 2024]. For example, a study by Trautwein *et al.* [2018] demonstrated that phytosterol intake led to triglyceride reductions ranging from 0.8% to 18%, with the greatest reductions observed in individuals who had elevated baseline triglyceride levels. Given their cholesterol-lowering properties in mammals, soluble phytosterols have been incorporated into various food products. Current dietary recommendations suggest an average phytosterol intake of approximately 250 mg/day for healthy nutrition [Nattagh-Eshtivani *et al.,* 2022]. Comparing the phytosterol profiles of seeds like chia, flax, pumpkin, and sesame [Alasalvar *et al.,* 2021], *N. sativa* seeds exhibit a similar composition, with β-sitosterol, stigmasterol, campesterol, and Δ^5-avenasterol as the primary compounds. Thus, *N. sativa* seeds represent a valuable plant-based source of phytosterols,

Table 2. Phytosterol composition (expressed in mg/100 g of oil) in *Nigella sativa* L. seed oils from four Tunisian regions (Nabeul, Kairouan, Sfax, and Gabès).

Phytosterol	Nabeul	Kairouan	Sfax	Gabès
Cholesterol	0.92±0.04^{a}	1.02±0.11^{a}	1.08±0.16^{a}	1.32±0.18^{a}
Campesterol	33.76±1.22^{a}	31.71±1.10^{b}	29.54±1.31^{c}	27.52±1.06^{d}
Stigmasterol	40.23±2.10^{d}	44.61±2.11^{c}	48.49±2.40^{b}	54.21±2.45^{a}
β-Sitosterol	100.46±5.35^{a}	98.23±5.12^{b}	92.55±4.62^{c}	88.19±4.15^{d}
Δ^{5}-Avenasterol	16.60±0.83^{c}	18.45±0.85^{b}	19.83±0.92^{b}	22.42±1.18^{a}
$\Delta^{5,24}$-Stigmastadienol	1.34±0.15^{a}	1.25±0.12^{a}	1.13±0.11^{a}	0.94±0.11^{b}
Δ^{7}-Stigmastenol	2.51±0.12^{d}	3.26±0.15^{c}	3.86±0.16^{b}	4.65±0.24^{a}
Δ^{7}-Avenasterol	5.23±0.30^{a}	4.85±0.29^{b}	4.18±0.26^{c}	3.67±0.16^{d}

Values are mean ± standard deviation, n=3. Values in row without a common letter are significantly different (p<0.05).

Table 3. Policosanol composition (expressed in mg/kg of oil) in *Nigella sativa* L. seed oils from four Tunisian regions (Nabeul, Kairouan, Sfax, and Gabès).

Policosanol	Nabeul	Kairaoun	Sfax	Gabès
Eicosanol (C20)	2.05±0.92^{c}	4.55±0.84^{b}	8.53±0.77^{a}	9.43±0.79^{a}
Docosanol (C22)	5.33±0.72^{a}	5.09±0.55^{a}	4.61±0.64^{a}	2.93±0.59^{b}
Tetracosanol (C24)	7.36±0.78^{a}	5.85±0.85^{a}	3.46±0.79^{b}	2.34±0.84^{b}
Pentacosanol (C25)	3.75±0.63^{a}	3.17±0.65^{a}	1.63±0.47^{b}	1.23±0.10^{b}
Hexacosanol (C26)	9.64±0.80^{a}	7.26±0.41^{b}	7.15±0.79^{b}	5.26±0.66^{c}
Heptacosanol (C27)	2.74±0.59^{c}	4.90±0.57^{b}	7.56±0.59^{a}	8.41±0.72^{a}
Octacossanol (C28)	9.59±1.18^{d}	12.22±0.17^{c}	16.21±0.73^{b}	19.19±0.76^{a}
Nonacosanol (C29)	4.26±0.83^{c}	7.73±0.78^{b}	9.43±0.94^{b}	13.41±0.73^{a}
Triacontanol (C30)	30.48±0.64^{a}	27.74±1.54^{ab}	25.36±1.47^{bc}	24.13±1.17^{c}
Hentricontanol (C31)	4.14±0.57^{a}	3.25±0.64^{ab}	2.61±0.57^{bc}	1.29±0.29^{c}
Dotriacontanol (C32)	10.79±1.24^{a}	7.85±0.32^{b}	4.42±0.63^{c}	3.66±0.65^{c}

Values are mean ± standard deviation, n=3. Values in row without a common letter are significantly different (p<0.05).

offering nutritional and health benefits, and can be classified as functional food seeds.

Policosanol composition of *N. sativa* seed oil

The analysis of the policosanol composition in *N. sativa* seed oils revealed the presence of eleven distinct compounds including eicosanol (C20), docosanol (C22), tetracosanol (C24), pentacosanol (C25), hexacosanol (C26), heptacosanol (C27), octacosanol (C28), nonacosanol (C29), triacontanol (C30), hentricontanol (C31), and dotriacontanol (C32) (**Table 3**). Among these, hexacosanol, octacosanol, triacontanol, and dotriacontanol were the major policosanols of *N. sativa* seed oil, accounting for more than 65% of the total policosanol content. Triacontanol was the most abundant, with contents of 30.48, 27.74, 25.36 and 24.13 mg/kg oil of seeds from Nabeul, Kairoun, Sfax, and Gabès, respectively. In contrast, policosanols with an odd number of carbon atoms (pentacosanol, heptacosanol, and hentricontanol) were detected in lower amounts.

The total policosanol content of *N. sativa* seed oil (average 90 mg/kg) placed it among other natural, rich sources of these bioactive compounds; when compared with previously reported values, this content was substantially higher than that found in brown beeswax (5.2 mg/kg) and yellow beeswax (12 mg/kg) [Jackson *et al.*, 2006], and it also exceeded the 17.4 mg/kg reported for whole sugar cane. Although sugar cane leaves and peels remain richer sources (181 and 270 mg/kg, respectively) [de Lucas

Table 4. Tocopherol isomer composition (expressed mg/100 g of oil) in *Nigella sativa* L. seed oils from four Tunisian regions (Nabeul, Kairouan, Sfax, and Gabès).

Region	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol
Nabeul	10.38±1.56^{d}	1.25±0.10^{d}	8.45±1.25^{a}	1.83±0.16^{a}
Kairouan	10.92±1.61^{c}	1.39±0.11^{c}	7.97±1.18^{b}	1.65±0.12^{b}
Sfax	11.01±1.61^{b}	1.51±0.12^{b}	7.86±1.12^{c}	1.57±0.12^{c}
Gabès	11.51±1.68^{a}	1.56±0.12^{a}	7.38±0.89^{d}	1.14±0.11^{d}

Values are mean ± standard deviation, n=3. Values in column without a common letter are significantly different (p<0.05).

Table 5. Total phenolic (TP), total flavonoid (TF), and total tannin (TT) contents of extracts of *Nigella sativa* L. seeds from four locations obtained using various solvents – ethanol, 80% (*v/v*) ethanol, and water.

Parameter	Solvent	Nabeul	Kairouan	Sfax	Gabès
Total phenolic content (mg GAE/g dry extract)	Water	60.24±0.64^{bC}	60.53±0.58^{bC}	60.8±0.64^{abC}	62.17±0.63^{aC}
	80% EtOH (*v/v*)	72.50±1.42^{aA}	72.82±1.18^{aA}	73.17±1.32^{aA}	74.35±1.23^{aA}
	EtOH	67.26±0.59^{bB}	67.39±0.62^{bB}	68.12±0.60^{abB}	69.57±0.64^{aB}
Total flavonoid content (mg QE/100 g dry extract)	Water	17.19±0.61^{cC}	18.62±0.63^{cC}	20.24± 0.46^{bB}	23.82±0.61^{aB}
	80% EtOH (*v/v*)	23.48±0.66^{cA}	24.67±0.63^{cA}	27.25±0.53^{bA}	29.47±0.91^{aA}
	EtOH	21.76±0.76^{bB}	22.20±1.38^{bB}	25.70±0.83^{aA}	27.82±1.01^{aA}
Total tannin content (mg CE/g dry extract)	Water	4.13±0.25^{aC}	4.63±0.54^{aC}	4.78±0.46^{aC}	5.16±0.43^{aB}
	80% EtOH (*v/v*)	9.13±0.41^{aA}	9.30±0.88^{aA}	9.81±0.85^{aA}	10.19±0.92^{aA}
	EtOH	7.18±0.62^{aB}	7.35±0.62^{aB}	7.82±0.83^{aB}	8.07±1.07^{aA}

Values are mean ± standard deviation, n=3. Values for each parameter (TP, TF and TT) followed by the different lowercase letter (a–c) in the same row (origin comparison) or uppercase letters (A–C) in the same column (solvent comparison) are significantly different (p<0.05). GAE, gallic acid equivalents; QE, quercetin equivalents; CE, (+)-catechin equivalents.

et al., 2007], the values obtained for *N. sativa* seeds oil indicate that these seeds are also a valuable and underutilized plant material in this respect.

Tocopherol composition of *N. sativa* seed oil

Tocopherols are exclusively synthesized by photosynthetic organisms and primarily act as antioxidants. They play a critical role in improving the oxidative stability of vegetable oils and enhancing the nutritional value of crop plants for human consumption. The tocopherol isomers identified in *N. sativa* seed oils (α, β, γ, and δ) and their respective contents in the samples from the four regions are presented in **Table 4**. The data indicate that α-tocopherol was the most abundant isomer, with relatively high contents ranging from 10.38 to 11.51 mg/100 g oil across all seed samples. γ-Tocopherol was also found in significant amounts, ranging from 7.38 to 8.45 mg/100 g oil. Conversely, β-tocopherol and δ-tocopherol were detected in smaller amounts, ranging between 1 and 2 mg/100 g oil, across the seeds from all regions. These results are consistent with those reported by Albakry *et al.* [2022] for *N. sativa* seeds obtained from an Iranian supermarket, which showed a comparable distribution of tocopherol isomers.

Tocopherols act as antioxidants because they can neutralize lipid-free radicals by donating a hydrogen atom from their phenolic group. In fact, it was reported that the hydrogen-donating capacity of tocopherol isomers follows the order: α>β>γ>δ [Kamal-Eldin & Appelqvist, 1996]. Additionally, Patterson [1981] demonstrated that one molecule of tocopherol can protect the oxidation of 103 to 106 molecules of unsaturated fatty acids. Natural α-tocopherol has been shown to effectively inhibit lipid oxidation, thereby improving the oxidative stability of lipid-rich food products during storage, often performing comparably or even better than certain synthetic antioxidants, such as tertiary butylhydroquinone (TBHQ) [Alizadeh *et al.,* 2019]. This underscores the further use of *N. sativa* seed oil, a valuable source of these lipophilic tocopherols, as a food additive.

Total phenolic, total flavonoid, and total tannin contents of *N. sativa* seed extracts

The TP content of the different extracts (aqueous, ethanolic, and hydroethanolic) of *N. sativa* seeds ranged from 60.24 to 74.35 mg GAE/g dry extract across the four studied localities (**Table 5**). The highest amount was recorded in the hydroethanolic

Table 6. Antioxidant activity determined in DPPH assay (expressed as IC_{50}, mg/mL) and ferric-reducing antioxidant power (FRAP) (expressed as mg Trolox equivalent, TE/g dry extract) of various solvent extracts of *Nigella sativa* L. seeds from four Tunisian regions (Nabeul, Kairouan, Sfax and Gabès).

Assay	Solvent	Nabeul	Kairouan	Sfax	Gabès
DPPH	Water	5.11 ± 0.02^{aA}	4.95 ± 0.11^{aA}	4.65 ± 0.06^{bA}	4.53 ± 0.06^{bA}
	80% EtOH (*v/v*)	3.25 ± 0.03^{aC}	3.10 ± 0.03^{bC}	2.93 ± 0.08^{cC}	2.82 ± 0.03^{cC}
	EtOH	3.62 ± 0.04^{aB}	3.42 ± 0.03^{bB}	3.33 ± 0.04^{cB}	3.21 ± 0.02^{dB}
FRAP	Water	187 ± 3^{bC}	204 ± 3^{bC}	289 ± 4^{aC}	316 ± 5^{aC}
	80% EtOH (*v/v*)	646 ± 7^{dA}	680 ± 8^{cA}	792 ± 11^{bA}	819 ± 14^{aA}
	EtOH	588 ± 6^{bB}	612 ± 7^{bB}	697 ± 8^{aB}	724 ± 11^{aB}

Values are mean ± standard deviation, $n=3$. Values followed by the different lowercase letter (a–c) in the same row or uppercase letters (A–C) in the same column (separately for each assay) are significantly different ($p<0.05$). IC_{50}, the concentration required to inhibit 50% of the DPPH radicals. The butylated hydroxytoluene (BHT) was used in DPPH assay as reference antioxidant, with an IC_{50} value of 0.46 mg/mL.

extract, ranging from 72.5 mg GAE/g (seeds from the Nabeul region) to 74.35 mg GAE/g (seeds from Gabès). These findings highlight the crucial role of solvent type and polarity in the extraction efficiency of total phenolic content. In fact, the water-ethanol mixture proved most effective for extracting phenolics due to the complementary polarities of the solvents, whereas distilled water and pure ethanol were less efficient, extracting only highly polar or moderately polar compounds, respectively. The results obtained using maceration were higher than those reported by Mariod *et al.* [2009], who found that the TP content of *N. sativa* seed extracts from Malaysia was 27.8 mg GAE/g of dry extract when methanol was used as the sonication solvent. However, using a Soxhlet apparatus, Zwolan *et al.* [2020] reported TP contents of 24.89 and 35.72 mg GAE/g of dry extract when water and ethanol were used as extraction solvents, respectively.

The total flavonoid content of *N. sativa* seed extracts ranged from 17.19 to 29.47 mg QE/100 g, depending on the extraction solvent used and the sampling region (**Table 5**). Similar to determining TF content, the ethanol-water mixture proved to be the most effective solvent for flavonoid extraction (yielding the highest amount 29.47 mg QE/100 g in dry extract of seeds from the Gabès region), followed by ethanol, and finally distilled water. However, the difference in TF content between the hydroethanolic and ethanolic extracts was less obvious in this case compared to TP content. Ravi *et al.* [2024] recorded a TF content of 7.31–8.15 mg QE/100 g for Indian cumin seeds. Ouattar *et al.* [2022] found that the methanol-water extract of Moroccan *N. sativa* seeds contained 25.8 mg QE/100 g of total flavonoids when maceration was used as the extraction method. These variations in total flavonoid content among *N. sativa* seeds can be attributed to several factors, including genotypic and environmental differences within species, the sampling period, and the analytical methods used for quantification.

The total tannin content of the different extracts (aqueous, ethanolic, and hydroethanolic) of *N. sativa* seeds ranged from 4.13 to 10.19 mg CE/g dry extract across the four studied localities (**Table 5**). As with total phenolics and flavonoids, the hydroethanolic solvent exhibited the highest extraction efficiency for total tannins. This can be attributed to the strong solubility of tannins, both hydrolyzable and condensed, in polar solvents, particularly water-alcohol mixtures. Similar to total phenolic (TP) and total flavonoid (TF) contents, *N. sativa* seeds from the Gabès region exhibit the highest total tannin content, regardless of the extraction solvent used. This is because *N. sativa* produces more secondary defense metabolites, including flavonoids, tannins, alkaloids, and thymoquinone, when exposed to a hot climate, enhancing its resilience and adaptability to these challenging conditions [Ibrahim *et al.,* 2023].

Antioxidant activity of *N. sativa* seed extracts

In the present study, the antioxidant potential of *N. sativa* seed extracts was evaluated using two complementary analytical assays: DPPH radical scavenging activity and ferric-reducing antioxidant power. The antioxidant activity of the seed extracts was expressed as IC_{50} and mg TE/g dry extract for the DPPH and FRAP assays, respectively (**Table 6**). The results indicated that the hydroethanolic extracts exhibited significantly ($p<0.05$) higher antioxidant activity compared to the ethanolic and aqueous extracts, regardless of the assay employed. The DPPH IC_{50} values for the hydroethanolic extracts of *N. sativa* seeds collected from the Nabeul, Kairouan, Sfax, and Gabès regions were 3.25, 3.10, 2.93, and 2.82 mg/mL, respectively. Previous study by Gueffai *et al.* [2022] showed that the DPPH IC_{50} value of the hydroethanolic (5:5, *v/v*) extract of *N. sativa* seeds was 1.96 mg/mL when obtained by maceration, whereas it decreased to 1.14 mg/mL under optimized ultrasound-assisted extraction conditions. The FRAP of *N. sativa* seed extracts ranged from 187 to 819 mg TE/g across the different sampling locations (**Table 6**). The lowest values were determined for the aqueous extracts (187–316 mg TE/g), whereas the intermediate (588–724 mg TE/g) and highest (646–819 mg TE/g) values were obtained when ethanol and aqueous ethanol were used as solvents, respectively. The highest FRAP (819 mg ET/g) was recorded in the hydroethanolic extract of *N. sativa* seeds from the Gabès region.

Table 7. Antimicrobial activity measured by the diameter of the inhibition zone (mm) of ethanolic extracts (10 mg/mL) of *Nigella sativa* L. seeds from four Tunisian regions (Nabeul, Kairouan, Sfax, and Gabès).

Region	*Escherichia coli*	*Salmonella enteritidis*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Nabeul	13.84±0.03^{d}	14.52±0.04^{d}	11.25±0.02^{d}	16.74±0.04^{d}
Kairouan	14.24±0.02^{c}	15.62±0.01^{c}	11.55±0.04^{c}	17.63±0.03^{c}
Sfax	14.61±0.04^{b}	15.81±0.06^{b}	11.94±0.02^{b}	17.84±0.02^{b}
Gabès	15.07±0.03^{a}	16.44±0.05^{a}	12.32±0.04^{a}	18.43±0.02^{a}
Gentamycin*	18.30±0.13	20.23±0.02	16.43±0.02	22.52±0.02

Values are mean ± standard deviation, n=3. Values followed by the different lowercase letter (a–d) in the same column are significantly different (p<0.05). *Gentamycin (10 mg/mL) was used as reference antibiotic.

Kadam & Lele [2017] reported that delipidated *N. sativa* seeds from India exhibited a FRAP of 1.85 mM TE/g (equivalent to 463 mg TE/g) when extracted with an ethanol-water (60:40, *v/v*). Overall, the FRAP obtained in the present study using ethanol--water (80:20, *v/v*) was higher than this reported by Kadam & Lele [2017]. This difference may be explained by the fact that during lipid extraction process, some secondary metabolites such as, phenolics may migrate into the oil fraction, thereby reducing the antioxidant potential measured in the remaining sample.

According to the experimental results, the hydroethanolic solvent has proven to be more effective at extracting maximum antioxidant compounds from *N. sativa* seeds, including phenolics, which are secondary metabolites known for their antioxidant properties. The ethanol effectively extracts also thymoquinone, a prominent biomolecule characteristic of *N. sativa* seeds [Ahmad *et al.,* 2021]. This bioactive compound has been shown to exhibit a wide range of therapeutic properties, including antioxidant, anti-inflammatory, antihistamine, antibacterial, anticancer, and encephalomyelitis effects in various *in vitro* and certain *in vivo* studies [Akter *et al.,* 2021; Alberts *et al.,* 2024; Kazemi *et al.,* 2024].

■ Antimicrobial activity of *N. sativa* seed ethanolic extract

Table 7 shows the inhibition zone diameters for the ethanolic extracts of *N. sativa* seeds from different locations, alongside those for gentamicin, a reference antibiotic, tested against four foodborne pathogens. The results indicate that all the examined bacterial strains exhibited sensitivity to the ethanolic extracts of *N. sativa* seeds. The diameter of the inhibition zone varied from 11.25 to 18.43 mm, depending on the bacterial strain and the region where the *N. sativa* seeds were harvested. Notably, the *S. aureus* strain demonstrated the highest sensitivity to the ethanolic extract, with inhibition zones ranging from 16.74 to 18.43 mm, compared to gentamicin which had an inhibition zone of 22.52 mm. This notable sensitivity can be attributed to *S. aureus* being a Gram-positive bacterium, which lacks an outer membrane, allowing the extract's compounds to penetrate more easily and inhibit its growth. In contrast, the strains *S. enteritidis* (14.52 to 16.44 mm), *E. coli* (13.84 to 15.07 mm), and *P. aeruginosa* (11.25 to 12.32 mm) exhibited varying levels of sensitivity to the ethanolic extract of *N. sativa* seeds. The obtained results also indicate that, for the same bacterial strain, the extract from *N. sativa* seeds harvested in the Gabès region demonstrated the most significant antibacterial effect, with inhibition diameters of 18.43 mm for *S. aureus*, 16.44 mm for *S. enteritidis*, 15.07 mm for *E. coli*, and 12.32 mm for *P. aeruginosa*. This result suggests that climatic conditions also influence the antibacterial potency, given the significant climatic differences among the regions studied. The accumulation of secondary metabolites is likely to explain the pronounced antibacterial activity of *N. sativa* seeds from the Gabès region compared to those from the remaining areas. In the literature, few studies address the antimicrobial activities of *N. sativa* seeds. Consistent with Bourgou *et al.* [2012], who demonstrated antibacterial activity of methanolic seed extracts against *S. aureus* and *E. coli*, our results also show notable inhibition of these strains by ethanolic extracts. Similarly, the antifungal properties reported by Nadaf *et al.* [2015] and the broad--spectrum antibacterial and antifungal activity of *N. sativa* oils documented by Tiji *et al.* [2021] corroborate the view that this seed is a rich source of bioactive compounds with antimicrobial potential. Importantly, our study demonstrates that the antibacterial potency of *N. sativa* seeds varies with geographic origin, with seeds from the Gabès region showing the strongest inhibitory effects. This suggests that environmental stressors such as high temperature, aridity, and solar radiation in Saharan regions enhance the accumulation of secondary defense metabolites, thereby boosting antimicrobial efficacy. These findings do not only highlight their consistency with earlier studies, but also the fact that agro-climatic conditions shape the bioactivity of *N. sativa*, reinforcing its potential as a natural antimicrobial source for food preservation and safety.

CONCLUSIONS

The study findings indicated that the *N. sativa* seeds are rich in bioactive lipids, including fatty acids, phytosterols, policosanols, and tocopherols, confirming their value as a functional food resource. The seed extracts showed notable antioxidant activity while ethanolic extracts particularly exhibited significant

antibacterial activity against key foodborne pathogens. Extracts of seeds from the Gabès region displayed the strongest activity, a finding that underscores the influence of harsh Saharan climatic conditions on the accumulation of secondary defense metabolites. Overall, these findings highlight that *N. sativa* seeds hold great potential for future applications, both in food preservation, as a natural alternative to synthetic additives, and as a valuable source of health-promoting compounds in the human diet. Future research should focus on isolating and characterizing the specific bioactive molecules responsible for these effects, clarifying their mechanisms of action, and testing their applications in food formulations and clinical settings. Such studies will further consolidate the position of *N. sativa* seeds as a valuable natural resource for nutrition, health, and food safety.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest to influence the work reported in this paper.

ORCID IDs

F. Sakouhi https://orcid.org/0000-0001-9708-0446

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Fractionation of the Maca (*Lepidium meyenii* Walp.) Leaf Extract Using Macroporous Resin Chromatography and Its Biological Properties

Eun J. Lee, Kyung Y. Yoon*

Department of Food and Nutrition, Yeungnam University, Gyeongsan, Republic of Korea

This study investigated the bioactivities of maca (*Lepidium meyenii* Walp.) leaf extract fractions obtained using deep eutectic solvent (DES)-based ultrasound-assisted extraction (UAE) followed by macroporous resin chromatography. Four fractions (Fr. 1 – Fr. 4) were obtained by elution with ethanol of varying concentrations (25%, 50%, 75%, and 100%, *v/v*), and subsequently total phenolic content (TPC), total saponin content (TSC), and individual phenolic contents as well as their anti-inflammatory, antidiabetic, and antioxidant activity were determined. Pearson correlation analysis revealed that TSC and TPC were strongly associated with biological activities, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activities, as well as α-glucosidase and nitric oxide (NO) inhibitory activities. Fr. 4, characterized by a high TSC (260.95 mg oleanolic acid equivalents (OAE)/g dry weight, DW), exhibited notable anti-inflammatory activity, inhibiting NO production by 36.30% at 60 µg/mL. Fr. 3, with TSC of 219.37 mg OAE/g DW and TPC of 53.45 mg gallic acid equivalents (GAE)/g DW, showed strong antidiabetic activity with an IC_{50} value of 0.24 mg/mL for α-glucosidase inhibition. Fr. 2, enriched in phenolic compounds and with high TPC of 104.78 mg GAE/g DW, demonstrated potent antioxidant activity, with IC_{50} values of 0.52 mg/mL and 0.38 mg/mL in DPPH and ABTS assays, respectively. These results indicate that macroporous resin chromatography is effective in obtaining fractions enriched in phenolic compounds and saponins from maca leaf extracts prepared by DES-based UAE, highlighting their potential application as functional food ingredients.

Keywords: deep eutectic solvent, ultrasound-assisted extraction, bioactive components, antioxidant activity, anti-inflammatory activity, antidiabetic activity

INTRODUCTION

Maca (*Lepidium meyenii* Walp.) is a cruciferous plant indigenous to the Andes, which is noted for its remarkable resilience, flourishing in adverse conditions, such as cold temperatures, strong winds, and drought. It produces diverse compounds to withstand these harsh environments, which also confer significant health benefits to humans [Wang & Zhu, 2019]. Morphologically, maca is a small herbaceous plant with a basal rosette growth form consisting of leaves ranging from oval to spatulate and an underground, swollen hypocotyl-root complex that functions as a storage organ [Gonzales *et al*., 2014]. Maca contains diverse secondary metabolites, including macaenes, macamides, glucosinolates, isothiocyanates, alkaloids, polyphenols, saponins, and sterols, which have been associated with metabolic regulation, gastrointestinal health, cardioprotection, antihypertensive effects, photoprotection, muscle growth, hepatoprotection, angiogenesis promotion, antithrombotic effects, and antiallergic activity [del Carpio *et al*., 2024]. Notably, maca leaves have been reported to

***Corresponding Author:**
tel.: +82-53-810-2878; fax: +82-53-810-4768; e-mail: **yoonky2441@ynu.ac.kr** (Prof. K.Y. Yoon)

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contain higher levels of total phenolics and total flavonoids than the roots, along with various polyphenols, such as kuwanon G, laricitin, and ligustroflavone [Lee & Chang, 2019]. In addition, saponins in maca are both more abundant and diverse in the leaves than in the roots. Despite being rich in phytochemicals, maca leaves are often discarded as agricultural by-products, accounting for approximately 20%–40% of the total biomass and leading to resource wastage and environmental concerns [Caicai *et al*., 2018]. Thus, more studies are essential to explore the potential of maca leaves as a functional resource.

To efficiently source bioactive compounds from plant materials, extraction and separation processes are crucial for minimizing the loss, deformation, and degradation of the target substances. Among these, ultrasonic-assisted extraction (UAE) is a cost-effective and simple technique that offers high yields, reduces the loss of heat-sensitive components, and can be applied to various solvents [Kadam *et al.,* 2013]. Recently, the application of deep eutectic solvents (DES) in UAE has gained increasing interest. DES is a eutectic mixture formed by strong interactions between a hydrogen-bond donor and a hydrogen-bond acceptor, serving as an efficient alternative solvent owing to its low cost, low toxicity, and biodegradability [Chandran *et al*., 2019]. After extraction, additional fractionation is often required to concentrate target bioactive compounds from complex mixtures, and macroporous resin chromatography is widely used. Macroporous resins exhibit high adsorption capacity, a stable structure, and recyclability [Lu *et al*., 2025], and chromatography applied to this type of resins is effective in separating and concentrating phenolic compounds and saponins through stepwise elution with aqueous ethanol solutions [Yang *et al*., 2016; Zhang *et al*., 2022].

It is documented in the literature that extracts of *L. meyenii* and related Brassicaceae plants exhibit antioxidant, anti-inflammatory, and antidiabetic activities, which have been largely attributed to their phenolic compounds and saponins [del Carpio *et al*., 2024; Gonzales *et al*., 2014; Wang & Zhu, 2019]. In our previous study, DES-based UAE of maca leaves resulted in a higher content of bioactive compounds and stronger antioxidant activity compared with conventional hot-water extraction [Lee & Yoon, 2025]. Accordingly, as a follow-up study aimed at enhancing the utilization of maca leaves as functional ingredients, the present study applied DES-based UAE to obtain maca leaf extracts, followed by fractionation using macroporous resin chromatography. The fractions were evaluated for total saponin content (TSC), total phenolic content (TPC), and biological activities, including antioxidant, α-glucosidase inhibitory, and nitric oxide (NO) inhibitory activities, to assess their potential application as functional food materials.

MATERIALS AND METHODS

Materials and reagents

Maca (*L. meyenii*) used in this study was cultivated in Cheongdo-gun, Gyeongsangbuk-do, Republic of Korea (35.6473° N, 128.7341° E; approximately 200 m above sea level). The plants were sown in early spring (March–April), and the leaves were harvested in July and purchased from the Youth Research Institute located in the same region. The plant material was identified as *L. meyenii* based on morphological characteristics according to standard taxonomic descriptions provided by the supplier. For sample preparation, the roots were removed, and the leaves were thoroughly washed and lyophilized. The dried leaves were then pulverized using a food mixer (FM-681C, Hanil, Incheon, Korea), passed through a 45-mesh sieve, and stored at −40°C in a deep freezer until further analysis.

Diaion HP20 resin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphoric acid, *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG), Griess reagent, lipopolysaccharide (LPS), and acarbose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A Folin–Ciocâlteu phenol reagent and oleanolic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Kita-ku, Tokyo, Japan), and 100% acetonitrile was purchased from Burdick & Jackson (Honeywell International Inc., Charlotte, NC, USA). Dulbecco's modified Eagle's medium (DMEM) was acquired from HyClone (Pittsburg, PA, USA), and fetal bovine serum (FBS) and penicillin/streptomycin were bought from Welgene Inc. (Gyeongsan, Korea). RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Choline chloride and glycerin were purchased from Daejung Chemical & Metals (Siheung, Korea), and all other chemicals used were of analytical grade.

Preparation of deep eutectic solvent

The DES used for the extraction of phytochemicals from maca leaves was formulated following the procedure described by Lee & Yoon [2025]. Briefly, choline chloride and glycerol were mixed at a molar ratio of 1:2 and heated in a shaking water bath (BS-11, JeioTech, Seoul, Korea) at 80°C with agitation at 120 rpm until a clear, homogeneous solution was obtained. After cooling, the prepared DES was stored at ambient temperature overnight to confirm its stability prior to use.

Preparation of maca leaf extract by deep eutectic solvent-based ultrasound-assisted extraction

The extract was obtained from maca leaves using a DES–based UAE method adapted from Lee & Yoon [2025]. In brief, maca leaf powder (5 g) was mixed with 200 mL of DES containing 30% (*v/v*) water. UAE was performed using a probe-equipped ultrasonicator (KFS-600N, Korea Process Technology, Seoul, Korea) at an output power of 300 W for 30 min, while the extraction temperature was controlled to remain below 40°C. The extract was then centrifuged at 9,896×*g* for 20 min at 4°C, after which the supernatant was collected, filtered, and subsequently used for macroporous resin chromatography.

Fractionation of maca leaf extract *via* macroporous resin chromatography

The bioactive compound-rich extract was subjected to fractionation using a Diaion HP20 resin column. Initially, 9.5 mL of distilled water were introduced into a glass column (20×200 mm)

fitted with a filter, then 25 mL of Diaion HP20 resin were added, and the mixture was allowed to settle until firm. Subsequently, 5 mL of the filtered extract were loaded onto the top of the resin column. To remove residual DES and non-target compounds from the extract, 80 mL of distilled water were first used as an eluent. The extract was then sequentially subjected to stepwise elution with 50 mL of 25%, 50%, 75%, and 100% (*v/v*) ethanol. Each ethanol-eluted fraction was concentrated using a rotary evaporator (N-1300, Eyela, Tokyo, Japan) and then lyophilized. The fractions eluted with 25%, 50%, 75%, and 100% ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

Yield and distribution ratio of fractions

The yield (%) of each fraction was calculated as the percentage of its dry weight relative to the dry weight of the maca leaf extract obtained by DES-based UAE. The distribution ratio (%) of each fraction was calculated as the proportion of dry weight of each fraction to the total dry weight of all fractions obtained after fractionation.

Determination of total saponin content

The TSC of each fraction was quantified using the vanillin–sulfuric acid colorimetric assay based on the method of Le *et al*. [2018]. Briefly, 0.25 mL of each fraction dissolved in ethanol at a concentration of 1.0 mg/mL was mixed with 0.25 mL of an 8% (*w/v*) vanillin solution in ethanol and 2.5 mL of 72% sulfuric acid. The reaction mixture was incubated at 60°C for 10 min in a water bath and subsequently cooled in an ice-water bath for 5 min. Absorbance was determined at 544 nm using a microplate reader (Epoch, BioTek Instrument Inc., Winooski, VT, USA). TSC was calculated from a calibration curve constructed with oleanolic acid and expressed as mg oleanolic acid equivalents (OAE)/g dry weight (DW) of the dried fraction powder.

Determination of total phenolic content

The TPC was measured using the Folin–Ciocâlteu colorimetric assay following the method described by Singleton *et al*. [1999]. Briefly, 0.2 mL of the Folin-Ciocâlteu reagent was added to 0.2 mL of a solution of fraction in distilled water at a concentration of 1.0 mg/mL. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 3 min, followed by the addition of 0.4 mL of 10% sodium carbonate (Na_2CO_3) and 4 mL of distilled water. The samples were then incubated in the dark at room temperature for 1 h. Absorbance was recorded at 720 nm using a microplate reader (Epoch, BioTek Instrument Inc.). TPC was calculated from a calibration curve prepared with gallic acid and expressed as mg gallic acid equivalents (GAE)/g DW of the dried fraction powder.

Determination of individual phenolic compound content

Phenolic compounds were analyzed by high-performance liquid chromatography (HPLC) following the procedure of Nour *et al*. [2013] with appropriate adjustments. Analyses were performed using an HPLC system (Waters 2695, Waters Co., Milford, MA, USA) equipped with a UV detector (Waters 2489, Waters Co.). Prior to analysis, each fraction was filtered through a 0.45-µm membrane filter (Millipore, Billerica, MA, USA). Separation was achieved on an Atlantis dC18 column (4.6×150 mm, 5 µm; Waters Co.) maintained at 34°C. The mobile phase consisted of 1% phosphoric acid in water (solvent A) and acetonitrile (solvent B). Elution was carried out using a gradient program as follows: 0 min, 90% A (*v/v*); 0–27 min, 60% A (*v/v*); 28–55 min, 56% A (*v/v*); and 56–60 min, 90% A (*v/v*). The flow rate was set at 1.0 mL/min, and the injection volume was 10 µL. Detection was conducted at 280 nm over a total run time of 60 min. External standard stock solutions of gallic acid, caffeic acid, catechin, cinnamic acid, epicatechin, ferulic acid, naringin, *p*-coumaric acid, and protocatechuic acid (Sigma-Aldrich Co., St. Louis, MO, USA) were prepared in HPLC-grade methanol. Working standard solutions at defined concentrations were obtained by serial dilution of the stock solutions and used to construct calibration curves based on the peak areas of the corresponding standards. Individual phenolic compounds were quantified and expressed as mg/g DW of the dried fraction powder.

Determination of DPPH radical scavenging activity

The DPPH$^{\bullet}$ scavenging activity of the fractions was evaluated using the method of Brand-Williams *et al*. [1995]. Briefly, 100 µL of each fraction solution at various concentrations (0.3, 0.6, 0.9, 1.2, and 1.5 mg/mL) prepared in distilled water were mixed with 200 µL of a 0.2 mM DPPH$^{\bullet}$ solution dissolved in ethanol in a microplate. The reaction mixture was incubated at 37°C for 30 min, after which absorbance was measured at 517 nm using a microplate reader (Epoch, BioTek Instruments Inc.). The radical scavenging activity (RSA) against DPPH$^{\bullet}$ was calculated according to Equation (1), and the results were expressed as the IC_{50} value, defined as the concentration of fraction solution required to scavenge 50% of DPPH radicals:

$$\text{RSA (\%)} = \left(1 - \frac{A - B}{C}\right) \times 100 \quad (1)$$

where: A is the absorbance of the sample–radical mixture, B is the absorbance of the blank sample (radical replaced with distilled water), and C is the absorbance of the radical control (sample replaced with distilled water).

Determination of ABTS radical cation scavenging activity

The ABTS$^{\bullet+}$ scavenging activity of the fractions was evaluated according to method described by Re *et al*. [1999]. The ABTS$^{\bullet+}$ was generated by reacting a 7 mM ABTS solution with 2.45 mM potassium persulfate in distilled water and allowing the mixture to stand in the dark for 12 h. Prior to analysis, the ABTS$^{\bullet+}$ solution was diluted with 80% ethanol to obtain an absorbance of 0.700±0.002 at 734 nm. For the assay, 15 µL of each fraction solution in distilled water were mixed with 300 µL of the diluted ABTS$^{\bullet+}$ solution in a microplate and incubated for 6 min at room

temperature. Fr. 1–3 were tested at 0.3–1.5 mg/mL, whereas Fr. 4 was tested at 0.5–3.0 mg/mL. Absorbance was measured at 734 nm using a microplate reader. $ABTS^{\bullet+}$ scavenging activity was calculated according to Equation (1) and expressed as the IC_{50} value, defined as the concentration of the fraction required to scavenge 50% of $ABTS^{\bullet+}$.

Determination of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was measured using the method of Lee *et al*. [2021] with slight modifications. Acarbose, used as a positive control, and each fraction were dissolved in distilled water. Acarbose and Fr. 3 were tested at concentrations of 0.2–1.0 mg/mL, whereas the remaining fractions were prepared at concentrations of 0.5–3.0 mg/mL. Next, 50 µL of a 0.1 U/mL α-glucosidase solution and 50 µL of 200 mM potassium phosphate buffer (pH 6.8) were mixed with a fraction solution (50 µL), and the mixture was incubated at 37°C for 15 min. Subsequently, 100 µL of 3 mM *p*-NPG were added, and the reaction was performed at 37°C for 10 min. The reaction was terminated by the addition of 50 µL of 0.1 M NaOH, and the absorbance was measured at 405 nm. α-Glucosidase inhibitory (AGI) activity was calculated using Equation (2) and expressed as IC_{50} value, which is the concentration of a fraction solution required to inhibit activity by 50%:

$$\text{AGI activity (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (2)$$

where: $A_{control}$ is the absorbance of the control (without sample), and A_{sample} is the absorbance of the reaction mixture with the test sample.

Determination of nitric oxide inhibitory activity using RAW264.7 cells

The inhibitory effect of maca leaf extract fractions on nitric oxide (NO) production were evaluated in LPS-induced RAW 264.7 cells using a Griess reagent-based colorimetric assay [Giustarini *et al*., 2008]. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator (MCO-17AIC, Sanyo, Osaka, Japan). They were seeded in 96-well plates at a density of 1×10^5 viable cells *per* well and cultured for 24 h in an incubator at 37°C and 5% CO_2. Subsequently, RAW 264.7 cells were stimulated with lipopolysaccharide (LPS, 0.1 µg/mL) for 2 h in an incubator at 37°C with 5% CO_2. The cells were then treated with 20 µL of each fraction solution, prepared in distilled water, at various concentrations (20, 40, and 60 µg/mL) and further incubated for 24 h. After incubation, 100 µL of the culture supernatant were mixed with an equal volume of the Griess reagent, allowed to react for 10 min, and then the absorbance was measured at 540 nm using a microplate reader. The NO inhibitory activity was expressed as the percentage inhibition of NO production relative to the LPS-treated control and calculated using Equation (3):

$$\text{NO inhibitory activity (\%)} = \left(1 - \frac{A - B}{C - B}\right) \times 100 \quad (3)$$

where: A represents the absorbance of the sample-treated group (LPS + reaction solution + sample), B represents the blank absorbance (reaction solution without LPS or sample), and C represents the absorbance of the control group (LPS + reaction solution).

Statistical analysis

The experimental results were expressed as the mean and standard deviation of three replicate measurements. Statistical analysis was performed using IBM SPSS Statistics ver. 23 (IBM Corp., Armonk, NY, USA), employing a one-way analysis of variance (ANOVA), with a significance level set at $p<0.05$. Significant differences between the mean values obtained from the experimental results were verified using Duncan's multiple-range test. The correlation between the bioactive compound contents and biological activity was analyzed using Pearson's correlation analysis.

RESULTS AND DISCUSSION

Yield and distribution ratio

After removal of the deep eutectic solvent, the dry weight of the extract was determined, yielding 32.7 g of dried extract obtained from 100 g of dried maca leaves. Based on this extract, the dry weights of the fractions were 3.84 g (Fr. 1), 1.73 g (Fr. 2), 0.76 g (Fr. 3), and 0.90 g (Fr. 4) (data not shown). The yields and distribution ratios calculated from these data are presented in **Table 1**. Total yield of fractions was 22.10%, with Fr. 1 showing the highest yield at 11.75%, followed by Fr.2 (5.28%), Fr. 4 (2.76%), and Fr. 3 (2.31%). The corresponding distribution ratios were 52.98% for Fr. 1, 24.02% for Fr. 2, 10.45% for Fr. 3, and 12.55% for Fr. 4. Since the yield and distribution patterns of fractions are strongly influenced by extraction and chromatography conditions, these values are provided to describe the fractionation profile rather than to imply a direct association with biological activity.

Table 1. Yields and distribution ratios of fractions obtained from the maca leaf extract by Diaion HP20 column chromatography.

Fraction	Yield (%)	Distribution ratio (%)
Fr. 1	11.75±1.90^{a}	52.98±3.72^{a}
Fr. 2	5.28±0.38^{b}	24.02±2.97^{b}
Fr. 3	2.31±0.22^{b}	10.45±0.79^{c}
Fr. 4	2.76±0.55^{b}	12.55±2.73^{bc}
Total	22.10±2.05	100

Results are shown as mean ± standard deviation (n=3). Values with different letters in the same column are significantly different at $p<0.05$ by Duncan's multiple range test. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

Table 2. Total saponin and total phenolic contents of the fractions obtained from the maca leaf extract by Diaion HP20 column chromatography.

Fraction	Total saponin content (mg OAE/g DW)	Total phenolic content (mg GAE/g DW)
Fr. 1	76.03±2.44^{d}	59.92±1.35^{b}
Fr. 2	151.11±2.71^{c}	104.78±3.62^{a}
Fr. 3	219.37±0.99^{b}	53.45±0.16^{c}
Fr. 4	260.95±0.48^{a}	32.78±0.87^{d}

Results are shown as mean ± standard deviation (n=3). Values with different letters in the same column are significantly different at p<0.05 by Duncan's multiple range test. OAE, oleanolic acid equivalent; GAE, gallic acid equivalent; DW, dry weight of fraction. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

Total saponin and total phenolic contents

The TSC and TPC of the maca leaf extract fractions are shown in **Table 2**. Saponins are known secondary metabolites found in various plants as molecules in which hydrophobic triterpenoid, steroid or alkaloid aglycones are linked with hydrophilic moieties of different sugars [El Aziz *et al*., 2019]. Their structures are diverse and complex, influenced by the aglycone structure and the attached side chains. They also exhibit a range of physiological activities, such as anti-inflammatory, antibacterial, antidiabetic, and antitumor effects, as well as blood lipid-reducing and blood cholesterol-reducing effects [Sharma *et al*., 2023]. In our study, the TSC in the fractions, expressed in oleanolic acid equivalents, increased with increasing ethanol concentration in the mobile phase used to elute the fractions. Specifically, Fr. 4 showed the highest saponin content (260.95 mg OAE/g DW), followed by Fr. 3 (219.37 mg OAE/g DW) and Fr. 2 (151.11 mg OAE/g DW), whereas Fr. 1 had the lowest saponin content (76.03 mg OAE/g DW). Ethanol, methanol, acetone, ethyl acetate, and *n*-butanol are used for saponin extraction, whereas ethanol and *n*-butanol are commonly used solvents [El Aziz *et al*., 2019]. Del Hierro *et al*. [2018] extracted saponins from various legumes using water, ethanol, methanol, and their aqueous mixtures as solvents, with ethanol extracts showing the highest saponin content. Deng *et al*. [2012] obtained fractions from the roots of *Polygonatum odoratum* (Mill.) Druce through ethanol extraction and *n*-butanol fractionation, followed by refractionation with H_2O, and 20%, 40%, 60%, and 80% ethanol using a D101 macroporous resin column chromatography. They determined the saponin content of each fraction and reported that it increased with rising ethanol concentrations, which is consistent with the results of the present study.

Phenolic compounds are secondary metabolites containing one or more hydroxyl groups on the aromatic rings and are known to exert various physiological effects such as antioxidant, anti-inflammatory, antidiabetic, and anticancer activities [Tsao, 2010]. The TPC in the maca leaf fractions was expressed using GA as a standard (**Table 2**). Fr. 2 exhibited the highest TPC (104.78 mg GAE/g DW), followed by Fr. 1 (59.92 mg GAE/g DW) and Fr. 3 (53.45 mg GAE/g DW), whereas Fr. 4 had the lowest TPC (32.78 mg GAE/g DW). Phenolic compounds are relatively hydrophilic and are extracted using water or polar organic solvents, such as methanol, ethanol, and acetone, or their aqueous mixtures [Tsao, 2010]. A mixture of ethanol and water is commonly used to extract phenolic compounds from plant materials, as aqueous–ethanol solvent systems efficiently solubilize phenolic acids, flavonoids, and other polyphenols [Kobus-Cisowska *et al*., 2020; Palaiogiannis *et al*., 2023; Plaskova & Mlcek, 2023]. Consistent with this general extraction behavior, del Hierro *et al*. [2018] reported that the total phenolic content was highest in extracts obtained using a 50% aqueous solvent compared with pure organic solvents, such as ethanol and methanol. Similar trends have also been observed in Brassicaceae plants; for instance, Reungoat *et al*. [2020] and Chadni *et al*. [2023] demonstrated efficient extraction of sinapine and related phenolic compounds from mustard (*Brassica juncea*) using ethanol–water mixtures.

Although direct quantitative comparisons are limited by differences in plant organs, cultivation environments, and extraction methods, previous studies have shown that the phytochemical composition of *L. meyenii* varies according to organ type and growing conditions [Gonzales *et al*., 2014; Lee & Chang, 2019]. In general, maca leaves contain higher levels of phenolics and saponins than roots, and environmental factors such as altitude and soil conditions can further influence their accumulation [Lee & Chang, 2020; Szakiel *et al*., 2011]. The phenolic compounds and saponins examined in this study are not unique to maca but are widely distributed across the species (*L. meyenii*), the genus (*Lepidium*), and the Brassicaceae family [Gonzales *et al*., 2014; Yang *et al*., 2018]. Therefore, the significance of the present study lies not in the identification of novel compounds, but in the selective enrichment of known phenolic and saponin compounds from maca leaves using DES-based UAE combined with macroporous resin fractionation, and in linking these enriched fractions to specific biological activities.

Individual phenolic compound content

The contents of individual phenolic compounds in the four fractions of maca leaf extract are presented in **Table 3**. A total of nine compounds were detected: gallic acid, catechin, caffeic acid, cinnamic acid, epicatechin, ferulic acid, *p*-coumaric acid, protocatechuic acid, and naringin. Notably, various phenolic compounds were detected in Fr. 1, Fr. 2, and Fr. 3, with Fr. 2 having the highest phenolic compound content, except for gallic acid. Catechin was particularly abundant, with contents of 43.43, 51.02, and 8.14 mg/g DW in Fr. 1, Fr. 2, and Fr. 3, respectively. Additionally, all fractions contained gallic acid in the range of 1.83–6.06 mg/g DW. Campos *et al*. [2013] optimized the ethanol extraction conditions to extract phenolics from maca roots and analyzed the phenolic compound content of the extract obtained under the optimal conditions. They showed the abundance of catechin derivatives and the presence of *p*-coumaric acid and protocatechuic acid, which aligned with the phenolics identified in maca leaf fractions in our study. Catechin is

Table 3. Content of individual phenolic compounds of the fractions (mg/g dry weight) obtained from the maca leaf extract by Diaion HP20 column chromatography.

Phenolic compound	Fr. 1	Fr. 2	Fr. 3	Fr. 4
Catechin	43.43±0.38^{b}	51.02±1.59^{a}	8.14±0.14^{c}	ND
Epicatechin	2.06±0.08^{b}	5.74±0.06^{a}	0.16±0.02^{c}	ND
Gallic acid	6.06±0.09^{a}	5.31±0.02^{b}	2.81±0.01^{c}	1.83±0.01^{d}
Cinnamic acid	ND	0.53±0.01^{a}	0.29±0.01^{b}	ND
Ferulic acid	0.99±0.05^{b}	5.34±0.00^{a}	0.58±0.00^{c}	0.06±0.00^{d}
Caffeic acid	1.54±0.21^{b}	2.09±0.01^{a}	0.26±0.00^{c}	ND
p-Coumaric acid	0.14±0.01^{b}	0.99±0.01^{a}	0.09±0.00^{c}	ND
Protocatechuic acid	0.57±0.02^{c}	5.19±0.07^{a}	1.16±0.01^{b}	ND
Naringin	0.32 ± 0.03^{b}	4.40 ± 0.51^{a}	0.07 ± 0.00^{b}	ND

Results are shown as mean ± standard deviation (n=3). Values with different letters in the same row are significantly different at p<0.05 by Duncan's multiple range test. ND, not detected. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

a flavonoid widely present in foods such as tea, wine, and various fruits, and has been reported to exhibit strong antioxidant properties, including scavenging activities against hydroxyl, peroxyl, superoxide, and DPPH$^{\bullet}$, as well as metal-chelating ability [Yilmaz, 2006]. Gallic acid, a phenolic acid abundantly found in various fruits and vegetables, is recognized for its biological effects, such as antibacterial, antioxidant, anticancer, anti-inflammatory, and antiviral effects [Hadidi *et al*., 2024]. Its strong antioxidant capacity is particularly noted for neutralizing free radicals, reducing oxidative stress, and protecting cells from damage.

Antioxidant activity

The antioxidant activity of maca leaf extract fractions determined by DPPH and ABTS assays and expressed as IC_{50} values is shown in **Table 4**. The IC_{50} value for DPPH$^{\bullet}$ scavenging activity was the lowest for Fr. 2 (0.52 mg/mL), followed by Fr. 1 (0.99 mg/mL), Fr. 4 (1.10 mg/mL), and Fr. 3 (1.20 mg/mL). Similarly, the IC_{50} value for the ABTS$^{\bullet+}$ scavenging activity was the lowest in Fr. 2 (0.38 mg/mL), followed by Fr. 3 (0.69 mg/mL), Fr. 1 (0.83 mg/mL), and Fr. 4 (2.63 mg/mL). Accordingly, Fr. 1 exhibited the highest antioxidant activity, and the inhibitory activity against ABTS$^{\bullet+}$ was higher than that against DPPH$^{\bullet}$ in all fractions, except Fr. 4. DPPH$^{\bullet}$ are scavenged by hydrogen atom donation, whereas ABTS$^{\bullet+}$ are scavenged by electron transfer [Li *et al*., 2012]. Therefore, all fractions, except Fr. 4, are considered to contain more bioactive compounds that can donate electrons. *Carica papaya* leaf extracts obtained under optimized saponin ethanol extraction conditions showed higher antioxidant activities with an increase in saponin and phenolic contents [Vuong *et al*., 2015]. Additionally, Naidu *et al*. [2011] determined the saponin and phenolic contents and antioxidant activities of extracts from buckwheat seeds, husks, and endosperm using ethanol and reported that the DPPH$^{\bullet}$ scavenging activity was more influenced by the phenolic content than by the saponin content, similar to the results of the present study. Thus, the high antioxidant activity of Fr. 2 is attributed to its high phenolic content, supporting the hypothesis that this fraction could be a potential source of antioxidants.

Table 4. IC_{50} value (mg/mL) of the fractions obtained from the maca leaf extract by Diaion HP20 column chromatography in antioxidant assays.

Fraction	DPPH assay	ABTS assay
Fr. 1	0.99±0.02^{c}	0.83±0.00^{b}
Fr. 2	0.52±0.02^{d}	0.38±0.02^{d}
Fr. 3	1.20±0.03^{a}	0.69±0.02^{c}
Fr. 4	1.10±0.03^{b}	2.63±0.08^{a}

Results are shown as mean ± standard deviation (n=3). Values with different letters in the same column are significantly different at p<0.05 by Duncan's multiple range test. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

α-Glucosidase inhibitory activity

α-Glucosidase is a digestive enzyme in the small intestine that converts polysaccharides into monosaccharides, which rapidly increases blood sugar levels. Thus, the use of α-glucosidase inhibitors can delay carbohydrate digestion and suppress postprandial hyperglycemia. Consequently, α-glucosidase inhibitory activity is employed as an indicator for evaluating substances that can control blood sugar [Hossain *et al*., 2020]. The IC_{50} values for the α-glucosidase inhibitory activity of each fraction obtained by varying ethanol concentrations are shown in **Figure 1**. Fr. 3 exhibited the lowest IC_{50} value (0.24 mg/mL), followed by Fr. 4 (1.18 mg/mL), Fr. 2 (2.23 mg/mL), and Fr. 1

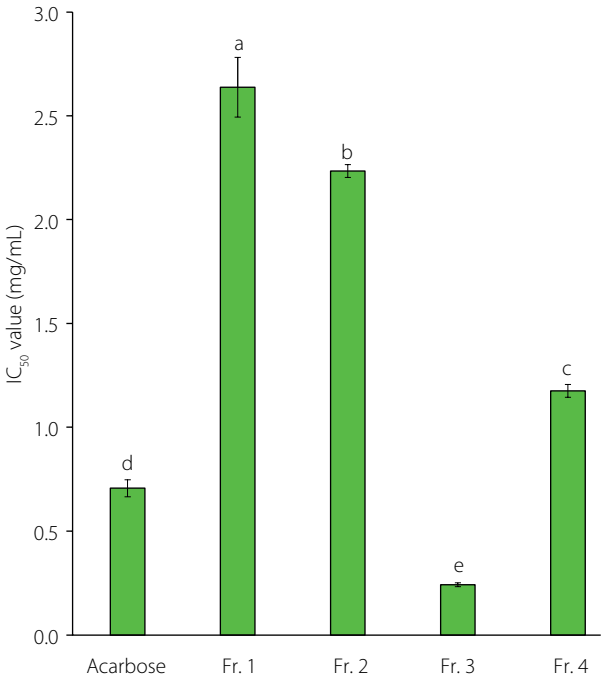

Figure 1. α-Glucosidase inhibitory activity of fractions obtained from the maca leaf extract using a Diaion HP20 resin column. The bar represents the mean and standard deviation (n=3). Values with different letters are significantly different at p<0.05 by Duncan's multiple-range test. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

(2.64 mg/mL), in order of an increasing IC_{50} value. Notably, the IC_{50} of Fr. 3 was significantly lower than that of acarbose (0.71 mg/mL), an antidiabetic agent (p<0.05), and Fr. 4 exhibited inhibitory activity corresponding to approximately 75% of acarbose activity. Maca leaves contain a wider variety of saponins than roots, such as triterpenes, esculin hydrate, tanshinone I, panaxytriol, lanatoside, marsdecoside B, colubrinoside, and rotundifolioside, with the leaves having higher saponin contents than the roots [Lee & Chang, 2019]. Studies have reported that these saponins have higher α-glucosidase inhibitory activity than acarbose [Deng *et al*., 2012; Luo *et al*., 2008]. Thus, the high α-glucosidase inhibitory activities of Fr. 3 and Fr. 4 are attributed to their high TSC. However, despite the high TSC of Fr. 4, its inhibitory activity was lower than that of Fr. 3, which is thought to be due to the type of saponin and phenolics that each fraction contained.

Nitric oxide inhibitory activity

LPS, a structural constituent of Gram-negative bacterial outer membranes, induces inflammatory activation of macrophages. Macrophages induced with inflammation by LPS produce proinflammatory mediators, such as NO and prostaglandin E2 [Yang *et al*., 2012]. The NO inhibitory effect of each fraction on LPS-induced NO release from RAW 264.7 cells is shown in **Figure 2**. In all fractions, NO production decreased in a concentration-dependent manner. In particular, Fr. 4 exhibited the highest inhibition of NO production (p<0.05), with 21.72% inhibition at 20 μg/mL, 31.44% at 40 μg/mL, and 36.30% at 60 μg/mL. Additionally, the NO inhibitory activities of Fr. 1, Fr. 2, and Fr. 3 were 12.48%, 14.42%, and 19.77% at 60 μg/mL, respectively, indicating that the inhibitory activity increased proportionally with the saponin content in the fraction. According to Jang *et al*. [2015], the saponin fraction extracted from ginseng inhibited NO production by suppressing the production of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β. Yan *et al*. [2016] found that steroid saponin extracted from *Trillium tschonoskii* Maxim also inhibited NO production. Therefore, the highest NO production inhibition effect observed in Fr. 4 is attributed to its high saponin content.

Correlations between the bioactive compound contents and bioactivities

Pearson correlation analysis was conducted to determine the relationships among TSC, TPC, and antioxidant (IC_{50} values in DPPH and ABTS assays), α-glucosidase inhibitory (IC_{50} values), and NO inhibitory activities of maca leaf extract fractions (**Table 5**). The $DPPH^{\bullet}$ scavenging activity significantly correlated with the TPC (r=–0.911, p<0.01) and all individual phenolic compound contents but not with TSC. The $ABTS^{\bullet+}$ scavenging activity significantly correlated with the TPC (r=–0.772, p<0.01) and with the TSC (r=–0.633, p<0.05). The ABTS assay values also significantly correlated with the contents of all phenolic compounds, except caffeic acid. The α-glucosidase inhibitory activity was strongly correlated with TSC (r=–0.801), TPC (r=–0.735), catechin (r=–0.807), GA (r=–0.843), cinnamic acid (r=–0.997), and caffeic acid (r=–0.948) (p<0.01), and significantly correlated with epicatechin (r=–0.761, p<0.05). The NO inhibitory activity was strongly correlated with the TPC (r=0.965, p<0.01), cinnamic acid (r=0.998, p<0.01), and caffeic acid (r=0.899, p<0.01), and significantly correlated with ferulic acid (r=0.602, p<0.05). Although cinnamic acid showed a high correlation with NO inhibitory activity, its low content in the fractions suggests a limited direct contribution. This correlation likely reflects its co-enrichment with other phenolic compounds and saponins, whereas caffeic acid, present at higher levels, may play a more direct role in NO inhibition.

TSC showed a significant correlation with both α-glucosidase and NO inhibitory activities (p<0.01) (**Table 5**). Additionally, TSC significantly correlated with the $ABTS^{\bullet+}$ scavenging activity (p<0.05) but its correlation with the $DPPH^{\bullet}$ scavenging activity was insignificant (p>0.05), which suggests that the hydrophilic saponins of the maca leaf fractions contributed more effectively to $ABTS^{\bullet+}$ scavenging. Although individual saponin species were not identified in this study, the significant correlation between TSC and NO inhibitory activity suggests that saponins of the maca leaf fractions may collectively contribute to anti-inflammatory effects, as saponins have been widely reported to suppress nitric oxide production in activated macrophages [Francis *et al*., 2002; Wang *et al*., 2008].

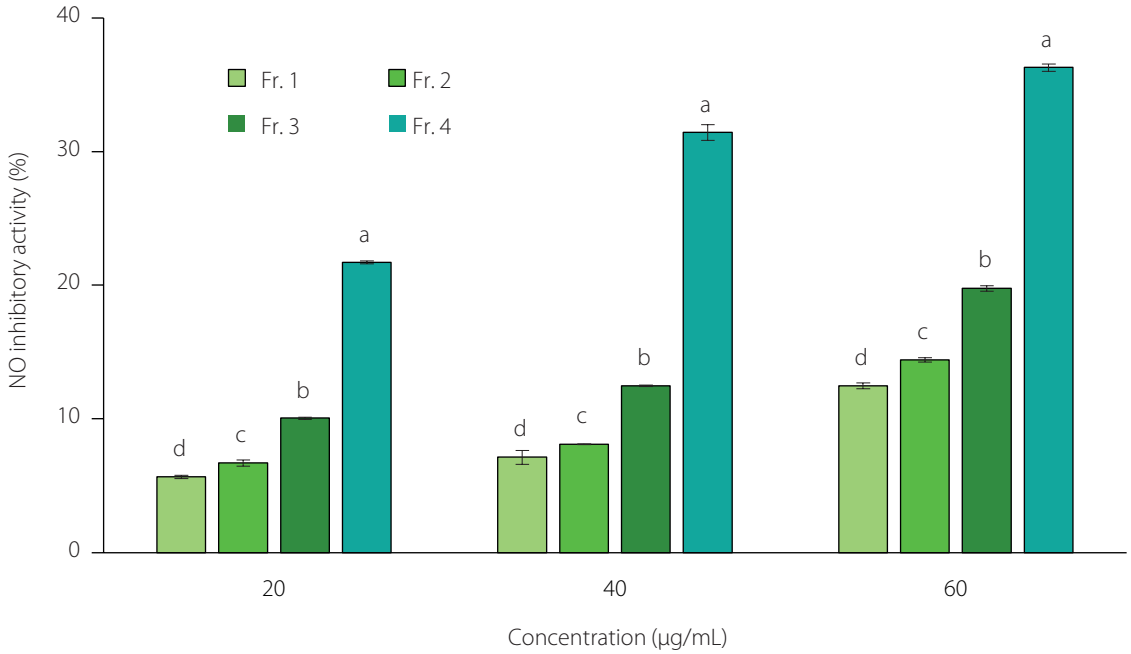

Figure 2. NO inhibitory activity of fractions of different concentrations obtained from the maca leaf extract using a Diaion HP20 resin column. The bar represents the mean and standard deviation (n=3). Values with different letters are significantly different at p<0.05 by Duncan's multiple-range test. The fractions eluted using 25%, 50%, 75%, and 100% (*v/v*) ethanol were designated as Fr. 1, Fr. 2, Fr. 3, and Fr. 4, respectively.

Table 5. Coefficients of Pearson's correlations among bioactive compound content, DPPH$^{\bullet}$ scavenging activity, ABTS$^{\bullet+}$ scavenging activity, α-glucosidase inhibitory activity and NO inhibitory activity.

Bioactive compounds	DPPH$^{\bullet}$ scavenging activity	ABTS$^{\bullet+}$ scavenging activity	α-Glucosidase inhibitory activity	NO inhibitory activity
Total saponins	–0.406	–0.633*	–0.801**	0.965**
Total phenolics	–0.911**	–0.772**	–0.735**	0.042
Catechin	–0.800**	–0.711**	–0.807**	0.308
Epicatechin	–0.996*	–0.783*	–0.761*	0.665
Gallic acid	–0.602*	–0.706*	–0.843**	0.302
Cinnamic acid	–0.998**	–0.988**	–0.997**	0.998**
Ferulic acid	–0.965**	–0.604*	–0.554	0.602*
Caffeic acid	–0.886*	–0.469	–0.948**	0.899**
p-Coumaric acid	–0.968*	–0.927**	–0.545	0.426
Protocatechuic acid	–0.912**	–0.977**	–0.395	0.267
Naringin	–0.956**	–0.919**	–0.538	0.421

DPPH$^{\bullet}$ scavenging activity, ABTS$^{\bullet+}$ scavenging activity and α-glucosidase inhibitory activity were expressed as IC_{50} values. Correlations are significant at p<0.05 (*) or p<0.01 (**).

The TPC exhibited a significant correlation with the DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging, and α-glucosidase inhibitory activities (p<0.01) (**Table 5**). These results aligned with the findings of Lee & Chang [2019], who reported a strong correlation between the TPC of the methanol extract from maca leaves and the DPPH$^{\bullet}$ scavenging activity. A previous study also reported that the TPC demonstrated a strong correlation with antioxidant activity measured by the DPPH assay (r=0.95, p<0.001) and a significant correlation with α-glucosidase inhibitory activity (r=0.73, p<0.05) [Tian *et al.*, 2025]. The strong association between TPC and radical scavenging activity can be explained by the redox properties of phenolic compounds, particularly the electron-donating ability of their hydroxyl groups and reducing ketone moieties, which enable effective neutralization of reactive species

and disruption of oxidative chain reactions [Millán-Laleona *et al*. 2023; Vyas *et al*., 2012]. The α-glucosidase inhibition of phenolic compounds is associated with polyphenol-induced conformational changes in the enzyme, including reductions in α-helix and β-sheet structures, which ultimately suppress enzymatic activity [Gong *et al*., 2020]. In addition, the magnitude of this inhibitory effect may depend on the presence and number of phenolic hydroxyl groups within the molecular structure of the compounds [Lee *et al*., 2019].

Contents of catechin, epicatechin, and gallic acid demonstrated significant correlations with both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities and α-glucosidase inhibitory activity. Moreover, caffeic acid showed a significant correlation with the $DPPH^{\bullet}$ scavenging, α-glucosidase inhibitory, and NO inhibitory activities. Consequently, these four compounds, in conjunction with saponins, were identified as the primary phenolic compounds that influence the biological activity of maca leaf extracts. In contrast, although cinnamic acid was strongly correlated with all bioactivities, it was present in only small quantities in Fr. 2 and Fr. 3. Therefore, concluding that cinnamic acid was the main component that affects the biological activity of maca leaf extracts is unjustified.

CONCLUSIONS

In this study, maca leaf extracts obtained by DES-based ultrasound-assisted extraction were fractionated using macroporous resin chromatography and analyzed for the distribution of bioactive compounds and related biological activities. Distinct functional characteristics were observed among the fractions. The fraction eluted with pure ethanol showed pronounced inhibition of nitric oxide production, which corresponded to its relatively high phenolic content. The fraction eluted with 75% (*v/v*) ethanol exhibited strong α-glucosidase inhibitory activity, likely associated with the combined presence of saponins and phenolic compounds, including appreciable amounts of caffeic acid and gallic acid. Meanwhile, the fraction eluted with 50% (*v/v*) demonstrated the highest antioxidant capacity, consistent with its elevated total phenolic content and chemically-diverse phenolic profile. Taken together, these results indicate that maca leaf fractions differ markedly in their functional properties depending on their phytochemical composition, and highlight the potential of phenolic- and saponin--enriched fractions as value-added ingredients for functional food applications. Further studies focusing on the underlying mechanisms of action would support more targeted utilization of these fractions in the food industry.

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CONFLICT OF INTERESTS

Authors declare no conflicts of interest.

ORCID IDs

E.J. Lee https://orcid.org/0009-0000-3001-6917
K.Y. Yoon https://orcid.org/0000-0003-0626-5563

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Physical and Antioxidant Characteristics, and Sensory Preference of Muffins Incorporated with Banana Inflorescence (*Musa* × *paradisiaca* L.) Powder

Nhi T.Y. Tran[1,2], Thy N.H. Ao[1,2], Linh T.K. Vu[3], Ngoc L. Le[1,2]*

[1]Department of Food Technology, International University, Quarter 33, Linh Xuan Ward, Ho Chi Minh City, Vietnam
[2]Vietnam National University, Ho Chi Minh City, Vietnam
[3]Department of Food Technology, Faculty of Chemical and Food Technology, Ho Chi Minh City University of Technology and Engineering, No. 1 Vo Van Ngan Street, Thu Duc Ward, Ho Chi Minh City, Vietnam

This study explored the potential of banana flower powder (BFP) as a nutritional ingredient in muffins by assessing its impact on physicochemical properties, bioactive contents, and sensory preference. BFP contained high levels of protein (20.54 g/100 g dry weight (DW)), crude fiber (28.57 g/100 g DW), and antioxidant compounds, including total phenolic content of 2,564 mg gallic acid equivalent/100 g DW, total flavonoid content of 164.6 mg rutin equivalent/100 g DW, and total anthocyanin content of 2,729 µg cyanidin 3-glucoside equivalent/100 g DW. Incorporating BFP into wheat flour (5–25% substitution, *w/w*) significantly altered the pasting behavior of composite flours, characterized by increased gelatinization temperature (57.4–58.1°C), and reduced peak viscosity (926–875 Brabender units (BU)) and final viscosity (1,084–956 BU) compared to wheat flour (57.3°C, 1,021 BU and 1,188 BU, respectively). These modifications suggested limited starch swelling and disrupted gluten network formation, which translated into notable changes in muffin texture, specifically, increased hardness and chewiness, and reduced cohesiveness. Microstructural analysis supported these findings, revealing denser crumb structures of muffins with BFP leading to a lower specific volume (from 1.85 to 1.71 mL/g compared to 2.09 mL/g for control muffins) and porosity (from 39.32 to 32.93% compared to 43.72% for control muffins). Although crumb color darkened with increasing BFP levels, sensory evaluation showed that muffins with up to 15% substitution of wheat flour by BFP (*w/w*) maintained high acceptability in terms of appearance, texture, and taste with the scores of >7.6 over 9.0. The findings demonstrated that BFP could be effectively utilized to produce nutrient-dense muffins while maintaining acceptable sensory quality, particularly texture, requires careful optimization of the BFP content within a specified threshold.

Keywords: antioxidants, banana flower powders, color, microstructure, muffins, texture profile

INTRODUCTION

The rising prevalence of nutrition-related diseases, such as obesity, heart disease, and certain cancers, has driven a surge in consumer demand for healthy and nutritious food options. The World Health Organization (WHO) advocates food fortification to address malnutrition, adding functional ingredients and nutrients without compromising safety, shelf-life, or consumer appeal [Dary & Hurrell, 2006]. The term "food fortification", as a public strategy, is the process of the intentional addition of nutrients or non-nutrient bioactive compounds to food products to balance the nutritional value or prevent nutrient intake shortfalls and associated deficiencies. Up to now, it functions mostly to increase low dietary intake and prevent malnutrition rather than prevent diagnosable conditions [Olson *et al.*, 2021]. Incorporating

***Corresponding Author:**
e-mail address: **lnlieu@hcmiu.edu.vn** (Prof. Ngoc Lieu Le)

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fruits and vegetables, rich in nutrients and antioxidants, can be considered as a key strategy for enhancing product quality and reducing the risk of chronic diseases [Hossain *et al.*, 2025].

With a 10.07% annual growth and widespread appeal, bakery goods are well-suited for fortification due to their convenience and palatability [Tasnim *et al.*, 2020]. Muffins are one of the most commonly consumed bakery products worldwide, often enjoyed as a breakfast item, snack, or dessert on various occasions [Kaur & Kaur, 2018; Mildner-Szkudlarz *et al.*, 2016]. However, standard muffin recipes, including wheat flour, vegetable oil, eggs, and milk, generally present a nutritional profile of low dietary fiber, limited key amino acids and minerals, while being high in sugars and lipids [Choi, 2015; Croitoru *et al.*, 2018]. With their low nutrient content and short shelf-life, fortification of muffins fosters potential to increase nutrient and bioactive compound intakes. Driven by a growing interest in nutrition and disease prevention, consumers increasingly demand value-added or functional foods rich in antioxidants and dietary fiber.

A wide range of natural-based nutritional ingredients, such as dietary fiber, protein, antioxidants, and phytochemicals, have been successfully incorporated to enhance muffin's nutritional value and extend their shelf-life, as evidenced by studies on red capsicum pomace powder [Nath *et al.*, 2018], dried lotus flour [Ashoka *et al.*, 2025], tomato pomace [Marak *et al.*, 2022], pomegranate peel [Giri *et al.*, 2024; Topkaya & Isik, 2019], brewer's spent grain flour [Bazsefidpar *et al.*, 2024; Shih *et al.*, 2020], and seaweed composite flour [Mamat *et al.*, 2018]. Nath *et al.* [2018] found that fortifying muffins with red capsicum pomace powder improved their overall quality and enhanced appearance, flavor, texture, fiber content, and antioxidant properties. Furthermore, the fortified muffins exhibited significantly lower hardness and staling rates than the control sample after 15 days of storage. According to Mehta *et al.* [2018], incorporating tomato pomace into muffins significantly improved their nutritional profile, resulting in higher levels of dietary fiber, vitamin C, antioxidants, and minerals. Despite these benefits, the literature also highlights significant technological trade-offs when incorporating such by-products. For instance, high fiber substitution may lead to texture hardening, loss of loaf volume due to gluten dilution, and color darkening, which may negatively affect consumer acceptance [Ashoka *et al.*, 2025; Gadallah *et al.*, 2022]. These limitations necessitate a careful balance between nutritional enrichment and the maintenance of standard sensory and structural profiles.

Banana (*Musa* spp.), a member of the family Musaceae, is one of the most significant agricultural commodities worldwide. Global banana trade has recorded high levels of around 20 million tonnes annually [FAO, 2025]. Vietnam contributes significantly to this landscape; as the country's largest fruit crop covers 163,000 hectares, and the sector produces 2.8 million tons *per* year [Diep, 2025]. In most areas of the world, the pulp of the banana is used and consumed because of its nutrient content and health advantages, while banana blossoms are mostly underexploited and discarded as agricultural waste. Banana flowers are an excellent source of high-quality protein, dietary fiber, vitamins, and trace minerals, including copper, iron, and magnesium [Lau *et al.*, 2020; Mostafa, 2021]. Additionally, they contain considerable levels of flavonoids, which are known for their bioactive properties [Chiang *et al.*, 2021]. This leads to their high antioxidant capability, which contributes to the mitigation of oxidative stress, a key factor in the development of various cardiovascular diseases. Following Bhaskar *et al.* [2012], banana flowers have also been proven to possess antihyperglycemic, antimicrobial, hypolipidemic, and anti-hypertensive activities. Therefore, they have been traditionally used in ethnomedicine to treat conditions such as diarrhea, ulcers, and bronchitis. Considering their favorable nutritional composition, banana flowers are used quite widely in Asian cuisines, especially in curries, soups, salads, and cutlets. To enhance both the nutritional value and economic utility of banana flowers, their integration into food products presents a promising avenue for value addition. For instance, Amornlerdpison *et al.* [2021] reported that an extract with bioactive compounds and antioxidant properties from banana inflorescence was a healthy food supplement in beverages for breastfeeding mothers. Tasnim *et al.* [2020] used banana blossom powder with various pretreatment methods to supplement plain cake to investigate the nutritional quality, structure, and sensory properties of the product. They demonstrated that banana blossom powder had moderate water (>4 g/g) and oil (>7 g/g) absorption capacities, low solubility and foaming capacity, and hence an appropriate substitution should be controlled to maintain consumer acceptability.

Although several studies have highlighted the nutritional richness of banana flower bracts and their application in various food systems [Gayathry & John, 2024; Senevirathna & Karim, 2024; Silva *et al.*, 2025], research specifically focusing on their integration into muffin formulations remains limited. Therefore, this study aimed to evaluate the effects of incorporating different levels of banana flower powder on muffins' quality characteristics, bioactive compound contents, and sensory properties, providing further insights into its potential as a functional ingredient in bakery products.

MATERIALS AND METHODS

Materials and chemicals

The inflorescence of fresh banana (*Musa* × *paradisiaca* L.), which was grown in Ho Chi Minh City, Vietnam, was collected 12 months post-planting between September and October. Commercial all-purpose wheat flour (Meizan, Calofic, Quang Ninh, Vietnam), sugar (Bien Hoa Co., Dong Nai, Vietnam), oil (Simply, Calofic, Quang Ninh, Vietnam), milk (Vinamilk, Ho Chi Minh City, Vietnam), baking powder (Arm & Hammer, Ewing, NJ, USA), eggs (Ba Huan, Long An, Vietnam), and salt were purchased from a local market in Ho Chi Minh City, Vietnam. All the chemicals were of analytical grade and purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), including sulfuric acid, acetone, hexane, sodium hydroxide, boric acid, potassium sulfate, *n*-octanol, potassium hydroxide, copper(II) sulfate, ethanol, Folin-Ciocalteu reagent, gallic acid, sodium carbonate, sodium nitrite, aluminum

Table 1. Formulas for muffins with wheat flour (WF) substituted with different ratios of banana flower powder (BFP).

Ingredient	BFP0	BFP5	BFP10	BFP15	BFP20	BFP25
WF (g)	54	51.3	48.6	45.9	43.2	40.5
BFP (g)	–	2.7	5.4	8.1	10.8	13.5
Baking powder (g)	3	3	3	3	3	3
Sugar (g)	30	30	30	30	30	30
Salt (g)	0.5	0.5	0.5	0.5	0.5	0.5
Oil (mL)	15	15	15	15	15	15
Egg (g)	20	20	20	20	20	20
Milk (mL)	40	40	40	40	40	40

BFP0, control sample; BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

chloride, rutin, potassium chloride, sodium acetate, acetic acid, and hydrochloric acid.

Preparation of banana flower powder

Banana flowers were washed under running tap water, and any damaged or wilted portions were removed. The cleaned flowers were manually sliced into 3-mm thick pieces and immersed in a 0.5% (*w/v*) citric acid solution for 1 h. Subsequently, the sliced flowers were blanched at 90°C for 3 min, drained and dried overnight at 55°C in a drying oven (Memmert GmbH & Co. KG, Schwabach, Germany) until the moisture content of ≤10% was achieved. The dried flowers were then pulverized into powder using a grinder and passed through a 60-mesh sieve. The resulting banana flower powder (BFP) was stored at –20°C for further analyses.

Muffin preparation

Six muffin formulations were prepared with varying BFP levels (**Table 1**) according to the method of Heo *et al.* [2019] with minor modifications. Wheat flour (WF) was substituted with BFP (*w/w*) at 0% (control, designated BFP0), 5% (BFP5), 10% (BFP10), 15% (BFP15), 20% (BFP20), and 25% (BFP25). Eggs, milk, and vegetable oil were combined and stirred manually for 2 min. The well-mixed dry ingredients (wheat flour, baking powder, BFP, and salt) were then added, and the mixture was hand-mixed for approximately 15 s. Equal portions of batter (30 g) were poured into muffin molds and baked at 170°C for 20 min in a pre-heated oven. The baked muffins were removed from the oven and allowed to cool at room temperature for 2 h. Muffins were placed in polypropylene zip-lock bags and stored in a dry and cool place for further analyses. Each batch of baking contained 10 muffins. The samples were prepared in triplicates.

Determination of chemical composition of wheat flour and banana flower powder

Chemical composition of WF and BFP was determined following the AOAC International methods, including moisture (AOAC method 934.01), protein (AOAC method 990.03), ash (AOAC method 942.05), crude fiber (AOAC method 978.10), and lipid content (AOAC method 920.39) [AOAC, 2005]. The total carbohydrate content was calculated based on the difference by subtracting the sum of protein, lipid, ash and crude fiber in 100 g from 100 (on dry weight basis).

Determination of pasting properties of blends of wheat flour and banana flower powder

The pasting properties of composite flours were determined using a micro visco-amylograph (Brabender GmbH & Co. KG, Duisburg, Germany). A 15% (*w/v*) flour suspension in water was subjected to the controlled heating and cooling cycle, including heating from 30°C to 93°C at 7.5°C/min, holding at 93°C for 15 min, cooling from 93°C to 30°C at 7.5°C/min, and holding at 30°C for 15 min [Krishnaiya *et al.*, 2016; Marti *et al.*, 2015]. Key pasting parameters were recorded, including gelatinization temperature (T, °C), peak viscosity (PV), which is the maximum viscosity reached during the heating cycle and reflects the water-binding capacity of starch and the degree of granule swelling, trough viscosity (TV), which is the minimum viscosity reached during the constant temperature (holding) phase and measures the ability of starch to withstand breakdown under shear and heat, and final viscosity (FV), which is the viscosity at the end of the cooling cycle and indicates the ability of starch to form a viscous paste or gel after cooling. Breakdown viscosity (BV) was calculated as the difference between peak viscosity and trough viscosity. A high breakdown value indicates that the starch is

less stable under heat and mechanical shear. Setback viscosity (SV) was calculated as the difference between final viscosity and trough viscosity. A higher setback value typically indicates a greater tendency for the gel to firm up or leak water (syneresis). All the viscosities were expressed in Brabender unit (BU), which is an empirical measure of torque resistance.

Determination of antioxidant contents of wheat flour, banana flower powder, and muffins

Total phenolic, flavonoid, and anthocyanin contents of WF, BPF, and muffins were determined. The crumb was used in these tests to minimize the interference from Maillard reaction products typically concentrated in the crust. Following the research of Nath *et al.* [2018], with slight modifications, the sample (1.5 g) was extracted with 15 mL of 80% (*v/v*) ethanol at 25°C for 1 h in a shaking incubator. The mixture was then centrifuged at 2,378×*g* and 4°C for 15 min. The supernatant was collected for measurements.

The total phenolic content (TPC) was determined using the Folin-Ciocalteu colorimetric method based on Singleton *et al.* [1999] with a minor modification. A 1 mL aliquot of the extract was mixed with 6 mL of distilled water and 1-mL of a 10% (*v/v*) Folin-Ciocalteu reagent and left in the dark at room temperature for 5 min. Subsequently, 1 mL of a 7.5% (*w/v*) Na_2CO_3 solution was added, and the mixture was vortexed for 10 s. After a 1-h incubation in the dark at room temperature, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (UVD-2960, Labomed Inc., Los Angeles, CA, USA). Gallic acid was used to establish the standard curve (y=0.5998x, R^2=0.9989). The results were expressed as mg of gallic acid equivalents (GAE) *per* 100 g of dry weight (DW).

The total flavonoid content (TFC) was determined using a method adapted from Saeed *et al.* [2012]. A 1-mL aliquot of the extract was combined with 4 mL of distilled water and 0.3 mL of a 5% (*w/v*) $NaNO_2$ solution, and allowed to stand for 5 min. Subsequently, 1 mL of a 10% (*w/v*) $AlCl_3$ solution, 2 mL of a 4% (*w/v*) NaOH solution, and 2.4 mL of distilled water were added, and the mixture was vortexed for 10 s. Absorbance was measured at 506 nm. Rutin was used to establish the standard curve (y=0.0104x, R^2=0.9981). Results were expressed as mg of rutin equivalents (RE) *per* 100 g DW.

The total anthocyanin content (TAC) was measured according to the method previously used by Saldaña *et al.* [2021] with modifications. A 1-mL extracted aliquot was mixed with 9 mL of a potassium chloride buffer (pH 1.0) or sodium acetate buffer (pH 4.5) with HCl. Absorbance of both mixtures was then measured at 510 nm and 700 nm. The TAC, expressed as cyanidin 3-glucoside equivalent (CGE), was calculated as follows (1):

$$\text{TAC (µg CGE/100 g DW)} = \frac{A \times MW \times DF \times V_1 \times V_0 \times 100}{\varepsilon \times L \times V_2 \times m \times (1 - MC)} \quad (1)$$

where: A was calculated as (absorbance at 520 nm – absorbance at 700 nm)$_{pH1.0}$ – (absorbance at 520 nm – absorbance at 700 nm)$_{pH4.5}$, MW is the molecular weight of cyanidin 3-glucoside (449.2 g/mol), DF is a dilution factor, V_1 is the volume of the mixture (mL), V_0 is the total volume of the extract (mL), V_2 is the volume of the extract used for measurement (mL), L is the pathlength (cm), ε is the molar extinction coefficient for cyanidin 3-glucoside (26,900 L/(mol×cm)), m is the weight of the sample subjected to extraction (g), and MC is the moisture content.

Determination of physical properties of muffins

The muffins' specific volume was determined by the seed replacement method [Çabuk, 2021]. The bulk density of the seeds was determined by filling a container of known volume with seeds and recording their weight. A muffin was then placed in the container, and the volume of displaced seeds was measured. The volume of a muffin was calculated from the weight of displaced seeds and the established bulk density. The specific volume was determined by dividing the muffin volume by its weight.

The muffins' crumb and crust colors were measured using a color device (RGB-1002, Lutron, Taipei, Taiwan), matching the CIE Lab color scales, determining L^* (brightness), a^* (green to red), and b^* (blue to yellow). The total color difference (ΔE) in comparison with the control muffin was then calculated following Equation (2) [Nath *et al.*, 2018]:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (2)$$

where: L^*, a^*, and b^* are color parameters of the tested muffins; L^*_0, a^*_0, and b^*_0 are color parameters of the control muffin.

The muffins' porosity was assessed through their halved scanned pictures. Using the ImageJ software (National Institutes of Health, Bethesda, MD, USA), color images were converted to grayscale, leveraging the resulting contrast between the dark void cells of muffin pores and the light crumb for porosity determination [Petrusha *et al.*, 2018].

Texture profile analysis

The texture profile of muffins, including hardness, cohesiveness, chewiness, and springiness, was obtained by Brookfield CT3 texture analyzer (Brookfield Engineering Labs, Middleboro, MA, USA) according to the method described by Topkaya & Isik [2019], with a minor modification. Muffins were cut into 2-cm cubes. The texture analysis was performed by pressing the probe down 30% of the muffin original height with an initial force of 0.05 N, with a pre-test speed of 2 mm/s, a test speed of 1 mm/s, and a post-test speed of 1 mm/s.

Sensory evaluation of muffins

The sensory evaluation was conducted in a standard sensory laboratory with individual booths, involving 30 untrained panelists

(22 women and 8 men) aged 19 to 35 years. Participants were recruited based on their regular consumption of bakery products (at least once a week) and were screened *via* a questionnaire to exclude smokers or individuals with known food allergies (specifically to wheat, eggs, dairy, or banana flower powder) after providing verbal informed consent. Before the session, panelists were briefed on the specific olfactory, taste, and textural attributes associated with banana flower powder to ensure high sensitivity to the modifications in the muffin samples. Each sample was coded with a three-digit random number and served in a randomized order. To prevent sensory fatigue and ensure accuracy, the panelists were required to rinse their mouths with water and rest for at least 30 s between samples to maintain a clean palate. The sensory evaluation was conducted using a 9-point hedonic scale (1 – dislike extremely; 9 – like extremely), and the panelists rated the muffins for appearance, color, texture, taste, and overall acceptability.

Statistical analysis

All samples were triplicated, and data were expressed as mean and standard deviation. Statistical analysis was performed using Minitab software at a 95% confidence level. Independent *t*-tests were used for two-sample comparisons, and one-way analysis of variance (ANOVA) with Fisher and Tukey post-hoc tests was used for multiple-sample comparisons. In cases where variance homogeneity was not satisfied, Welch's ANOVA and Games-Howell tests were employed.

RESULTS AND DISCUSSION

Chemical composition and antioxidant properties of wheat flour and banana flower powder

The chemical composition of WF and BFP is shown in **Table 2**. BFP had significantly higher levels of ash, protein, lipid, and crude fiber but lower contents of moisture and carbohydrates when compared to WF. The protein, lipid, and ash contents of BFP were 20.54, 7.76, and 7.83 g/100 g DW, which were comparable to those reported by Wickramarachchi & Ranamukhaarachchi [2005]. With its high protein and lipid contents (>50% higher than those of WF), the banana flower powder could deliver enough levels of necessary nutrients. Ash content quantifies the overall quantity of minerals or inorganic components in food. The report by Basumatary & Nath [2018] had already demonstrated that banana flower contained various minerals necessary for the human body's physiological and biological activities. It contained both macro-elements, including potassium, sodium, calcium, and magnesium (Mg), as well as micro-elements, such as iron, zinc, copper, and manganese [Kang *et al.*, 2014]. Notably, the crude fiber content of BFP was 28.57 g/100 g DW, *i.e.*, approximately six-fold higher than that of WF (**Table 2**). These values demonstrated higher nutrient contents of BFP compared to WF.

Regarding antioxidants, **Table 2** lists the TPC, TFC, and TAC of BFP and WF. These values for BFP were 2,564 mg GAE/100 g DW; 164.6 mg RE/100 g DW; and 2,729 µg CGE/100 g DW, respectively, which were approximately 20-, 2- and 40-fold higher than those of WF. These findings imply BFP can be a rich source of antioxidants, which is consistent with previous reports. For example, Lau *et al.* [2020] concluded from numerous studies that banana flowers were found a rich source of simple phenolics, such as gallic, *p*-hydroxybenzoic, protocatechuic, gentisic, vanillic, caffeic, syringic, ferulic, *p*-coumaric, chlorogenic, and sinapic acids, vanillin, and catechol, as well as flavonoids, including catechin, epicatechin, quercetin, and rutin. These bioactive compounds with free radical scavenging, anti-allergic, antibacterial, anticoagulant, and antimutagenic properties, could potentially contribute to cancer chemoprevention [Lau *et al.*, 2020].

Table 2. Proximate composition and antioxidant contents of wheat flour (WF) and banana flower powder (BFP).

Parameters	WF	BFP
Moisture (%)	11.54±0.11^a	5.27±0.10^b
Ash content (g/100 g DW)	0.56±0.00^b	7.83±0.06^a
Protein content (g/100 g DW)	13.58±0.21^b	20.54±0.05^a
Lipid content (g/100 g DW)	4.91±0.33^b	7.76±0.17^a
Crude fiber content (g/100 g DW)	4.82±0.63^b	28.57±0.38^a
Carbohydrate content (g/100 g DW)	76.13±0.56^a	35.31±0.10^b
TPC (mg GAE/100 g DW)	119±14^b	2,564±18^a
TFC (mg RE/100 g DW)	84.7±3.6^b	164.6±5.6^a
TAC (µg CGE/100 g DW)	70.94±0.35^b	2,729±14^a

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences between WF and BFP ($p<0.05$). TPC, total phenolic content; TFC, total flavonoid content; TAC, total anthocyanin content; DW, dry weight; GAE, gallic acid equivalent; RE, rutin equivalent; CGE, cyanidin 3-glucoside equivalent.

Pasting properties of composite flours

The pasting characteristics of WF and its blends with BFP are presented in **Table 3**. Pasting properties are important in determining the flour quality for cooking and baking purposes. The BFP fortification significantly influenced the pasting properties of starch in the composite flours. The pasting temperature, also referred as gelatinization temperature (T), is the point at which starch granules begin to swell [Malomo *et al.*, 2011]. In this study, the gelatinization temperature of flour blends varied significantly ($p<0.05$), exhibiting a gradual increase from 57.3°C in WF to 58.1°C with the incorporation of 25% (*w/w*) BFP. The progressive increase in BFP content interfered with the gelatinization and swelling processes, which required a higher temperature to initiate gelatinization. Furthermore, the highest recorded values for peak viscosity (PV), trough viscosity (TV), and final viscosity (FV) were recorded for WF without BFP fortification, which were 1,021 BU; 676 BU; and 1,188 BU, respectively. However, with increasing BFP incorporation, the viscosities of the flour blends decreased, reaching the lowest values of 875

Table 3. Pasting properties of wheat flour (WF) and its blends with banana flower powder (BFP).

Flour/blend	Gelatinization temperature (°C)	Peak viscosity (BU)	Trough viscosity (BU)	Final viscosity (BU)	Breakdown viscosity (BU)	Setback viscosity (BU)
WF	57.3±0.3^{d}	1,021±14^{a}	676±11^{a}	1,188±13^{a}	408±11^{a}	325±10^{a}
BFP5	57.4±0.3^{cd}	926±7^{b}	616±9^{b}	1,084±18^{b}	394±16^{ab}	319±4^{a}
BFP10	57.7±0.2^{bc}	915±10^{bc}	519±11^{c}	1,047±16^{c}	384±13^{ab}	324±6^{a}
BFP15	57.8±0.1^{abc}	908±7^{bc}	483±5^{d}	1,015±12^{d}	376±7^{b}	317±8^{a}
BFP20	58.0±0.3^{ab}	896±13^{c}	404±13^{e}	998±16^{d}	392±34^{ab}	313±6^{a}
BFP25	58.1±0.1^{a}	875±11^{d}	354±10^{f}	956±19^{e}	407±4^{ab}	314±7^{a}

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences among flour and blends (p<0.05). BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

BU for PV, 354 BU for TV, and 956 BU for FV at the highest BFP content. The observed trends were consistent with the findings from previous studies examining the effects of replacing wheat flour with water chestnut and perennial wheatgrass [Krishnaiya *et al.*, 2016; Marti *et al.*, 2015]. They could be explained by the presence of fiber, protein, and lipid components in the BFP, as shown in **Table 2**, which could compete with starch for water absorption, hindering starch gelatinization, weakening the gluten network, and ultimately reducing viscosity [Feng *et al.*, 2025]. Moreover, the lipid content in the composite flour could partially affect the viscosity, as it hinders starch molecule interactions and reduces their swelling ability. Additionally, the lower starch content in the BFP-incorporated blends, indicated by the carbohydrate levels in **Table 2**, also acted as a significant factor in these observed structural modifications.

Among pasting characteristics, breakdown viscosity (BV) serves as an indicator of the degree of disintegration of starch granules, and, in contrast, setback viscosity (SV) refers to the viscosity produced by a decrease in temperature, which causes starch molecules to retrograde or reassociate. BV exhibited mostly no significant differences (p≥0.05) among the samples, except for BFP15, in which it was significantly lower than in the WF (**Table 3**). This reduction is likely attributed to the presence of fiber, protein, and lipids in BFP, which compete with starch for water absorption and restrict starch granule swelling, thereby reducing granule breakdown during heating [Feng *et al.*, 2025]. Similar non-linear trends in BV have been reported in starch systems supplemented with fiber-rich powders [Lou *et al.*, 2022]. Meanwhile, SV showed no significant differences (p≥0.05) among the flour blends with and without BFP. Overall, with the appropriate addition of BFP, the moderate peak viscosity in this study indicated its sutiability for making bakery products with desirable starch swelling and batter viscosity during baking, which allows greater cake expansion. However, an over-incorporation may lead to an excessive decrease in batter viscosity, which may hinder the stabilization of the cake structure during baking, potentially leading to the collapse or formation of a dense, underbaked interior.

Moisture and antioxidant contents of muffins

Table 4 presents the moisture and antioxidant contents of muffins prepared from WF and different flour blends with BFP, while **Figure 1** illustrates their appearance. The moisture of the control muffin was 32.95%. The substitution of WF by BFP up to 15% (*w/w*) did not significantly influence the moisture content (p≥0.05), but its further amounts at 20% and 25% (*w/w*) slightly increased it to 34.74%. This may be because BFP had more fiber than WF (as shown in **Table 2**). Fiber-rich ingredients have been demonstrated to retain water and raise the moisture content of muffins after baking [Mehta *et al.*, 2018; Nath *et al.*, 2018]. Similar findings were reported for muffins with kale powder added [Choi, 2015].

On the other hand, there were statistically significant increases (p<0.05) in TPC, TFC, and TAC in the muffins with increasing BFP content (**Table 4**). The control sample had the lowest TPC (757 mg GAE/100 g DW), which increased progressively with higher percentages of BFP, reaching the highest value of 1,055 mg GAE/100 g DW at 25% (*w/w*) BFP. Additionally, a significant increase in TFC was observed in the fortified muffins, rising from 89.9 mg RE/100 g DW in the control sample to 124.8 mg RE/100 g DW in the muffins produced from the blend with 25% (*w/w*) substitution of WF by BFP. Similarly, TAC improved from 86.7 µg CGE/100 g DW to 297.4 µg/100 g DW. These enhancements were due to the abundant presence of these antioxidants in BFP (as discussed from **Table 2**) and their notable retention in the baked product. The increasing trend of total phenolic, total flavonoid, and total anthocyanin contents was also demonstrated in the report by Croitoru *et al.* [2018], upon increasing the amount of black rice flour in muffins. Therefore, the muffins fortified with BFP were expected to potentially offer numerous health benefits to consumers.

Physical properties, textural profile, and color attributes of muffins

Parameters of the physical properties and textural profile of muffins are shown in **Table 5**. The specific volume, a parameter indicative of dough swelling, showed a significant decrease

Table 4. The moisture and antioxidant contents of muffins produced using wheat flour (WF) and its blends with banana flower powder (BFP).

Muffin	Moisture (%)	TPC (mg GAE/100 g DW)	TFC (mg RE/100 g DW)	TAC (µg CGE/100 g DW)
BFP0	32.95±0.92^{b}	757±5^{f}	89.9±4.7^{e}	86.7±1.4^{f}
BFP5	32.09±0.49^{b}	845±3^{e}	110.6±3.2^{d}	165.7±7.7^{e}
BFP10	32.79±0.09^{b}	936±3^{d}	113.5±1.1^{cd}	189.9±3.7^{d}
BFP15	33.07±0.98^{b}	977±1^{c}	117.4±2.1^{bc}	226.1±7.6^{c}
BFP20	34.25±0.41^{a}	1,036±6^{b}	121.2±2.5^{ab}	265.4±6.4^{b}
BFP25	34.74±0.41^{a}	1,055±12^{a}	124.8±4.3^{a}	297.4±28.7^{a}

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences among muffins (p<0.05). TPC, total phenolic content; TFC, total flavonoid content; TAC, total anthocyanin content; DW, dry weight; GAE, gallic acid equivalent; RE, rutin equivalent; CGE, cyanidin 3-glucoside equivalent; BFP0, control sample (without BFP); BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

Figure 1. The appearance of muffins produced using wheat flour (WF) and its blends with banana flower powder (BFP). BFP0, control sample (only WF); BFP5, 5% substitution of WF by BFP (*w/w*), BFP10; 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

Table 5. The physical properties and textural profile of muffins produced using wheat flour (WF) and its blends with banana flower powder (BFP).

Muffin	Specific volume (mL/g)	Porosity (%)	Hardness (N)	Cohesiveness	Chewiness (N)	Springiness
BFP0	2.09±0.09^{a}	43.72±0.64^{a}	3.31±0.08^{f}	0.82±0.02^{a}	2.85±0.27^{d}	1.05±0.10^{a}
BFP5	1.85±0.03^{b}	39.32±0.78^{b}	3.79±0.08^{e}	0.79±0.01^{b}	3.03±0.12^{d}	1.02±0.03^{ab}
BFP10	1.82±0.03^{bc}	38.00±0.16^{b}	4.48±0.06^{d}	0.77±0.00^{b}	3.45±0.02^{c}	1.00±0.02^{ab}
BFP15	1.88±0.07^{b}	36.77±1.09^{bc}	5.36±0.06^{c}	0.74±0.02^{c}	3.85±0.13^{b}	0.97±0.02^{ab}
BFP20	1.75±0.05^{cd}	34.70±0.28^{c}	5.75±0.05^{b}	0.71±0.01^{d}	4.02±0.14^{b}	0.99±0.03^{ab}
BFP25	1.71±0.01^{d}	32.93±0.25^{d}	6.69±0.06^{a}	0.69±0.01^{d}	4.50±0.08^{a}	0.97±0.02^{b}

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences among muffins (p<0.05). BFP0, control sample (without BFP); BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

from 2.09 mL/g to 1.71 mL/g as the BFP content increased from 0 to 25% (*w/w*) in the flour blend. The finding aligned with those previously reported by Lee & Chung [2013] and Choi [2015] who pointed out that the muffins' specific volume decrease occurred as a result of partial substitution of WF with freeze-dried apricot powder and kale powder, respectively. Another parameter reflecting the capacity of muffin to retain trapped carbon dioxide and air bubbles introduced during mixing is porosity. **Figure 2** depicts the cross-sectional images of muffin, indicating that the crumb structure became denser with the increase in BFP amount. Subsequently, the porosity exhibited a decreasing trend from 43.72% to 32.93% when muffin fortification with BFP

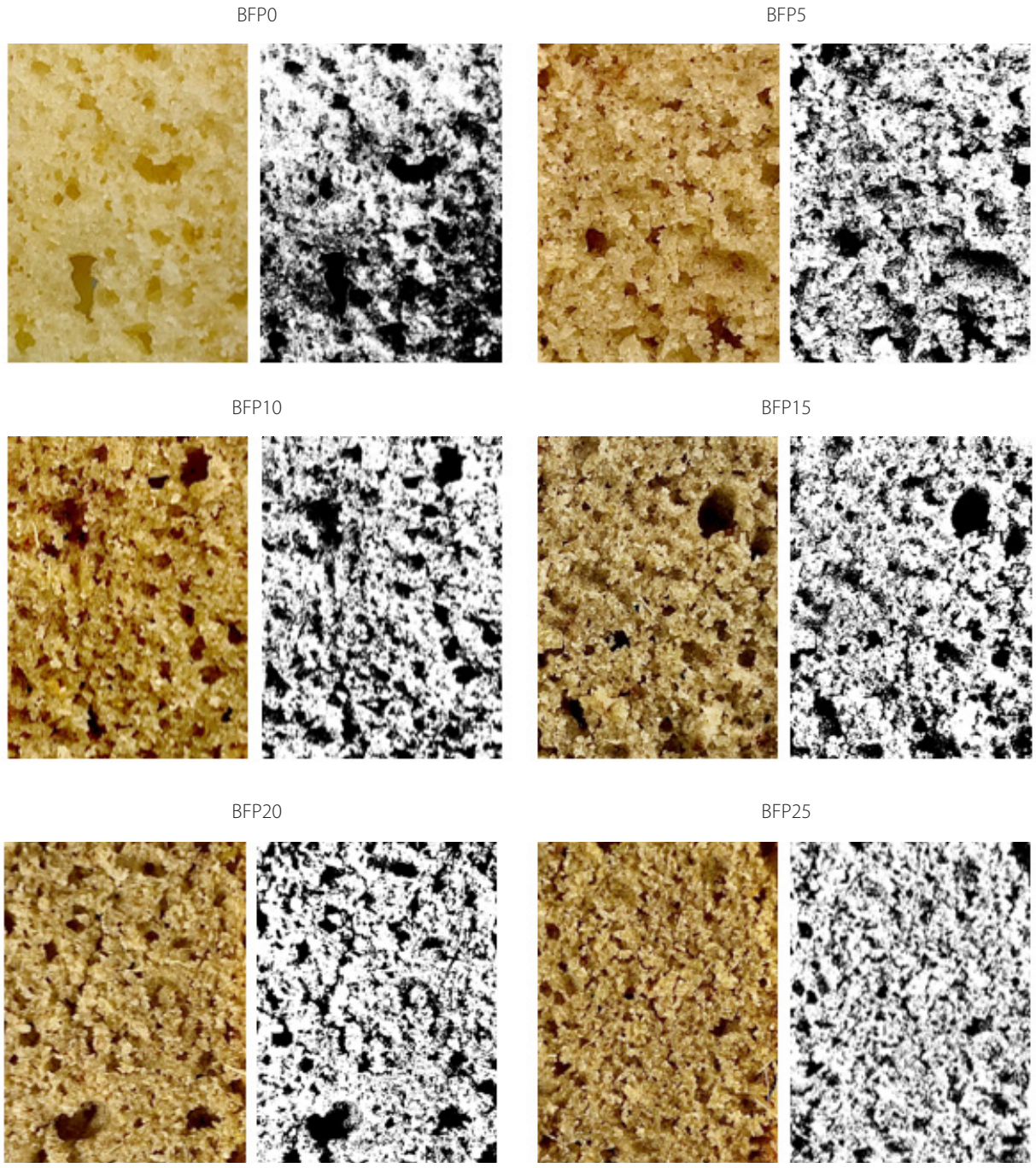

Figure 2. Cellular structure of muffin crumb with different levels of banana flower powder (BFP). Left: scanned images of the cross section of muffin crumb, right: modified binary images. BFP0, control sample (only wheat flour, WF); BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

increasing to 25% (**Table 5**). Similar observations were reported by Konuk Takma *et al.* [2021], where the incorporation of fig seed pomace flour produced less porous cupcakes. The decreases in specific volume and porosity were consistent with the reduction in viscosity of the flour blends as shown in **Table 3**, which was attributed to the elevated content of fiber and the lessened amount of starch. These changes could disrupt and weaken the starch-gluten matrix, limiting its capacity to retain air bubbles during baking [Sandrine *et al.*, 2022].

There was also a correlation between structure density with the textural profile of muffins. As presented in **Table 5**, the control muffin with the highest specific volume and porosity presented the lowest hardness (3.31 N) and chewiness (2.85 N). With an increasing content of BFP in the flour blend, the fortified muffins exhibited significantly higher values ($p<0.05$) of these two attributes up to 6.69 N and 4.50 N, respectively, with a dense and tightly packed crumb structure. The compact microstructure due to the lack of large air pockets increased crumb density,

making it more resistant to deformation and more difficult to chew. These results matched those of earlier research conducted by Topkaya & Isik [2019] and Lee *et al.* [2020], in which muffins incorporated with pomegranate peel and Kamut exhibited an increase in hardness and chewiness. On the other hand, **Table 5** presents a decrease in cohesiveness as the BFP content increased. This attribute refers to the strength of internal bonds within a food's structure, which relies on its capacity to endure deformation [Noorlaila *et al.*, 2017]. Its decline was probably caused by the dilution and weakening of a gluten network, as demonstrated by the pasting properties of composite flours in **Table 3**. Meanwhile, springiness, a measure of muffin's elastic recovery, exhibited a small variation among the samples, ranging from 0.97 to 1.05.

Table 6 displays the color parameters (L^*, a^*, b^*) of muffin crust and crumb, which may significantly influence consumer acceptance. Increasing BFP incorporation led to a statistically significant decrease ($p<0.05$) in muffin lightness (L^*) compared to the control sample. Specifically, crust and crumb lightness decreased from 63.78 to 45.8 and from 84.5 to 56.2, respectively. This change was consistent with their appearance observed in **Figure 1**. Crust redness (a^*) showed no apparent trend with increasing BFP content in the flour blends; however, crumb redness significantly increased from −1.8 to 4.7, and in general BFP-incorporated muffins exhibited higher a^* values than the control. The control muffin displayed the highest yellowness (b^*) values, with 43.9 for the crust and 35.9 for the crumb. Crust yellowness also showed no consistent trend with an increasing BFP content in the flour blends. Conversely, crumb b^* significantly decreased with increasing BFP addition. These trends could be attributed the intrinsic darkness and color of BFP. A similar observation was reported previously by Tasnim *et al.* [2020] and Topkaya & Isik [2019], which showed an increased a^* value and decreased b^* value for fortified plain cake. In addition, **Table 6** displays that the BFP incorporation led to a significant increase in the total color difference for both the crust and the crumb of the muffins compared to the control sample (BFP0). This increase reflected a significant shift toward a darker and more intense coloration. In all fortified samples, the ΔE values exceeded the threshold of 3.0, indicating that the color changes induced by BFP were easily perceptible to the human eye [Nath *et al.*, 2018], which agreed with their optical images in **Figure 1**. Despite the common perception that darker colors are less desirable in food, a correlation between darker muffins and perceived health benefits exists among certain consumers [Walker *et al.*, 2014].

Sensory scores of muffins

The sensory scores for muffins in terms of appearance, color, texture, taste, and overall acceptability are shown in **Table 7**. The general findings indicate that all samples received good scores for all attributes, ranging from 6.67 to 7.80 on a 9-point scale. The control muffin had the mean scores above 7, ranging from 7.07 to 7.90, corresponding to "like moderately". The BFP incorporation up to 20% of WF (*w/w*) did not significantly influence ($p\geq0.05$) consumer preferences for all sensory attributes, except for the BFP20, with a lower score for texture. Texture was the most sensitive sensory parameter, especially at 20% and 25% substitution levels. This result aligned with the texture profile, as presented in **Table 5**, which showed increased hardness and chewiness. Muffins with 25% (*w/w*) substitution of WF by BFP consistently received the lower scores for all attributes, including appearance (6.83), color (6.87), texture (6.77), taste (7.17), and overall acceptability (6.77). This reduction of BFP25 mufin could be attributed to its high moisture content (**Table 4**), high values of hardness and chewiness (**Table 5**), and excessively dark color (**Table 6**). These results imply that substituting wheat flour with BFP up to 15% (*w/w*) maintained a balanced sensory profile; however, at the 20% (*w/w*) level, while the overall acceptability remained comparable, a significant decline in texture scores was observed, suggesting a potential sensory trade-off at higher fortification levels.

Table 6. Color attributes of the crust and crumb of muffins produced using wheat flour (WF) and its blends with banana flower powder (BFP).

Muffin	Crust				Crumb			
	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE
BFP0	63.7±0.9^{a}	6.7±0.5^{e}	43.9±0.5^{a}	–	84.5±0.6^{a}	−1.8±0.8^{d}	35.9±0.6^{a}	–
BFP5	51.2±0.3^{b}	15.9±0.8^{a}	42.9±0.3^{b}	15.6±1.3^{ab}	71.2±0.6^{b}	2.4±0.6^{c}	33.6±0.4^{b}	14.0±1.1^{d}
BFP10	50.3±0.2^{b}	10.5±0.5^{c}	41.3±0.6^{c}	14.0±0.9^{b}	63.5±0.5^{c}	2.5±0.2^{bc}	33.5±0.5^{b}	21.4±0.9^{c}
BFP15	49.4±0.6^{c}	13.6±0.8^{b}	37.4±0.3^{f}	15.9±1.6^{ab}	61.0±0.4^{d}	3.3±0.6^{b}	31.6±0.6^{c}	24.0±1.0^{b}
BFP20	47.4±0.6^{d}	9.9±0.4^{c}	40.5±0.3^{d}	16.7±0.9^{a}	58.6±0.0^{e}	4.4±0.4^{a}	30.5±0.3^{d}	26.6±0.7^{a}
BFP25	45.8±0.1^{e}	7.9±0.1^{d}	39.0±0.5^{e}	18.1±1.0^{a}	56.2±0.3^{f}	4.7±0.4^{a}	30.3±0.5^{d}	29.0±1.2^{a}

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences among muffins ($p<0.05$). BFP0, control sample (without BFP); BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*). L^*, lightness; a^*, greenness/redness; b^*, blueness/yellowness; ΔE, total color difference.

Table 7. Sensory scores of muffins produced using wheat flour (WF) and its blends with the banana flower powder (BFP).

Muffin	Appearance	Color	Texture	Taste	Overall Acceptability
BFP0	7.70±1.15^{a}	7.90±1.27^{a}	7.07±1.05^{ab}	7.20±1.06^{ab}	7.23±0.57^{ab}
BFP5	7.63±0.85^{a}	7.63±0.85^{ab}	7.17±1.02^{ab}	7.70±1.06^{ab}	7.63±0.93^{a}
BFP10	7.73±0.94^{a}	7.67±0.71^{ab}	7.47±1.04^{ab}	7.43±1.31^{ab}	7.53±0.73^{a}
BFP15	7.77±0.94^{a}	7.73±1.29^{a}	7.63±0.81^{a}	7.67±1.28^{a}	7.80±1.06^{a}
BFP20	7.03±1.00^{ab}	7.47±1.20^{ab}	6.90±0.61^{b}	7.40±0.97^{ab}	7.17±1.00^{ab}
BFP25	6.83±1.29^{b}	6.87±1.11^{b}	6.77±1.10^{b}	7.17±0.87^{b}	6.67±0.88^{b}

Results are shown as mean ± standard deviation. Means with different lowercase letters indicate significant differences among muffins ($p<0.05$). BFP0, control sample (without BFP); BFP5, 5% substitution of WF by BFP (*w/w*); BFP10, 10% substitution of WF by BFP (*w/w*); BFP15, 15% substitution of WF by BFP (*w/w*); BFP20, 20% substitution of WF by BFP (*w/w*); BFP25, 25% substitution of WF by BFP (*w/w*).

CONCLUSIONS

This study confirmed that BFP is a nutrient-dense ingredient, rich in dietary fiber, protein, and specific bioactive compounds (with high contents of total phenolics, total flavonoids, and total anthocyanins), which significantly enhanced the nutritional profile of the fortified muffins. The incorporation of BFP altered the pasting properties of the flour blends by increasing gelatinization temperature and reducing peak and final viscosities, indicating restricted starch swelling and a weakened gluten network. These physicochemical changes resulted in a more compact muffin structure, characterized by a decreased specific volume, lower porosity, and increased hardness. Sensory evaluation identified 15% (*w/w*) BFP as the optimal substitution level for maintaining a favorable balance of texture and taste. Although overall acceptability remained comparable at 20% (*w/w*), this level should be considered the maximum tolerable threshold due to a significant decline in texture scores. While BFP addition significantly increased the total antioxidant content, further research is required to evaluate the bioaccessibility and bioavailability of these compounds to validate their actual health-related benefits. Additionally, future studies should focus on the thermal degradation kinetics of bioactive markers during baking and utilize specialized antioxidant activity assays to provide a more comprehensive functional assessment.

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CONFLICT OF INTERESTS

There are no conflicts of interest related to the publication of this article.

ORCID IDs

N.L. Le https://orcid.org/0000-0002-4634-7267
N.T.Y. Tran https://orcid.org/0009-0004-6210-4240
L.T.K. Vu https://orcid.org/0000-0002-5029-4866

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Analysis of Human Milk Leptin and Ghrelin in Relation to Maternal Factors and Infant Weight Gain Within Six Months

Bożena Kulesza-Brończyk[1]†, Agnieszka Bzikowska-Jura[2,3]*†, Agnieszka Bień[4], Aleksandra Mołas[3], Aleksandra Wesołowska[3], Tomasz Molcan[5,6], Dominika Gutkowska-Kawka[7], Monika M. Kaczmarek[5,7]

[1]Department of Obstetrics, Gynecology and Maternity Care, Medical University of Bialystok, Szpitalna 37, 15-295 Bialystok, Poland
[2]Department of Clinical Dietetics, Medical University of Warsaw, Ciołka 27, 01-445 Warsaw, Poland
[3]Laboratory of Human Milk and Lactation Research, Department of Medical Biology, Medical University of Warsaw, Litewska 14/16, 00-575 Warsaw, Poland
[4]Chair of Obstetrics Development, Medical University of Lublin, Staszica 4/5, 20-081 Lublin, Poland
[5]Molecular Biology Laboratory, InLife Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Trylińskiego 18, 10-683 Olsztyn, Poland
[6]Computational Biology Core Facility, InLife Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Trylińskiego 18, 10-683 Olsztyn, Poland
[7]Programming of Fertility and Development Team, InLife Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Trylińskiego 18, 10-683 Olsztyn, Poland

This study examined leptin and ghrelin concentrations in human milk (HM) in the first six months of lactation and their associations with maternal characteristics, milk composition, and infant weight gain. Twenty exclusively breastfeeding mothers were recruited from the University Hospital of Białystok, Poland. HM samples were collected at six postpartum intervals, and hormone concentrations were measured using the enzyme-linked immunosorbent assay (ELISA). Maternal pre-pregnancy body mass index (BMI) was recorded, and milk macronutrient composition was analyzed with an HMA analyzer. Mean HM leptin concentrations ranged from 0.30 to 0.41 ng/mL within the first six months of lactation, while mean ghrelin concentrations ranged from 23 to 30 pg/mL. Maternal pre-pregnancy BMI correlated positively with ghrelin concentrations at the second postpartum interval but not with leptin levels. Leptin showed a moderate negative correlation with a true protein concentration at 7–8 weeks postpartum. Infant weight gain over the six months postpartum ranged from 370 to 790 g *per* month, with no significant associations identified between weight gain and hormone levels. Although maternal BMI was associated with ghrelin concentration, this correlation did not translate into measurable differences in infant growth trajectories. The relatively stable concentrations of leptin and ghrelin throughout the first six months of lactation underscore the need for further research on their potential roles in infant growth, gut development, and metabolic programming.

Keywords: hormones, human milk composition, infant growth, maternal BMI, metabolic programming

INTRODUCTION

Human milk (HM) is widely recognized as the optimal source of nutrition for newborns and infants, particularly for preterm babies, facilitating a critical transition from intrauterine to extrauterine life. HM provides unique nutritional, immunological, and trophic properties vital during life's early stages. As a result, global child health and nutrition organizations advocate exclusive breastfeeding for the first six months of life [Perrella *et al*., 2021; WHO, 2022]. Although initiation of breastfeeding is relatively high in many European countries, the prevalence

***Corresponding Author:**
e-mail address: **abzikowska@wum.edu.pl** (Dr. A. Bzukowska-Jura)
†These authors contributed equally to this work.

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of exclusive breastfeeding in the first six months of life remains below international recommendations. According to data from the WHO European Region, the prevalence of exclusive breastfeeding up to six months of age is lower than in most other WHO regions worldwide. In many European countries, the proportion of infants exclusively breastfed during the first six months of life ranges from approximately 13% to 39% [Theurich *et al.,* 2019]. In Poland, although nearly all mothers initiate breastfeeding after birth, national data from the PITNUTS 2016 study indicate that only 5.9% of the infants were exclusively breastfed at six months of age [Weker *et al.,* 2017].

The composition of HM is dynamically tailored to meet the evolving needs of the infant. This adaptive nature ensures that dietary requirements are met through precisely-designed contents of energy, macronutrients, and micronutrients, along with a diverse array of biologically-active compounds, such as enzymes, hormones, growth factors, anti-infective factors, and blood cells [Andreas *et al.,* 2015; Demmelmair & Koletzko, 2022]. The contents of these components can vary significantly depending on maternal physiology, environmental influences, and other factors [Fields *et al.,* 2016].

HM is pivotal in regulating hunger and satiety, driven by hormones and growth factors such as leptin and adiponectin, ghrelin, resistin, obestatin, and insulin-like growth factor [Savino *et al.*, 2012]. These bioactive components mediate the interaction between the gastrointestinal system and hypothalamic centers responsible for energy homeostasis. Recent studies have emphasized the significance of early-life nutrition, including breastfeeding, in reducing the risk of overweight and obesity later in life [Carrello *et al.,* 2025; Ma *et al.,* 2020; Sun *et al.,* 2024; Yan *et al.,* 2014].

Polypeptide hormones in HM regulate energy balance and are thought to originate from two primary sources: local synthesis and secretion by mammary epithelial cells and transfer from maternal plasma [Savino *et al.,* 2012]. Among these, leptin plays a key role in energy homeostasis. Synthesized primarily by adipose tissue and the intestinal mucosa, leptin interacts with hypothalamic receptors to suppress hunger and promote energy expenditure [Palou & Pico, 2009]. Leptin in HM has been implicated in appetite suppression and enhanced energy expenditure in breastfed infants. It is suggested that leptin in HM may enter the systemic circulation of newborns and infants through leptin receptors located on epithelial cells of the stomach and small intestine, potentially influencing early energy balance and metabolism [Cannon *et al.*, 2015; Flier & Maratos-Flier, 2017]. It is worth noting that leptin concentrations in HM exhibit circadian variation, with levels declining in the afternoon and rising overnight, peaking at around 05:00 h. Notably, higher concentrations have been reported between 22:00 and 04:00 h [Suwaydi *et al.*, 2023], which is consistent with the nocturnal rise in circulating leptin observed in humans [Saad *et al.,* 1998].

Ghrelin, often called the "hunger hormone", is present in HM and produced by the mammary gland, mammary epithelial cells, and the placenta. It is crucial in regulating an infant's appetite [Fields *et al.,* 2016]. During feeding, ghrelin levels increase, stimulating hunger and ensuring that the infant consumes the necessary nutrients and energy for optimal growth [Karatas *et al.,* 2011]. Although diurnal fluctuations of ghrelin have been documented in saliva [Aydin *et al.,* 2006], there is currently no evidence demonstrating circadian variation of ghrelin concentrations in HM, and studies addressing this issue are lacking.

Leptin and ghrelin concentrations are closely linked to lactation. Several longitudinal studies have shown that leptin levels are highest in colostrum and generally decrease over the first months of lactation [Ilcol *et al.,* 2006; Schuster *et al.,* 2011; Yu *et al*., 2018], while ghrelin levels tend to remain stable or increase in mature milk [de Fluiter *et al.,* 2021; Kon *et al*., 2014]. Leptin concentrations are initially higher in term than in preterm colostrum, but they decline more rapidly, leading to comparable levels in both groups by six weeks postpartum [Bielicki *et al.,* 2004]. Other investigations, however, have reported lower leptin concentrations in mature term milk, likely reflecting differences in the postpartum interval before sample collection [Vass *et al.,* 2020].

Both hormones are thought to contribute to regulating growth and development during neonatal and infancy periods and to likely play a role in protecting infants from the development of obesity. Additionally, previous studies have suggested that leptin and ghrelin are involved in metabolic programming [Fields *et al*., 2016; Flier & Maratos-Flier, 2011; Savino *et al.*, 2010], a process where prenatal and early postnatal conditions significantly influence the development and function of tissues and organs, potentially shaping long-term metabolic regulation.

Building on the understanding of the dynamic composition of HM and the pivotal roles of leptin and ghrelin in energy balance and infant development, this study aimed to investigate these hormones in the context of lactation and early growth. Specifically, it sought to examine the variability in leptin and ghrelin concentrations in HM over the first six months of lactation and to identify the maternal factors, *e.g.*, age, body mass index (BMI), that may affect their levels. This research has the potential to deepen our understanding of the interplay between maternal factors, hormonal regulation, and infant growth, ultimately contributing to the development of strategies for optimizing early-life nutrition and long-term health outcomes.

MATERIALS AND METHODS

Participants' recruitment

Participants were recruited at the University Hospital of Białystok, Poland, between January and September 2022. During the initial screening, 35 breastfeeding mothers were evaluated for eligibility. Nine individuals did not meet the inclusion criteria, which required participants to be at least 18 years old, in good health with no chronic illnesses, non-smokers during and after pregnancy, exclusively breastfeeding, and producing an adequate milk supply. Additionally, six more participants were unable to complete the study due to circumstances such as transitioning to formula feeding, subsequent pregnancy, or relocation.

Notably, no participants withdrew from the study due to health complications, as mothers and infants remained in good health throughout the observation period. Ultimately, complete datasets and human milk samples from 20 participants were included in the final analysis.

The study adhered to rigorous ethical standards, consistent with the Declaration of Helsinki. The protocol was approved by the Bioethics Committee of the Medical University of Białystok, Poland (approval no. AKP.002.501.2021). All participants were informed about the research objectives and provided written informed consent before enrollment. Furthermore, they voluntarily contributed milk samples for analysis, ensuring that the study met its goals while upholding ethical integrity.

Anthropometric measurements

Maternal anthropometric data included pre-pregnancy weight and height, which were self-reported at enrollment and used to calculate pre-pregnancy BMI. Maternal weight at delivery was obtained from medical records, and gestational weight gain was calculated as the difference between pre-pregnancy and delivery weight.

Infant anthropometric data comprised birth weight, collected from hospital records, and subsequent monthly body weights, which were recorded during routine pediatric visits and verified at study appointments. Infant body weights were expressed in kilograms and converted to age- and sex-specific weight-for-age z-scores (WAZ) using WHO Child Growth Standards with the anthro package, version 1.0.1 (WHO, Geneva, Switzerland).

Human milk collection and nutrient concentration analysis

Milk samples were collected between 7:00 and 9:00 AM, with a minimum interval of one hour since the last breastfeeding session. Participants were provided with sterilized collection tubes. To maintain hygiene, mothers were advised to wash their hands and clean the chest area before collection. Approximately 20 mL of milk were collected as a composite sample, consisting of equal volumes of pre-feed (foremilk) and post-feed (hindmilk) milk. These fractions were pooled prior to centrifugation and hormone analysis to obtain a representative mixed milk sample for each feeding. After collection, the milk samples were stored at 4°C for a few hours, but no longer than five, before being transported to the laboratory. Then, each sample was divided into two equal portions, processed on the day of collection, frozen within 24 h, and stored at –20°C until analysis. To ensure consistency, all samples were analyzed after a standardized storage period of three to four months, and each sample was subjected to a single freeze–thaw cycle to minimize peptide degradation.

Milk samples were collected at six specific postpartum time points: 3–4 weeks, 7–8 weeks, 11–12 weeks, 15–16 weeks, 19–20 weeks, and 23–24 weeks, resulting in a total of 120 samples. At the first collection time point (3–4 weeks postpartum), additional data on maternal and infant characteristics were gathered for further analysis.

The energy, lactose, fat, and protein concentrations of HM were measured using the MIRIS human milk analyzer (HMA) (Miris, Uppsala, Sweden), following a validated protocol. The analysis utilized mid-infrared (MIR) transmission technology, with wavelengths specific to different macronutrient bonds: lactose (C–OH stretch at 9.61 µm), fat (C=O at 5.73 µm and C–H at 3.48 µm), and protein (CO–N stretch at 6.46 µm). The energy value of HM was calculated *per* 100 mL based on the measured concentrations of lactose, protein, and fat, which were conventionally expressed *per* 100 mL of human milk. Energy value was estimated using specific Atwater conversion factors for human milk: 4.0 kcal/g for lactose, 4.4 kcal/g for protein, and 9.25 kcal/g for fat. In addition to macronutrient concentrations, the MIRIS HMA also provided a measurement of dry mass. This parameter reflects the proportion of solid components in milk (fat, protein, and carbohydrates) after subtraction of the water content and was automatically generated during mid-infrared analysis.

The total protein content was determined by the MIRIS HMA based on the total nitrogen (N) content and calculated using a nitrogen-to-protein conversion factor of 6.38, as specified by the manufacturer. Because total protein includes non-protein nitrogen (NPN) compounds, which account for approximately 20–25% of the total nitrogen in HM, true protein values were estimated by applying a correction factor of 0.8, according to the MIRIS methodology, using Equation (1):

$$\text{True protein (g/100 mL)} = \text{Total protein (g/100 mL)} \times 0.8 \quad (1)$$

For each sample, three aliquots (~12 mL in total) were analyzed for macronutrient concentrations, and the final value was calculated as the mean of three measurements.

Analysis of leptin and ghrelin concentrations in human milk samples

For hormonal analysis, the HM samples were thawed at room temperature and centrifuged at 1,770×*g* and 4°C for 15 min using a 5702R centrifuge with an F-45-24-11 rotor (Eppendorf, Hamburg, Germany). The fat and cellular layers were removed, and the supernatant (skim milk) was used for hormone analysis. Leptin and ghrelin concentrations were determined using enzyme-linked immunosorbent assay (ELISA). All samples were processed under identical conditions and subjected to a single freeze–thaw cycle, and all assays were performed in duplicate.

Hormone concentrations were determined using commercial ELISA kits: human leptin ELISA DEE007 kit (Demeditec, Kiel, Germany; assay range: 0.25–100 µg/L; intra-assay variance ≤15%) and human ghrelin ELISA kit (cat. no. orb561916, Biorbyt, Cambridge, UK; assay range: 1.875–120 pg/mL; sensitivity: 1.125 pg/mL). Optical density was measured with a Synergy HTX multimode microplate reader (BioTek, Winooski, VT, USA), and data analysis was performed using Gen5 Data Analysis Software (BioTek). Concentrations of leptin and ghrelin were expressed in ng and pg *per* 1 mL of milk, respectively.

An attempt was also made to determine the concentrations of acylated ghrelin using a commercially available Bertin Bioreagent ELISA kit (#A05106.96, Bertin Technologies, Montigny-le-Bretonneux, France; assay range: 2–250 pg/mL). However, this assay did not yield reliable results in the analyzed human milk samples.

Statistical analysis

Continuous variables were expressed as mean and standard deviation (SD) for normally distributed or as median and SD for non-normally distributed data. The normal distribution of continuous variables was tested using the Shapiro-Wilk test. The categorical data were presented as numbers and percentages. Z-scores were derived based on child weight, age (in days), and sex, using the lambda-mu-sigma (LMS) method. Changes in milk compound concentrations over time were analyzed using a Bayesian hierarchical model with random intercepts for individual subjects and an autoregressive (AR1) correlation structure to account for repeated measurements. The associations between leptin and ghrelin concentrations and maternal characteristics (*e.g.*, body mass index, body weight, age and ΔWAZ) were evaluated using Bayesian multilevel regression models. All models were fitted in R using the *brms* package (v2.23.0; R Foundation for Statistical Computing, Vienna, Austria) [Bürkner, 2021], specifying a Student-*t* likelihood. Weakly informative priors were used for the fixed effects (Normal (0, 5)) and for the residual standard deviation (Student-*t* distribution with 3 degrees of freedom, mean 0, scale 10). Posterior distributions were estimated using four Markov chain Monte Carlo (MCMC) chains, each with 4,000 iterations, including 1,000 warm-up iterations. Convergence was assessed using R-hat statistics (<1.01), effective sample sizes, and visual inspection of trace plots. To facilitate interpretation, estimated marginal means and pairwise comparisons were obtained with the *emmeans* package (v1.11.2-8; R Foundation for Statistical Computing). Correlations between hormone concentration and milk parameters were assessed using Spearman's rank correlation. Trend analysis for milk compound concentrations over time was performed using the Mann-Kendall test. All statistical analyses were conducted using the R environment (v. 4.4.1; R Foundation for Statistical Computing).

RESULTS AND DISCUSSION

Characteristics of the study group

Maternal age averaged 32.0 years (**Table 1**). The mean pre-pregnancy weight was 62.4 kg, and the mean pre-pregnancy BMI was 22.4 kg/m^2. Sixteen women (75.0%) had normal weight, while four (25.0%) were classified as overweight. Mean gestational age at delivery was 40.0 weeks, and 9 participants (45.0%) delivered by cesarean section. Mean pregnancy weight gain was 13.9 kg. Regarding parity, 8 women (40.0%) were primiparous, 6 (30.0%) were in their second pregnancy, and 6 (30.0%) in their third pregnancy. Socio-economic status was reported as high in 1 participant (5.0%), middle in 13 (65.0%), and low in 6 (30.0%). Infant birth weight averaged 3,679 g (**Table 1**), and the calculated monthly weight gain ranged from 370 to 790 g *per* month across the observation period.

Human milk composition

The summary of the composition of HM over the first six months of lactation was presented in the Supplementary **Table S1**. Statistical analysis using the Mann-Kendall test revealed no significant temporal changes in the concentrations of key components, including energy value, and concentrations of total protein, true protein, fat, lactose, dry mass, leptin, and ghrelin (*p*-values ranging from 0.070 to 1.000). Mean leptin levels ranged from 0.30 to 0.41 ng/mL (p=0.603), while ghrelin concentrations varied between 23 and 30 pg/mL (p=1.000).

The HM leptin concentrations were in a similar range compared to other studies [de Fluiter *et al.*, 2021; Ilcol *et al.,* 2006; Schuster *et al.,* 2011; Yu *et al.*, 2018]; however, significantly higher than values reported by Bronsky *et al.* [2011] (0.1 ng/mL, 3 months postpartum), and on the other hand lower than in the American study [Young *et al.,* 2017] (7.1 ng/mL – for normal weight women, 4 months postpartum). Ghrelin concentrations in HM vary across studies, likely due to methodological differences [Andreas *et al.,* 2016; Aydin *et al.,* 2007; Cesur *et al.,* 2012]. In our study, the observed levels were lower than those reported by Yu *et al.* [2018] (147.25–381.88 pg/mL, 3 months postpartum) and Khodabakhshi *et al.* [2015] (133–156 pg/mL, 2 to 5 months postpartum). However, they were higher than those measured by Kon *et al.* [2014], who reported concentrations of 5.06 pg/mL at 2 months and 0.71 pg/mL at 3 months postpartum.

In our cohort, neither leptin nor ghrelin concentrations showed significant longitudinal changes between 1 and 6 months postpartum. This contrasts with several previous studies that described decreasing leptin concentrations over the course of lactation [Ilcol *et al.,* 2006; Schuster *et al.,* 2011] and increasing or changing ghrelin levels [Cesur *et al.,* 2012; de Fluiter *et al.,* 2021, Kon *et al.,* 2014]. The absence of a clear trend in our data may reflect the relatively small sample size, the homogeneous characteristics of the study population, or methodological differences such as sampling strategy and analytical techniques. Collectively, our findings indicate that the stability of these hormones during early lactation is also possible and highlight the heterogeneity of results reported in the literature.

Due to the absence of statistically significant trends observed for the analyzed factors, a comparative analysis was performed between specific time points. The changes in the total protein concentration throughout the lactation period were analyzed using Bayesian generalized (non-)linear multivariate multilevel models. The total protein at 3–4 weeks was significantly higher than at all other measured time points (**Figure 1A**). The difference between 3–4 and 11–12 weeks was estimated at 0.203 (95% higher posterior density, HPD: 0.107–0.304), with a posterior probability of the effect being greater than zero equal to 1, indicating very strong evidence for a true positive effect. Similarly, comparisons of 3–4 weeks with 7–8, 15–16, 19–20, and 23–24 weeks showed positive differences of 0.143 (0.054–0.233), 0.154 (0.057–0.255),

Table 1. General characteristics of the study group (*n*=20).

Parameter		Mean ± standard deviation	Median (quartile ranges)	Number (percentage)
Maternal characteristics				
Age (years)		32.0±4.8	34.0 (26.8–36.0)	
Pre-pregnancy weight (kg)		62.4±9.5	58.5 (55.0–70.0)	
Pre-pregnancy body mass index (kg/m^2)		22.4± 2.8	22.4 (20.3–23.9)	
Pre-pregnancy nutritional status: Normal weight/Overweight				16 (75%)/4 (25%)
Pregnancy weight gain (kg)		13.9±4.6	13.5 (10.8–15.5)	
Pregnancy order: 1st/2nd/3rd				8 (40%)/6 (30%)/6 (30%)
Socio-economic status: High/Middle/Low				1 (5%)/13 (65%)/6 (30%)
Prenatal data				
Gestational age (weeks)		40.0±1.0	40.0 (39.0–41.0)	
Mode of delivery: Vaginal/C-section				11 (55.0%)/9 (45.0%)
Infant characteristics				
Birth weight (g)		3,679±682	3,675 (3,375–3,810)	
Infant body weight (g)	Month 1	4,588±704	4,470 (4,268–4,900)	
	Month 2	5,377±806	5,255 (4,890–5,593)	
	Month 3	6,156±810	6,108 (5,788–6,453)	
	Month 4	6,744±810	6,650 (6,290–7,123)	
	Month 5	7,115±868	7,255 (6,768–7,823)	
	Month 6	7,728±882	7,785 (7,090–8,300)	

0.207 (0.113–0.307), and 0.254 (0.157–0.355), respectively. Comparisons among later weeks also revealed smaller but significant differences, including 7–8 *vs.* 23–24 weeks (0.110, 0.018–0.205) and 15–16 *vs.* 23–24 weeks (0.099, 0.011–0.189).

True protein concentration at 3–4 weeks was significantly higher than at all other time points (**Figure 1B**). The difference between 3–4 and 11–12 weeks was estimated at 0.169 (95% HPD: 0.090–0.250), with a posterior probability of the effect being greater than zero equal to 1, indicating very strong evidence for a positive effect. Comparisons of 3–4 weeks with 7–8, 15–16, 19–20, and 23–24 weeks also showed significant positive differences of 0.113 (0.043–0.186), 0.136 (0.056–0.218), 0.162 (0.084–0.239), and 0.202 (0.122–0.283), respectively. Among later weeks, 7–8 *vs.* 23–24 weeks revealed a weaker yet significant difference (0.088, 0.017–0.162).

Lactose concentration at 3–4 and 11–12 weeks was generally lower than at later time points (**Figure 1C**). Specifically, the difference between 3–4 and 19–20 weeks was estimated at −0.160 (95% HPD: −0.323 to −0.003), with a posterior probability of the effect being less than zero of 0.978, indicating strong evidence for a negative effect. Similarly, 3–4 *vs.* 23–24 weeks showed a difference of −0.151 (−0.301 to −0.006). Among later weeks, 11–12 *vs.* 19–20 weeks and 11–12 *vs.* 23–24 weeks also exhibited significant negative differences of −0.201 (−0.364 to −0.049) and −0.192 (−0.349 to −0.043), respectively.

A significant decrease in dry mass was observed between 15–16 and 19–20 weeks, with an estimated difference of −0.835 (95% HPD: −1.590 to −0.082), indicating strong evidence for a negative effect (**Figure 1D**).

Factors affecting human milk composition

A correlation analysis evaluated the relationship between nutritional composition, maternal factors, and hormone concentrations across six time points. Overall, no consistent statistically significant correlations were observed between energy, total or true protein, or milk fat concentrations and leptin or ghrelin levels across the study period (**Figure 2**). However, at the second measurement time point, a statistically significant

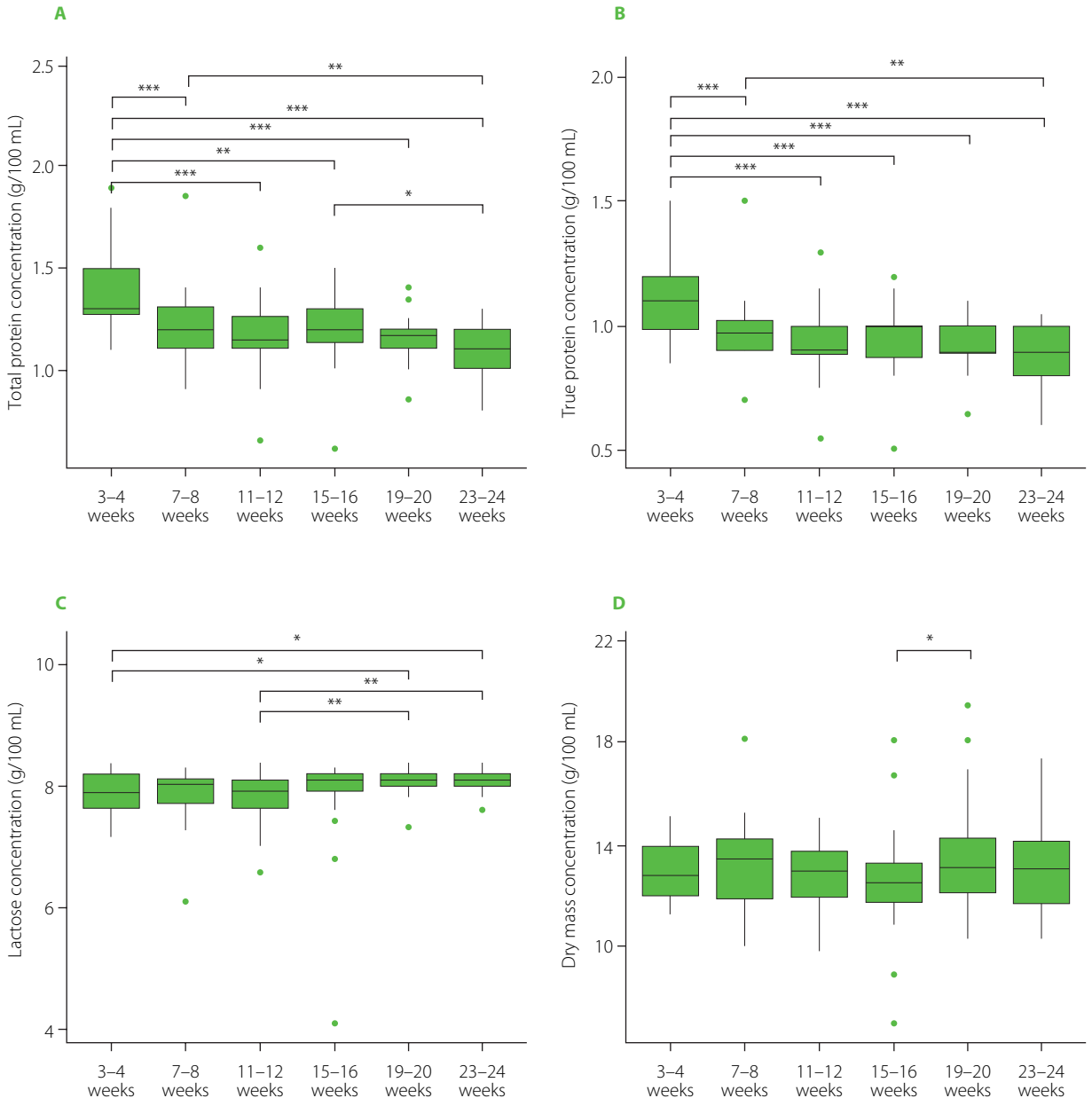

Figure 1. Concentrations of total protein (**A**), true protein (**B**), lactose (**C**), and dry mass (**D**) in human milk at different time points during the lactation period. In each boxplot, the central line represents the median, boxes indicate the interquartile range (IQR), and whiskers denote 1.5 × IQR. Asterisks indicate statistically significant differences between time points (*p<0.05, **p<0.01, ***p<0.001).

moderate negative correlation was identified between total and true protein concentrations and leptin levels. This inverse relationship may reflect distinct regulatory pathways influencing hormonal and macronutrient secretion in the mammary gland. Although limited, previous research has suggested that leptin and milk macronutrient content may be modulated by separate maternal metabolic signals [Lönnerdal, 2017; Qureshi *et al.,* 2024]. It is also possible that this association represents a transient physiological adaptation during early lactation. To the best of our knowledge, such a negative correlation has not been reported in the literature, which highlights the need for further studies to elucidate its biological relevance and reproducibility in more diverse populations. It is noteworthy that these associations were observed only during the second postpartum month, with no consistent patterns at other time points, possibly reflecting transient regulatory mechanisms in lactation.

The association between BMI and ghrelin was evaluated using a Bayesian multilevel regression model, adjusting for relevant covariates and repeated measures. The results indicated a statistically significant positive correlation between BMI and ghrelin levels, with higher standardized BMI values associated with higher ghrelin concentrations (posterior mean = 5.63, 95% credible interval from 1.86 to 9.32). None of the other maternal factors (*e.g.*, age, total weight gain during pregnancy) was correlated with HM leptin and/or ghrelin concentrations.

Figure 2. The correlation matrix between human milk nutritional composition, hormone (leptin, ghrelin) concentrations, and maternal pre-pregnancy body mass index (BMI) at six-time points: first month (**A**), second month (**B**), third month (**C**), fourth month (**D**), fifth month (**E**) and sixth month (**F**) of the lactation. ρ, Spearman rank correlation coefficient. Asterisks indicate statistically significant correlations (*p<0.05, **p<0.01, ***p<0.001).

Guler *et al.* [2022] reported that obese mothers (*n*=20) had significantly higher ghrelin levels in foremilk compared to those with normal weight (*n*=20; *p*=0.025) at 2 months postpartum, which aligns with our findings. Conversely, Yu *et al.* [2018] examined ghrelin concentrations in colostrum and mature milk collected on postpartum days 42 and 90 and reported an inverse association between maternal BMI – both pre-pregnancy (*p*=0.031) and during lactation (*p*<0.001) – and ghrelin levels. Meanwhile, Khodabakhshi *et al.* [2018] examined the correlation between maternal body composition and hormone levels in 80 participants but found no significant association between maternal fat mass or BMI and ghrelin concentrations in HM. Notably, their study assessed hormone levels at 6 months postpartum. Collectively, these findings suggest a complex relationship between maternal anthropometric parameters and ghrelin concentrations in HM, which may be influenced by the timing of milk collection and lactation stage.

We identified only one study [Chan *et al.,* 2018] in which maternal age was analyzed as a potential factor that impacts HM leptin concentration. The authors reported that HM leptin level was lower in older women (aged 35–44 years) than those <35 years (*p*=0.03). Numerous studies have explored the relationship between maternal weight and BMI as potential factors influencing leptin concentrations in HM [Chan *et al.,* 2018; Christensen *et al.,* 2022; Cortés-Macías *et al.,* 2023; Khodabakhshi *et al.,* 2018; Larsson *et al.,* 2018; Lemas *et al.,* 2016; Sadr Dadres *et al.,* 2019]. Lemas *et al.* [2016] reported that at two weeks postpartum in obese mothers (*n*=12), HM leptin concentration was twice as high as that of normal-weight mothers (*n*=18). Similarly, Young *et al*. [2017] observed consistently elevated leptin concentrations in HM among 22 overweight or obese mothers compared to 26 mothers of normal weight at both two weeks and four months postpartum. A literature review by Qureshi *et al.* [2024] indicated that 20 out of 21 studies found a positive correlation between maternal BMI and leptin levels in HM. Interestingly, in the remaining study, the authors reported a negative correlation in normal-weight mothers (*p*=0.03) [Chan *et al.,* 2018].

Leptin and ghrelin concentrations and infants' growth

Figure 3 illustrates the infant weight gain within the first six months of life and HM leptin and ghrelin concentrations, showing no apparent trends or consistent associations. Within the first six months of life, no evidence was found for an association between HM concentrations of leptin or ghrelin and short-term changes in infant growth. In Bayesian mixed-effects models adjusting for age interval and infant sex and accounting for repeated measurements within infants, the overall association between HM leptin concentration and changes in infant weight-for-age z-scores (ΔWAZ) was weak and not statistically significant (posterior mean = −0.39, 95% credible interval from −1.33 to 0.50). In additional sensitivity analyses, we allowed the association between leptin concentration and ΔWAZ to differ across specific age intervals. These analyses produced similar findings, as the age-specific posterior estimates remained imprecise and their credible intervals consistently included zero. Similarly, HM ghrelin concentration was not associated with ΔWAZ in Bayesian mixed-effects models adjusted for age interval and infant sex (posterior mean = −0.04, 95% credible interval from −0.43 to 0.36). Models permitting age-specific effects did not reveal consistent associations at any measurement interval.

Spearman rank correlation analyses supported these findings. No statistically significant correlations were observed between infants' ΔWAZ and HM leptin. Interval-specific Spearman correlations between hormone concentrations and ΔWAZ were weak and non-significant across most time points (**Figure 4** and **Figure 5**). Although a moderate positive correlation between HM ghrelin concentration and ΔWAZ was observed at 7–8 weeks of age (ρ=0.45, *p*=0.049), this isolated result was not supported by the mixed-effects models.

The relationships between leptin and ghrelin levels in human milk and infants' growth patterns have also been explored in other studies [Christensen *et al.,* 2022; Cortés-Macías *et al*., 2023; Khodabakshi *et al.,* 2018; Kugananthan *et al*., 2017]. Interestingly, some studies have suggested that HM leptin may influence infant gut microbiome development, potentially contributing indirectly to growth outcomes [Lemas *et al.,* 2016]. A study involving 18 normal-weight mothers and 12 obese mothers, along with their exclusively breastfed infants, demonstrated that higher HM leptin concentrations at 2 weeks postpartum were associated with lower bacterial protease activity, a marker of gastrointestinal inflammation in infants [Lemas *et al.,* 2016]. Considering these results, the authors suggested that leptin may play a significant role in infant growth and should be considered within the broader framework of factors influencing early development. In turn, a recent study by Kebbe *et al.* [2024] reported no significant associations between HM leptin and the infant gut microbiome, highlighting the need for cautious interpretation. Taken together, these findings indicate that while HM leptin may play a role in infant growth, its effects could be mediated by multiple pathways, including but not limited to direct anthropometric outcomes.

Our study did not find any relationship between maternal pre-pregnancy BMI and infants' weight gain during the first six months of life. In turn, Chan *et al.* [2018] reported that elevated HM leptin concentrations were correlated with a lower infant weight-for-length z-score at both 4 months (β of −0.65, 95% CI (confidence interval): −1.13, −0.16; *p*=0.0009 for highest *vs*. lowest quintile) and 1 year of age (β of −0.58, 95% CI: −1.02, −0.14; *p*=0.0009 for highest *vs*. lowest quintile) after adjusting for factors such as multiparity and exclusive breastfeeding, which were themselves associated with reduced leptin levels in HM. In contrast, an earlier study [Khodabakhshi *et al.,* 2015] comparing exclusively breastfed overweight/obese infants (*n*=40) and normal-weight infants (*n*=40) found no significant differences (*p*>0.05) in exposure to HM leptin concentrations between 2 and 5 months postpartum. Additionally, the relationship between HM hormones and the infant gut microbiome has been explored.

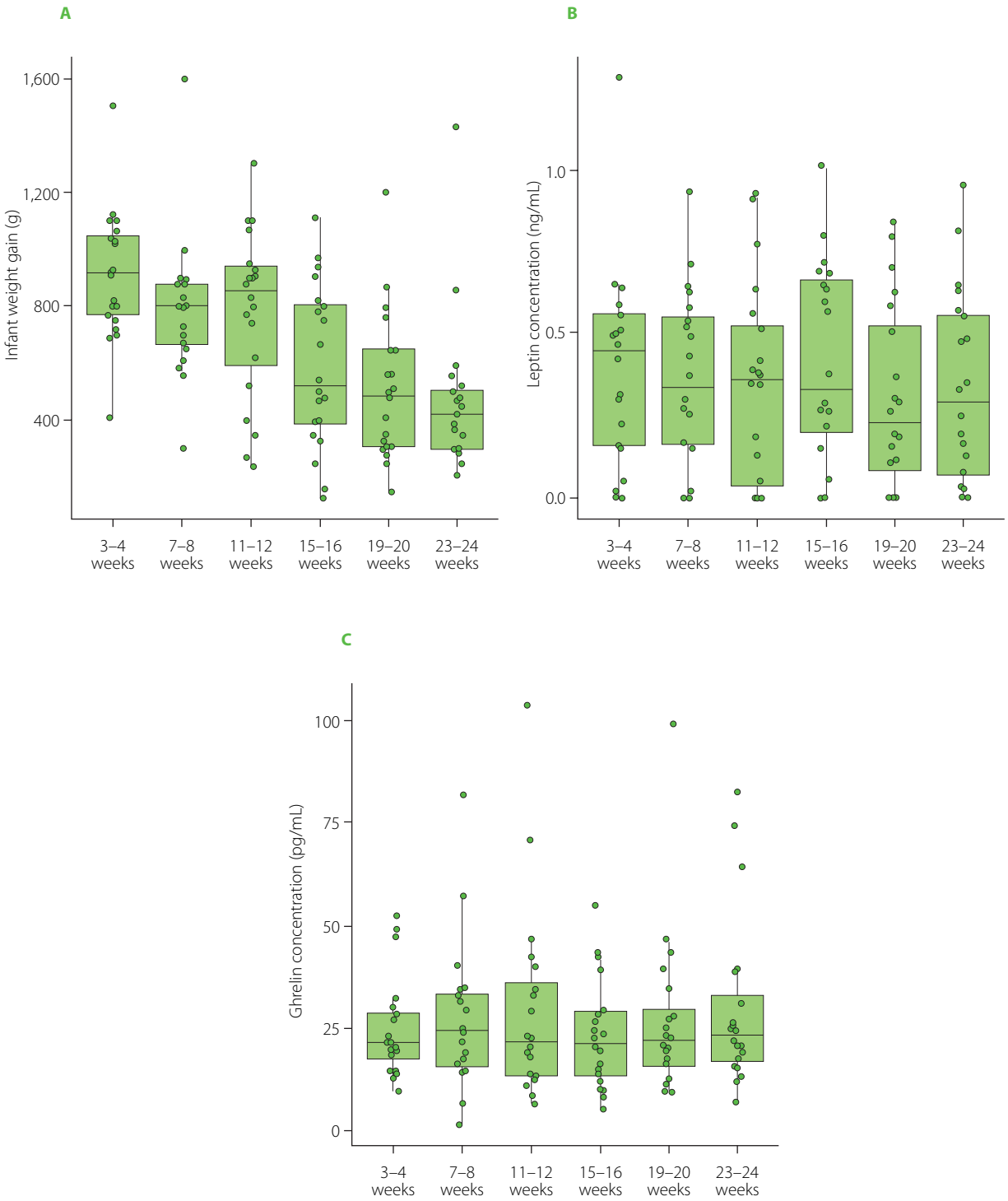

Figure 3. Infants' weight gain during the first six months of life (**A**), and leptin (**B**) and ghrelin (**C**) concentrations in milk collected from lactating mothers. In each boxplot, the central line represents the median, boxes indicate the interquartile range (IQR), and whiskers denote 1.5 × IQR.

As an appetite-stimulating hormone, ghrelin, is thought to influence feeding behavior and infant weight gain. Cesur *et al.* [2012] reported a significant positive correlation ($p<0.05$) between HM ghrelin levels at 4 months postpartum and infant weight gain during the same period. Similarly, Kon *et al.* [2014] found that at the first month of lactation, ghrelin levels in HM were significantly higher in a group of infants with normal weight gain ($n=40$) compared to those with low weight gain ($n=18$). However, in a group of infants with high weight gain ($n=45$), ghrelin concentrations were lower than in those with normal weight gain. These findings contrast with our study, where no significant association was observed between infant weight gain and ghrelin concentrations in HM. Additionally, Yu *et al.* [2018] reported no relationship between total ghrelin levels and infant head circumference or weight-for-height z-score. Interestingly, formula-fed infants ($n=100$) have been shown to exhibit

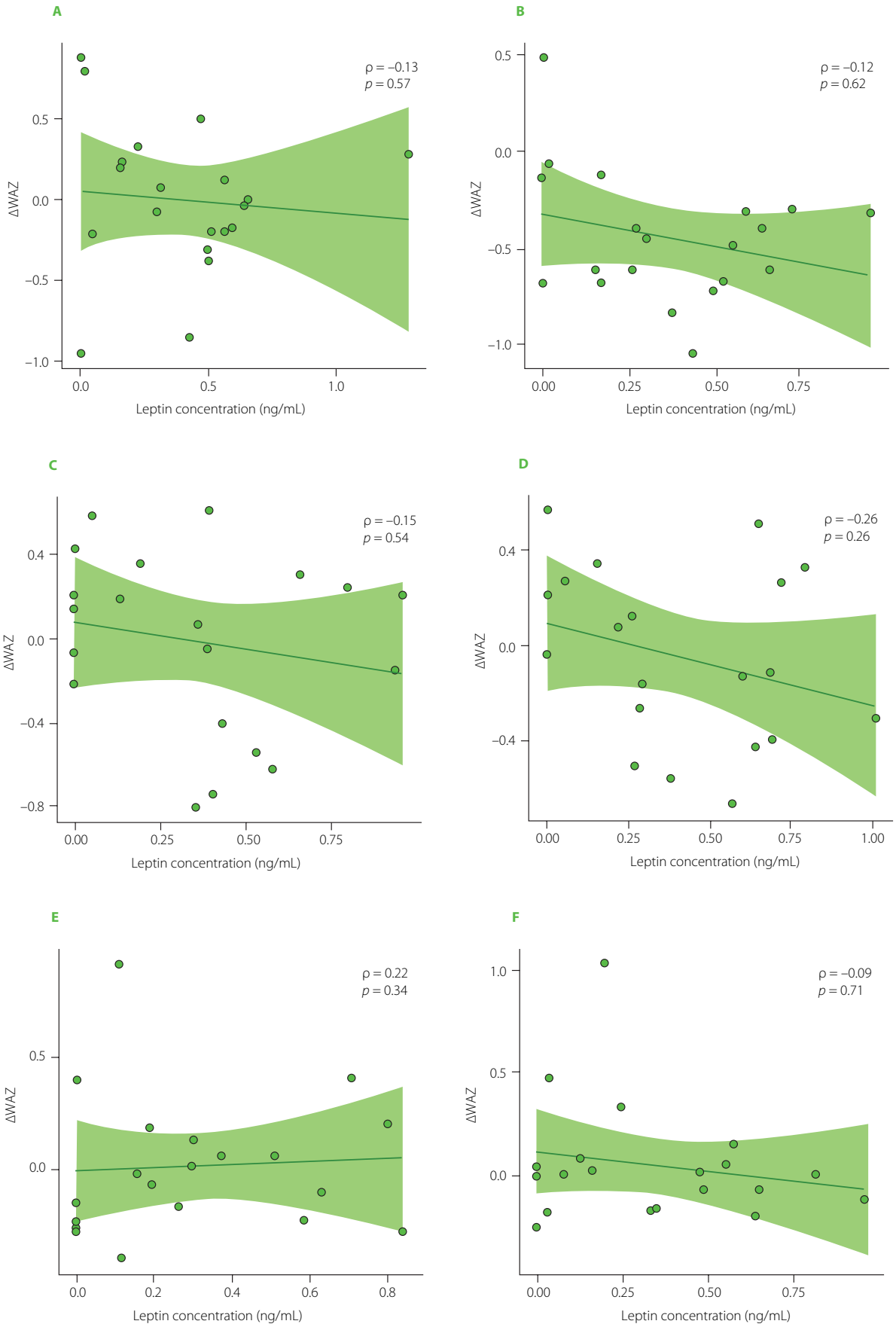

Figure 4. Spearman rank correlations between leptin concentration and changes in weight-for-age z-scores (ΔWAZ) at six time points during lactation: first month (**A**), second month (**B**), third month (**C**), fourth month (**D**), fifth month (**E**), and sixth month (**F**). The Spearman rank correlation coefficient (ρ) and the corresponding *p*-value are shown in the corner of each graph.

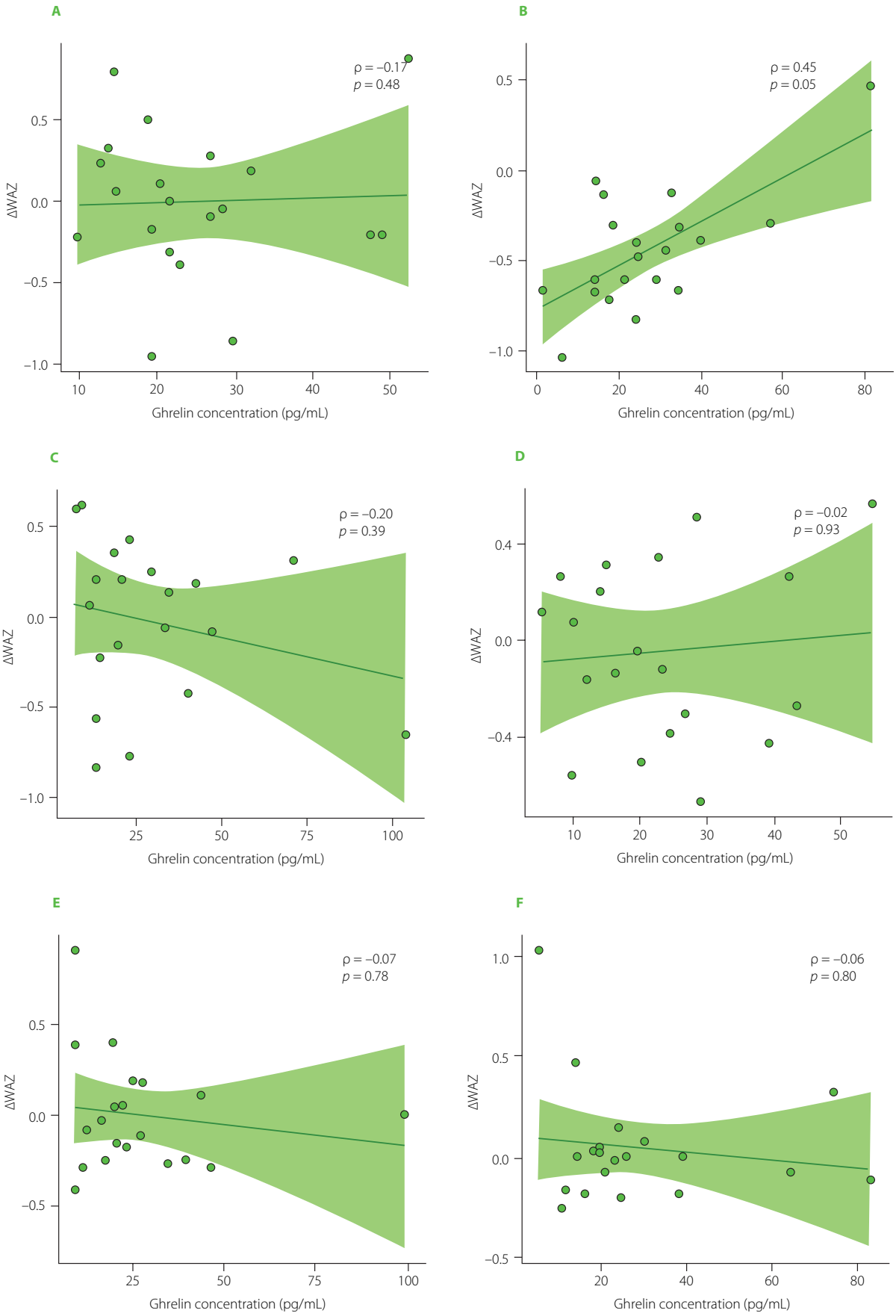

Figure 5. Spearman rank correlations between ghrelin concentration and changes in weight-for-age z-scores (ΔWAZ) at six time points during lactation: first month (**A**), second month (**B**), third month (**C**), fourth month (**D**), fifth month (**E**), and sixth month (**F**). The Spearman rank correlation coefficient (ρ) and the corresponding *p*-value are shown in the corner of each graph.

higher plasma ghrelin levels than breastfed infants (n=106). While anthropometric measurements did not differ between these groups, Savino *et al.* [2005] found a negative correlation between ghrelin and BMI in formula-fed infants, suggesting that the feeding mode may play a role in ghrelin regulation and its impact on metabolic programming.

Beyond methodological considerations, it is also important to acknowledge the conceptual limitations of interpreting human milk hormone research based solely on concentration measurement. It should be noted that hormone concentration may not accurately represent the dose ingested by the infant when the exact volume of HM consumed is not measured. What is more, leptin and ghrelin levels capture only one potential bioactivity, while they do not account for different molecular forms (*e.g.*, acylated *vs*. deacylated ghrelin), binding to carrier proteins, or the influence of milk fraction (foremilk *vs*. hindmilk) on bioavailability [Karatas *et al.,* 2011]. Moreover, the ultimate effect is shaped by interactions with other bioactive milk components and by infant-specific factors, such as enzymatic modification in the gastrointestinal tract, epithelial transport, and receptor expression, which can modulate absorption and activity [Lönnerdal *et al.,* 2017]. Discrepancies among studies may therefore reflect not only methodological differences (*e.g.*, assay sensitivity, timing of collection) [Andreas *et al.,* 2016; Suwaydi *et al*., 2023] but also these broader biological influences. Importantly, previous reviews emphasize that maternal metabolic status and systemic hormone levels may act as mediators between milk composition and infant outcomes [Qureshi *et al.,* 2024]. In addition, milk hormones may interact with other milk-derived signals, such as the microbiome and immune factors, influencing infant development beyond direct hormone exposure [Lemas *et al.,* 2016]. To better clarify these relationships, future research should complement concentration data with analyses of hormone isoforms and functional assessments (*e.g.*, digestion stability, bioactivity in cell-based models), incorporate simultaneous maternal plasma and infant biomarkers, and work toward standardized sampling and reporting protocols. Such multidimensional approaches will strengthen biological validity and improve comparability across studies.

Strengths and limitations

One of the main strengths of this study is its longitudinal design, with six standardized milk collection time points within the first six months of lactation. All samples were collected in the morning under controlled conditions, with consistent handling and storage protocols, reducing potential pre-analytical variability. Macronutrient analysis was performed using validated method, allowing reliable results of nutritional milk composition. Moreover, hormone measurements were performed using ELISA kits intended for use with various liquid matrices, following prior adaptation of the method to human milk samples. However, our findings must be interpreted considering certain limitations. The relatively small sample size and the homogeneous study population (all participants were highly educated and from a single geographic area) may limit the extent to which the findings can be generalized. Small sample size restricted statistical power and may have contributed to the absence of significant associations. Recruitment was limited to mothers exclusively breastfeeding up to six months postpartum, a population group that is relatively rare in Poland, which further constrained sample size. Additionally, while we identified some correlations at specific time points, these may reflect transient or chance findings rather than consistent physiological patterns. A further limitation is that, although leptin and ghrelin concentrations were quantified in HM, individual milk intake volumes were not measured. Thus, we could not estimate infants' total hormonal exposure, which may have influenced the observed associations. Another notable limitation of this study is the lack of data on more nuanced growth indicators, such as weight-for-length z-scores and BMI-for-age, which restricts the depth of our growth assessments and their interpretability. Furthermore, foremilk and hindmilk fractions were not analyzed separately, as pooled samples were used to reflect average hormonal exposure during feeding; therefore, potential differences in leptin and ghrelin concentrations between milk compartments could not be assessed. Finally, although we assessed several maternal and infant variables, other unmeasured factors, such as maternal diet, stress, and metabolic markers, may also influence milk composition and merit further investigation in future studies.

CONCLUSIONS

In this longitudinal study, leptin and ghrelin concentrations in human milk remained relatively stable during the first six months of lactation, and no association was detected in this cohort with the available infant growth metrics (including weight gain). These findings suggest that, within the limits of our sample and the absence of milk intake data, HM leptin and ghrelin are unlikely to be major independent determinants of early postnatal weight accretion.

However, given the well-established physiological roles of these hormones in appetite regulation, gut maturation, and metabolic signaling, and considering that their biological effects may not be fully reflected by concentration measures alone, further studies in larger and more diverse populations, incorporating functional and mechanistic outcomes, are advised to clarify their potential contributions to infant development and metabolic programming.

RESEARCH FUNDING

The study did not receive any external funding.

CONFLICT OF INTERESTS

None of the authors declares any conflict of interest, and all have approved the final version of the manuscript.

INFORMED CONSENT

The study adhered to rigorous ethical standards, consistent with the Declaration of Helsinki. The protocol was approved by the Bioethics Committee of the Medical University of Białystok

(approval no. AKP.002.501.2021). All participants were informed about the research objectives and provided written informed consent before enrollment

SUPPLEMENTARY MATERIALS

The following are available online at **https://journal.pan.olsztyn.pl/Analysis-of-Human-Milk-Leptin-and-Ghrelin-in-Relation-to-Maternal-Factors-and-Infant,218306,0,2.html**; **Table S1**. Human milk composition over the first six months of lactation.

ORCID IDs

A. Bień **https://orcid.org/0000-0002-5268-6900**
A. Bzikowska-Jura **https://orcid.org/0000-0001-5181-3771**
D. Gutkowska-Kawka **https://orcid.org/0009-0009-6074-1357**
M.M. Kaczmarek **https://orcid.org/0000-0002-8213-4381**
B. Kulesza-Brończyk **https://orcid.org/0000-0002-1859-018X**
T. Molcan **https://orcid.org/0000-0002-0380-0156**
A. Mołas **https://orcid.org/0000-0002-2680-9946**
A. Wesołowska **https://orcid.org/0000-0002-7270-5910**

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Cultivar- and Growth-Stage-Dependent Variability of Saponins in Roots and Leaves of *Beta vulgaris* L. Characterized by Liquid Chromatography Coupled with Mass Spectrometry

Anna Tekieli[1]†, Kamila Frącek[1]†, Giorgia Perelli[2]†, Szymon Janecki[1], Aneta Spórna-Kucab[1]*

[1]Department of Chemical Technology and Environmental Analysis, Faculty of Chemical Engineering and Technology, Cracow University of Technology, Warszawska 24, 31-155 Kraków, Poland
[2]Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy

Beta vulgaris L. is an important food crop and a rich source of bioactive triterpene saponins. This study evaluated cultivar- and growth-stage-dependent variability of saponins in leaves and roots of three beet cultivars (Round Dark Red, Cylindra, and Snow Ball) and in leaves of Swiss chard (Rhubarb Chard), harvested at seven harvest dates between June and September 2024. Saponins were identified and quantified using liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS). A total of 32 triterpene saponins representing oleanane-type, akebonoic acid-, hederagenin-, and gypsogenin-derived aglycones were detected. Pronounced organ-dependent differences were observed. Total saponin content ranged from 386 to 10,414 mg/kg fresh weight (FW) in leaves and from 1,170 to 23,298 mg/kg FW in roots. Two-way analysis of variance confirmed highly significant effects of cultivar, harvest time, and their interaction on total saponin levels in both leaves and roots (all $p<0.0001$). Roots exhibited a broader content range and a pronounced mid-season maximum, whereas leaf saponin levels generally peaked in the mid-to-late season in a genotype-dependent pattern. Major saponins (Act-UrA--akebonoic acid and betavulgarosides II, III, IV, and VII) predominated in the quantitative profile and exhibited coordinated seasonal variation. Multivariate analyses (principal component analysis and hierarchical cluster analysis) clearly separated samples according to plant organ and further resolved cultivar- and season-related patterns. Overall, saponin accumulation in *B. vulgaris* is strongly regulated by organ type, genotype, and growth stage, emphasizing the importance of cultivar selection and harvest timing for maximizing bioactive potential.

Keywords: oleanane-type aglycones, plant metabolomics, seasonal variation, secondary metabolites, triterpene glycosides

INTRODUCTION

Beta vulgaris L. is a plant of considerable economic and nutritional importance, comprising four main cultivated forms: red beet, Swiss chard, fodder beet, and sugar beet [Mroczek, 2015]. It is a rich source of bioactive compounds, including polyphenols, betalains, and triterpene saponins, which are increasingly recognized for their health-promoting and disease-preventive properties [Biswas & Dwivedi, 2019]. Among these compounds, triterpene saponins have attracted considerable attention owing to the broad range of their biological activities, including antiviral, anticancer, antimicrobial, anti-inflammatory, antihypertensive, and antidiabetic effects [Jolly *et al*., 2024; Kaur *et al*., 2024].

***Corresponding Author:**
e-mail: **aneta.sporna-kucab@pk.edu.pl** (Prof. A. Spórna-Kucab)
†These authors had equal contribution to this work

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Saponins are composed of a sugar moiety (glycone) and an aglycone, also referred to as sapogenin. Their classification is primarily based on the structure of the aglycone – which may have either a triterpenoid or steroidal skeleton – as well as on the number and type of sugar units present in the glycone [Vincken *et al*., 2007]. The glycone consists of a linear or branched oligosaccharide chain, typically containing hexoses and pentoses such as glucose, galactose, arabinose, rhamnose, or xylose, and may also include uronic acids, most commonly glucuronic acid [Jolly *et al*., 2024].

The saponin composition and content in plants exhibit substantial variability and depend on multiple factors, including species, plant organ, environmental conditions, and developmental stage. Environmental stressors, such as soil salinity, drought, nutrient deficiencies, and elevated temperatures, can markedly reduce saponin levels [Kaur *et al*., 2024; Sharma *et al*., 2022]. In addition, saponin accumulation is strongly species- and growth-stage-dependent: in tea plants, saponin levels increase during early maturation and later stabilize, whereas in yam and *Lycium barbarum* peak accumulation occurs during tuber development or fruiting stages [Kregiel *et al*., 2017; Yu & He, 2018]. This highlights the need to consider the biological and environmental context when interpreting saponin variability.

Previous research on *B. vulgaris* saponins has primarily focused on their composition in mature plants and across different cultivars, whereas information on their variation across different growth stages is currently lacking [Mikołajczyk-Bator *et al*., 2016a, 2024; Mroczek *et al*., 2012, 2019, 2021]. The aim of the present study was to investigate organ-, cultivar-, and growth-stage-dependent variation in saponin content in *B. vulgaris* using liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS), with particular emphasis on seasonal changes in total and individual saponins.

MATERIALS AND METHODS

Solvents and reference compounds

Respective standards from a previous study on *B. vulgaris* (Red Sphere cultivar) were used for saponin identification [Spórna-Kucab & Wybraniec, 2020], along with extracts of red, yellow, and white *B. vulgaris* cultivars – Ceryl, Chrobry, Forono, Tytus, and Boldor [Spórna-Kucab *et al*., 2022]. Oleanolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography–mass spectrometry (LC–MS)-grade acetonitrile and formic acid (purity ≥98%) were obtained from Sigma-Aldrich. Acetone was purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). All chemicals and solvents were of analytical grade and used as received. Water utilized throughout the experiments with a resistivity of 18.0 mΩ/cm at 295 K was deionized through a Milli-Q purification system (Merck Millipore, Burlington, MA, USA).

Plant materials

Roots and leaves of *Beta vulgaris* L. cultivars Round Dark Red, Cylindra, and Snow Ball, as well as leaves of Swiss chard (cv. Rhubarb Chard), were collected from plants grown under standard agronomic conditions in a temperate climate zone on fertile, neutral soil in Zręcin, southeastern Poland, without the application of fertilizers. Seeds of Rhubarb Chard and the white cultivar Snow Ball were purchased from Torseed (Toruń, Poland), while seeds of the red cultivars Cylindra and Round Dark Red were obtained from Legutko (Jutrosin, Poland).

Plants were harvested at two-week intervals from June to September 2024. At each harvest date, plant material was collected from multiple individual plants *per* cultivar, constituting independent biological replicates. Immediately after harvesting, fresh roots and leaves were washed, weighed, and subjected directly to extraction.

Preparation of plant extracts

The roots and leaves of beets were individually blended in a household blender. A 100-g portion of each sample was extracted by maceration with 400 mL of 50% (*v/v*) acetone for 30 min at room temperature. The extraction was performed three times using a fresh portion of the solvent for each plant part. After each extraction, the resulting extracts were combined, partially concentrated at 25°C under reduced pressure, and then freeze-dried. Finally, the freeze-dried extracts were weighed and used for further studies on the quantitative and qualitative profile of saponins by the LC–ESI-MS/MS system. All extraction and analytical steps were carried out identically for all biological replicates.

LC–ESI-MS/MS – instrumentation and conditions

Analyses were conducted by means of high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry on an LCMS-8030 system (Shimadzu, Kyoto, Japan), equipped with a DGU-20A5R degasser, CBM-20A controller, Nexera LC-20ADXR binary pump, and SIL-20ACXR autosampler, operated *via* LabSolutions software (version 5.60 SP1, Shimadzu). Chromatographic separation was performed on a Kinetex C_{18} column (150×4.6 mm, 5.0 µm) with a matching guard column (Phenomenex, Torrance, CA, USA), maintained at 40°C. The flow rate was set to 0.5 mL/min. A binary mobile phase consisting of 2% (*v/v*) aqueous formic acid (solvent A) and acetonitrile (solvent B) was used. For saponin analysis, the gradient program was as follows: 38–60% B for 15 min, 60–99% B for 1 min, 99% B for 2 min, followed by re-equilibration to 38% B; the total run time was 19 min. For oleanolic acid, the gradient consisted of 20–99% B for 7 min, 99% B for 8 min, and re-equilibration to 20% B, giving a total run time of 19 min.

Tandem mass spectrometry (MS/MS) analysis was performed on a triple-quadrupole mass spectrometer with an electrospray ion source. The following ESI-MS/MS parameters were applied: curved desolvation line (CDL) and heat-block temperatures of 230°C, nebulizing gas flow of 1.5 L/min, electrospray voltage of 4.5 kV, and capillary temperature of 250°C, with nitrogen used as nebulizing and drying gas. Collision-induced dissociation (CID) was carried out with argon as the collision gas, using a collision

energy of 35 V. Data were acquired in a negative-ion mode using full-scan (*m/z* 100–2,000) and selected-ion monitoring (SIM).

Analysis of saponins

Prior to instrumental analysis of saponins, 10 mg of each freeze-dried extract were dissolved in 200 µL of demineralized water and centrifuged at 3,000×*g* for 5 min using microcentrifuge type 320 (UNIPAN, Warsaw, Poland). Subsequently, 150 µL of the supernatant were collected for further analysis. Aliquots of 15 µL of each sample and 5 µL of the standard solution were injected into the LC–ESI-MS/MS system.

Each extract, originating from an independent biological replicate, was analyzed in three independent technical replicates. Saponin identification was based on characteristic MS/MS fragmentation patterns, accurate mass data, and comparison with data previously reported for *B. vulgaris* triterpene saponins [Tekieli *et al*., 2026]. Diagnostic aglycone fragment ions and neutral losses of sugar moieties were used for structural assignment. Individual saponins as well as the total saponin content were quantified using an external calibration curve prepared with oleanolic acid in the concentration range of 1.25–20.0 µg/mL. Oleanolic acid was selected as the calibration standard because it represents the aglycone of the major oleanane-type saponins reported in *B. vulgaris*, including betavulgarosides, and due to the limited availability of individual saponin reference standards.

The calibration curve, constructed based on MS peak area *vs.* concentration, exhibited excellent linearity (R^2=0.9996). The content of saponins was expressed as mg of saponin *per* kg of fresh weight of root or leaf material (mg/kg FW). All samples were processed under identical analytical conditions to ensure comparability.

Statistical analysis

All quantitative results are presented as mean and standard deviation (SD) calculated from two independent biological replicates (material collected from different individual plants for each cultivar, organ, and harvest date). Each biological replicate was independently extracted and analyzed in three technical LC–ESI-MS/MS replicates; technical replicates were averaged prior to statistical analysis.

The effects of cultivar, harvest time, and their interaction on the total saponin content and on the contents of individual saponins were evaluated using two-way analysis of variance (ANOVA), performed separately for leaves and roots. When significant main effects or interactions were detected, post hoc multiple comparisons were conducted using Tukey's honestly significant difference (HSD) test. A significance level of $p \leq 0.05$ was applied. Normality of residuals was verified using the Shapiro-Wilk test, and homogeneity of variances was assessed prior to ANOVA.

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were applied as exploratory multivariate tools to visualize relationships among samples based on the full set of 32 quantified saponins. Statistical analyses were conducted using RStudio (version 2023.13.0+386, Posit Software, PBC, Boston, MA, USA).

RESULTS AND DISCUSSION

Identification of triterpene saponins in *B. vulgaris*

In the present study, LC–ESI–MS/MS analysis of extracts from four *B. vulgaris* cultivars (Round Dark Red, Cylindra, Rhubarb Chard, and Snow Ball) revealed a total of 32 triterpene saponins, reflecting substantial diversity in aglycone structures and sugar moieties, including uronic acid (UrA), pentoses (Pen), hexoses (Hex), and substituted acetal (Act) and dioxolane (Diox) residues. The detected saponins comprised oleanane-type, akebonoic acid–based, hederagenin- and gypsogenin-derived aglycones. Because closely related oleanane-type aglycones may generate highly similar MS/MS fragmentation patterns, aglycone assignments based solely on LC–ESI-MS/MS data were discussed at the oleanane-type level unless supported by authentic standards or previously reported nuclear magnetic resonance (NMR) data, particularly in the case of features consistent with GOTCAB-type (glycosides of oleanane-type triterpenoid carboxylic acid bisdesmosides) structures [Arslan, 2017, 2020; Arslan & Cenzano, 2021]. Most compounds were identified using precursor ion masses, MS/MS spectra, and reference standards, while the remaining features were tentatively annotated based on literature fragmentation data. Detailed analytical parameters are summarized in **Table 1**. Representative chemical structures of the dominant and structurally confirmed saponins identified in the investigated cultivars are shown in **Figure 1**.

Oleanane-type saponins

Oleanane-type saponins predominated in the saponin profile and represented the most structurally diverse group, comprising 20 detected compounds (**Table 1**). Oleanane-type cores were detected predominantly in glycosylated form, as indicated by a characteristic neutral loss of 176 Da corresponding to a uronic acid residue [Mroczek *et al*., 2012, 2019; Spórna-Kucab & Wybraniec, 2020; Wen *et al*., 2023]. The simplest oleanane-type saponins (**31** and **32**; *m/z* 631) showed diagnostic fragments at *m/z* 455 and a neutral loss of 176 Da, which was consistent with a uronic acid–substituted oleanane core, and were confirmed by comparison with reference compounds previously isolated from *B. vulgaris*.

The structural diversity of oleanane-type saponins is largely generated by modifications of the core unit composed of an oleanane-type aglycone and uronic acid (*m/z* 631), which varies through the attachment of additional sugar residues. Accordingly, several structural variants were identified, including mono- and diglycosylated derivatives bearing pentose, hexose, dioxolane, or acetal substituents (saponins **5** and **26–30**). Selected structures were confirmed using reference standards (saponins **26–30**) or literature data (saponin **5**).

Oleanane-type saponins bearing two sugar groups were observed in several configurations, including Hex–Pen (saponins **12**

Table 1. Chromatographic and mass-spectrometric data of identified saponins in the *Beta vulgaris* L. extracts.

No.	Saponin	Trivial name	Molecular formula	t_R (min)	*m/z* $[M-H]^-$	*m/z* from MS^2 of $[M-H]^-$
1	Hex-Hex-Hex-UrA-oleane-type		$C_{53}H_{82}O_{25}$	5.59	1,117	955; 793; 631; 455
2	Diox-Hex-UrA-hederagenin		$C_{47}H_{70}O_{21}$	6.20	969	925; 867; 849; 809; 647; 471
3	Act-Hex-Hex-UrA-oleane-type	Betavulgaroside V	$C_{53}H_{82}O_{25}$	6.30	1,117	997; 955; 835; 793; 455
4	Act-Hex-UrA-akebonoic acid	Betavulgaroside VIII	$C_{46}H_{68}O_{20}$	6.36	939	819; 777; 657; 615; 553; 439
5	Hex-UrA- oleane-type		$C_{42}H_{66}O_{14}$	6.80	793	673; 631; 569; 455
6	Act-Hex-Pen-UrA-oleane-type	Betavulgaroside IX	$C_{52}H_{80}O_{24}$	6.80	1087	967; 925; 805; 763; 455
7	Act-Hex-Hex-UrA-oleane-type	Betavulgaroside V	$C_{53}H_{82}O_{25}$	7.00	1,117	997; 955; 835; 793; 455
8	Act-Hex-UrA-oleane-type	Betavulgaroside III	$C_{47}H_{72}O_{20}$	7.80	955	835; 793; 673; 631; 569; 455
9	Hex-Pen-UrA-oleane-type		$C_{47}H_{74}O_{18}$	7.84	925	805; 763; 701; 569; 455
10	Hex-UrA-hederagenin		$C_{42}H_{66}O_{15}$	8.00	809	749; 689; 647; 629; 585; 471
11	Diox-Hex-UrA-oleane-type	Betavulgaroside I	$C_{47}H_{70}O_{20}$	8.00	953	909; 851; 793; 631; 455
12	Hex-Pen-UrA-oleane-type		$C_{47}H_{74}O_{18}$	8.30	925	805; 763; 701; 569; 455
13	Act-Hex-UrA-akebonoic acid	Betavulgaroside VIII	$C_{46}H_{68}O_{20}$	9.24	939	819; 777; 657; 615; 553; 439
14	Hex-Pen-UrA-akebonoic acid	Betavulgaroside X	$C_{46}H_{70}O_{18}$	10.00	909	789; 747; 685; 597; 553; 439
15	Pen-UrA-hederagenin		$C_{41}H_{64}O_{14}$	10.20	779	647; 629; 585; 567; 471
16	Act-UrA-hederagenin	Betavulgaroside VII	$C_{41}H_{62}O_{16}$	10.40	809	689; 647; 471
17	Act-Hex-UrA-oleane-type	Betavulgaroside III	$C_{47}H_{72}O_{20}$	10.60	955	835; 793; 673; 631; 569; 455
18	UrA-hederagenin		$C_{36}H_{56}O_{10}$	10.70	647	615; 535; 471
19	Act-Hex-UrA-oleane-type	Betavulgaroside III	$C_{47}H_{72}O_{20}$	11.40	955	835; 793; 673; 631; 569; 455
20	Pen-UrA-akebonoic acid		$C_{40}H_{60}O_{13}$	11.74	747	615; 535; 439
21	Act-Pen-UrA-oleane-type		$C_{46}H_{70}O_{19}$	11.84	925	763; 569; 631; 455
22	Act-UrA-akebonoic acid		$C_{40}H_{58}O_{15}$	11.91	777	657; 615; 439
23	Act-UrA-gypsogenin		$C_{41}H_{60}O_{16}$	11.95	807	687; 645; 469
24	Act-Pen-UrA-oleane-type		$C_{46}H_{70}O_{19}$	12.27	925	763; 569; 631; 455
25	UrA-akebonoic acid		$C_{35}H_{52}O_9$	12.70	615	539; 493; 439
26	Pen-UrA-oleane-type		$C_{41}H_{64}O_{13}$	13.80	763	631; 587; 455
27	Act-UrA-oleane-type	Betavulgaroside IV	$C_{41}H_{62}O_{15}$	13.81	793	673; 631; 455
28	Pen-UrA-oleane-type		$C_{41}H_{64}O_{13}$	14.24	763	631; 587; 455
29	Diox-UrA-oleane-type	Betavulgaroside II	$C_{41}H_{60}O_{15}$	14.50	791	747; 631; 455
30	Act-UrA-oleane-type	Betavulgaroside IV	$C_{41}H_{62}O_{15}$	14.55	793	673; 631; 455
31	UrA-oleane-type		$C_{36}H_{56}O_9$	14.60	631	587; 455
32	UrA-oleane-type		$C_{36}H_{56}O_9$	15.50	631	587; 455

Aglycone annotations are based on LC–ESI–MS/MS data. Due to the well-recognized similarity of fragmentation patterns among closely related oleanane-type saponins – including structures consistent with GOTCAB saponins (oleanane-type triterpenoid carboxylic acid 3,28-bisdesmosides) – compounds yielding characteristic oleanane-type fragments are reported at the oleanane-type level unless supported by authentic standards or literature NMR data. For defined betavulgarosides (I–X), names and aglycone identities are discussed in the text based on previously reported NMR evidence. t_R, Retention time; *m/z*, mass-to-charge ratio.

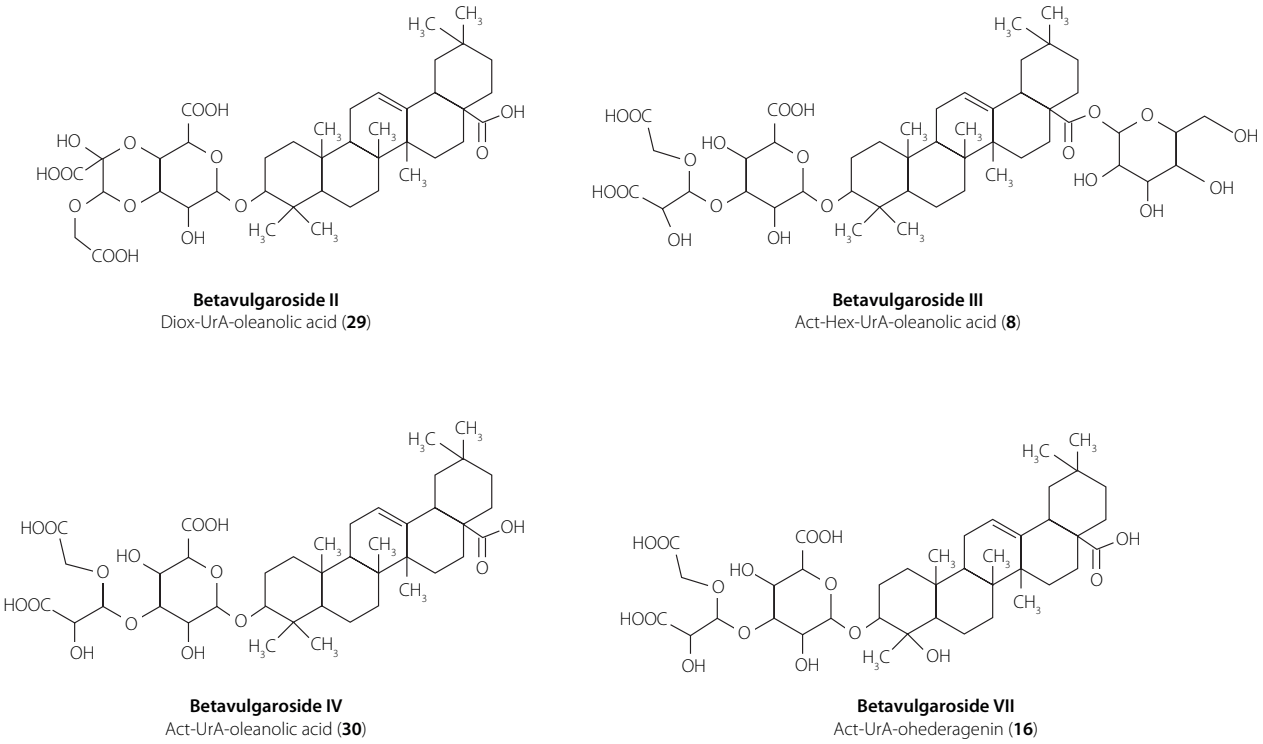

Figure 1. Chemical structures of the dominant saponins in *Beta vulgaris* L. cultivars: Round Dark Red, Cylindra, Snow Ball, and Rhubarb Chard.

and **19**), Diox–Hex (saponin **11**), Act–Pen (saponins **21** and **24**), and Act–Hex (saponins **8**, **17**, and **19**). Saponins **8**, **11**, **17**, and **19** were confirmed using authentic standards or previously reported NMR data [Yoshikawa *et al*., 1996], whereas saponins **12** and **19** were confirmed by comparison with reference compounds, and saponins **21** and **24** were assigned based on literature data [Mroczek *et al*., 2021]. These saponins have been previously reported in *B. vulgaris* tissues [Mroczek *et al*., 2012, 2019, 2021; Mikołajczyk-Bator *et al*., 2016a, 2024].

Triglycosylated oleanane-type saponins were also detected, including Act–Hex–Hex (saponins **3** and **7**), Hex–Hex–Hex (saponin **1**), and Act–Hex–Pen (saponin **6**). An acetal substituent derived from a hexose unit was confirmed for saponins **3**, **6**, and **7** using reference standards, whereas saponin **1** was tentatively identified based on literature MS data [Mroczek *et al*., 2012, 2019]. Saponins **3** and **7** (betavulgaroside V) are widely distributed in *B. vulgaris* cultivars and have also been reported in *Achyranthes fauriei* [Edelmann *et al*., 2020a,b; Ida *et al*., 1995; Mroczek *et al*., 2012, 2019, 2021; Mikołajczyk-Bator *et al*., 2016, 2024; Yoshikawa *et al*., 1998].

Saponins with akebonoic acid aglycone

In addition to oleanane-type saponins, *B. vulgaris* contained triterpene saponins with akebonoic acid as the aglycone, identified by a diagnostic fragment ion at *m/z* 439 under negative ESI conditions (**Table 1**). The simplest derivative, saponin **25** (*m/z* 615), was assigned as an akebonoic acid–uronic acid conjugate based on a characteristic neutral loss of 176 Da from the C-3 position of the aglycone and represents a compound not previously reported in *B. vulgaris*.

Single-glycosylated akebonoic acid derivatives were detected as saponin **20** (*m/z* 747; pentose substituent), tentatively assigned based on its MS/MS fragmentation pattern, and saponin **22** (*m/z* 777; acetal substituent), which was unambiguously identified by comparison with reference standards. More complex derivatives included saponin **14** (*m/z* 909; betavulgaroside X), identified based on sequential sugar losses and previously reported NMR data [Yoshikawa *et al*., 1998], as well as saponins **4** and **13** (*m/z* 939; betavulgaroside VIII), both confirmed using reference standards and previously reported in *B. vulgaris* and related beet cultivars [Edelmann *et al*., 2020a,b; Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2021; Yoshikawa *et al*., 1998].

Hederagenin and gypsogenin aglycones of saponins

B. vulgaris contained a minor group of triterpene saponins with hederagenin and gypsogenin aglycones (**Table 1**), detected at much lower levels than oleanane- and akebonoic acid–based derivatives and characterized by diagnostic ions at *m/z* 471 and 469, respectively. Saponin **18** (*m/z* 647) was identified as a hederagenin monoglycoside bearing a uronic acid residue based on a neutral loss of 176 Da and has not been previously reported in *B. vulgaris*. Compounds **10**, **15**, and **16** (*m/z* 779 and 809) were identified as hederagenin-based saponins containing a uronic acid residue and additional hexose and/or pentose units or an acetal substituent, as confirmed by a diagnostic fragment at *m/z* 471. Saponin **16** (betavulgaroside VII) is the most thoroughly characterized saponin in this group, with its structure elucidated by NMR [Yoshikawa *et al*., 1998] and reported across numerous *B. vulgaris* cultivars [Mikołajczyk-Bator *et al*., 2016a,b,

2024]. Saponins **15** and **16** were confirmed using reference standards, whereas saponin **10** was assigned based on literature data [Mroczek *et al*., 2012; Mikołajczyk-Bator *et al*., 2016a,b, 2024].

Saponin **2** (*m/z* 969) was identified as a hederagenin-based saponin bearing a uronic acid, a hexose, and a dioxolane substituent and has previously been reported only in sugar beet cultivars [Mikołajczyk-Bator *et al*., 2016b]. Saponin **23** (*m/z* 807) was the sole gypsogenin-based saponin detected, as confirmed by a diagnostic fragment at *m/z* 469, and was identified by comparison with reference standards; this compound has been previously reported in sugar beet and Swiss chard cultivars [Mikołajczyk-Bator *et al*., 2016b; Mroczek *et al*., 2021].

Analytical precision and variability assessment

Instrumental precision and extraction repeatability were evaluated using three independent samples prepared from the same homogeneous plant material and subjected to the complete extraction procedure. Each resulting extract was analyzed three times by LC–ESI-MS/MS to assess instrumental variability.

The relative standard deviation (RSD) obtained from repeated LC–ESI-MS/MS analyses of the same extract (n=3) ranged from 2.7% to 4.3%, indicating satisfactory instrumental precision. Extraction repeatability was assessed by comparing the results of three independent extractions performed on identical plant material. The RSD values calculated between these extractions ranged from 2.3% to 9%, which was considered acceptable given the complexity of plant matrices. Biological variability was assessed based on the two independent biological replicates collected for each cultivar, organ, and harvest date and expressed as biological RSD (%). The biological RSD values exhibited a relatively broad range. For individual saponins, biological RSD values generally ranged from approximately 5% to 16%, with the majority of compounds showing variability between 7% and 14%, depending on cultivar, organ, and harvest time.

The relative standard deviation (RSD) values for total saponins (calculated as the sum of individual saponins for each biological replicate) most commonly fell within the range of approximately 8–11%, although slightly higher values were occasionally recorded at specific harvest dates or for particular cultivars (**Tables S1–S8** in Supplementary Materials).

The reduced variability of the total saponin content reflects the cumulative nature of this parameter, whereby variability in individual saponins partially compensates when summed at the level of biological replicates. In cases where a given saponin was not detected in either biological replicate (mean value equal to zero), calculation of RSD was not applicable and was therefore omitted.

Saponin quantitative analysis

The total saponin content in leaves and roots of *B. vulgaris* cultivars harvested at different times is shown in **Figure 2**, and the relative proportions of the 32 identified compounds in **Figure 3**. Additionally, the contents of individual saponins in the fresh weight of the plant materials are presented in **Tables S1–S8**. Quantitative LC–ESI-MS/MS analysis demonstrated that saponin accumulation in *Beta vulgaris* L. was influenced by plant organ, harvest date and cultivar. Two-way ANOVA showed highly significant effects of cultivar, harvest time, and their interaction on total saponin content in leaves (f=137.73 for cultivar, f=68.26 for time, f=25.50 for cultivar × time; all p<0.0001) and in roots (f=77.45, 69.36, 31.56, respectively; all p<0.0001). Similarly strong effects were observed for most individual saponins (typically p<0.0001). Detailed two-way ANOVA outputs for individual saponins and total saponins in leaves and roots are provided in **Table S9** in Supplementary Materials.

Organ-dependent quantitative trends in total saponin content

Across cultivars and harvest dates, roots accumulated higher total saponin levels than leaves, with a much wider range of values (**Figure 2** and **Figure 3**). Total saponin content ranged from 386 to 10,414 mg/kg FW in leaves and from 1,170 to 23,298 mg/kg FW in roots. These values are consistent with those reported previously [Spórna-Kucab *et al*., 2022], where up to 20,810 mg/kg FW were found in beetroot peels of various cultivars. Although Mroczek *et al*. [2019] reported higher total saponin levels in leaves for some cultivars, their study also demonstrated that, depending on genotype, roots may accumulate equal or even higher concentrations. Beyond the overall differences between organs, distinct temporal patterns were also evident. Seasonal dynamics were organ-specific: total saponin content in leaves peaked in mid- to-late season (depending on cultivar), whereas roots exhibited a pronounced mid-season maximum followed by a decline (**Figure 2** and **Figure 3**). This pattern likely reflects developmental regulation of secondary metabolism and tissue-specific biosynthesis, with preferential accumulation in protective outer tissues. Indeed, Spórna-Kucab *et al*. [2022] demonstrated significantly higher saponin concentrations in beet peel compared to the flesh, supporting their enhanced deposition in tissues directly exposed to environmental stressors.

Cultivar-specific patterns of total saponin accumulation

In leaves, genotype-related differences were pronounced and strongly dependent on harvest date. The cultivar Rhubarb Chard showed the highest total saponin content, reaching 10,414 mg/kg FW on 25 August. The highest value for the cultivar Round Dark Red was recorded on 28 July (7,010 mg/kg FW), for the cultivar Cylindra on 11 August (5,060 mg/kg FW), and for the cultivar Snow Ball on 14 July (3,861 mg/kg FW) (**Figure 2** and **Figure 3**). These results are consistent with Mroczek *et al*. [2019], who demonstrated significant variability in saponin content among *B. vulgaris* genotypes.

Root tissues also displayed clear genotype-dependent accumulation patterns. The greatest accumulation capacity was observed for the cultivar Round Dark Red, which reached 23,290 mg/kg FW on 28 July. The cultivar Cylindra exhibited a pronounced mid-season maximum (17,330 mg/kg FW on 28 July), followed by a sharp decline to 1,170 mg/kg FW on 8 September.

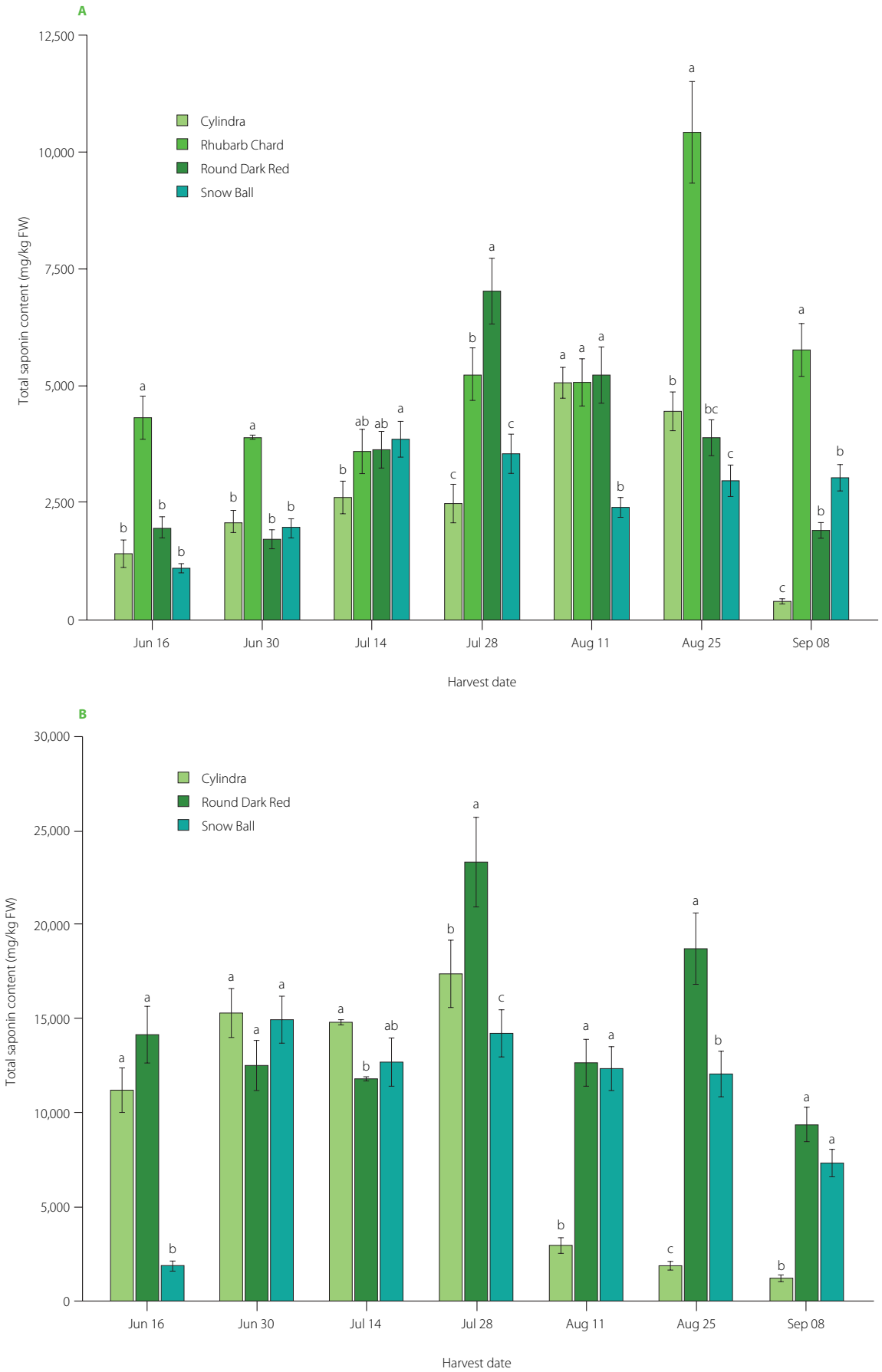

Figure 2. Total saponin content in leaves (**A**) and roots (**B**) of four *Beta vulgaris* L. cultivars across seven harvest dates. Data are expressed as mean and standard deviation. Different lowercase letters indicate significant differences among cultivars within each harvest date (Tukey's HSD test, $p \leq 0.05$). FW, fresh weight.

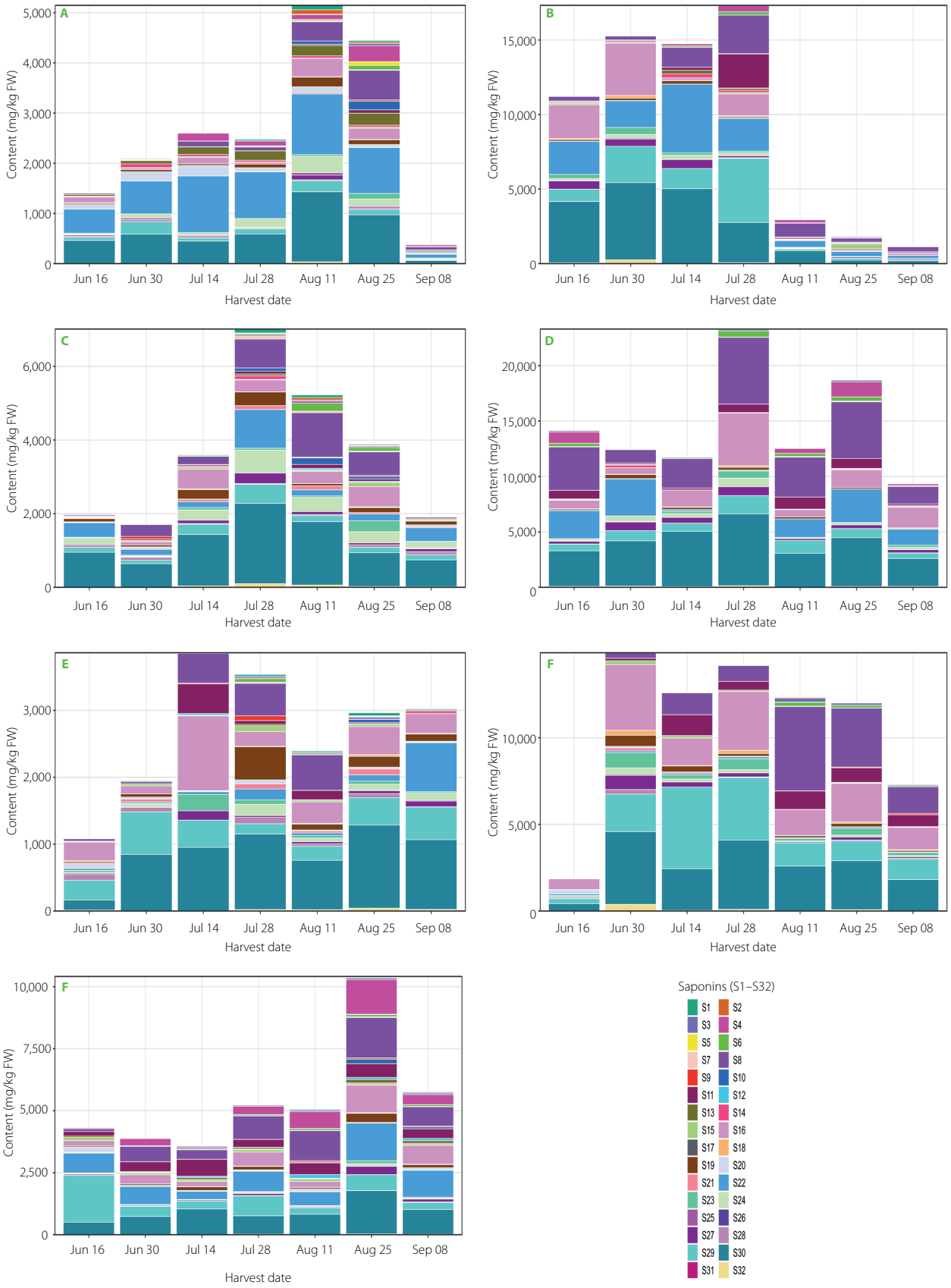

Figure 3. Stacked bar plots showing the total content and relative proportions of 32 saponins in leaves and roots of four *Beta. vulgaris* L. cultivars collected across seven harvest dates. Panels: (**A**) Cylindra leaves, (**B**) Cylindra roots, (**C**) Round Dark Red leaves, (**D**) Round Dark Red roots, (**E**) Snow Ball leaves, (**F**) Snow Ball roots, and (**G**) Rhubarb Chard leaves. Bar height represents total saponin content, and colors indicate individual saponins S1–S32, which are listed in **Table 1**. FW, fresh weight.

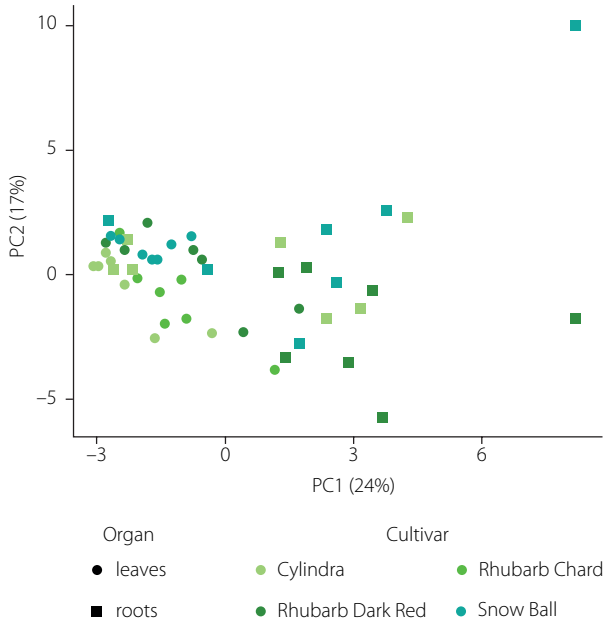

Figure 4. Principal component analysis (PCA) score plot of saponin profiles in leaves (circles) and roots (squares) of four *Beta vulgaris* L. cultivars collected across seven harvest dates. PC1 explains 24% and PC2 explains 17% of the total variance. Colors indicate cultivars.

The cultivar Snow Ball showed elevated early-to-midseason total saponin content (14,910 mg/kg FW on 30 June), gradually decreasing to 7,310 mg/kg FW by 8 September (**Figure 2** and **Figure 3**). These trends are consistent with previous reports; Spórna-Kucab *et al*. [2022] documented approximately 20,810 mg/kg in the peel of yellow beet cultivar Boldor, compared with only 500 mg/kg in the flesh of white Snow Ball, indicating strong tissue-specific distribution. Likewise, Mikołajczyk-Bator *et al*. [2024] reported significantly higher saponin content in root skins than in the flesh. Such findings support the mid-season maxima observed here and likely reflect preferential accumulation in outer protective tissues, followed by dilution or metabolic redistribution at later developmental stages.

Quantitative variability of individual saponins

Individual saponins showed pronounced heterogeneity in abundance as well as in temporal and cultivar-dependent patterns (**Figure 3**, **Tables S1–S8**). Many low-abundance compounds were detected only intermittently, whereas a limited number of high-abundance saponins dominated the overall profile, including in particular, betavulgarosides II (**29**), III (**8**), IV (**30**), VII (**16**) and Act-UrA-akebonoic acid saponin (**22**). These major compounds exhibited coordinated seasonal variation in both temporal occurrence and abundance, including cultivar-dependent maxima. Two-way ANOVA showed highly significant effects of cultivar, harvest date, and their interaction on these and most other saponins ($p<0.0001$), supporting the idea of a differentiated biosynthetic network (**Table S9**). This aligns with Mroczek *et al*. [2019], who described various accumulation rates of specific saponins and spatially differentiated biosynthesis across organs.

Principal component and hierarchical clustering analyses

PCA performed on the dataset comprising 32 saponins clearly separated the samples according to plant organ (**Figure 4**). The first principal component (PC1), explaining 24% of the total variance, distinctly discriminated root samples (positive PC1 values) from leaf samples (negative PC1 values), while the second principal component (PC2), accounting for 17% of the variance, further differentiated samples according to harvest date.

The separation along PC1 and PC2 was primarily driven by four high-loading saponins, corresponding to betavulgaroside III (**8**), betavulgaroside VII (**16**), Act–UrA–akebonoic acid saponin (**22**), and betavulgarosides II (**29**) and IV (**30**), previously described in beet [Mroczek *et al*., 2019]. Their consistent contribution across samples indicates that these compounds constitute the principal sources of variability within the dataset. Hierarchical clustering analysis (heatmap visualization) fully supported the PCA results. Samples were grouped primarily according to plant organ (root *vs*. leaf), followed by harvest date and cultivar, revealing a clear underlying structure in saponin distribution patterns (**Figure 5**).

Biological significance of triterpene saponins identified in *B. vulgaris*

The triterpene saponins identified in *B. vulgaris* constitute a structurally diverse and biologically relevant group of secondary metabolites with well-established functional and pharmacological importance. The predominance of oleanane-type saponins observed in this study suggests that this compound class may play a central role in determining the biological and functional properties of beet-derived tissues and products. In particular, the presence of NMR-confirmed betavulgarosides [Yoshikawa *et al*., 1996, 1998], previously associated with anti-inflammatory, hepatoprotective, antimicrobial, antioxidant, and anticancer activities, underscores their potential contribution to the health-promoting effects of *B. vulgaris* [Castellano *et al*., 2022; Günther & Bednarczyk-Cwynar, 2025; Gupta, 2022; Lisiak *et al*., 2021; Liu *et al*., 2019; Sen, 2020; Yoon & Choi, 2010].

The biological activity of oleanolic acid–based saponins is strongly influenced by their glycosylation patterns. The presence of uronic acid residues together with additional hexose or pentose units modulates key physicochemical properties, including solubility, membrane interactions, and bioavailability [Francis *et al*., 2002]. The predominance of glycosylated oleanane-type cores detected in the present study is consistent with earlier reports indicating that biologically-active forms of oleanolic acid occur predominantly as saponins rather than as free aglycones in plant tissues [De Tommasi *et al*., 1991; Kinjo *et al*., 1999].

Beyond oleanane-type compounds, the identification of akebonoic acid–based saponins further expands the biological relevance of the saponin profile of *B. vulgaris*. Akebonoic acid, a 30-noroleanane triterpenoid, has been reported to exhibit pronounced anticancer, antibacterial, and antidiabetic activities [Dirir *et al*., 2021; Wang *et al*., 2014]. Although the biological functions of its glycosylated derivatives remain insufficiently

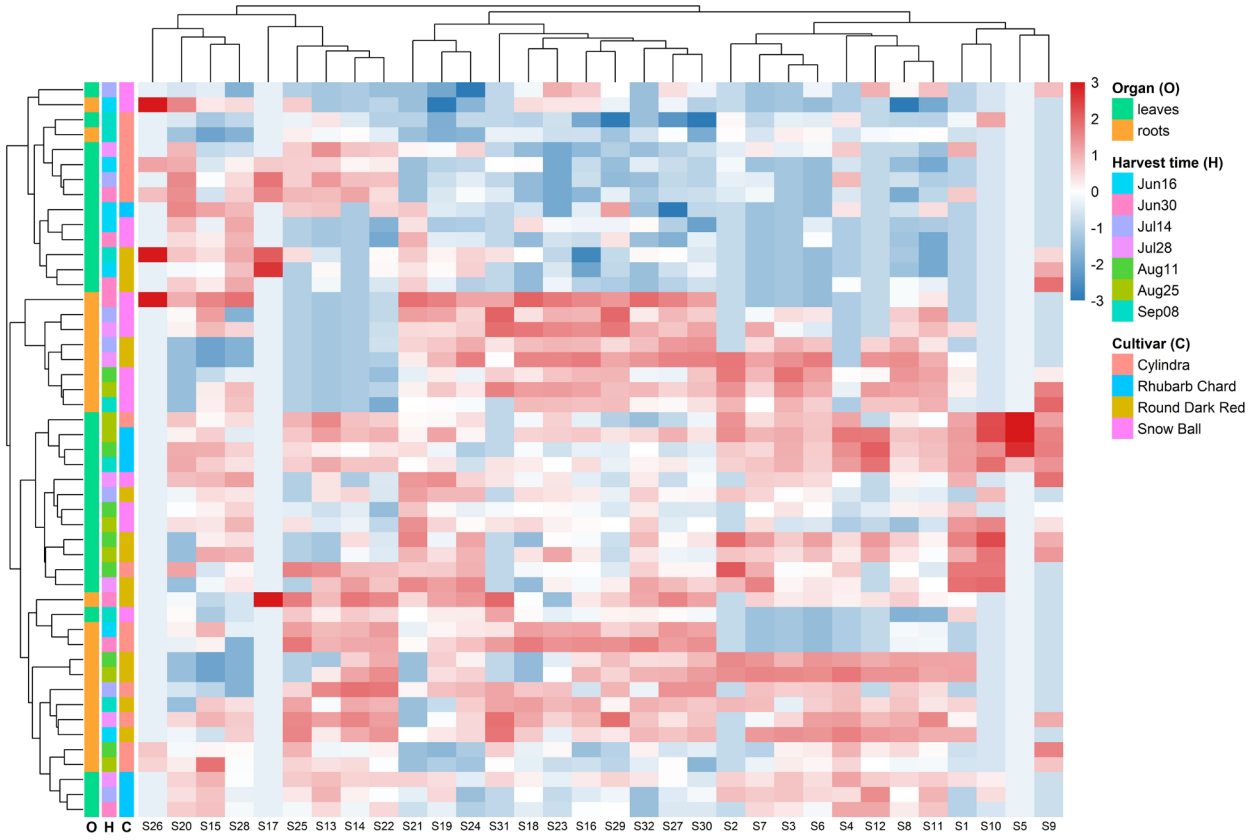

Figure 5. Hierarchical clustering and heatmap of 32 saponins detected in leaves and roots of different *Beta vulgaris* L. cultivars across seven harvest dates. The heatmap presents normalized relative liquid chromatography–mass spectrometry signal intensities (z-scores) of individual saponins across all samples (red = higher relative abundance, blue = lower relative abundance). Rows correspond to samples and columns to individual saponins. Colored side bars indicate harvest date (time), plant organ (leaves or roots), and cultivar. Dendrograms represent hierarchical clustering of samples (left) and saponins (top), illustrating similarities in saponin profiles and co-variation patterns among compounds.

characterized, their detection in beet tissues suggests that *B. vulgaris* may represent a valuable source of structurally--unique saponins with largely unexplored bioactivity. The occurrence of simple monoglycosidic derivatives may additionally reflect early biosynthetic intermediates or compounds with enhanced membrane-disrupting potential [Böttger & Melzig, 2013].

Minor saponins containing hederagenin and gypsogenin aglycones further contribute to the functional complexity of the saponin mixture in *B. vulgaris*. Both aglycones are known for their cytotoxic, antimicrobial, antifungal, and antiviral properties [Cheng *et al*., 2018; Ciftci *et al*., 2018; Engholm *et al*., 2017; Favel *et al*., 1994; Krasteva *et al*., 2014; Su *et al*., 2024; Sun *et al*., 2023; Xie *et al*., 2023; Zeng *et al*., 2018]. Despite their lower abundance compared to oleanane- and akebonoic acid–based saponins, these compounds may still be biologically-relevant due to potential additive or synergistic interactions within complex saponin assemblies.

Taken together, the diversity of aglycone scaffolds and glycosylation patterns observed in *B. vulgaris* highlights this species as a rich source of triterpene saponins with multifaceted biological potential. The tissue-specific and season-dependent variability in saponin composition demonstrated in this study may have important implications for the nutritional value, functional characteristics, and potential health benefits of beet-derived products. Nevertheless, further investigations employing purified compounds and targeted bioassays are required to establish direct links between saponin composition, concentration, and biological activity.

CONCLUSIONS

This study demonstrates that saponin accumulation in *Beta vulgaris* L. is strongly dependent on plant organ, cultivar, and harvest time, with significant interaction effects between these factors. Roots accumulated higher total saponin levels than leaves and exhibited a pronounced mid-season maximum followed by a decline, whereas leaf saponin contents generally peaked in mid-to-late season depending on genotype.

Quantitative variability was largely driven by a limited group of dominant saponins, which showed coordinated seasonal and cultivar-dependent dynamics. Multivariate analyses confirmed that plant organ represents the primary source of variance in the dataset, with additional structuring according to harvest date and genotype.

Overall, the results indicate that saponin biosynthesis in *B. vulgaris* is developmentally-regulated and spatially-differentiated between organs. From a practical perspective, both cultivar selection and optimized harvest timing are critical parameters for maximizing total saponin yield and shaping the qualitative composition of beet-derived plant material.

RESEARCH FUNDING

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

The following are available online at **https://journal.pan.olsztyn.pl/Cultivar-and-Growth-Stage-Dependent-Variability-of-Saponins-in-Roots-and-Leaves-of,218708,0,2.html**. **Table S1.** Content of saponins 1–8 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S2.** Content of saponins 9–16 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S3.** Content of saponins 17–24 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S4.** Content of saponins 25–32 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S5.** Content of saponins 1–8 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S6.** Content of saponins 9–16 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S7.** Content of saponins 17–24 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S8.** Content of saponins 25–32 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S9.** Summary of two-way ANOVA results showing the effects of cultivar, harvest date, and their interaction on individual saponins (S1–S32) and total saponin content in leaves and roots of *Beta vulgaris* L.

ORCID IDs

S. Janecki https://orcid.org/0009-0002-3596-9471
A. Spórna-Kucab https://orcid.org/0000-0002-6653-3702
A. Tekieli https://orcid.org/0000-0003-1147-9136

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