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Anti-Inflammatory Effects of Enzymatically Hydrolyzed Pectin from *Premna ligustroides* Hemsl. Leaves in Gouty Rats Through NLRP3 Inflammasome Inhibition

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Gout is a rheumatic inflammatory disease resulting from abnormal uric acid metabolism. This study investigated the anti-inflammatory effects and mechanisms of enzymatically hydrolyzed pectin from *Premna ligustroides* Hemsl. leaves (EHPPL) in gout-induced rats. Serum and organ function markers, along with ankle tissue hematoxylin-eosin staining and NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) expression, were analyzed. In gouty rats, EHPPL significantly reduced uric acid (from 73.38 to 54.85 μmol/L) and urea nitrogen levels (from 11.97 to 4.02 mmol/L), normalized liver and kidney functions, and decreased key inflammatory markers, including tumor necrosis factor-α, monocyte chemoattractant protein-1, interleukin (IL)-18, IL-1β, lipopolysaccharides, NLRP3, and cysteinyl aspartate specific proteinase-1. Additionally, EHPPL upregulated anti-inflammatory IL-10 levels. These effects were attributed to the inhibition of a toll-like receptor (TLR) 2/TLR4/myeloid differentiation primary response protein 88/nuclear factor-κB signaling, suggesting that EHPPL may serve as a potential dietary intervention for acute gouty arthritis.

Keywords: inflammation inhibition, joint swelling, NOD-like receptor, polysaccharides, uric acid

ABBREVIATIONS

ABCG2, ATP-binding cassette subfamily G member 2; ALT, alanine aminotransferase; AST, asp artate aminotransferase; Caspase-1, cysteinyl aspartate specific proteinase-1; EHPPL, enzymatically extracted pectin from *Premna ligustroides* Hemsl. leaves; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hematoxylin-eosin; IL, interleukin; LPS, lipopolysaccharides; MCP-1, monocyte chemoattractant protein-1; MSU, monosodium urate; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor-κB; NLRP3, NOD-like receptor thermal protein domain associated protein 3; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene-fluoride; SOP, standard operating procedures; TBST, tris-buffered saline with Tween 20; TLR, toll-like receptor; PDZK1, PDZ domain-containing protein 1; TNF-α, tumor necrosis factor-α; NSAIDs, nonsteroidal anti-inflammatory drugs.

INTRODUCTION

Dysregulation of purine metabolism or reduced uric acid excretion can lead to elevated blood uric acid levels, resulting in crystal-induced inflammatory joint diseases due to the deposition of monosodium urate (MSU) crystals [Dalbeth *et al.*, 2021]. Symptoms include hyperuricemia, redness, swelling, fever in bones and joints, intermittent joint pain, gout, kidney stones, and joint

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deformities, all of which significantly affect patients' daily lives [Perez-Ruiz *et al*., 2015]. The incidence of these conditions is rising, with a notably higher prevalence among younger individuals [Dehlin *et al*., 2020]. Gout is primarily caused by purine metabolism disorders and abnormal inflammatory pathways [Pascart & Lioté, 2019]. MSU crystals stimulate the release of inflammatory mediators, which induce and perpetuate inflammation [Roddy & Choi, 2014]. Research has demonstrated that even in the absence of gout, soluble uric acid can trigger inflammation in renal cells by activating NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasomes [Kim *et al*., 2015]. Furthermore, soluble uric acid increases the expression of PDZ domain-containing protein 1 (PDZK1) and ATP-binding cassette subfamily G member 2 (ABCG2) in intestinal cells *via* the toll-like receptor (TLR) 4 and NLRP3 inflammasome signaling pathway [Chen *et al*., 2018]. TLR2 and TLR4 are critical in the production of interleukin (IL)-1β in response to MSU crystals [Guo *et al*., 2019]. High uric acid levels can also damage the intestinal mucosal barrier in mice, increasing intestinal permeability and enabling the translocation of intestinal bacteria, inflammatory cytokines, and lipopolysaccharides (LPS) into the bloodstream. LPS in the gut further elevates xanthine oxidase (XOD) activity, contributing to increased blood uric acid levels [Guo *et al*., 2021]. The TLR4/nuclear factor-κB (NF-κB) and NLRP3 signaling pathways, which involve key cytokines and inflammatory mediators such as TLR4, myeloid differentiation primary response protein 88 (MyD88), NLRP3, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), are crucial targets for the treatment of hyperuricemia and gout [Sun *et al*., 2024]. The NF-κB pathway is particularly implicated in the release of inflammatory cytokines like TNF-α, IL-6, and IL-1β in the context of gout.

During acute gout episodes, first-line treatments such as nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, glucocorticoids, and allopurinol may lead to adverse reactions [Dalbeth *et al*., 2021; Shi *et al*., 2020]. Diet plays a pivotal role in reducing systemic inflammation and influencing the progression of chronic diseases. Numerous studies suggest that certain food components or natural ingredients can alleviate arthritis, gout, and hyperuricemia [Huang *et al*., 2022; Li *et al*., 2021; Li *et al*., 2025]. Therefore, identifying effective compounds from edible sources for gout management is of significant importance. *Premna ligustroides* Hemsl., a shrub from the Verbenaceae family (genus *Premna* L.), also known as *Premna microphylla* Turcz. [Liu *et al*., 2021b], thrives in the mountainous regions of Sichuan, Chongqing, and Hubei, China. The *P. ligustroides* is known for its antioxidant potential due to content of phenolics, diterpenoids and alkaloids [Duan *et al*., 2022]. Leaves of *P. ligustroides* are also rich in pectin with the content of 35 g/100 g [Liu *et al*., 2021b]. Research by Song *et al*. [2021] revealed that polysaccharides derived from this plant reduce inflammation by enhancing intestinal resistance. *P. ligustroides* pectin can be effectively extracted with water after pectinase and cellulase treatment [Liu *et al*., 2023]. Such enzymatically hydrolyzed pectin from *P. ligustroides* leaves (EHPPL) showed potent anti-inflammatory properties [Deng *et al*., 2025].

The aim of this study was to examine the impact of EHPPL on ankle joint swelling in rats with MSU-induced acute gouty arthritis and understanding how EHPPL can alleviate gout symptoms. Study findings may provide valuable insights into improving uric acid crystal metabolism through dietary intervention.

MATERIALS AND METHODS

Materials

The leaves of *P. ligustroides* were purchased from Mianyang Changshan Agricultural Technology Co., Ltd. (Sichuan, China). Allopurinol tablets were obtained from Hefei Jiulian Pharmaceutical Co., Ltd. (Anhui, China). Sodium urate crystals were sourced from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

Twenty male specific pathogen-free (SPF) Sprague-Dawley (SD) rats, aged 5 weeks and weighing 200–220 g, were acquired from Hunan Slake Jingda Experimental Animal Co., Ltd. (Hunan, China) under License No.: SCXK (Xiang) 2019-0004. The animals were housed in the SPF-level barrier facility of the Experimental Animal Center of Pharmacy, Southwest University (Facility license No.: SYXK (Yu) 2020-0006), under controlled conditions: relative humidity of 53±2%, temperature of 23±1°C, and a 12 h light/dark cycle. The animal procedures adhered to ARRIVE (Animals in Research: Reporting *In Vivo* Experiments) guidelines and were approved by the Laboratory of Animal Welfare and Ethics Committee of Southwest University (IACUCI No.: IACUC-20230425-02).

Biochemical kits for determinations of uric acid, creatinine, urea nitrogen, and enzyme-linked immunosorbent assay (ELISA) kits for determinations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, urea nitrogen, TNF-α, monocyte chemoattractant protein-1 (MCP-1), LPS, NLRP3, and caspase-1 were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Biochemical kits for IL-18, IL-10, and IL-10 were obtained from Quanzhou Ruixin Biotechnology Co., Ltd. (Fujian, China). Antibodies for NLRP3 and NF-κB p65 were purchased from Affinity (Jiangsu, China), and TLR4 antibodies and anti-fluorescence quenching sealing tablets were from Wuhan Sanying (Hubei, China). Antibodies for TLR2 and p-NF-κB p65 were obtained from Beijing Bioss Biotechnology Co., Ltd. (Beijing, China), MyD88 from Boster Biological Technology Co., Ltd. (USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Hangzhou Xianzhi Biotechnology Co., Ltd. (Zhejiang, China), and fluorescent (CY3)-labeled sheep anti-mouse IgG from Wuhan Doctoral Biotechnology Co., Ltd. (Hubei, China).

Preparation of enzymatically hydrolyzed pectin from *P. ligustroides* leaves

The EHPPL was prepared as described in our previous publications [Deng *et al*., 2025; Liu *et al*., 2023]. The leaves were soaked in hot water at a 1:10 ratio (*w/w*), ground into a paste, and treated with an enzyme mixture consisting of pectinase and cellulose at a 1:1 ratio (*w/w*) at 50°C for 5 h. The ratio of the enzyme mixture to the leaf paste was 1:100 (*w/w*). After hydrolysis and suction filtration, the filtrate was further passed

through a reverse osmosis membrane. The next step of purification was precipitation with ethanol (50–70%) and drying, followed by macroporous resin adsorption to achieve a molecular weight range of 5,000–35,000 Da. The composition of EHPPL was determined in our previous study [Liu *et al*., 2023]. In 100 g, it contained 1.33 g of protein, 1.13 g of ash, and 45.13 g of galacturonic acid. A degree of esterification was 12.56 g/100 g. The monosaccharide composition was 48.08% of glucose, 33.48% of galacturonic acid, 10.17% of arabinose, 3.03% of galactose, 1.39% of mannose, 1.25% of rhamnose, and 2.56% of xylose, with a rhamnose to galacturonic acid ratio of 0.04%.

Modeling, intervention and sampling in an *in vivo* experiment

After 7 days of adaptive feeding, the rats were randomly assigned to a normal control (NC) group (n=5) and a model group (n=15). The model was induced according to the method described by Zhuge *et al*. [2025], with some modifications. Under ether anesthesia, the right lower limb of each rat was positioned at a right angle to fully expose the ankle joint. A 4.5 injection needle was inserted into the joint cavity, guided by the posterior ligament at a 35° angle. The model group received 0.25 mL of a 5% (*w/v*) MSU suspension (finely ground MSU crystals were suspended in phosphate-buffered saline, PBS) into the joint cavity, while the NC group was injected with 0.25 mL of PBS. The model group was then randomly divided into three subgroups: M group, PC group, and T group (n=5 each).

Four hours after modeling, the PC group was administered allopurinol (9 mg/kg, equivalent to the human dose) orally, while the T group received EHPPL (450 mg/kg, based on preliminary experimental results) orally. The M and NC groups received an equivalent dose of saline orally. The treatment lasted for 7 days, with free access to food and water. The rats' gait was monitored daily, and ankle joint circumference was measured.

Blood was collected from the eye socket 12 h after the last intervention. Urine was collected 24 h prior to the experiment's conclusion. At the end of the experiment, the rats were euthanized under ether anesthesia, and blood was drawn from the abdominal aorta. After allowing the blood to stand at room temperature for 30 min, it was centrifuged at 1,000×*g* for 15 min at 4°C to obtain serum. Liver and kidney tissues were weighed, and a 0.5 cm × 0.5 cm section from above the ankle joint was harvested. The right part of the tissue was fixed in 4% paraformaldehyde for hematoxylin-eosin (HE) staining, while the left part was stored in liquid nitrogen for western blot analysis.

Histological observation of the ankle joint

The right ankle joint samples were used for histological examination. Standard operating procedures (SOP) for pathological testing were followed, including dehydration, embedding, slicing, staining, and sealing. Samples were examined at 20× and 200× magnifications using the Pannoramic DESK (3DHISTECH, Budapest, Hungary) and CaseViewer 2.4 software (3DHISTECH).

Ankle joint swelling was assessed daily for 7 days post-modeling using a 2–3 mm paper strip and vernier caliper. Joint swelling degree was calculated according to Equation (1):

$$\text{Joint swelling degree (\%)} = \frac{A_1 - A_2}{A_2} \times 100\% \quad (1)$$

where: A_1 represents joint circumference at different time points and A_2 represents initial circumference.

Liver and kidney index calculation

Liver and kidney indexes were calculated using Equation (2):

$$\text{Organ index (\%)} = \frac{\text{Total organ weight (g)}}{\text{Body weight (g)}} \times 100\% \quad (2)$$

Determination of biochemical indicators of serum and urine

Biochemical indicators, including serum uric acid, urea nitrogen, creatinine, AST, ALT, TNF-α, MCP-1, IL-18, IL-1β, IL-10, LPS, NLRP3, and caspase-1, as well as uric acid content in urine, were measured according to the reagent kit instructions.

Western blot analysis

Ankle joint tissue was placed in a 2 mL Eppendorf tube, to which clean steel balls were added. Then, 200 µL of lysis buffer (containing 2 µL of phenylmethanesulfonyl fluoride (PMSF) and 2 µL of phosphatase inhibitor) were added for tissue lysis. The tube was homogenized using a tissue disruptor (SCIENTZ-24, Ningbo Xinzhi Biotechnology Co., Ltd., China). After homogenization, the tube was placed on ice for 30 min to ensure complete lysis, followed by centrifugation at 15,000×*g* for 5 min at 4°C. The supernatant was 20-fold diluted with PBS, and protein concentration was measured using a Multiskan FC microplate reader (Thermo Scientific, Waltham, MA, USA). For protein denaturation and membrane transfer, the polyvinylidene-fluoride (PVDF) membrane was soaked in tris-buffered saline with Tween 20 (TBST) with 5% skim milk powder and sealed on a shaker at room temperature for 2 h. After blocking, the membrane was incubated overnight at 4°C with specific primary antibodies (rabbit polyclonal antibodies for TLR2, MyD88, NF-κB, and NLRP3 at a 1:1,000, *v/v*, dilution, and TLR4 at a 1:4,000, *v/v*, dilution) in blocking buffer. After five 5-min washes with TBST, the membrane was incubated with secondary antibodies (1:10,000, *v/v*, dilution) for 2 h, followed by five additional 5-min washes with TBST. The membrane was then dried and scanned using the SH-523 chemiluminescence imaging system (Hangzhou Shenhua Technology Co., Ltd., China), and grayscale values were analyzed. Band intensities were compared to the standard protein – GAPDH.

Statistical analysis

Data were analyzed using Statistix software, version 9.0 (Analytical Software, Tallahassee FL, USA) with completely randomized analysis of variance (ANOVA) and all-pairwise comparisons Tukey's honestly significant difference (HSD) test. Results were

plotted using Excel 2010 (Microsoft, Redmond, WA, USA), and differences between groups were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Serum level of uric acid and creatinine, and ankle joint condition at 12 h after modeling

At 12 h post-modeling, the right ankle joints of the rats exhibited varying degrees of redness, swelling, loss of bone markers, limited movement, curled feet, and a three-legged gait. Significant increases were found in serum uric acid and creatinine levels ($p<0.05$) after 12 h of modeling, with serum uric acid reaching 95.10 μmol/L (**Figure 1**). MSU crystals were metabolized and entered the bloodstream, elevating uric acid levels, which in turn triggered systemic inflammation [Perez-Ruiz & Becker, 2015]. Excess uric acid crystallized, causing localized inflammation and tissue damage [Kushiyama *et al.*, 2016].

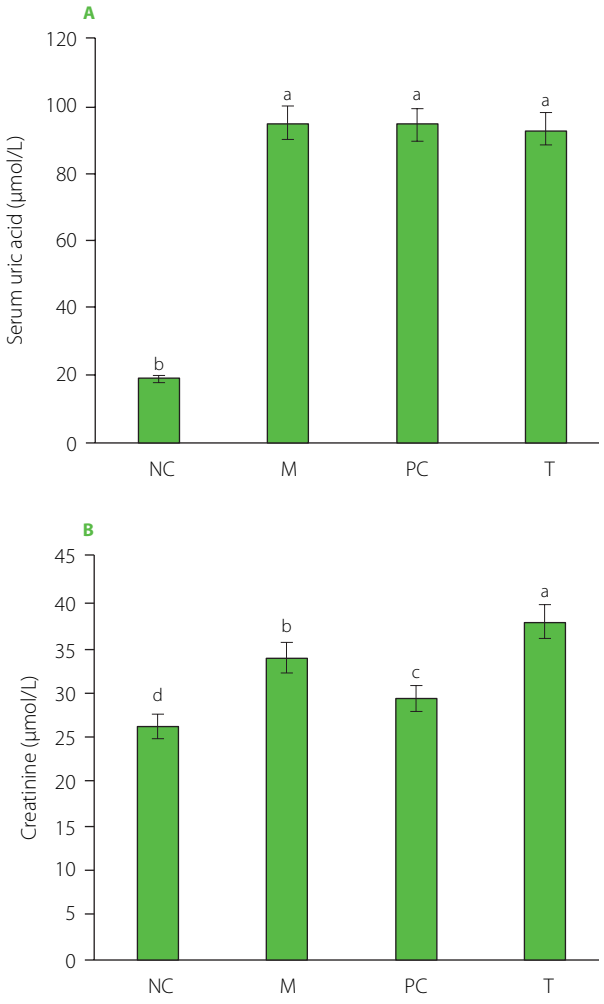

Figure 1. Serum uric acid (**A**) and creatinine (**B**) levels at 12 h after modeling. Data are presented as mean ± standard deviation ($n=3$). Different lowercase letters indicate significant differences ($p<0.05$) between groups. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

Changes in the body weight of rats during the intervention period

During the feeding adaptation phase, the rats appeared healthy, exhibiting shiny fur, good general condition, and normal eating habits. After MSU injection, they showed reduced activity and appetite. However, rats in the T and PC groups gradually resumed normal activity after receiving EHPPL or allopurinol. As presented in **Figure 2**, the changes in body weight remained stable, with no significant fluctuations, and there were no significant differences in the final body weight of rats from different groups ($p\geq0.05$), indicating the relative safety of EHPPL.

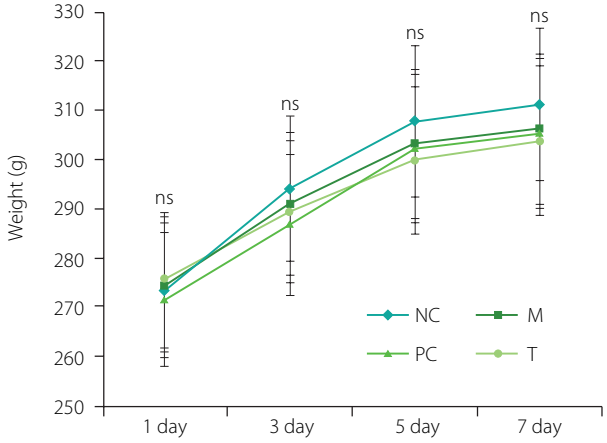

Figure 2. Changes in rat body weight during the intervention period. Data are presented as mean ± standard deviation ($n=5$). ns denotes no significant differences ($p\geq0.05$) between groups on the same day. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

Swelling degree and pathological analysis of ankle joints in rats

As presented in **Figure 3A**, on the 4th day post-modeling, rats in the M group exhibited ankle redness and swelling. In contrast, the PC and T groups showed nearly normal conditions. As presented in **Figure 3B**, ankle joint swelling degree peaked on the second day post-modeling, with the M group displaying significantly greater swelling than the NC group ($p<0.05$). Treatment with EHPPL and allopurinol reduced ankle redness and joint swelling degree returning it nearly to normal by day 4 ($p\geq0.05$). Gait gradually improved, normalizing by day 7.

Pathological sections of ankle joints are shown in **Figure 3C**. The NC group exhibited normal structure, smooth cartilage, and uniform tissue staining with no signs of synovial proliferation or inflammation. The M group displayed severe synovial connective tissue proliferation, neovascularization (yellow arrow), and infiltration by lymphocytes and macrophages (blue arrow). The PC group showed slight synovial proliferation, neovascularization (yellow arrow), and immune cell infiltration (blue arrow). The T group exhibited normal ankle joint structure with no pathological abnormalities.

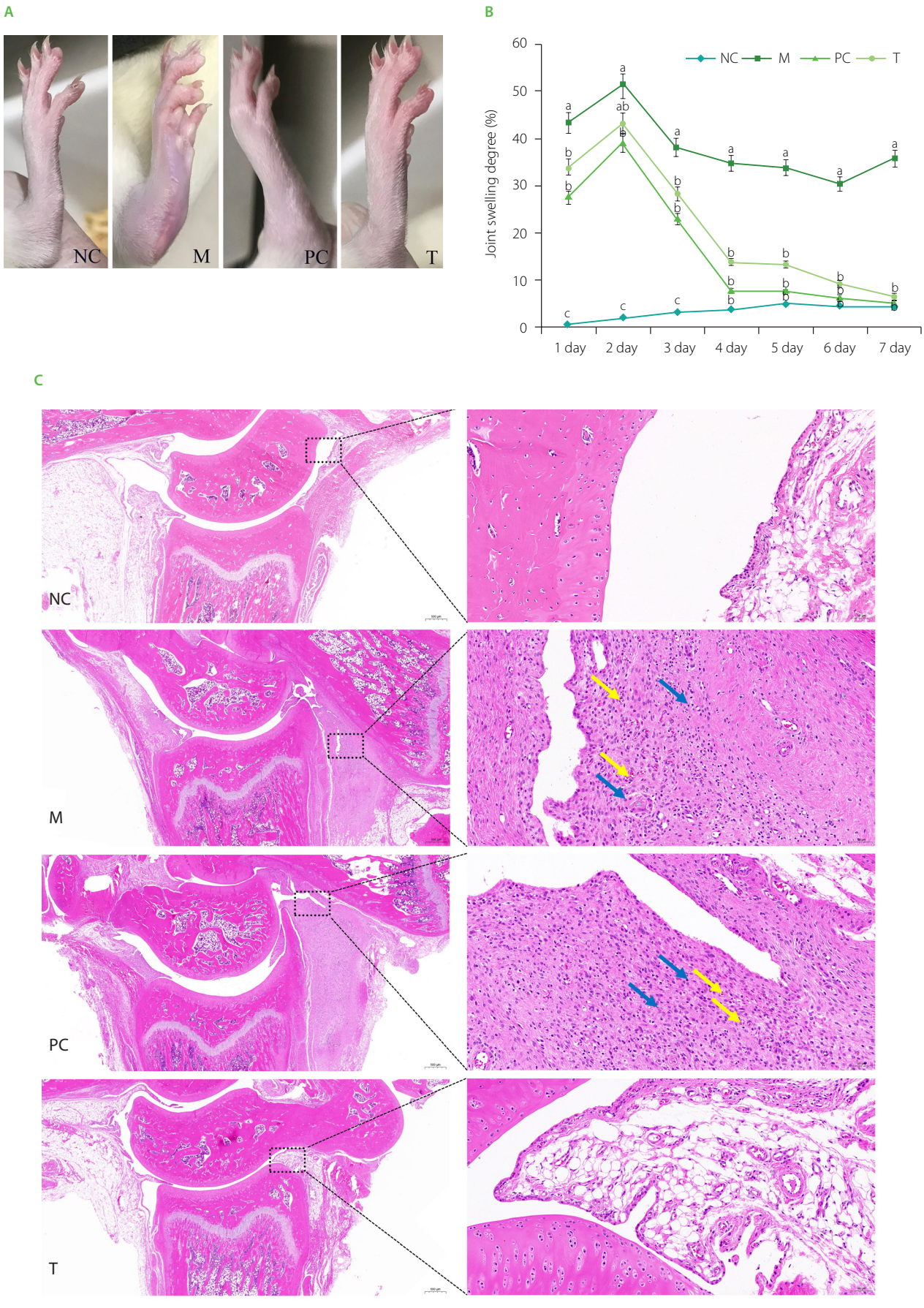

Figure 3. Ankle joint condition after 4 days of intervention (**A**), joint swelling degree (**B**), and histology images of the ankle joint at 20× (left images) and 200× (right images) magnifications (**C**). Data are presented as mean ± standard deviation (n=3). Different lowercase letters indicate significant differences (p<0.05) between groups on the same day. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

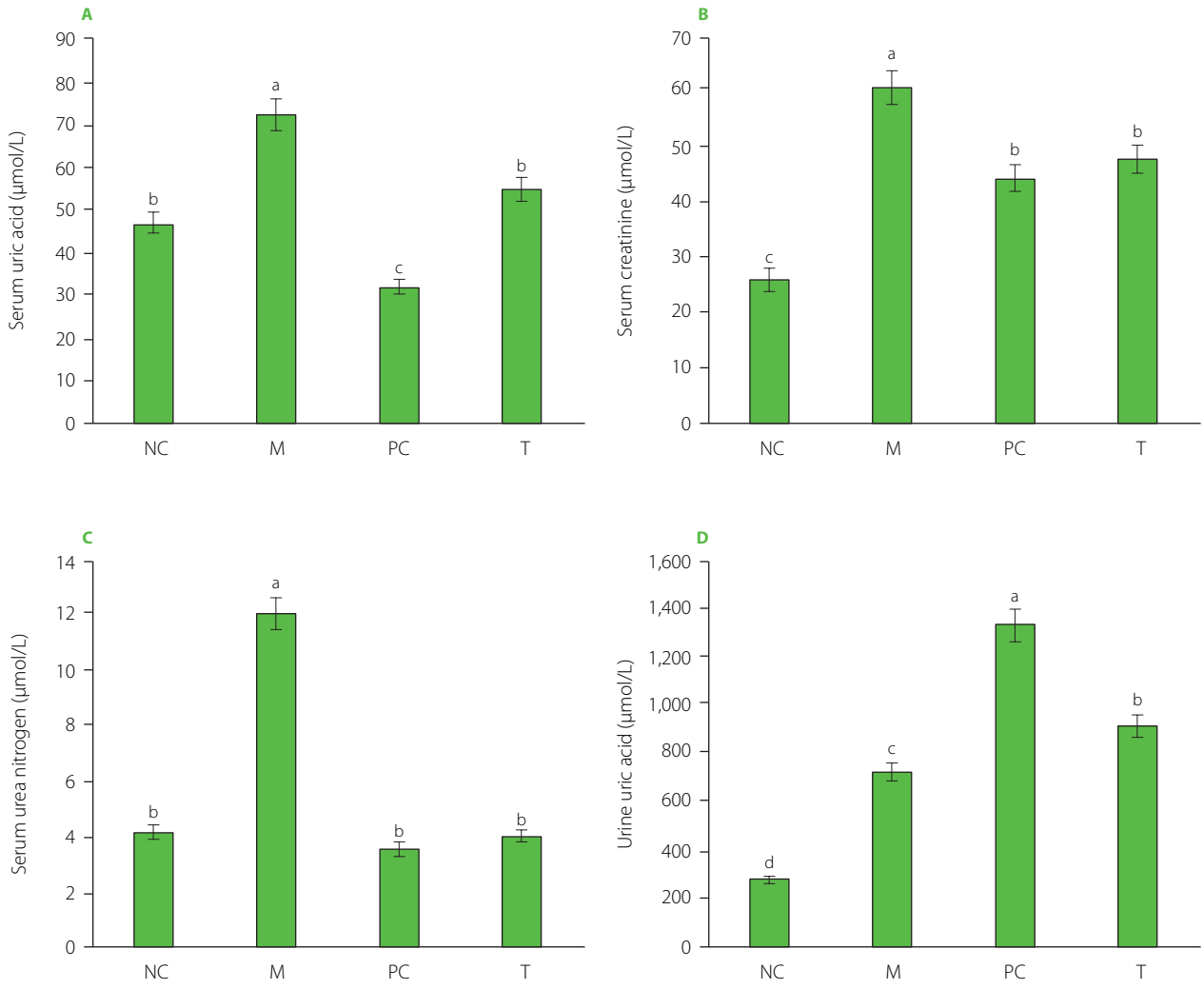

Figure 4. Serum uric acid (**A**), creatinine (**B**), and urea nitrogen (**C**) levels, and uric acid content in urine (**C**) of rats. Data are presented as mean ± standard deviation (n=3). Different lowercase letters indicate significant differences (p<0.05) between groups. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

Serum levels of uric acid, creatinine, and urea nitrogen, and the uric acid content in urine

As shown in **Figure 4A–C**, the M group exhibited significantly elevated serum uric acid, creatinine, and urea nitrogen levels compared to the NC group (p<0.05). However, after the treatment with EHPPL and allopurinol, the T group and PC groups showed a significant reduction in these indicators (p<0.05). EHPPL reduced serum uric acid from 73.38 to 54.85 μmol/L and urea nitrogen levels from 11.97 to 4.02 mmol/L (p<0.05), returning levels to those similar to the NC group. Serum creatinine concentration is a key indicator of renal function [Zhang *et al*., 2018], suggesting that MSU may potentially damage the kidneys, while EHPPL could offer renal protection. Additionally, **Figure 4D** shows a significant increase in urinary uric acid excretion (p<0.05) in the PC and T groups, indicating that both EHPPL and allopurinol facilitated uric acid elimination *via* urine. Numerous studies have confirmed that polysaccharides can influence uric acid metabolism. For instance, polysaccharides from γ-irradiated and non-irradiated *Schizophyllum commune* have demonstrated kidney uric acid-lowering effects in Kunming mice [Yin *et al*., 2022]. Li *et al*. [2021] found that a polysaccharide from *Enteromorpha prolifera*, containing rhamnose, glucuronic acid, galactose, arabinose, and xylose, significantly reduced serum uric acid, serum urea nitrogen, serum XOD, and liver XOD levels in hyperuricemic mice. Similarly, Wang *et al*. [2021] showed that neutral polysaccharides extracted from corn silk effectively decreased blood uric acid levels, reduced liver XOD activity, improved kidney damage, and enhanced uric acid excretion in hyperuricemic mice.

Liver and kidney indexes and serum enzyme activity

As presented in **Figure 5A** and **5B**, the liver and kidney indexes in the M group were significantly elevated (p<0.05) compared to the other groups. High uric acid levels are known to induce oxidative stress, leading to considerable damage, particularly to the kidneys and liver [Liu *et al*., 2021a; Packer *et al*., 2020]. In

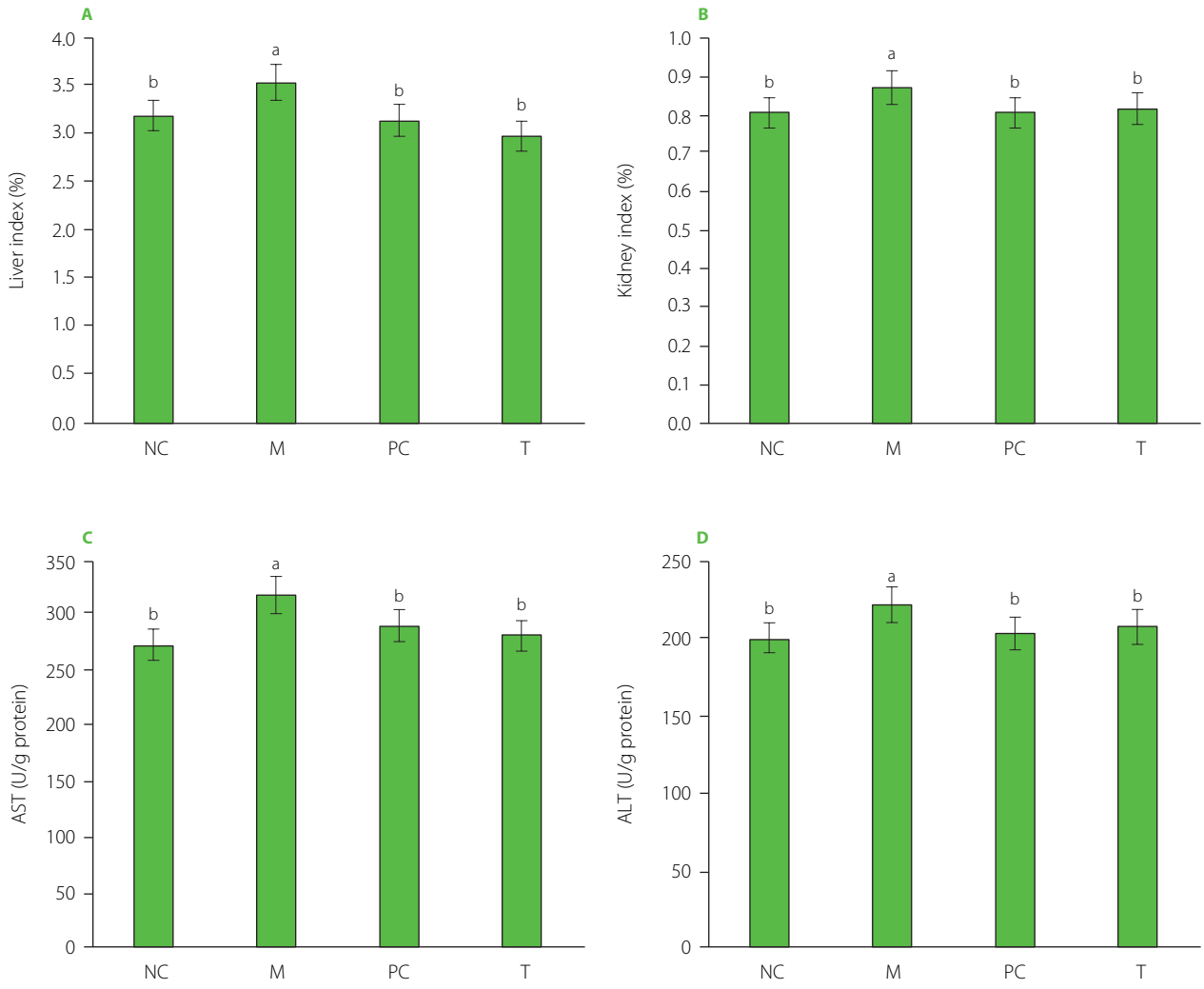

Figure 5. Liver index (**A**) and kidney index (**B**), serum aspartate aminotransferase (AST) levels (**C**), and alanine aminotransferase (ALT) levels (**D**) of rats. Data are presented as mean ± standard deviation (n=3). Different lowercase letters indicate significant differences (p<0.05) between groups. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

our experiment, MSU injection resulted in a significant increase in uric acid levels in the M group, reaching 95.10 µmol/L, which was five times higher than in the NC group (**Figure 1A**), potentially contributing to organ damage. Moreover, as presented in **Figure 5C** and **5D**, serum AST and ALT activity levels were notably elevated in the M group, indicating liver damage in this group. When liver cells are damaged, enzymes like AST and ALT are released into the bloodstream. Impaired liver and kidney function can disrupt uric acid metabolism and excretion, further exacerbating gout symptoms.

Our prior research demonstrated that EHPPL could reduce the abnormal elevation of the liver index in mice with hypercholesterolemia [Deng *et al*., 2025]. Seabuckthorn polysaccharides have been shown to alleviate insulin resistance in diabetic rats, reducing liver and kidney damage. This effect is attributed to the inhibition of the PERK/ATF4/CHOP pathway activity [Yao *et al*., 2021]. Polysaccharides from *Salvia miltiorrhiza* Bunge residue exert anti-liver injury effects and immune enhancement [Jiang *et al*., 2020]. Additionally, sulfate polysaccharides from brown algae have been demonstrated to lower blood uric acid levels in hyperuricemic mice, regulate urea nitrogen and creatinine levels, improve kidney function, reduce oxidative damage to liver and kidney tissues, and help maintain normal organ function [Jiao *et al*., 2011]. Neutral polysaccharides in corn silk also exhibit anti-hyperuricemia effects by inhibiting XOD activity, reducing blood uric acid production, protecting kidney function, and promoting uric acid excretion [Yuan *et al*., 2022]. Furthermore, the green alga *Enteromorpha prolifera* polysaccharides reduce uric acid accumulation in mice by enhancing ABCG2 protein expression [Li *et al*., 2021]. These findings suggest that EHPPL may effectively improve liver and kidney function in rats by enhancing uric acid clearance.

Serum inflammatory markers

The M group exhibited significantly elevated levels of inflammatory markers, including TNF-α, MCP-1, IL-18, IL-1β, LPS, NLRP3, and caspase-1, compared to the NC group (p<0.05) (**Figure 6**). Following EHPPL treatment, the T group showed a pronounced

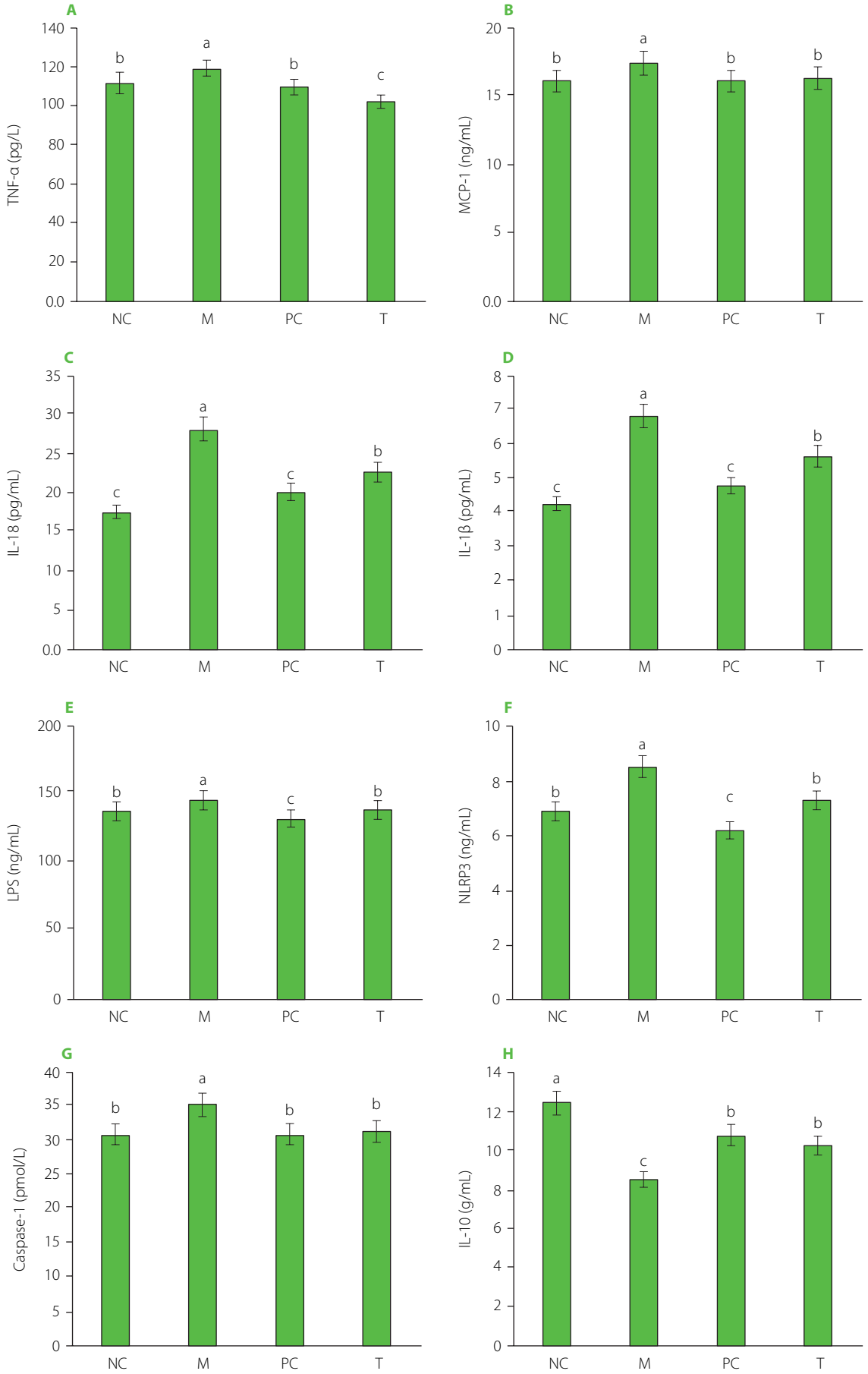

Figure 6. Levels of TNF-α (**A**), MCP-1 (**B**), IL-18 (**C**), IL-1β (**D**), LPS (**E**), NLRP3 (**F**), caspase-1 (**G**), and IL-10 (**H**) in rat serum. Data are presented as mean ± standard deviation (n=3). Different lowercase letters indicate significant differences (p<0.05) between groups. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

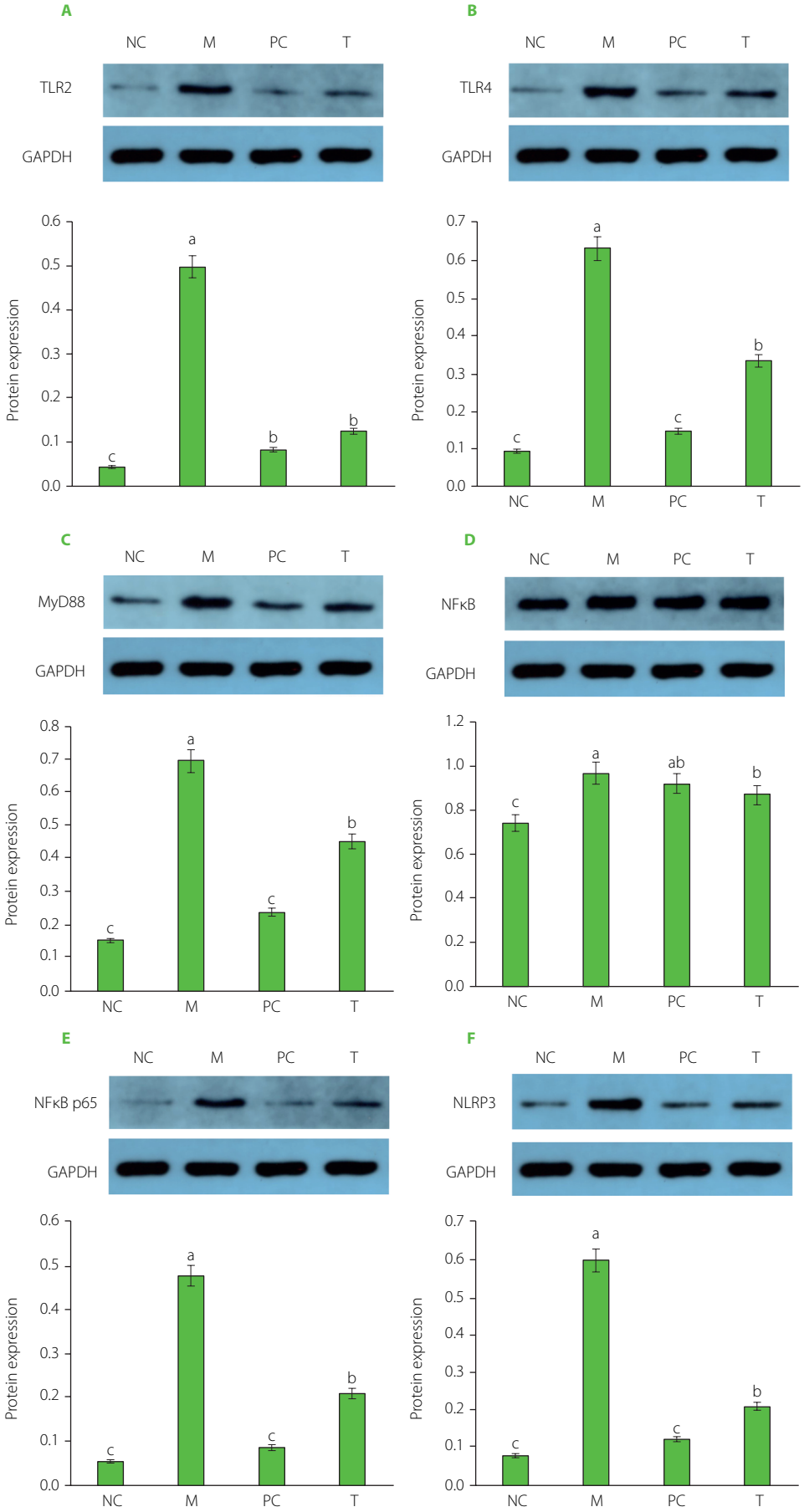

Figure 7. Protein expression of TLR2 (**A**), TLR4 (**B**), MyD88 (**C**), NF-κB (**D**), NF-κB p65 (**E**), and NLRP3 (**F**). Data are presented as mean ± standard deviation (n=3). Different lowercase letters indicate significant differences (p<0.05) between groups. NC, normal control group; M, model group (gout-induced rats); PC, allopurinol group; T, group receiving enzymatically hydrolyzed pectin from leaves of *Premna ligustroides* Hemsl. (EHPPL).

reduction in these markers compared to the M group ($p<0.05$). Acute gout is often characterized by joint inflammation driven by the secretion of inflammatory mediators and immune cell infiltration. The NLRP3 inflammasome plays a pivotal role in activating caspase-1, leading to the maturation of IL-1β and IL-18, both of which are critical in gout pathogenesis. Specifically, IL-1β release triggers inflammation, and excessive NLRP3 inflammasome activation is a primary source of IL-1β in gout [Kim, 2022]. In the present study, MSU stimulated the secretion of inflammatory markers such as TNF-α, MCP-1, IL-18, IL-1β, LPS, NLRP3, and caspase-1, while simultaneously reducing the anti-inflammatory cytokine IL-10. Natural ingredients in food, such as chrysanthemum extract [Peng *et al*., 2019], moringa leaf hydrolysate [Tian *et al*., 2021], rhizomes of *Smilax glabra* Roxb. [Wang *et al*., 2019], celery seed extract [Li *et al*., 2019], *Dendrobium candidum* leaf macroporous resin extract [Lou *et al*., 2020], and *Dendrobium loddigesii* Rolfe extract [Zhang *et al*., 2020], can alleviate arthritis and hyperuricemia by reducing inflammatory responses. In this context, EHPPL was found to inhibit MSU-induced NLRP3 activation, thereby improving gout symptoms through the reduction of inflammatory mediator secretion and immune cell infiltration, as confirmed by HE staining (**Figure 3C**).

■ TLR2, TLR4, MyD88, NF-κB, and NLRP3 signaling pathway

The results regarding key proteins involved in the TLR2/TLR4/MyD88/NF-κB pathway are shown in **Figure 7**. In the M group, the relative expression levels of TLR2, TLR4, MyD88, NF-κB, and NLRP3 in ankle joint tissue were significantly elevated compared to the NC group ($p<0.05$). After EHPPL treatment, these proteins were significantly downregulated ($p<0.05$) compared to the M group. High uric acid levels can activate inflammatory responses, triggering the NLRP3 inflammasome *via* the TLR4 pathway, which promotes the production of caspase-1 and IL-1β. The TLR4/NF-κB signaling pathway plays a critical role in regulating inflammatory factors and immune responses. TLR4, a transmembrane receptor, responds to inflammatory stimuli by activating the MyD88 protein, which, in turn, enhances NF-κB transcriptional activity. NF-κB then translocates to the nucleus and activates the expression of pro-inflammatory cytokines [Wang *et al*., 2018]. These findings suggest that EHPPL mitigates the inflammatory response induced by high uric acid levels by inhibiting the TLR2/TLR4/MyD88/NF-κB pathway.

Under normal conditions, inflammatory cells typically express low levels of NLRP3 protein. However, external stimuli activate NF-κB, leading to increased NLRP3 expression, which subsequently activates caspase-1 and promotes the expression of IL-1β and IL-18 precursors [Kim, 2022]. IL-1β plays a central role in joint inflammation, contributing to the swelling and pain characteristic of acute gout. The NLRP3 inflammasome drives IL-1β release, exacerbating the inflammatory response by inducing vasodilation and attracting neutrophils to the site of urate crystal deposition [Guo *et al*., 2021; Zeng *et al*., 2020]. Furthermore, activated caspase-1 promotes the release of pro-inflammatory mediators, leading to pyroptosis [Aglietti & Dueber, 2017]. Inhibiting IL-1β and caspase-1 generation effectively alleviates inflammation, as demonstrated by EHPPL's anti-inflammatory effects in gout rats. These results suggest that EHPPL's anti-inflammatory properties are likely due to its ability to inhibit NLRP3 inflammasome activation.

CONCLUSIONS

This study demonstrated that EHPPL significantly reduced inflammation in gout rats by decreasing pro-inflammatory cytokines, limiting inflammatory cell infiltration, and inhibiting NLRP3 inflammasome activation. Its anti-inflammatory effects were closely associated with the regulation of the NLRP3 inflammasome. However, further research is needed to fully elucidate the interactions between EHPPL, uric acid, oxidative stress, and the NLRP3 pathway. EHPPL shows promise as a dietary intervention for managing gouty arthritis.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Effect of Prickly Pear (*Opuntia ficus-indica* L.) Peel Incorporation to Meat Batter on the Quality of Functional Chicken Sausages

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While representing a valuable source of high-quality protein, meat products have certain limitations, including their susceptibility to lipid oxidation and a general lack of complex carbohydrates, like dietary fiber. This study explores the potential of prickly pear peel (PPP), an agricultural byproduct rich in dietary fiber and phenolic compounds, as a functional food additive to address these drawbacks. In this study, functional chicken sausages were prepared with varying contents of PPP (2%, 4%, 6%, and 8% of the total meat batter, *w/w*), and their nutritional value, cooking properties, content of thiobarbituric acid reactive substances (TBARS), microbial quality, and sensory acceptability were assessed. PPP incorporation to meat batter significantly enhanced the nutritional value of the sausages, as evidenced by an increase in both ash and dietary fiber contents. Furthermore, PPP addition improved the cooking yield from 85.96% to 89.47% and the water holding capacity from 25.36% to 66.29%. The TBARS value decreased as the total phenolic content of the sausages increased. Notably, after 21 days of refrigeration, the sausages supplemented with 8% PPP exhibited significantly lower total plate counts (4.62×10^5 CFU/g) compared to the control samples (5.54×10^5 CFU/g). Sensory evaluation revealed that the control samples and the sausages with 2% PPP achieved the highest overall acceptability scores among all treatments. These findings support the potential of PPP as a value-added ingredient to improve the nutritional quality, functional properties, and storage stability of meat products.

Keywords: dietary fiber, functional food, meat product, nutritional value, prickly pear fruits

INTRODUCTION

Consumer demands in the food production sector have changed dramatically in recent years. Modern consumers expect food to not only provide essential nutrients but also address nutritional deficiencies and promote well-being. Functional foods, which can be either natural or industrially processed, play a crucial role in meeting these new expectations [Alongi & Anese, 2021]. Meat, such as chicken, is a rich source of protein, omega-3 fatty acids, minerals, and vitamins. Consumption of chicken meat has increased significantly over the last few decades and is expected to rise further. Chicken meat is not only nutrient-dense but also relatively low in calories, making it an excellent choice for those seeking a healthy diet. Additionally, its mild flavor, consistent texture, and light color make it suitable for various processing methods [Petracci *et al*., 2013]. However, despite the nutritional benefits of chicken meat, it does have certain drawbacks. For instance, it lacks dietary fiber, and its high polyunsaturated fatty acid (PUFA) content makes it prone to lipid oxidation, which can lead to changes in nutritional value, color, texture, and flavor [Das *et al*., 2020]. Furthermore, meat products, in general, have

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been associated with high cholesterol, obesity, diabetes, and cardiovascular diseases. Consequently, there is a growing interest in developing "healthier meat products" by reducing unhealthy compounds like nitrates, salt, and saturated fats, while simultaneously enhancing antioxidant capacity and preserving nutritional value [Akram *et al*., 2022]. Numerous recent studies have highlighted the important role of plant-derived materials and their bioactive compounds, including phenolics, in preventing lipid oxidation by neutralizing free radicals [Bai *et al*., 2025; de Oliveira *et al*., 2025]. The meat industry has recognized the protective effects of these plant-based materials, making them an appropriate choice for preserving meat products and lowering the risk of development of various human diseases [Bhat *et al*., 2020].

One such plant with potential health benefits is the prickly pear (*Opuntia* spp.), which is found in arid and semiarid regions of Latin America, the Mediterranean region, and South Africa [Sipango *et al*., 2022]. Prickly pear fruit (*Opuntia ficus-indica* L.) has gained popularity in recent years due to its nutritional and antioxidant properties. The fruit can be green, red, or purple, depending on the presence of pigments, like betalains [García-Cayuela *et al*., 2019]. Notably, the peel of the prickly pear fruit accounts for 30% to 50% of the total fruit, depending on the cultivar [Gómez-Salazar *et al*., 2022].

Fruit peels, often discarded as waste, are a valuable and cost-effective source of phytochemicals with significant functional potential. Prickly pear peels (PPP) contain cellulose, hemicellulose, pectin, proteins, minerals, and antioxidants, making them suitable for various food applications [Barba *et al*., 2017]. Despite being considered waste in many cultures, PPP contain numerous bioactive compounds that elicit benefits to human health and can be utilized in various food products. These peels are rich in antioxidants, which protect the body from oxidative stress and free radicals. Moreover, the phenolics and betalains present in PPP have demonstrated antimicrobial, anti-inflammatory, antioxidant, and anticancer properties [Melgar *et al*., 2017; Reguengo *et al*., 2022]. Another bioactive compound found in PPP are phytosterols, which can help lower cholesterol levels and reduce the risk of heart disease [Amaya-Cruz *et al*., 2019; Reguengo *et al*., 2022]. Additionally, PPP is high in dietary fiber and vitamin C [Amaya-Cruz *et al*., 2019; Jiménez-Aguilar *et al*., 2015].

Dietary fiber, a non-starch polysaccharide that cannot be broken down and absorbed by human digestive enzymes, offers various health benefits. However, for high-fiber products to be appealing, they should also possess improved technological characteristics. With its water retention ability, lack of distinct flavor, and ability to reduce cooking loss, fiber is an excellent component for the development of meat products [Akram *et al*., 2022; Zaini *et al*., 2020].

While the use of PPP has been explored in improving pan bread quality [Anwar & Sallam, 2016], biscuit formulations [Bouazizi *et al*., 2020], and sustainable baker's yeast production [Diboune *et al*., 2019], there is limited research on its application in processed meat products. Therefore, the aim of this study was to investigate the impact of PPP incorporation on the quality of functional chicken sausages. The study also examined the nutritional and chemical properties of the sausages, as well as their storage stability and sensory characteristics.

MATERIALS AND METHODS

Materials

Prickly pear fruits (*Opuntia ficus-indica* L.) were collected randomly from different plants at multiple locations from the Haifan Directorate in southern Taiz City, Yemen (latitude 13°16′06″ N, longitude 44°18′16″ E). Boneless chicken breast was purchased from a local supermarket in Yibin City, Sichuan, China. Ingredients for sausage formulation, including chicken skin fat, soy protein isolate, ground black pepper, and non-iodized salt, were obtained from local markets in Yibin City. All chemicals and reagents used were of analytical grade.

Prickly pear peel powder preparation

To ensure cleanliness, prickly pear fruits were thoroughly washed under running tap water to remove dust and debris. The pulp and peel were then manually separated using a knife. The separated peels were sliced into small pieces (approximately 2×2 cm^2), which were then soaked in a solution of sodium hypochlorite (40 mg/L) for 30 min to reduce microbial load [Bouazizi *et al*., 2020]. Following this, the peels were thoroughly rinsed three times with distilled water to remove any residual hypochlorite. The rinsed PPP were then oven-dried at 40°C for 48 h using an electrothermal blast drying oven (WLG-45B, Tianjin, China). The drying process continued until the powder's moisture content was reduced to 6.36±0.71 g/100 g. The dried peels were subsequently ground into a fine powder using a hammer mill, then sieved through a 60-mesh screen. The resulting peel powder was placed in polyethylene bags and stored at 4°C for future use.

Preparation of chicken sausages and their storage

The sausages were made with boneless chicken breast following the procedure of Manzoor *et al*. [2022] with a few alterations. In a bowl chopper, 1,000 g of boneless chicken breast was blended with 200 g of chicken skin fat, 20 g of soy protein isolate, 150 mL of ice water, 2 g of ground black pepper, and 10 g of non-iodized salt. These ingredients were mixed for 35 s to achieve a highly homogeneous meat batter. The prepared PPP powder was then incorporated into the meat batter at four different levels (*w/w*, based on the total meat batter weight): 2%, 4%, 6%, and 8%. A control sample (0% PPP) was prepared using the same method, but without PPP addition. The meat batters (both control and PPP-supplemented) were filled into cellulose casings using a sausage stuffer and labeled. The sausages were cooked in a conventional electric oven at approximately 200°C for 15 min, followed by 30 min of steaming at 75±2°C in a steamer, and then 20 min of dipping in cold water at 15°C. After draining, the sausages were placed in airtight nylon-polyethylene bags and stored at 4°C for up to 21 days. Their content of thiobarbituric acid reactive substances (TBARS) and microbiological quality were determined at regular intervals throughout the storage period (on days 0, 7, 14, and 21).

All other parameters, including physical, chemical, and cooking properties, were measured only once on day 0.

Proximate analysis

Chemical composition of the sausages was determined using the methods recommended by AOAC International [AOAC, 2007]. The oven-drying method at 105°C was used to determine the moisture content (method no. 950.46). The protein content was determined using the Kjeldahl procedure (method no. 981.10], with the total nitrogen content multiplied by a conversion factor of 6.25. The lipid content was determined using the Soxhlet method by utilizing a solvent extraction system with petroleum ether (method no. 960.39). The total ash content was determined using an incinerator at 525°C (method no. 920.153). The carbohydrate content was calculated by subtracting from 100 the sum of moisture, lipid, protein, and ash contents expressed in g *per* 100 g of sausage.

Total dietary fiber analysis

The total dietary fiber (TDF) content of both the sausages and PPP powder was determined using the enzymatic-gravimetric analysis proposed by the AOAC International [AOAC, 2005]. A gram of defatted dried sample was weighed and digested with a series of enzymes. First, *α*-amylase was added, and the combination was heated for 15 min at 98–100°C. This was followed by the addition of protease and amyloglucosidase, and a 30-min incubation at 60°C. The residue was filtered, washed with acetone and 95% ethanol before being dried and weighed. The protein content was determined using the Kjeldahl method, and the ash content was determined by heating a similar sample to 525°C in a muffle furnace. By subtracting the weight of protein and ash from the weight of the residue, the TDF content was calculated and expressed in g *per* 100 g of sausage or PPP powder.

pH determination

In 50 mL of distilled water, 10 g of chicken sausages were homogenized for 30 s, and the pH of the homogenate was measured using a pH meter (Mettler Toledo FE20/EL20, Shanghai, China).

Water-holding capacity determination

The centrifugation technique was used to estimate the water-holding capacity (WHC), according to the procedure by Zhuang *et al*. [2007] with some modifications. A 10-g sample of sausage (w_{sample}) was thoroughly homogenized in a laboratory blender with 15 mL of a 0.6 M NaCl solution. The mixture was then centrifuged at 3,000×*g* and 4°C for 15 min. After centrifugation, the supernatant was carefully discarded, and the remaining residue was weighed ($w_{residue}$). The WHC was calculated using Equation (1):

$$WHC\ (\%) = \frac{W_{residue} - W_{sample}}{W_{sample}} \times 100 \quad (1)$$

Cooking yield determination

To assess the cooking yield, the sausages were weighed before ($w_{initial}$) and after (w_{final}) the complete three-step cooking and cooling process, following the method described by Choi *et al*. [2014]. The cooking yield was calculated using Equation (2):

$$Cooking\ yield\ (\%) = \frac{W_{final}}{W_{initial}} \times 100 \quad (2)$$

Color coordinate measurement

A Konica Minolta CR-400 chromameter (Tokyo, Japan) was applied to measure the color of sausages. Six perpendicular measurements were recorded, and photographs of various surfaces of the sausage were taken. The results were reported as CIE *a** (redness), *L** (lightness), and *b** (yellowness). The instrument was calibrated using a standard white tile before measurements. The illuminant used was D65, and the observer angle was 10°.

Total phenolic content determination

The method with the Folin-Ciocalteu reagent was used to determine the total phenolic content (TPC) of both the sausages and PPP powder, as described by Özünlü *et al*. [2018], with some alterations. Chicken sausages or PPP powder (1 g) were homogenized and extracted overnight at 4°C in 10 mL of methanol with continuous agitation at 180 rpm. The mixture was then centrifuged at 3,000×*g* for 10 min, and the supernatant was collected. Then, 0.5 mL of the chicken sausage extract, 2.5 mL of the Folin-Ciocalteu reagent (10-fold diluted), and 2 mL of a sodium carbonate solution (75 g/L) were combined and left for 30 min at room temperature. The absorbance at 765 nm was measured using a UV-visible spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China), and gallic acid was used as the standard. The results were expressed as mg of gallic acid equivalent (GAE) *per* 100 g of sausage or PPP powder.

Texture profile analysis

Texture profile analysis (TPA) was conducted using a TA.XT Plus texture analyzer (Stable Micro Systems, Godalming, UK) on six replicates of each sample. The cooked sausage samples were allowed to cool to room temperature before analysis [Choe *et al*., 2013]. Each sausage was cut into standardized cylindrical pieces measuring 20 mm in height and 25 mm in diameter. To ensure consistent and reproducible contact with the compression probe, the samples were sliced horizontally to create uniform, flat cross-sectional surfaces. A cylindrical aluminum probe was used to perform a two-cycle compression test. The pre-test, test, and post-test speeds were set at 2.0, 1.0, and 5.0 mm/s, respectively. Samples were compressed to 50% of their original height, and a trigger force of 5 g was applied. Data were collected and analyzed for hardness (N), springiness, cohesiveness, and chewiness (N).

Determination of the content of thiobarbituric acid reactive substances

The content of TBARS was determined on days 0, 7, 14, and 21 following the procedure proposed by Manzoor *et al*. [2023] with some alterations. Chicken sausage samples (5 g) were homogenized in a blender with 25 mL of 7.5% trichloroacetic acid (TCA)

for 2 min. After a 10-min centrifugation at 3,500×*g*, the supernatant was filtered and mixed with 5 mL of a 0.02 M thiobarbituric acid (TBA) solution. The samples were then immersed in a 95°C hot water bath for 35 min. The reaction products between TBA and the oxidized substances were measured at 532 nm using a UV-1800 PC spectrophotometer (Mapada Instruments Co., Ltd., Shanghai, China). The TBARS values were calculated from a standard curve with 1,1,3,3-tetraethoxypropane (TEP) and presented as mg of malondialdehyde (MDA) equivalent *per* kg of sausage (mg MDA/kg).

Microbiological analysis

The microbial load of the chicken sausage treatments was determined on days 0, 7, 14, and 21 by estimating the total plate count using the method proposed by Akram *et al.* [2022]. Chicken sausage samples (1 g) were aseptically diluted in 9 mL of sterile peptone water (0.1%, *w/v*). The samples were homogenized using a stomacher for 60 s to ensure proper dispersion. An appropriate serial 10-fold dilutions were prepared. From these dilutions, aliquots were surface-plated onto plate count agar using the streak plate technique. The plates were then incubated aerobically at 37°C for 48 h. The findings were presented as colony forming units *per* g of sausage (10^5 CFU/g).

Sensory evaluation

A trained sensory panel consisting of 30 regular sausage consumers, all experienced in the sensory evaluation of meat products, assessed the chicken sausage samples. Panelists were selected based on their consistent consumption of sausages and their proven ability to identify and differentiate basic tastes and textures. This ability was confirmed through a series of taste and texture identification tests conducted prior to the main evaluation. Before the study, they participated in a brief training session to familiarize themselves with the specific attributes to be evaluated: appearance, color, odor, taste, hardness, juiciness, and overall acceptability. They also learned to use the seven-point hedonic scale for their evaluations. The sensory evaluation took place in individual, well-lit booths designed to minimize distractions. Samples were prepared by removing the casings, cutting the sausages into slices approximately 3 mm thick, and serving them at room temperature on white plastic plates, each randomly numbered with three-digit codes [Stajić *et al.*, 2018]. The sausages were evaluated immediately on the first day of preparation. Three slices of each product were served sequentially in a monadic manner. Panelists used water and unsalted crackers to cleanse their palates between samples. Each attribute (appearance, color, odor, taste, hardness, juiciness) and overall acceptability were rated using a seven-point hedonic scale, defined as follows: 1 (extremely dislike), 2 (dislike), 3 (slightly dislike), 4 (neither dislike nor like), 5 (slightly like), 6 (like), 7 (extremely like).

Statistical analysis

All experiments were conducted in triplicate, and the results were reported as mean and standard deviation. A one-way analysis of variance (ANOVA) with Duncan's multiple range test was performed using SPSS version 24.0 (IBM Corp., Armonk, NY, USA) to evaluate the impact of PPP on the sensory and physicochemical attributes of the chicken sausages. A significance level of $p<0.05$ was set to determine the differences between the means for the various attributes.

RESULTS AND DISCUSSION

Proximate composition of chicken sausages

The proximate composition of chicken sausages is illustrated in **Table 1**, and the results indicate that there were no significant variations ($p\geq0.05$) in moisture, protein, or lipid content among the sausages. However, as the amount of PPP added to the meat batter increased, the ash content in sausages also significantly increased ($p<0.05$). This increase in ash content can be attributed to the high levels of TDF, resistant starch, and minerals in the PPP [El-Beltagi *et al.*, 2023; Parafati *et al.*, 2020]. The mineral content influences the ash content, indicating that the inclusion of PPP increases the nutritional value in terms of mineral content [Park *et al.*, 2011]. Similar findings were reported by López-Vargas *et al.* [2014] in their study on the impact of passion fruit albedo on pork burgers and by Zaini *et al.* [2020] in their investigation on the influence of banana peel powder on chicken sausages.

The dietary fiber content of sausages

Because of a low dietary fiber content of meat, its consumption has been linked to chronic diseases. However, the inclusion of PPP in chicken sausages significantly increased the TDF content ($p<0.05$), as shown in **Table 1**. The TDF content of PPP used in this study was 31.70± 0.01 g/100 g. This high fiber content accounts

Table 1. Proximate composition (g/100 g) of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*).

Parameter	Control	2% PPP	4% PPP	6% PPP	8% PPP
Moisture	71.03±0.42^{a}	68.96±0.14^{a}	67.53±0.24^{a}	67.74±0.18^{a}	67.65±0.24^{a}
Protein	14.26±0.27^{a}	14.57±0.12^{a}	14.94±0.34^{a}	15.03±0.43^{a}	14.82±0.44^{a}
Lipid	8.29±0.17^{a}	8.07±0.35^{a}	8.43±0.45^{a}	8.56±0.15^{a}	8.36±0.35^{a}
Ash	3.39±0.09^{c}	3.59±0.17^{b}	3.58±0.24^{b}	4.52±0.14^{ab}	4.84±0.10^{a}
TDF	1.68±0.17^{e}	2.42±0.12^{d}	3.81±0.15^{c}	4.34±0.10^{b}	5.39±0.30^{a}

All values are means of triplicate determinations ± standard deviation. Means within the same row with different letters are significantly different at $p<0.05$. TDF, total dietary fiber.

for the significant increase in TDF determined in the sausages with added PPP, which ranged from 1.68 g/100 g for the control to 5.39 g/100 g for the 8% PPP sausage. The presence of fiber in the control sausage may be attributed to the other ingredients in the formulation, such as soy protein isolate and spices. The inclusion of fiber in meat products improves their health benefits. The presence of fiber in meat shortens the time it spends in the intestines, minimizing the exposure of colon cells to carcinogenic substances. Thus, the presence of fiber in meat could mitigate its carcinogenic impact [Arias-Rico *et al*., 2025]. Similar to our findings, Zaini *et al*. [2019] observed an increase in crude fiber content in fish patties when banana peel powder was added.

pH of sausages

The pH value is a crucial variable to measure as it affects the color, texture, shelf-life, and water-holding capacity of meat products. As displayed in **Table 2**, the incorporation of 2%, 4%, 6%, and 8% PPP to chicken sausages led to a significant decrease in pH values ($p<0.05$) compared to the pH value of the control sausages. Manzoor *et al*. [2022] reported lower pH values in chicken sausages supplemented with various levels of mango peel extract compared with the control sample, which is consistent with our findings. Furthermore, Mahmoud *et al*. [2017] discovered lower pH values in burgers supplemented with various amounts of orange peel when compared with the control sample. These findings can be explained by the presence of organic acids in the PPP [Tunç *et al*., 2025]. The reduction in pH is beneficial as it hinders microbial growth under lower pH conditions.

Cooking properties of chicken sausages

WHC is a crucial quality characteristic affecting meat products' texture and overall sensory properties. It is primarily influenced by the muscle pH and protein structure [Mahmoud *et al*., 2017]. Water loss poses a major concern for the meat industry, as it reduces product weight and can negatively impact quality [Hautrive *et al*., 2008]. In our study, the addition of PPP significantly increased ($p<0.05$) the WHC of the sausages (**Table 2**). A significant increase was observed at the 4%, 6%, and 8% PPP supplementation levels, with WHC values of 34.5%, 54.7%, and 66.3%, respectively, compared to the control. This increase can be attributed to the high TDF content of the PPP, which forms a gel-like network that effectively traps and holds water within the meat matrix.

Table 2. The pH values and cooking properties of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*).

Sausage	pH	WHC (%)	Cooking yield (%)
Control	6.17±0.03^{a}	25.4±1.8^{d}	85.96±0.75^{d}
2% PPP	5.98±0.02^{b}	25.9±1.7^{d}	87.69±0.37^{c}
4% PPP	5.87±0.02^{c}	34.5±1.4^{c}	88.26±0.49^{b}
6% PPP	5.80±0.03^{d}	54.7±1.9^{b}	88.84±0.28^{ab}
8% PPP	5.72±0.02^{e}	66.3±1.7^{a}	89.47±0.37^{a}

All values are means of triplicate determinations ± standard deviation. Means within the same column with different letters are significantly different at $p<0.05$. WHC, water-holding capacity.

As shown in **Table 2**, the cooking yield of all sausages with PPP was significantly ($p<0.05$) higher compared to the control (85.96%), and it increased from 87.69% to 89.47% as the amount of PPP in sausages increased from 2% to 8%, respectively. Zaini *et al*. [2020] discovered that adding 6% banana peel powder to sausages increased the cooking yield to 99.54%, compared to 96.96% determined for the control sausage. Similarly, Mahmoud *et al*. [2017] discovered that adding 10% orange peel powder increased the cooking yield of burger by up to 57.61% compared to the control sausages (46.53%). The increased water retention capacity can be attributed to the fiber network in meat products, which prevents water loss during cooking. This improvement enhances the texture and sensory properties of the final product.

Color coordinates of chicken sausages

The color of meat and meat products is often used to assess their freshness. Natural plant-based antioxidants can play a vital role in preserving the color of cooked meat products by mitigating lipid oxidation, which can lead to color degradation [Lavado & Cava, 2025].

The color values of chicken sausages are shown in **Table 3**. The incorporation of PPP significantly ($p<0.05$) reduced the lightness value (L^*) of the sausages compared to the control samples. The darker color of the chicken sausages is likely due to the presence of natural red and yellow pigments, specifically betalains, in PPP [Smeriglio *et al*., 2021]. The inclusion of PPP significantly ($p<0.05$) raised the redness (a^*) and reduced the yellowness (b^*) values of chicken sausages. Increasing the levels of PPP from 2% to 4% resulted in an increase in a^* values (from 3.47 to 4.57) and a decrease in b^* values (from 12.98 to 11.15) but further increasing the PPP content of sausages had no significant ($p\geq0.05$) effect on these color coordinates. These results are consistent with findings from other studies on the use of PPP in food products. For instance, Parafati *et al*. [2020] reported that adding PPP as a functional ingredient to bread increased its a^* value while decreasing the L^* and b^* values. Similarly, Bouazizi *et al*. [2020] found that incorporating PPP powder into biscuits significantly decreased both the L^* and b^* values.

Textural properties of chicken sausages

The textural characteristics of chicken sausages with PPP are provided in **Table 3**. The inclusion of PPP in meat batter significantly ($p<0.05$) increased chewiness and hardness of the chicken sausages, while decreased their cohesiveness. However, no significant ($p\geq0.05$) variation was discovered in springiness. Similar results were reported by Younis *et al*. [2021] in their study on buffalo meat sausages, where the inclusion of mosambi peel powder increased hardness while decreasing springiness. A variety

Table 3. The color coordinates and textural properties of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*).

Sausage	Color coordinate			Textural property			
	*L**	*a**	*b**	Hardness (N)	Springiness	Cohesiveness	Chewiness (N)
Control	60.89±1.19^{a}	2.34±0.18^{c}	15.25±0.32^{a}	58.29±1.25^{e}	0.85±0.02^{a}	0.75±0.02^{a}	12.17±0.69^{d}
2% PPP	53.72±0.95^{b}	3.47±0.56^{b}	12.98±0.28^{b}	63.72±0.75^{d}	0.84±0.01^{a}	0.71±0.05^{b}	17.07±1.10^{c}
4% PPP	48.36±1.29^{c}	4.57±0.24^{a}	11.15±0.54^{c}	67.26±1.09^{c}	0.82±0.04^{a}	0.66±0.04^{c}	19.35±0.88^{b}
6% PPP	44.84±0.82^{d}	4.72±0.13^{a}	10.24±0.47^{d}	73.64±0.92^{b}	0.79±0.03^{a}	0.63±0.04^{d}	22.16±1.45^{a}
8% PPP	42.36±0.92^{e}	4.77±0.54^{a}	10.72±0.13^{cd}	76.16±1.85^{a}	0.81±0.03^{a}	0.61±0.03^{d}	22.47±1.22^{a}

All values are means of triplicate determinations ± standard deviation. Means within the same column with different letters are significantly different at $p<0.05$. *L**, lightness; *a**, redness; *b**, yellowness.

of factors influence the texture of meat products, including water and lipid content, lean meat particle size, non-meat ingredients, and others [Santhi *et al*., 2017]. Han & Bertram [2017] noted that the impact of fiber on meat product texture depended on the type of fiber present. They discovered that soluble fiber could increase meat product strength, whereas insoluble fiber triggered the opposite effect. The soluble dietary fiber in PPP can form a three-dimensional gel network. This network can alter the interactions between proteins and water, which in turn influences the tenderness and overall structure of the meat product [Ahmad *et al*., 2020; Parafati *et al*., 2020]. Conversely, the insoluble fraction of dietary fiber may lead to a hard and brittle texture by drawing water away from surrounding molecules [Han & Bertram, 2017]. This highlights the importance of considering the type of fiber present in meat products when evaluating its impact on texture and overall quality.

Cohesiveness refers to the internal bond strength within a food product, describing how effectively its components hold together. The findings that higher levels of PPP led to a reduction in cohesiveness are consistent with results of a study conducted by Zaini *et al*. [2020], where the addition of banana peel powder reduced the cohesiveness of chicken sausages. Moreover, Ktari *et al*. [2014] reported that removing fiber and adding fat to meat products increased their cohesiveness. When PPP was added, the chewiness value increased compared to the control samples. According to Barretto *et al*. [2015], chewing fiber-rich food requires more energy. Furthermore, chewiness is influenced by hardness, with chewiness increasing as texture hardness increases.

Total phenolic content of chicken sausages

Phenolic compounds have strong antioxidant activity as they can donate hydrogen atoms to interrupt radical chain reactions and convert free radicals into stable molecules, thereby preventing fat rancidity [Santos-Sánchez *et al*., 2019]. The TPC of the PPP powder used in this study was 734.20±0.63 mg GAE/100 g. The TPC of the chicken sausage samples is displayed in **Figure 1**. In the chicken sausage samples supplemented with PPP, it ranged from 134.41 to 252.74 mg GAE/100 g, and was significantly higher ($p<0.05$) than in the control sausage (129.62 mg GAE/100 g). When 8% PPP was added to the meat batter, the TPC of sausages was the highest. This finding aligns with that of Bouazizi *et al*. [2020], who discovered that the PPP powder used as a biscuit ingredient improved the total phenolic content of the product. The findings indicate a significant relationship between PPP additives and the phenolic content of chicken sausages, with increasing PPP levels leading to an increase in the total phenolic content.

Oxidative stability of chicken sausages

Lipid oxidation results in the production of primary (hydroperoxides) and secondary (carbonyl compounds) products. The latter can be measured using thiobarbituric acid. Unstable hydroperoxides are susceptible to degradation, leading to carbonyl molecules that are capable of interacting with substances like amino acids, peptides, and proteins [Hęś, 2017]. Lipid oxidation negatively affects meat quality and acceptability [Domínguez *et*

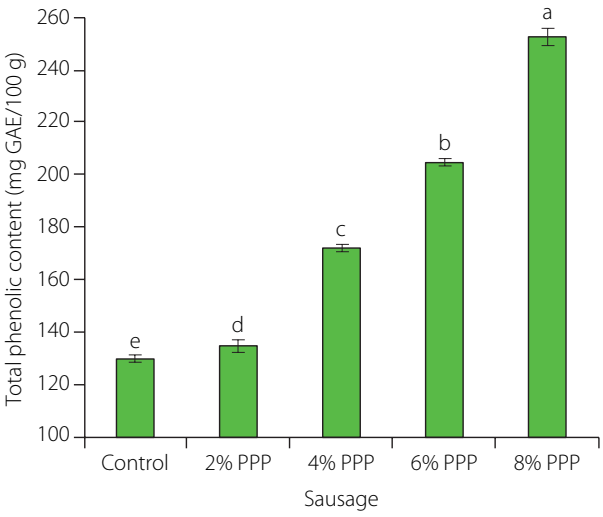

Figure 1. Total phenolic content of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*). Data are presented as the mean and standard deviation of three independent measurements. Different letters above the columns indicate significant differences at $p<0.05$. GAE, gallic acid equivalent.

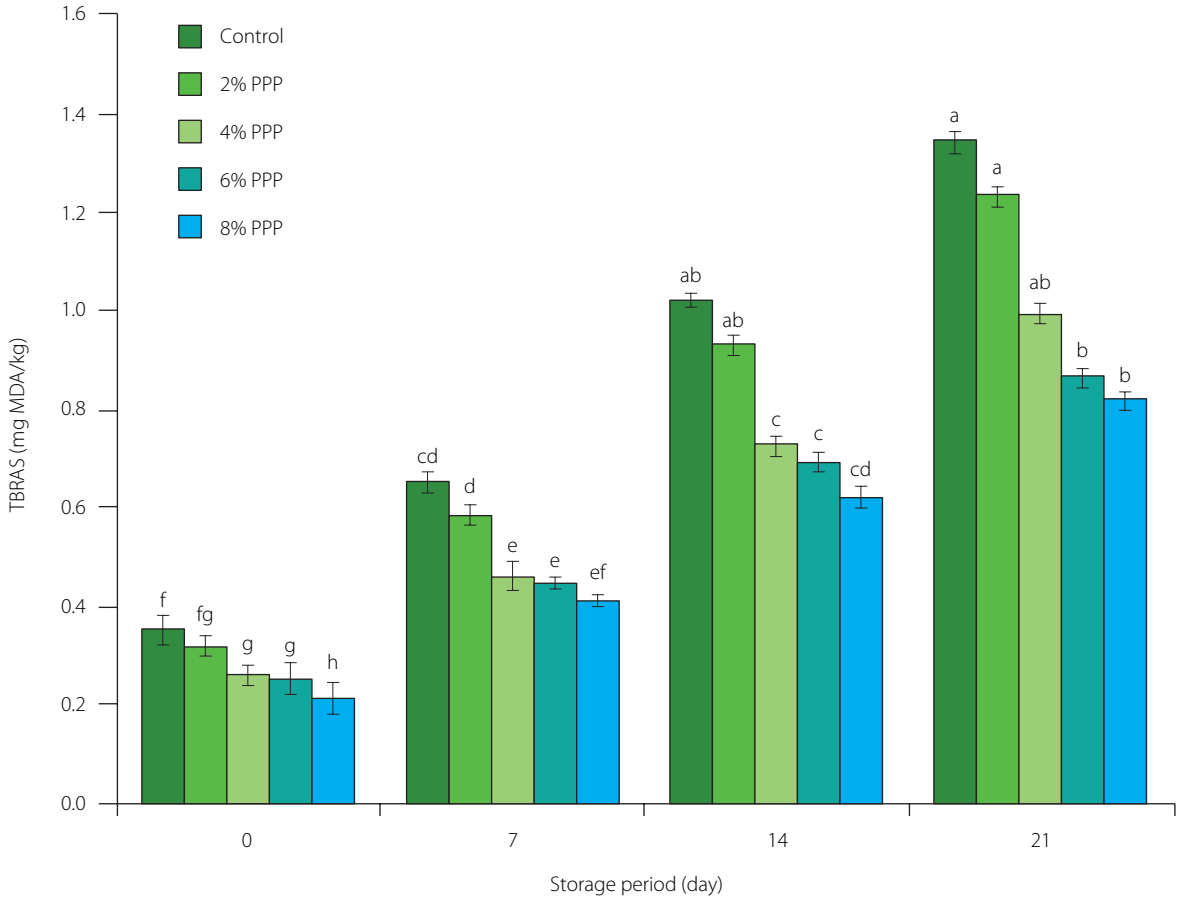

Figure 2. Thiobarbituric acid reactive substance (TBARS) values of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*) during storage. Data are presented as the mean and standard deviation of three independent measurements. Different letters above the columns indicate significant differences at $p<0.05$. MDA, malondialdehyde equivalent.

al., 2019]. The secondary oxidation products, including MDA, are associated with undesirable meat flavor and odor [Domínguez *et al*., 2019]. The TBARS values of the chicken sausages containing four different levels of PPP and control samples are shown in **Figure 2**. During storage, the sausages containing 4%, 6%, and 8% PPP exhibited significantly lower TBARS values ($p<0.05$) compared to the control sample. At the end of the storage period, the control sample had the TBARS value of 1.344 mg MDA/kg, while the sausage sample with 8% PPP had the value of 0.816 mg MDA/kg. This demonstrates that PPP effectively suppressed lipid oxidation compared to the control by slowing down lipid oxidation throughout the storage period. The antioxidant properties of PPP, attributed to their phenolic content, may be responsible for the decrease in TBARS values. Phenolic compounds possess redox properties that enable them to act as antioxidants by scavenging free radicals, quenching singlet oxygen, and decomposing peroxides [Bai *et al*., 2025]. Similar findings were reported by Amrane-Abider *et al*. [2023], who discovered that PPP extract significantly increased the oxidative stability of margarine. Likewise, Gonçalves *et al*. [2024] found that adding freeze-dried prickly pear pulp, which includes peel components, improved the oxidative stability of chicken patties.

Microbiological stability of chicken sausages

Figure 3 depicts the bacterial load of chicken sausage samples incorporated with PPP. The bacterial load was measured immediately after sausage formulation (0 day), and on days 7, 14, and 21 of cold storage. As expected, the bacterial load values successively increased with storage time. However, the incorporation of PPP significantly delayed bacterial growth compared to the control samples. The PPP-supplemented chicken sausages exhibited lower microbial counts, with values of 2.81×10^5, 2.65×10^5, 2.58×10^5, and 2.47×10^5 CFU/g for 2%, 4%, 6%, and 8% PPP, respectively. In contrast, the control group had a microbial count of 2.94×10^5 CFU/g. After 21 days of cold storage, the sausages with 8% PPP had the lowest total microbial count (4.62×10^5 CFU/g); while the control product had 5.54×10^5 CFU/g. This can be attributed to the inhibitory effect of PPP's phenolic compounds on spoilage bacteria. The antimicrobial properties of PPP resulting from the activity of phenolic compounds have been previously reported [Melgar *et al*., 2017]. Akram *et al*. [2022] observed a similar decreasing trend in bacterial load values when banana peel powder was added to chicken nuggets. Similarly, Abdel-Naeem *et al*. [2022] found a similar trend in the microbial load with the addition of fiber-rich peels to meat products.

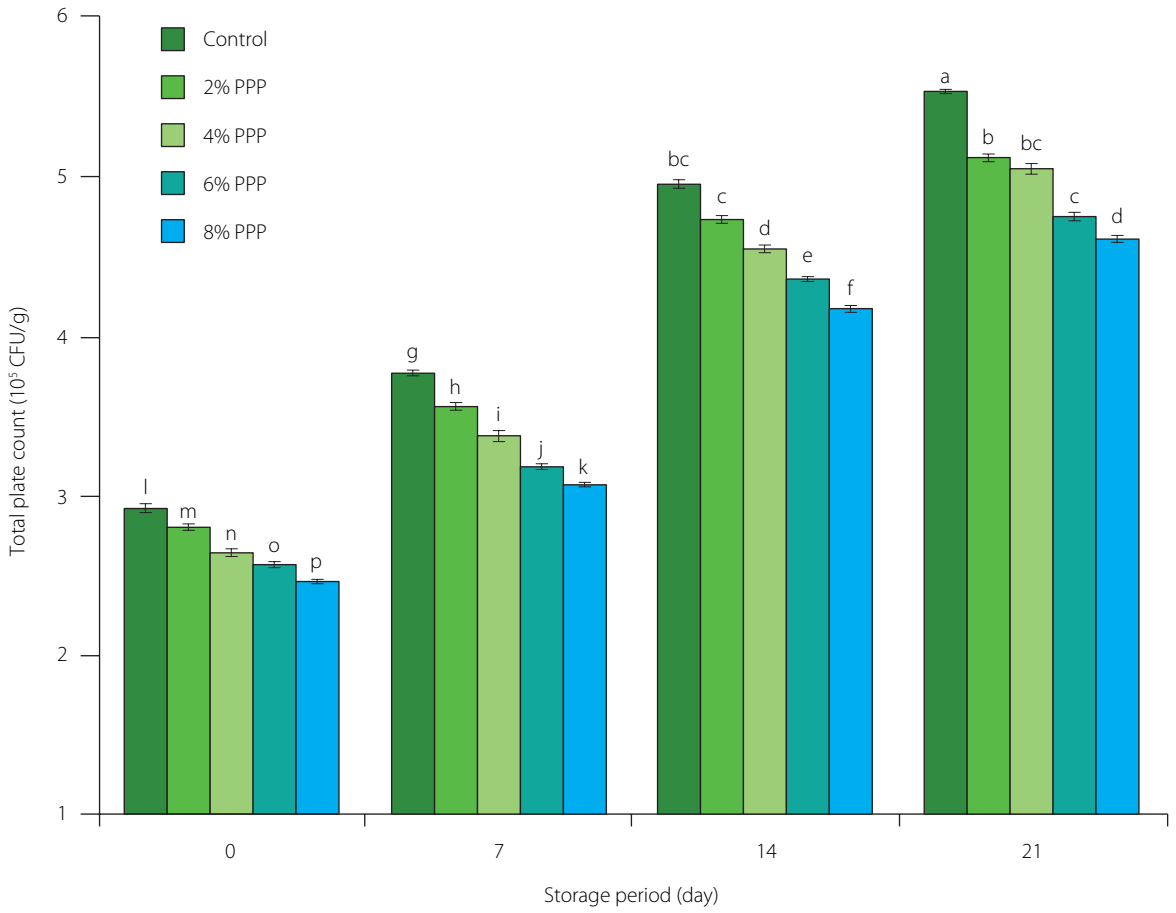

Figure 3. Total plate count of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*) during storage. Data are presented as the mean and standard deviation of three independent measurements. Different letters above the columns indicate significant differences at $p<0.05$.

Sensory evaluation of chicken sausages

The sensory scores of the functional chicken sausages containing varying levels of PPP are displayed in **Table 4**. Various sensory parameters, such as appearance, color, odor, taste, juiciness, hardness, and overall acceptability were assessed using a seven-point hedonic scale. The sensory evaluation indicated that the overall acceptability of the chicken sausages with 2% PPP was rated 4.82, which was not significantly different ($p\geq0.05$) from the control, indicating a similar level of preference among panelists. However, as the incorporation level of PPP increased to 4% and 6%, the overall acceptability scores significantly decreased ($p<0.05$) to 4.47 and 4.17, respectively. The sausages with 8% PPP had the lowest overall acceptability score of 3.84, which falls within the "slightly dislike" to "neither dislike nor like" range on the seven-point hedonic scale. The increasingly higher content of PPP resulted in increasingly lower scores for appearance, color, odor, taste, and juiciness of the chicken sausages. However, the 2% PPP chicken sausages were rated significantly higher ($p<0.05$) in terms of hardness compared to the control sausage. Sensory evaluation scores align with findings from

Table 4. Sensory scores of chicken sausages with different levels of prickly pear peel (PPP) powder (2–8% of the total meat batter, *w/w*).

Sausage	Appearance	Color	Odor	Taste	Hardness	Juiciness	Overall acceptability
Control	4.98±1.23^{a}	4.85±1.02^{a}	5.15±0.62^{a}	5.25±0.67^{a}	4.55±0.77^{b}	5.08±0.75^{a}	4.94±0.82^{a}
2% PPP	4.84±0.85^{ab}	4.73±0.87^{ab}	4.71±0.85^{b}	4.62±0.46^{b}	4.83±1.02^{a}	5.17±0.85^{a}	4.82±0.94^{ab}
4% PPP	4.26±1.12^{b}	4.45±1.04^{b}	4.46±0.74^{c}	3.87±0.49^{c}	4.33±0.41^{c}	4.92±0.68^{b}	4.47±0.48^{b}
6% PPP	4.03±0.92^{bc}	3.89±0.93^{c}	4.13±0.66^{d}	3.73±0.57^{cd}	4.07±0.72^{d}	4.64±0.92^{c}	4.17±0.55^{c}
8% PPP	3.86±1.05^{c}	3.55±0.68^{d}	3.75±1.03^{e}	3.38±0.97^{d}	3.47±0.85^{e}	4.18±0.74^{d}	3.84±0.77^{d}

All values are means of triplicate determinations ± standard deviation. Means within the same column with different letters are significantly different at $p<0.05$. A seven-point hedonic scale was used: 1 (extremely dislike), 2 (dislike), 3 (slightly dislike), 4 (neither dislike nor like), 5 (slightly like), 6 (like), and 7 (extremely like).

a previous study conducted by Chappalwar *et al*. [2022], which reported similar effects of banana peel powder and flour on the organoleptic properties of chicken patties. In contrast, Zaini *et al*. [2020] found that incorporating banana peel powder at concentrations exceeding 2% resulted in a decrease in the sensory perception of chicken sausages. Additionally, Parafati *et al*. [2020] reported that bread made with 10% PPP flour received the highest total sensory evaluation scores, which decreased at PPP flour incorporation levels of 15% and 20%.

CONCLUSIONS

PPP, with its fiber and phenolic compounds, contributes to the health-promoting properties and improved quality of chicken sausages. Supplementation of chicken sausages with 2%, 4%, 6%, and 8% PPP significantly delayed microbial proliferation and suppressed lipid oxidation throughout the storage period, indicating improved product stability. Furthermore, the addition of PPP improved product quality parameters, such as WHC and cooking yield. Sensory evaluations revealed that the control samples and the sausages with 2% PPP achieved comparable overall acceptability scores. Conversely, higher incorporation level of PPP (4–8%) resulted in a significant decline in sensory attributes. In summary, incorporating PPP into chicken sausages offers an effective strategy for enhancing their nutritional value. However, it is crucial to carefully consider the inclusion level to ensure consumer acceptability. The 2% PPP content in meat batter (*w/w*) represents an optimal balance between enhancing nutritional benefits and preserving desirable sensory qualities of sausages.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Application of Polyamide Microfilters as an Alternative to Cheesecloth in Labneh Production: Effects on Processing Efficiency and Product Quality

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This study aimed to evaluate the effectiveness of polyamide microfilters (MFs; 10–75 µm pore size) as an alternative process enhancement to traditional cheesecloth for labneh production. Fresh cow's milk was fermented into set yogurt and strained using either cheesecloth (control) or MFs. Labneh yield ranged from 21.2 g/100g (control) to 29.8 g/100 g (MF10), with MF10 showing significantly higher yield. The total solid content varied from 20.5 g/100 g (MF10) to 32.8 g/100 g (control), while fat content ranged between 7.9 and 12.5 g/100 g. Microbiological analysis revealed lower mesophilic aerobic count (3.3–4.6 $\log_{10}$ cfu/g) and yeast and mold count (1.1–3.6 $\log_{10}$ cfu/g) in the MF-treated samples, indicating enhanced hygiene. Microscopic imaging revealed that MFs had uniform, smooth surfaces, whereas cheesecloth consisted of loosely arranged cellulose fibers, which accounted for the differences in microbial retention. Whey drainage kinetics fit a linear model well (R^2>0.99), demonstrating predictable separation behavior. Apparent viscosity decreased from 8.33 to 0.10 Pa×s with increasing shear rate (36–3,600 s^{-1}), confirming pseudoplastic flow behavior. Texture analysis showed hardness ranging from 4.3 N (MF10) to 24.7 N (MF75), and hardness work between 2.7 and 18.8 N×s, with the control showing intermediate values. Texture analysis profile revealed that the control had a more spiked curve, indicating a less uniform internal structure. Sensory evaluation revealed no significant differences among treatments, except for granulation, which was higher in the control. Overall, labneh produced using MFs exhibited generally consistent quality characteristics, with MF57 being the most comparable to cheesecloth in total solids, fat content, hardness, hardness work, and adhesion. These findings confirm that MFs can improve process efficiency and microbial safety while preserving the desirable qualities of traditional labneh.

Keywords: dairy product, filtration, rheological properties, texture analysis, traditional production, whey drainage

INTRODUCTION

Labneh (also spelled labaneh) is a semi-solid dairy product derived from yogurt by partial removal of whey. Originating from the Arabic word "laben" (meaning fermented yogurt), it is widely consumed across the Middle East, Turkey, the Balkans, and parts of Europe. Its appeal lies in its unique texture, nutritional profile, and extended shelf life, which make it a staple in many household diets and an economically important fermented dairy product with growing market potential and consumer acceptance [Elkot *et al.*, 2025]. Labneh is typically enjoyed with bread and olive oil or used in culinary dishes. It is recognized for its white to creamy appearance, smooth and spreadable texture, low syneresis, and acidic taste, positioned between sour cream and cottage cheese in flavor and consistency [Atamian *et al.*,

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2014]. Compared to yogurt, labneh provides higher protein and mineral contents, a lower lactose level due to fermentation, and a higher count of viable probiotic bacteria, which enhances its nutritional and functional value [Aloğlu & Öner, 2013; Tawfek & Ali, 2022].

Whey separation is a critical step in labneh production. Traditionally, cheesecloth made from cellulose fibers is used to strain set yogurt, while modern methods involve centrifugation, ultrafiltration, and reverse osmosis [Dharaiya *et al.,* 2019; Leu *et al*., 2017]. Although traditional straining is labor-intensive and less efficient, it is often preferred for producing labneh with desirable texture and sensory characteristics. However, this method carries significant hygienic risks due to microbial retention in the cheesecloth, especially when reused. Cotton-based fabrics readily absorb moisture, creating an environment conducive to microbial growth and biofilm formation. Repeated use and inadequate sanitation practices can result in contamination by psychrotrophic yeasts and molds, ultimately reducing labneh's shelf life to about two weeks at ≤6°C [Bhaskaracharya *et al*., 2024].

To combat spoilage, interventions such as coating packaging films with potassium sorbate and incorporating essential oils have been explored [El-ssayad *et al*., 2025]. Potassium sorbate has shown higher antifungal efficacy, while essential oils yielded limited results and could affect sensory properties. Recent studies have also demonstrated that laser irradiation can enhance the microbial stability of probiotic labneh [Elshaghabee *et al*., 2022]. Despite efforts to modernize the whey separation process, high equipment costs, membrane fouling, and subsequent cleaning continue to limit widespread adoption of ultrafiltration and reverse osmosis [Dharaiya *et al*., 2019]. The use of superabsorbent polymers has also been investigated, but long dewatering times and adverse flavor impacts restrict their utility [Fauzi *et al*., 2020; Muncke *et al*., 2017]. Moreover, regulatory constraints on food contact materials pose further challenges. In large-scale production, many dairy companies choose centrifugation to enhance processing efficiency and hygiene, avoiding cheesecloth methods due to microbial contamination risks and yield losses. To address these issues while maintaining the traditional product identity, the integration of microfilter technology has emerged as a promising alternative. Microfilters (MFs) can be combined with mild centrifugation or hydrostatic pressure steps to optimize whey drainage without compromising sensory or structural attributes [Leu *et al*., 2017; Reig *et al*., 2021].

Artificial microfilters made of polyamide (nylon) are approved by the United States Food and Drug Administration (FDA) for food contact applications, offering high heat resistance, tensile strength, and resistance to microbial adhesion. Polyamide MFs are particularly well-suited for repeated cleaning and do not retain whey, reducing microbial growth. Their use in whey separation could significantly enhance labneh production by improving hygiene, yield, and consistency while maintaining the desired texture and identity of the traditional product [Huang *et al*., 2022]. Mechanical and rheological properties such as hardness, spreadability, adhesion, and viscoelasticity characteristics of labneh could be affected by processing conditions [Bayarri *et al*., 2012]. This study aimed to assess the feasibility of replacing traditional cheesecloth with microfilters in labneh production. Comparative analysis was focused on yield, chemical composition, microbiological quality, rheological behavior, mechanical, sensory attributes, and whey drainage kinetics.

MATERIALS AND METHODS

Labneh production and microfilter application

Labneh was produced following traditional protocols at the dairy pilot plant of the University of Jordan, Amman, Jordan. In total, 500 L of fresh cow's milk were filtered, heat-treated at 85±2°C for 30 min to ensure microbial safety, then cooled to 43±1°C before inoculation. The milk was inoculated with 2 g/100 mL of a direct-set, freeze-dried yogurt starter culture (LC DY223; 5U, Batch No. B28941, BDF Natural Ingredients SL, Girona, Spain), containing a defined symbiotic blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, essential for acidification and flavor development [Papadaki & Roussis, 2022]. Fermentation proceeded at 43±1°C until the pH reached 4.6, indicating proper gel formation. The set yogurt was then cooled to 5°C for 16 h and salted with sodium chloride (0.7 g/100 g) to enhance flavor and facilitate whey separation. For straining, 5-kg portions of set yogurt were placed into either traditional cheesecloth bags (control) or synthetic polyamide microfilter bags (50×70 cm). The bags were suspended and allowed to drain under gravity at 5±1°C for approximately 20 h. The resulting labneh was collected, packed into low-density polyethylene (LDPE) containers (500 mL), sealed, and stored at 5±1°C for up to 15 days, complying with the Jordanian Standard (JS) no. 108:2003 [JS 108, 2003] for labneh.

The MFs were fabricated from food-grade polyamide monofilaments sourced from Hebei Macrokun Trading Co., Ltd (Shijiazhuang, China) and classified by nominal pore size: 10 µm (MF10), 20 µm (MF20), 40 µm (MF40), 57 µm (MF57), and 75 µm (MF75). The MFs were certified for food contact under FDA regulations and deemed suitable for both single and repeated use [SGS Test Report, 2019]. The cheesecloth was supplied locally (Al-Ahli Co., Amman, Jordan). To ensure comparable processing conditions, all filtration bags had identical dimensions and drawstring closures. Before each use, all filtration materials underwent a standardized cleaning and sanitization protocol: washing in a commercial washer for 50 min at 40°C with a sodium lauryl sulfate-based detergent, followed by a 15-min sanitization cycle at 40°C using a food-grade disinfectant (Est-eem Evans, Preston, UK; 250 mL *per* cycle). The materials were then air-dried under mild sunlight and ambient airflow. Each washing cycle included 10 fabric pieces to ensure consistent hygiene standards.

Labneh yield calculation

Labneh yield (g/100 g) was calculated according to the method described by Elssadig *et al*. [2020], using Equation (1):

$$\text{Yield} = \frac{\text{Weight of labneh}}{\text{Weight of milk used to make the labneh}} \times 100 \quad (1)$$

Determination of total solids and fat contents, pH, and titratable acidity of labneh and whey

Standardized protocols were followed to ensure consistency and accuracy in the determination of total solids, fat content, pH, and titratable acidity of labneh and whey [AOAC, 2016]. The content of total solids (TS) of labneh and whey was determined using the oven-drying method. Approximately 3 g of a homogenized sample were weighed into pre-dried aluminum dishes and dried in a hot air oven (FD56, Binder GmbH, Tuttlingen, Germany) at 115°C for labneh and 90°C for whey until a constant weight was achieved. Samples were then cooled in a desiccator and weighed using a precision electronic balance (±0.01 g accuracy). TS content (g/100 g) was calculated following Equation (2):

$$TS = \frac{W_2}{W_1} \times 100 \quad (2)$$

where: W_1 is the initial labneh or whey weight and W_2 is the final weight after drying.

Fat content in labneh was determined using the Gerber method. For pH measurement, 5 g of labneh were diluted (1:1, *w/v*) with distilled water, homogenized, and subjected to measurements at room temperature using a pH meter (HI 8414, Hanna Instruments, Inc., Woonsocket, RI, USA). Titratable acidity was determined by titrating 5 g of the slurry against a standardized NaOH solution, with results calculated using Equation (3) and expressed as g lactic acid/100 g labneh:

$$\text{Titrable acidity} = \frac{V_{NaOH} \times C_{NaOH} \times 90}{w_{sample} \times 1000} \times 100 \quad (3)$$

where: V_{NaOH} is the volume (mL) of NaOH solution used for titration, C_{NaOH} is the concentration (M) of NaOH solution, w_{sample} is the weight (g) of sample, and 90 is the equivalent weight of lactic acid.

The pH and titratable acidity were monitored over 15 days of storage at 5±1°C as indicators of microbial stability and to assess the shelf-life quality of the labneh.

Microbiological analysis

Microbiological quality was assessed following the Bacteriological Analytical Manual [BAM, 2024]. The pour plate technique was used to enumerate mesophilic bacteria, yeasts, and molds. Homogenized labneh samples were serially diluted up to 10^{-4} in sterile peptone water. For mesophilic aerobes, aliquots were plated on plate count agar (PCA; HiMedia, Mumbai, India) and incubated at 37°C for 48 h under aerobic conditions. Yeasts and molds were plated on PCA supplemented with 100 µg/mL each of chloramphenicol and chlortetracycline-HCl (PanReac AppliChem, Darmstadt, Germany) and incubated at 25°C for 5 days. Results were expressed as colony-forming units *per* gram (cfu/g) and converted to log values ($\log_{10}$ cfu/g) for analysis. Additionally, microbiological assessments were conducted on cheesecloth and MFs after four cycles of use, cleaning, and sanitization, as described earlier. Swab samples were collected under aseptic conditions to evaluate surface contamination and material hygiene ($\log_{10}$ cfu/cm^2).

Microscopic imaging

Representative sections (2×2 cm) of the cheesecloth and selected microfilters (MF10 and MF57) were examined under a light microscope (Model SFX-31, Optika Microscopes, Ponteranica, Italy) at 2× and 4× magnifications. Images were captured using a mounted digital camera (SN 451524, Optika Microscopes). This analysis aimed to qualitatively compare the structural characteristics and pore morphology of the traditional and synthetic filtration materials used in labneh production.

Analysis of whey drainage kinetics

Whey drainage kinetics were evaluated following a modified procedure from Ebid *et al*. [2022]. Freshly set yogurt (5 kg) was transferred into sanitized cheesecloth or MFs bags, tied securely, and suspended for gravity-driven drainage. Whey weight was recorded at 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min. All samples were maintained at 5±1°C throughout to reduce temperature-induced variability and simulate refrigerated industrial straining conditions.

Analysis of apparent flow behavior and viscosity of labneh

The apparent viscosity of labneh was measured using a rotational viscometer (SNB-AI Digital Viscometer, Shandong, China) under steady shear conditions at 5±1°C. To avoid air incorporation, samples were gently transferred into the measurement vessel and analyzed within 10 min of removal from cold storage. Shear rates were applied incrementally (36; 120; 360; 720; 1,800; and 3,600 s^{-1}). At each shear rate (γ), steady-state shear stress (τ) was recorded after equilibrium was achieved, and apparent viscosity (η_a, Pa×s) was calculated as the ratio of shear stress to shear rate. Flow behavior was characterized by plotting τ *vs*. γ and fitting the data to the Herschel–Bulkley model desired by Equation (4), which extends the Power-law model by incorporating a yield stress (τ_o) representing the minimum force required to initiate flow.

$$\tau = \tau_o + m\gamma^n \quad (4)$$

where: τ is the shear stress (Pa), τ_o is the yield stress (Pa), m is the consistency coefficient (Pa×s^n), γ is the shear rate (s^{-1}), and n is the flow behavior index.

The model parameters, m and n, were derived from the intercept and slope, respectively, of the log-log plot of shear stress *vs*. shear rate [Biglarian *et al*., 2022]. The resulting flow and viscosity curves provided insight into the gel structure and spreadability of the labneh formulations.

Texture analysis

Texture analysis was performed using a CT3 texture analyzer (Brookfield, Middleboro, MA, USA) equipped with a 25-kg load cell. The TA-STF fixture, consisting of a TA15/1000 45° male cone probe and corresponding female cone holder, was used, as this setup is suited for assessing the mechanical properties of semi-fluid foods [Brighenti *et al*., 2008; Khule *et al*., 2024]. Labneh

samples were equilibrated at 5±1°C for 4 h before testing to ensure uniform thermal conditions. The samples were carefully loaded into the female cone to eliminate air pockets and levelled to create a uniform surface. Test parameters included a target penetration depth of 47 mm, a trigger load of 1 g, and a cross-head speed of 3 mm/s. During compression, the probe descended into the sample, simulating spreading, and then withdrew to complete the single-stroke cycle. Force–time data were recorded using TexturePro CT software (Version 1.10, Brookfield). Texture analysis profile (load *vs*. time) was recorded and hardness (maximum force during compression), hardness work (HW; area under the positive portion of the force–distance curve), and adhesion (area under the negative portion during withdrawal) were determined. Hardness (N) reflected firmness. HW (N×s) represented the energy required to shear and spread the sample. Adhesion quantified the work needed to overcome adhesive forces [Ahmed *et al*., 2020; Bayarri *et al*., 2012; Dejeu *et al*., 2022; Ziarno *et al*., 2023]. Coefficients of variation (CV) below 2% indicated good sample homogeneity, whereas higher values suggested internal variability.

Sensory evaluation

Sensory evaluations followed a 9-point hedonic scale, as described by Elshaghabee *et al*. [2022]. Thirty panelists (aged 24–50 years), including faculty and graduate students at the University of Jordan, evaluated attributes such as overall acceptability, softness, color, taste, granulation, and saltiness. Before testing, the panelists received brief training to familiarize them with the 9-point hedonic scale and the evaluation attributes specific to labneh, using reference samples for calibration (*e.g*., softness, taste, granulation). These reference samples were employed during training to ensure consistent scoring prior to the formal sensory evaluation. Labneh samples were presented on coded polyester plates with an accompanying structured questionnaire. Panelists worked in quiet, odor-free, and well-lit individual booths and received instructions to cleanse their palates with bottled water between samples. Samples were served at ~5°C in randomized order using three-digit codes. Panelists scored each attribute on a scale from 1 ("dislike extremely") to 9 ("like extremely"). Evaluations were conducted over three non-consecutive days to account for temporal variability and reduce panelist fatigue. A structured sensory evaluation questionnaire was specifically designed and utilized to systematically assess all quality attributes. Approval for the sensory evaluation procedure was granted by the Scientific Committee of the Deanship of Research at the University of Jordan (No. 1/2021/1539), in accordance with the principles outlined in the Declaration of Helsinki.

Statistical analysis

All experiments and measurements were conducted in triplicate and results were reported as mean and standard deviation. Statistical analysis was performed using IBM SPSS Statistics (version 23.0, IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by post-hoc comparisons using the least significant difference (LSD) tests at a 95% confidence level were used to determine significant differences ($p<0.05$) among treatments (MF10–MF75 and the control) and across multiple storage time points. Pearson correlation analysis was conducted to examine the relationship between fat content and total solids, and results were expressed as correlation coefficient (r).

RESULTS AND DISCUSSION

Yield and total solids

Labneh produced using cheesecloth exhibited significantly lower ($p<0.05$) yield (21.2 g/100 g) compared to that obtained with MF10 (29.8 g/100 g) and all other MFs (**Figure 1A**). No significant differences ($p\geq0.05$) in yield were observed among the MF treatments (MF10 to MF75). ANOVA confirmed that replacing cheesecloth with microfilters improved recovery by approximately 30% compared to the control. This improvement is technologically meaningful, as a higher yield directly enhances processing efficiency and profitability. The yield values obtained in this study align with those previously reported for labneh made from full-fat cow's and goat's milk using cheesecloth, which typically range between 23 and 32 g/100 g [Ayyad *et al*., 2015; Elssadig *et al*., 2020].

The lowest TS content was observed in labneh produced using MF10 (20.5 g/100 g), followed by MF20 (23.8 g/100 g). The MF40 and MF57 treatments showed no significant differences ($p\geq0.05$) in TS content compared to the control (32.8 g/100 g), while MF75 exhibited a significantly higher TS content (38.7 g/100 g) (**Figure 1B**). Comparable TS values, reaching up to 36 g/100 g, have been reported for labneh produced from goat's milk, as well as for labneh made from bovine, caprine, ovine, and mixed cow's milk sources [Aloğlu & Öner, 2013; Atamian *et al*., 2014; Bhaskaracharya *et al*., 2024; Tawfek & Ali, 2022]. Shamsia & El-Ghannam [2012] reported 22.2 g/100 g for labneh produced *via* ultrafiltration. Additionally, Habib *et al*. [2017] found TS content ranging from 23 to 29 g/100 g of labneh processed using cotton bags.

The high yield and low TS content observed in MF10 and MF20 treatments may be attributed to the smaller pore sizes, which likely became partially blocked by aggregated casein–fat complexes and other suspended solids. In contrast, serum proteins (3–6 nm) readily pass through even the finest MF (MF10) membranes [Reig *et al*., 2021]. This partial clogging likely resulted in greater moisture retention within the curd. While the control (cheesecloth) allowed for more efficient whey drainage and would theoretically produce higher TS content, this was not observed due to strong curd adhesion to the cheesecloth fibers, leading to product loss and lower overall yield. In contrast, labneh was easily released from the MF bags without significant losses.

The final TS content of labneh strained through cheesecloth can vary considerably depending on several factors, including the type of milk, starter culture composition, and straining duration, all of which may differ widely among processors [Bhaskaracharya *et al*., 2024]. Nevertheless, all labneh samples in the present study met the minimum TS requirement of 23 g/100 g for full-fat labneh made from cow's milk, as specified by the Jordanian Standard for fresh labneh [JS 108, 2003].

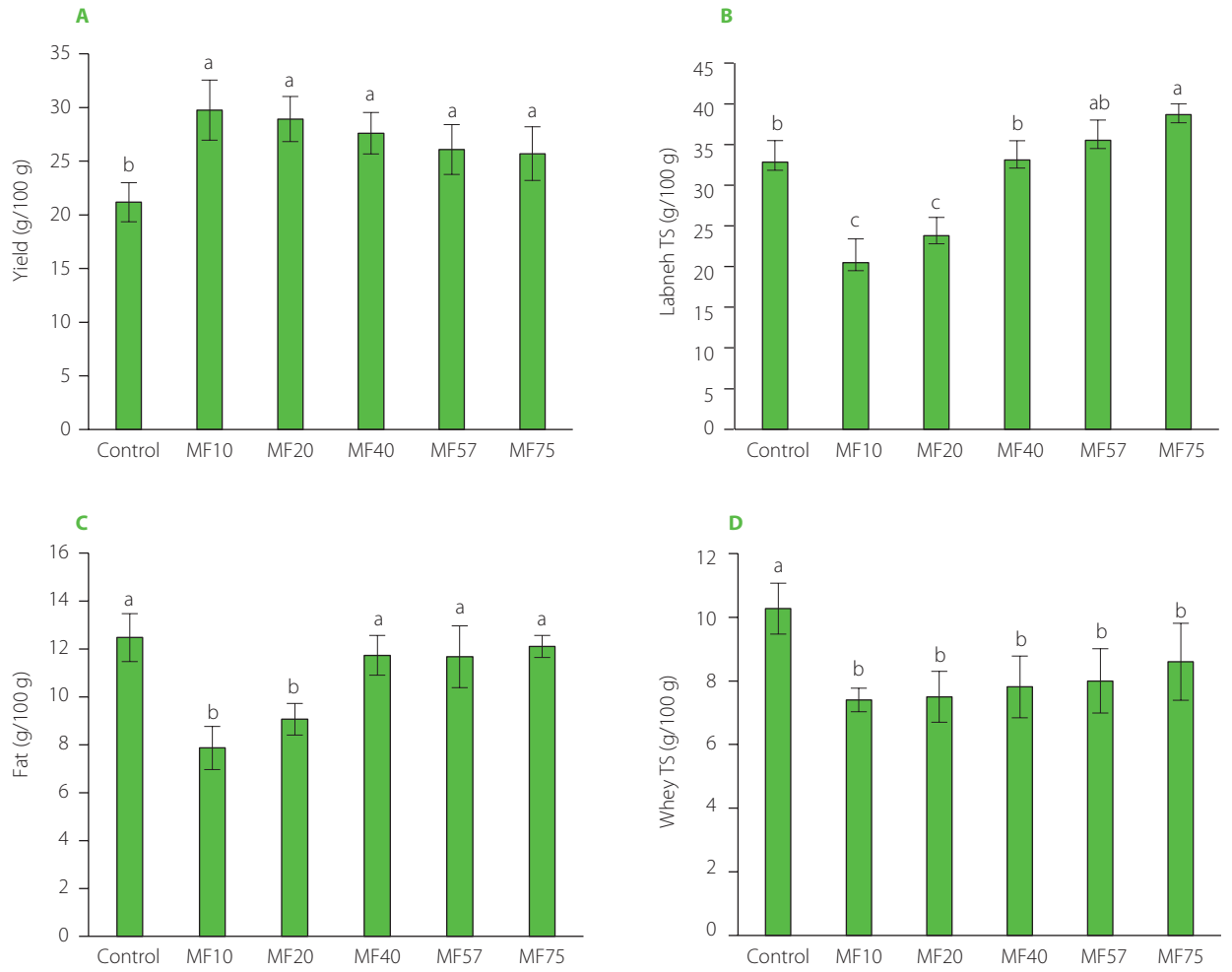

Figure 1. Effect of using cheesecloth (control) and microfilters (MFs) with different pore sizes (10–75 µm) on yield (**A**) and contents of total solids (TS) (**B**) and fat (**C**) of the labneh and TS content of strained whey (**D**). Different letters above bars indicate significant differences ($p<0.05$) among treatments for each quality parameter.

Fat content of labneh and content of total solids of whey

The fat content in the labneh samples ranged from 7.9 g/100 g in MF10 to 12.5 g/100 g in the control sample (**Figure 1C**) and showed a strong positive relationship with TS content (r=0.95). No significant differences ($p\geq0.05$) in fat content were observed among treatments, except for MF10 and MF20, which exhibited significantly lower ($p<0.05$) fat content. The fat content reported here is consistent with findings from previous studies. Atamian *et al.* [2014] reported fat contents of 9.18 g/100 g for full-fat, 4.79 g/100 g for reduced-fat, and 0.35 g/100 g for low-fat labneh prepared using cloth bags. Shamsia & El-Ghannam [2012] found no significant differences in fat content (8.6–8.7 g/100 g) between traditionally strained and ultrafiltered labneh. A broad range of fat contents (5.5–45.6 g/100 g) was recently reported by Bhaskaracharya *et al.* [2024] in a market survey of 116 labneh products from multinational and small-to-medium enterprises in the United Arab Emirates. These products were categorized as high-fat (17–18 g/100 g), full-fat (7.1–8 g/100 g), and low-fat (3.5–4.5 g/100 g), depending on their composition.

The TS content of whey did not differ significantly ($p\geq0.05$) among MF treatments, but all significantly differed ($p<0.05$) from the control (**Figure 1D**). These findings suggest that while pore size influences moisture and whey retention in the labneh curd, it does not significantly alter the composition of the expelled whey (*e.g.*, whey proteins, lactose, or lactic acid residues). This indicates that MFs primarily affect the quantity of retained whey rather than its composition.

There was a noticeable trend of increasing TS and fat content in labneh with larger pore sizes. This pattern is likely due to a dilution effect, where greater whey retention in treatments with finer pore sizes leads to lower TS and fat content in the final product. Conversely, larger pore sizes promote more effective whey drainage, resulting in higher TS and fat content relative to the retained solids. These findings are further supported by the consistent TS values observed in the whey across all treatments, indicating

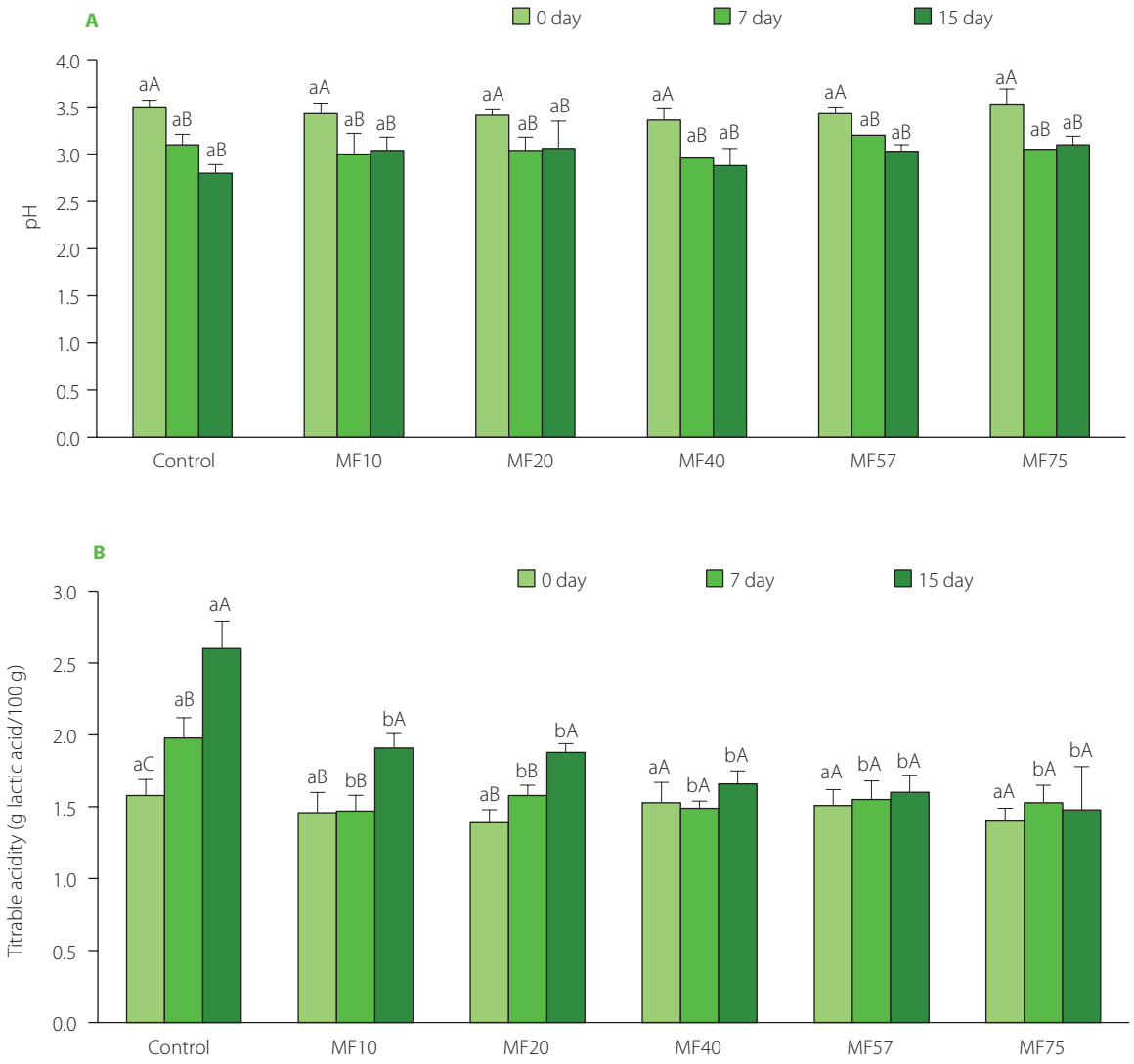

Figure 2. pH (**A**) and titratable acidity (**B**) of labneh strained through cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm) during storage at 5°C for 15 days. Different lowercase letters above bars indicate significant differences ($p<0.05$) among treatments within the same storage day, while different uppercase letters indicate significant differences ($p<0.05$) among storage days within the same treatment.

that pore size primarily influences the amount of whey retained within the curd rather than altering whey composition.

■ pH and titratable acidity

On day 0, pH values ranged from 3.4 to 3.5 (**Figure 2A**). A similar pattern was observed throughout storage, with pH values declining significantly ($p<0.05$) to 3.0–3.2 by day 7, followed by a slight decrease to 2.8 to 3.1 by day 15. A general trend was evident across all treatments: a pH reduction by day 7 and 15, likely due to continued acid production by residual lactic acid bacteria. However, no significant differences ($p\geq0.05$) in pH were detected among treatments or compared to the control at any time point during storage. These pH fluctuations may be attributed to the acid–base buffering capacity of milk proteins, as well as changes in protein structure and solubility following heat treatment and acid development [Lange *et al*., 2020]. The pH values recorded in this study were slightly lower than those reported in earlier works. For instance, Atamian *et al*. [2014] documented pH values around 3.7, while Hassabo [2017] observed values near 4.63 in labneh produced using traditional methods. However, the pH readings in the current study were more comparable to those reported by Haddad *et al*. [2017], who found values around 3.6 for both low-sodium (0.5 g NaCl/100 g) and full-sodium (1 g NaCl/100 g) labneh.

Titratable acidity ranged from 1.39 to 1.58 g lactic acid/100 g on day 0, from 1.47 to 1.98 g lactic acid/100 g on day 7, and from 1.48 to 2.60 g lactic acid/100 g on day 15 (**Figure 2B**). These values align with the range reported by Sumarmono *et al*. [2019], who found acidity levels between 1.30 and 1.45 g/100 g in labneh prepared using in-bag straining. Tawfek & Ali [2022] reported lower initial acidity values (0.95 g/100 g) for fresh cow's milk labneh, which increased to 1.45 g/100 g after 40 days of storage at 5°C. In contrast, Shamsia & El-Ghannam [2012] reported higher acidity levels (1.54–2.47 g/100 g) in labneh produced *via* ultrafiltration. While no statistically significant differences ($p\geq0.05$) in pH or titratable acidity were detected among treatments throughout

storage, the control product showed significantly higher acidity values ($p<0.05$) between days 7 and 15. This suggests that microfiltration may moderate acid development during storage by limiting microbial activity. Similar findings were reported by El-Sayed & El-Sayed [2021], who attributed higher acidity in control ultrafiltered labneh to more active lactic acid bacteria compared to samples treated with essential oil nanoemulsions.

All acidity values in this study complied with the Jordanian Standard for fresh labneh [JS 108, 2003], which specifies a maximum titratable acidity of 3.5 g/100 g as lactic acid. The increase in acidity observed in the control group after 7 days may be attributed to the metabolic activity of contaminating yeasts, which exhibit high oxidative capacity by generating additional organic acids [Abd El-Montaleb *et al*., 2022; Ayyad *et al*., 2015; El-Sayed & El-Sayed, 2021]. By contrast, Habib *et al*. [2017] reported stable acidity (1.25 g/100 g) in sage-fortified labneh stored at 5°C for 20 days, underscoring the role of additives and microbial composition in modulating acid development during storage. Overall, the consistent acidity trends observed in this study suggest microbial and chemical stability in MF-treated labneh samples throughout the storage period, regardless of pore size.

Microbiological analysis

Mesophilic aerobe counts in labneh samples ranged from 3.31 $\log_{10}$ cfu/g in the MF10 treatment to 4.63 $\log_{10}$ cfu/g in the control. Yeast and mold counts varied from 1.11 $\log_{10}$ cfu/g (MF10) to 3.58 $\log_{10}$ cfu/g (control) (**Table 1**). ANOVA confirmed significantly higher microbial loads in the control compared to all MF treatments ($p<0.05$), with differences reaching up to 1.2 $\log_{10}$ cfu/g for mesophilic aerobes and 2.3 $\log_{10}$ cfu/g for yeasts and molds. These statistically significant differences are relevant for product safety and shelf life. In contrast, no significant variation ($p\geq0.05$) was detected among MF10–MF75, underscoring the consistent hygienic performance of polyamide filters regardless of pore size. These results suggest that, despite the higher TS content in MF57 and MF75-treated labneh (**Figure 1B**), which could theoretically promote yeast growth due to increased nutrient availability and reduced water activity, the use of microfilters markedly improves the microbial quality of labneh relative to traditional cheesecloth methods. This improvement is likely attributable to the superior hygienic properties of synthetic polyamide MFs, which resist microbial adhesion and facilitate more thorough cleaning and sanitation protocols.

The microbial counts observed in this study were generally lower than those reported in previous research. Hassabo [2017], investigating labneh supplemented with palm oil, reported total bacterial counts ranging from 4.8 to 6.9 $\log_{10}$ cfu/g, with the highest levels found in control samples made from milk with standardized fat content of 4 g/100 g. Similarly, Elkot & Khalil [2022] and Tawfek & Ali [2022] documented total bacterial loads between 5.5 and 7.5 $\log_{10}$ cfu/g in traditionally strained labneh. However, unlike our findings, these studies reported no detectable yeast or mold growth in fresh samples. Ayyad *et al*. [2015] also observed no yeast or mold growth during 24 days of refrigerated storage in labneh produced *via* in-bag straining. Gharaibeh [2017] reported substantially higher microbial loads, with aerobic plate counts between 7.7 and 8.5 $\log_{10}$ cfu/mL and yeast and mold counts ranging from 6.0 to 7.1 $\log_{10}$ cfu/mL, in unbranded labneh samples collected from local markets in Irbid, which were produced at a small scale. Furthermore, Tawfek & Ali [2022] found that yeast and mold counts remained undetectable in traditionally strained labneh until day 20 of storage at 5°C. These discrepancies among studies may be attributed to differences in production scale, post-pasteurization handling, sanitation practices, and storage conditions.

To further investigate the role of straining tools in microbial contamination, mesophilic aerobes and yeast and mold counts were measured on cheesecloth and MFs after four consecutive cycles of use, cleaning, and sanitization. As shown in **Table 1**, microbial loads were significantly higher ($p<0.05$) in the cheesecloth compared to the MFs, indicating inadequate sanitization and higher microbial retention in traditional materials. This suggests that cheesecloth can serve as a reservoir for cross-contamination between batches, whereas MFs, made from hydrophobic

Table 1. Mesophilic aerobes (MA) and yeasts and molds (Y&M) counts in fresh labneh strained through cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm), and microbial counts on cheesecloth and MF surfaces after four consecutive cycles of use, cleaning, and sanitization.

Treatment	Labneh		Surface	
	MA ($\log_{10}$ cfu/g)	Y&M ($\log_{10}$ cfu/g)	MA ($\log_{10}$ cfu/cm^2)	Y&M ($\log_{10}$ cfu/cm^2)
Control	4.63±0.216^a	3.58±0.05^a	3.03±0.08^a	2.25±0.45^a
MF10	3.31±0.04^b	1.11±0.06^b	0.33±0.04^c	<10
MF20	3.43±0.19^b	1.22±0.10^b	0.69±0.05^b	<10
MF40	3.38±0.08^b	1.21±0.12^b	0.78±0.08^b	0.12±0.01^b
MF57	3.35±0.13^b	1.24±0.08^b	0.92±0.07^b	0.18±0.01^b
MF75	3.38±0.19^b	1.15±0.10^b	0.72±0.09^b	0.21±0.03^b

Values within the same column followed by different superscript letters within the same test type differ significantly ($p<0.05$). <10, Counts below the detection limit.

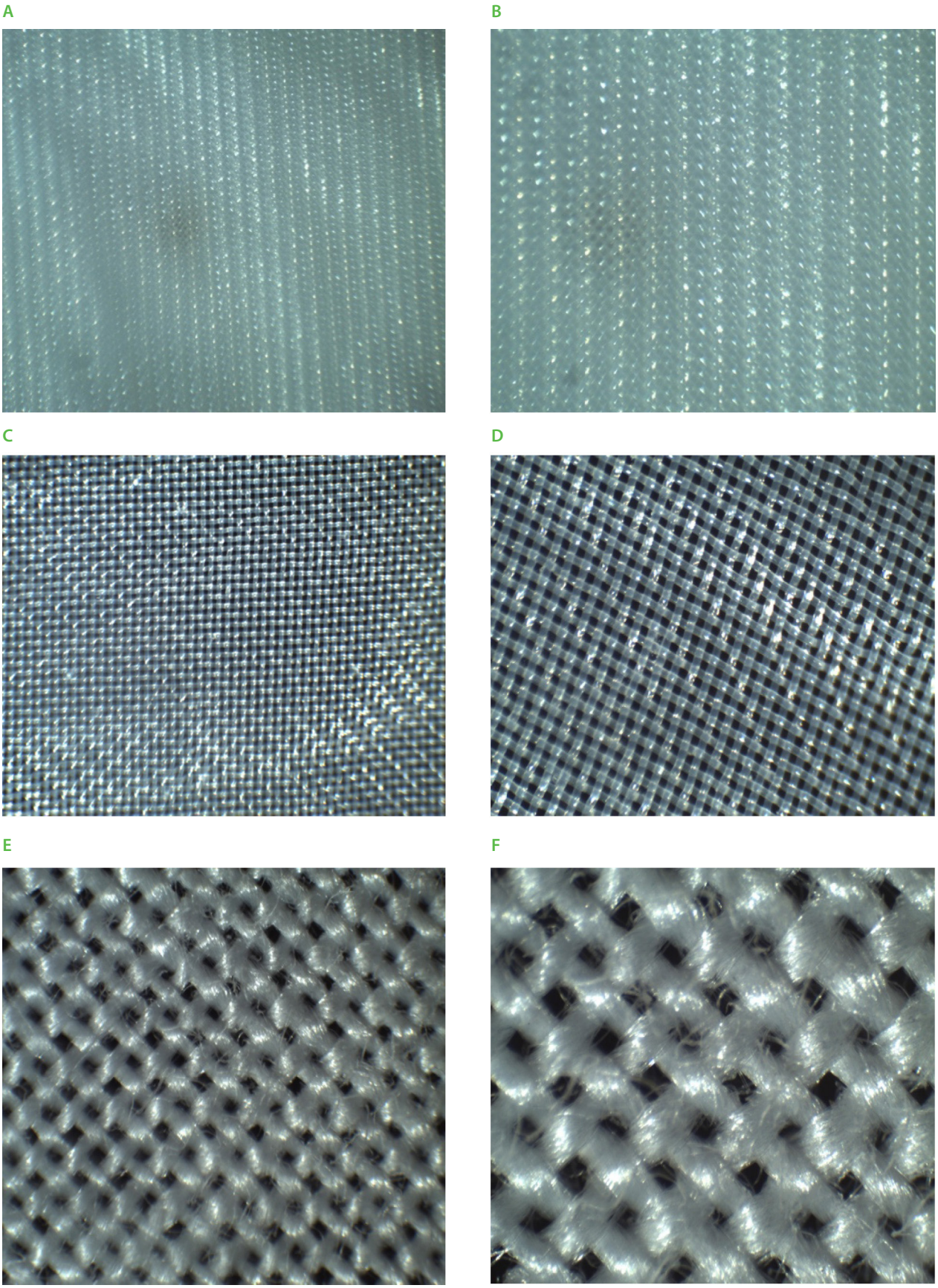

Figure 3. Microscopic images of polyamide microfilters with pore sizes of 10 µm, MF10 (**A** and **B**), and 75 µm, MF57 (**C** and **D**), and cheesecloth (**E** and **F**) at 2× and 4× magnification, respectively.

synthetic polyamide and certified for food contact, provide a more hygienic alternative. These findings underscore the importance of enhanced hygiene control in traditional labneh processing and support the adoption of synthetic microfiltration tools to improve food safety and product consistency.

Microscopic observations of cheesecloth and polyamide microfilters

Representative microscopic images of MF10, MF57, and traditional cheesecloth (control) at 2× and 4× magnifications are shown in **Figure 3**. The visual comparison indicates that the pore size of MF57 was approximately ten times smaller than that of the cheesecloth at both magnification levels. These differences became even more pronounced when comparing the finer-structured MF10 membranes with the control, highlighting substantial variation in pore geometry among the materials.

The MFs displayed uniform, smooth surfaces without visible fiber bundles or microfibers. In contrast, the cheesecloth was composed of loosely arranged cellulose fibers and microfibers, resulting in an inherently variable and rough pore architecture. These structural differences have important implications for whey separation performance and microbial contamination. The cellulose-based cheesecloth, being hydrophilic, absorbs whey during straining, causing fiber swelling that reduces the effective pore size over time and potentially impairs drainage efficiency. This swelling likely contributed to the lower yields and the higher product adhesion observed in the control samples (**Figure 1A**).

Conversely, polyamide-based microfilters, which are hydrophobic and resistant to moisture absorption, maintained stable pore dimensions throughout the straining process. This stability likely contributed to the higher and more consistent labneh yields achieved with MF treatments. Furthermore, the smooth, non-fibrous surfaces of the microfilters likely inhibited microbial attachment and biofilm formation during repeated cycles of use, cleaning, and sanitization. In contrast, the rough, porous surface of cheesecloth fibers provides an ideal environment for microbial colonization, especially within microfibers and microcracks that are difficult to reach with standard cleaning protocols. Surface roughness and porosity are well-documented factors that enhance microbial adhesion and biofilm development, reducing the effectiveness of conventional sanitization measures [Cheng *et al*., 2019; Zheng *et al*., 2021]. This phenomenon may also help explain the significantly higher microbial counts observed in the cheesecloth samples compared to those treated with microfilters (**Table 1**).

Kinetics of whey drainage

Although statistical analysis revealed no significant differences ($p \geq 0.05$) in the volume of whey drained among treatments at individual time points up to 180 min, a clear overall pattern emerged: microfilters with larger pore sizes promoted faster whey separation (**Figure 4**). This trend is consistent with the results shown in **Figure 1B**, where MFs with smaller pore sizes retained more moisture, leading to reduced whey drainage and consequently lower TS and fat content in the final product. In contrast, MF75 and the traditional cheesecloth demonstrated greater drainage efficiency, facilitating faster whey removal and yielding products with higher TS and fat contents.

The cotton-based cheesecloth, composed of hydrophilic cellulose fibers, displayed dynamic pore behavior during straining. As whey was absorbed by the fibers, the cellulose swelled,

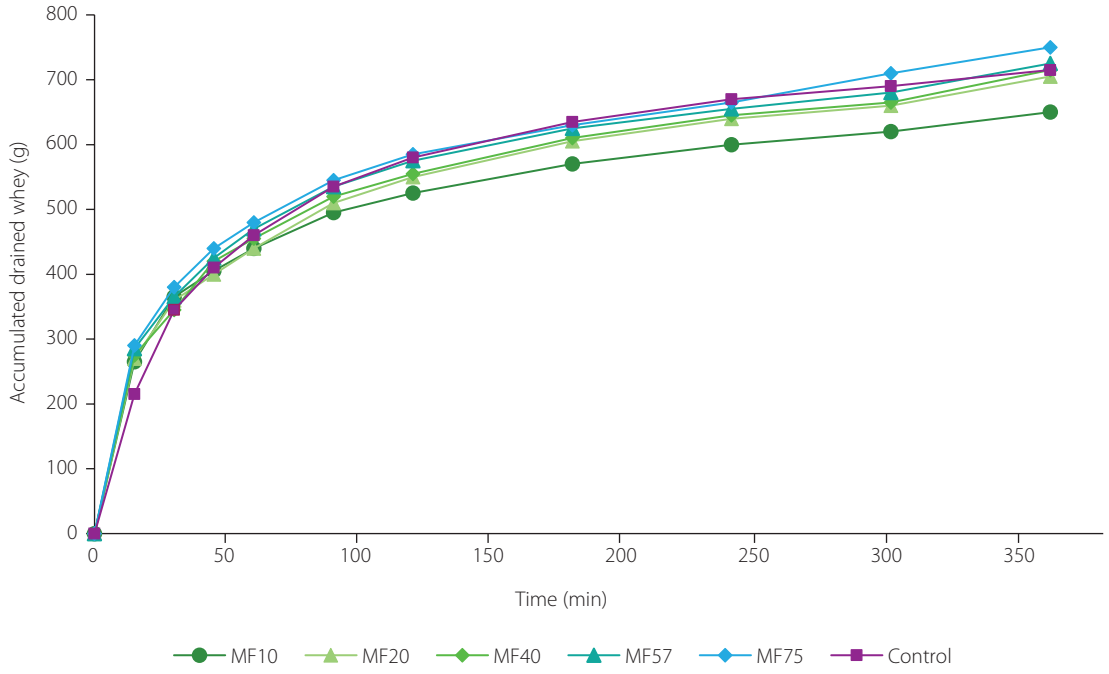

Figure 4. Kinetics of whey drainage of labneh produced by straining through polyamide microfilters (MFs) with different pore sizes (10–75 µm) and the traditional cheesecloth (control).

Table 2. Linear regression parameters of whey drainage kinetics for labneh strained using cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm).

Parameter	Control	MF10	MF20	MF40	MF57	MF75
Slope	156.67	117.81	131.09	137.66	137.75	141.07
Interception	–188.32	–43.22	–75.76	–106.70	–93.58	–98.19
R-square	0.9925	0.9975	0.9976	0.9961	0.9969	0.9973

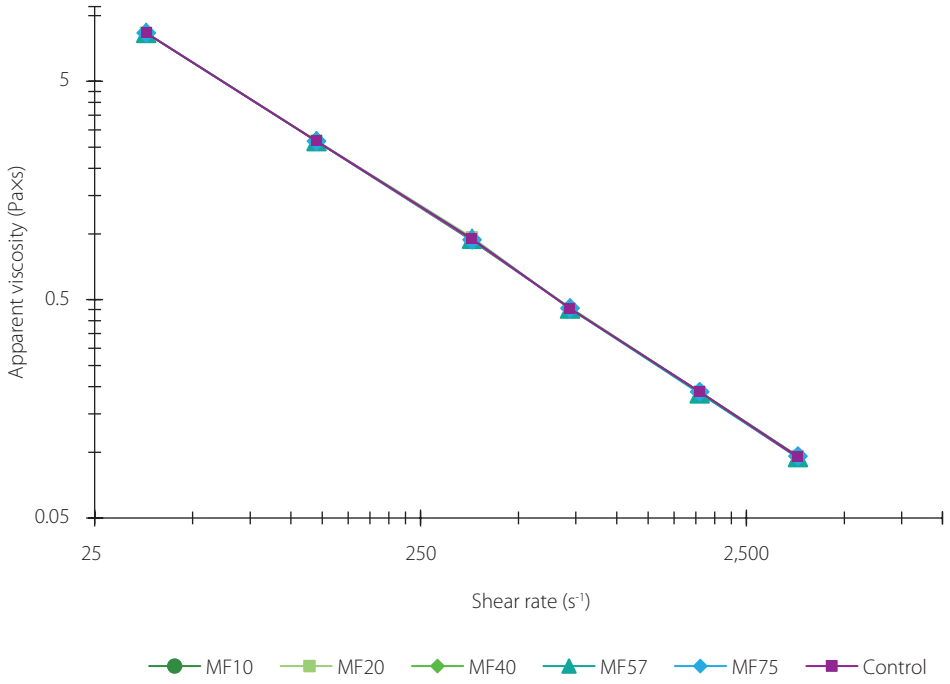

Figure 5. Apparent viscosity of labneh prepared by straining through cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm).

effectively reducing the pore size and slowing the drainage rate over time. This phenomenon likely explains the more pronounced slope reduction of the drainage curve during the whey separation period compared to the MF treatments (**Figure 4**). Nevertheless, the control sample maintained the highest whey drainage rate beyond 120 min, suggesting that despite fiber swelling, the effective pore size remained larger than that of most synthetic MFs. Whey separation *via* cheesecloth may also be influenced by capillary and osmotic forces acting across the hydrophilic membrane. The continuous diffusion of whey through the cellulose matrix, possibly driven by concentration gradients and osmotic flow, adds to the complexity of the drainage mechanism [Fauzi *et al.*, 2020].

Regardless of the treatment, the drainage curves (**Figure 4**) consistently showed a declining slope over time, reflecting reduced whey flow as the labneh structure became denser. This effect was especially pronounced in MFs with larger pores (MF40–MF75), where increased gel network strength, resulting from progressive whey loss, reduced the availability of free water and slowed syneresis. This mechanism is consistent with findings in similar dairy gels, where gel strengthening and reduced gravitational flow over time were associated with lower whey mobility and syneresis [Reig *et al.*, 2021]. Results of regression analysis in **Table 2** show that the cheesecloth demonstrated the highest drainage rate (slope =156.67), whereas MF10 showed the lowest one (117.81), highlighting an inverse relationship between filter fineness and whey flow rate. All treatments exhibited excellent linear fit (R^2>0.99), confirming that a linear model could accurately describe drainage behavior over time. The intercept values further suggest lower initial whey loss when using finer-pore filters.

Apparent viscosity and flow behavior

All samples exhibited non-Newtonian shear-thinning behavior (n<1), with viscosity decreasing from 8.33 Pa×s at 36 s^{-1} to 0.10 Pa×s at 3,600 s^{-1} (**Figure 5**). This pattern is characteristic of pseudoplastic (shear-thinning) flow behavior, where increasing shear disrupts the internal structural network, reduces resistance, and enhances spreadability [Yang *et al.*, 2021].

The observed decline in apparent viscosity at higher shear rates can be attributed to the progressive disruption of weak electrostatic and hydrophobic interactions within the protein matrix, leading to the breakdown of the gel structure and facilitating easier flow. Comparable behavior has been reported in dairy emulsions, where shear disrupts fat globule membranes and protein aggregates, resulting in reduced viscosity under high shear conditions [Biglarian *et al*., 2022]. No significant differences ($p \geq 0.05$) were observed in the viscosity-shear rate trends between MF-treated and control samples, suggesting that the overall structural integrity remained consistent regardless of filtration method. This observation is consistent with previous studies on traditional labneh produced by cheesecloth, which also exhibited shear-thinning and thixotropic behavior, fitting well with power-law rheological models [Mohameed *et al*., 2004].

TS content is a critical factor influencing labneh's rheological characteristics. Mohameed *et al*. [2004] reported that a 5 g/100 g increase in TS nearly doubled the apparent viscosity (from 26 to 60 Pa×s at 2.2 s^{-1}), highlighting the importance of solids concentration. In the present study, although TS varied slightly due to differences in filter pore size, these variations were statistically insignificant ($p \geq 0.05$) and did not substantially affect viscosity outcomes. Bhaskaracharya *et al*. [2024] confirmed that labneh rheology is strongly influenced by fat content, moisture level, and hydrocolloid presence. Furthermore, Vareltzis *et al*. [2016] emphasized the principal role of the casein matrix in water retention and viscosity maintenance, suggesting that whey separation is predominantly controlled by protein network dynamics rather than by the diffusion of free water.

Texture properties

During texture analysis, the probe was initially positioned 7 mm above the sample surface and moved downward to a depth of 47 mm, corresponding to the bottom of the test container. As the probe compressed the sample, the force required to deform it was continuously recorded over the deformation time. The peak force observed during this downward motion represents the hardness (F1) at the time-force curve (**Figure 6**). Following maximum compression, the probe began to withdraw from the sample. During this phase, two additional mechanical parameters were derived: hardness work (HW), measured as the area under the positive region of the curve (A1), and adhesion, represented by the negative area (A2). The representative time-force profiles of control and labneh produced using MF57 (**Figure 6**) showed similar overall patterns; however, the control sample exhibited a more spiked curve during compression, suggesting a less uniform internal structure and the presence of local weaker points [Ahmed *et al*., 2020]. This elevated variability likely reflects inconsistencies in the internal structure and reduced compositional uniformity in labneh produced using cheesecloth. In contrast, MF57 samples demonstrated more consistent textural measurements, indicating improved homogeneity and reproducibility.

Key texture attributes, hardness, HW, and adhesion, were significantly influenced by the pore size of the microfilters. Hardness ranged from 4.3 N (MF10) to 24.7 N (MF75), with the control sample showing an intermediate value of 11.6 N (**Figure 7A**). The control's hardness was significantly ($p<0.05$) higher than that of MF10 and MF20 but lower than that of MF75, and not significantly different from that of MF40 and MF57. In comparison, lower hardness values (0.3–1.6 N) have been reported for commercial labneh and for overrun dairy cream (1.1–4.5 N), likely due to their lower total solids content, simpler structural composition, differences in processing conditions, and the influence of product temperature at the time of measurement [Bhaskaracharya *et al*., 2024; Biglarian *et al*., 2022].

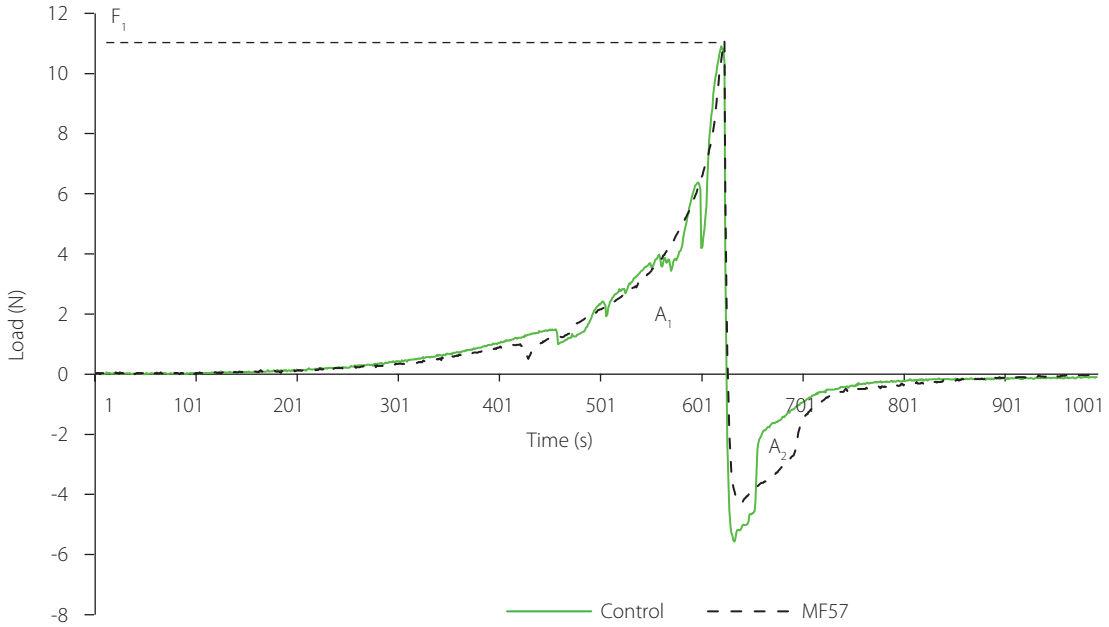

Figure 6. Texture analysis profile (load *vs.* time) of labneh prepared by straining through cheesecloth (control) and polyamide microfilter with pore size 57 µm (MF57). Hardness (F_1) corresponds to the peak force, hardness work (HW) is the area under the positive curve (A_1), and adhesion is the negative area (A_2).

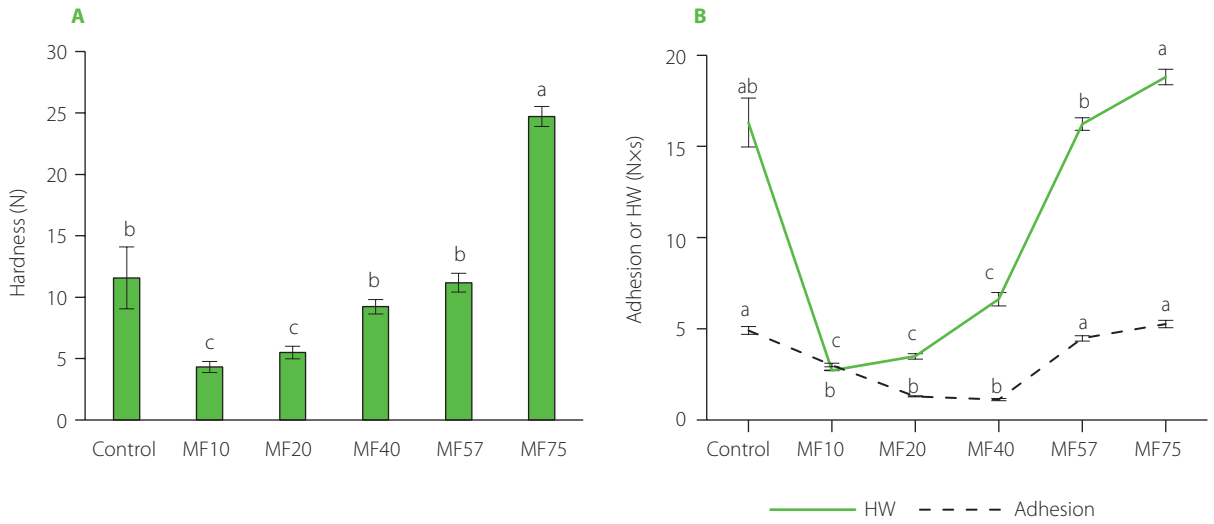

Figure 7. Texture parameters of labneh prepared by straining through cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm): (**A**) hardness and (**B**) hardness work (HW) and adhesion. Different letters indicate significant differences (p<0.05) among treatments within the same parameter.

HW for labneh samples ranged from 2.7 N×s (MF10) to 18.8 N×s (MF75) (**Figure 7B**). Statistical analysis demonstrated that HW, likewise hardness, increased significantly with larger pore size (p< .05) from MF40 to MF75, suggesting that smaller pore sizes enhance spreadability. The control exhibited the value of 16.3 N×s, which was not significantly different (p≥0.05) from those of MF57 and MF75, suggesting these filters most closely replicate traditional texture. In contrast, MF10 and MF20 had significantly lower (p<0.05) HW, consistent with their higher moisture retention (**Figure 1B**). These findings align with ranges reported for commercial full-fat cream cheese, where HW varies from 10.6 to 85 N×s and hardness from 3.7 to 26.5 N depending on fat content and temperature [Brighenti *et al*., 2008]. Moisture content played a critical role; samples with lower TS exhibited reduced hardness and HW, supporting the plasticizing effect of moisture [Singh *et al*., 2013]. The control labneh also demonstrated greater variability, with coefficients of variation of 21.7% for hardness and 8.2% for HW, whereas the MF-treated samples displayed lower variability, suggesting more consistent texture and internal structure in the MF-treated groups.

Adhesion of labneh ranged from 1.1 N×s (MF40) to 5.3 N×s (MF75) (**Figure 7B**). The control sample showed an adhesion value of 4.9 N×s, not significantly different (p≥0.05) from MF57 and MF75 (4.5 N×s). Comparable adhesion ranges (2.5–14.2 N×s) have been reported for spreadable processed goat cheese, while lower values (0.05–0.79 N×s) have been observed in commercial labneh [Bhaskaracharya *et al*., 2024; Burgos *et al*., 2020]. The observed variations in adhesion are influenced by chemical composition, test conditions, measurement parameters, and fixture geometry [Khule, *et al.,* 2024].

Sensory analysis

Sensory attributes assessed included overall acceptability, softness, color, taste, granulation, and saltiness (**Figure 8**). Overall acceptability scores ranged from 6.63 to 7.30 out of 9, while softness, taste, and saltiness also fell within relatively narrow ranges, indicating comparable sensory performance across treatments. All samples and the control exhibited a clean, natural white appearance, with color scores ranging from 7.73 to 8.13. No statistically significant differences (p≥0.05) were observed among treatments for most sensory attributes, except for granulation, where the control sample scored significantly higher (p<0.05) than the MF-treated samples. This indicates that the use of MFs not only maintained labneh's sensory quality but also contributed to a reduction in granulation, enhancing overall textural smoothness.

These findings are consistent with previous studies. Shamsia & El-Ghannam [2012] found no significant sensory differences between labneh prepared by traditional methods and labneh made from ultrafiltration (UF) retentate. In turn, Khider *et al*. [2022] reported that labneh produced *via* UF concentration was favored for its smooth texture, appealing appearance, and superior organoleptic properties than the traditional labneh. These results suggest that substituting cheesecloth with MFs does not compromise labneh's sensory profile. The strong sensory acceptability observed across MF-treated samples supports their potential for maintaining consumer satisfaction. Moreover, the relatively low perception of acidity despite lower pH and higher titratable acidity values (**Figure 2**) may be explained by the masking effect of higher fat content, which has been shown to suppress sour flavor perception in dairy products [Zhou *et al*., 2022].

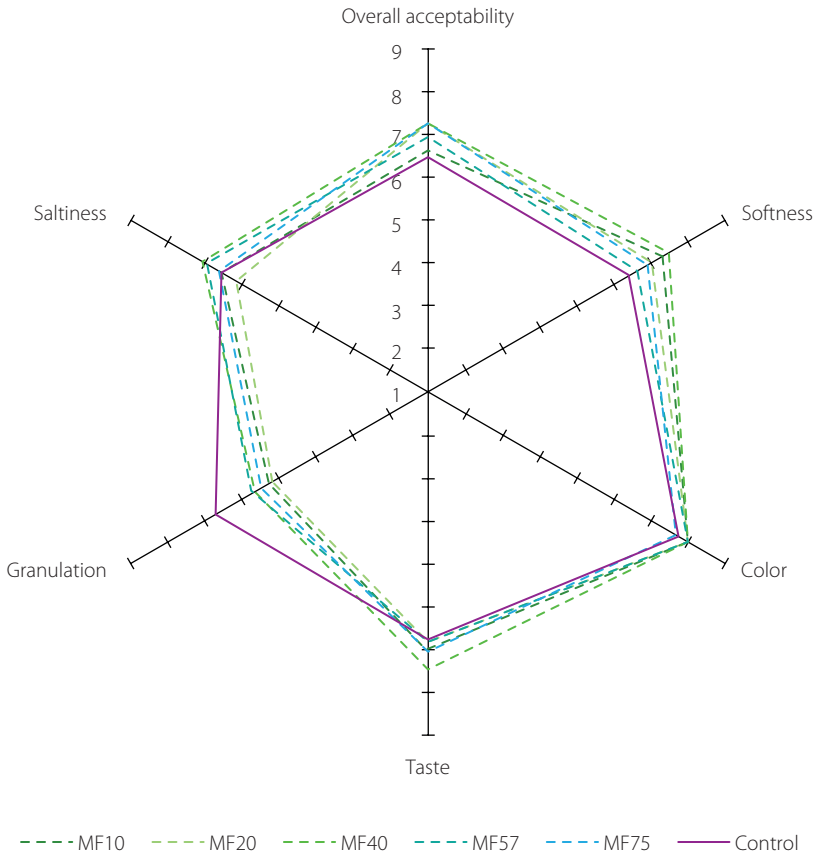

Figure 8. Sensory evaluation scores of labneh produced by straining through the cheesecloth (control) and polyamide microfilters (MFs) with different pore sizes (10–75 µm).

CONCLUSIONS

This study demonstrates that polyamide microfilters (MFs) provide a novel and superior alternative to traditional cheesecloth in labneh production. Their use improved yield recovery, enhanced hygienic performance, and preserved desirable sensory qualities without compromising product identity, while also providing more uniform hardness and spreadability. Labneh produced using MFs demonstrated, in general, consistent quality across treatments. Among them, MF57 was the most comparable to cheesecloth in terms of total solids, fat content, hardness, hardness work, and adhesion. In other attributes, all MFs either matched the performance of cheesecloth (*e.g.*, drainage kinetics and apparent viscosity) or outperformed it (*e.g.*, microbial quality). In addition to these quality improvements, polyamide MFs offer clear economic and operational advantages. Unlike cheesecloth, which deteriorates quickly, MFs are durable, reusable, and withstand repeated cleaning and sanitization, thereby reducing replacement costs and chemical use. Their standardized pore sizes and mechanical strength enable integration into automated dairy lines, supporting industrial scale-up without compromising quality. Furthermore, FDA food-contact certification and resistance to microbial adhesion enhance safety and compliance, positioning MFs as a sustainable and scalable solution for commercial labneh production. Future work should validate these results under industrial-scale conditions and evaluate long-term cost savings to support commercial adoption.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Physicochemical Properties and Antioxidant Capacity of Tryptic Hydrolysates of a Pea Protein Isolate: Influence of the Degree of Hydrolysis

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Enzymatic pea protein hydrolysates offer potential health benefits because of their content of bioactive peptides, which have been released from the protein by the action of proteases. This study examined how the degree of hydrolysis (DH) of pea protein with trypsin influences physicochemical parameters and antioxidant capacity of the resulting hydrolysates. The molecular weight (MW) distribution of a pea protein isolate and its hydrolysates at the DHs of 2%, 5%, 8%, and 12% was determined using size-exclusion chromatography. Surface hydrophobicity was evaluated by two fluorescent probe assays, namely 8-anilino-1-naphthalenesulfonic acid (ANS) and *cis*-parinaric acid (CPA). Antioxidant potential was assessed as $ABTS^{\bullet+}$ scavenging capacity, oxygen radical absorbance capacity ($ORAC_{FL}$), antioxidant capacity of water-soluble and lipid-soluble compounds in the photochemiluminescence assay (PCL-ACW and PCL-ACL, respectively), and the ability to inhibit the oxidation of β-carotene-linoleic acid emulsion. With increasing DH, the contribution of fractions with MWs of 2–4 kDa and 4–7 kDa in the hydrolysates increased. However, the relative content of peptides with MWs less than 2 kDa remained below 10% in all of them. The $ABTS^{\bullet+}$ scavenging capacity and $ORAC_{FL}$ also increased with DH, and the highest values, 0.111 and 0.320 mmol Trolox equivalent/g, respectively, were obtained for the hydrolysate at a DH 12%. Surface hydrophobicity increased only to DH 5%. Hydrolysates at DHs of 8% and 12% were characterized by gradually lower values. The trend of surface hydrophobicity changes was consistent with that of PCL-ACL. Additionally, principal component analysis showed an association between surface hydrophobicity and antioxidant capacity in the model emulsion. Overall, tryptic pea protein hydrolysates had improved antioxidant properties compared to the isolate, and the degree of hydrolysis was a parameter that allowed optimizing these properties under different conditions of antioxidant action.

Keywords: antiradical activity, molecular weight distribution, protein hydrolysis, size-exclusion chromatography, surface hydrophobicity, trypsin treatment

INTRODUCTION

Nowadays, with increasing knowledge about nutrition and growing health awareness, consumers pursuit foods that not only nourish but also provide additional health benefits. Enzymatic hydrolysis processes can contribute to the health-promoting properties of food proteins, which are one of the main nutrients in a well-balanced diet. Polypeptides and peptides with various biological activities are released during enzymatic hydrolysis of proteins, providing hydrolysates with, among others, antioxidant, antihypertensive, antidiabetic, immunomodulatory,

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and acetylcholinesterase and butyrylcholinesterase-inhibiting properties [Asen & Aluko, 2022; Liu *et al.*, 2024; Malomo *et al.*, 2020; Nguyen *et al.*, 2024; Zhao *et al.*, 2025]. The type and intensity of activity depend on the composition and amino acid sequence of the released peptides, their molecular weight and other physicochemical properties, *e.g.*, hydrophobicity [Awosika & Aluko, 2019; Irankunda *et al.*, 2025; Pownall *et al.*, 2010]. All these characteristics are affected by the degree of hydrolysis (DH) defined as the ratio of the number of peptide bonds cleaved during hydrolysis to the total number of peptide bonds of a substrate [Adler-Nissen, 1986]. This parameter is commonly used in monitoring the progress of hydrolysis [Jamdar *et al.*, 2010; Sulewska *et al.*, 2022; Trigui *et al.*, 2021] and easily controlled by modifying the hydrolysis conditions, *i.e.*, the initial protein concentration, enzyme to substrate ratio, pH, temperature, and time [Karamać *et al.*, 2002; Konieczny *et al.*, 2020b; Suarez *et al.*, 2021]. However, the maximum DH that can be achieved for a defined protein-enzyme system is determined by the protein structure (dependent on the protein source) and specificity of the enzyme used [Asen & Aluko, 2022; Sareen *et al.*, 2023].

Pea (*Pisum sativum* L.) seeds can be considered among protein-rich sources. Costantini *et al.* [2021] found that the protein content of different pea accessions was in the range of 24.42–27.76 g/100 g of dry weight. Globulins are the major storage proteins found in pea (65–80% of the total proteins) [Owusu-Ansah & McCurdy, 1991]. They consist of legumin with a molecular weight (MW) of 360–400 kDa, vicilin (160–200 kDa), and convicilin (280 kDa). Albumins are the second most abundant protein fraction, accounting for 20–35% of pea proteins. Yang *et al.* [2021] reported that the ratio between these fractions in pea protein isolates was dependent on the extraction method used.

Pea proteins have been treated with various enzymes, including plant, animal, and microbial proteases, to obtain hydrolysates with antioxidant capacity [Aguilar *et al.*, 2020; Asen & Aluko, 2022; Girgih *et al.*, 2015; Irankunda *et al.*, 2025; Pownall *et al.*, 2010; Žilić *et al.*, 2012]. Hydrolysis with these enzymes released polypeptides and peptides capable of scavenging free radicals, chelating pro-oxidant metal ions, and exhibiting reducing power [Asen & Aluko, 2022; El Hajj *et al.*, 2023; Girgih *et al.*, 2015; Pownall *et al.*, 2011]. Inhibition of linoleic acid oxidation by pea protein hydrolysates has also been reported [Pownall *et al.*, 2010, 2011]. Trypsin was one of the enzymes that effectively produced pea protein hydrolysates with antioxidant capacity [Asen & Aluko, 2022]. Tryptic hydrolysates had a similar hydroxy radical scavenging activity as hydrolysates obtained with pancreatin and chymotrypsin, but lower than proteins treated with Alcalase, Flavorzyme or pepsin [Asen & Aluko, 2022]. However, the antioxidant potential of trypsin-treated proteins at different degrees of hydrolysis has not been compared. Based on this background, this paper examines the influence of the DH on physicochemical parameters and antioxidant capacity of tryptic hydrolysates of a pea protein isolate. To this end, hydrolysates of a pea protein isolate at different DHs were obtained and assayed for molecular weight (MW) distribution, surface hydrophobicity, and antioxidant capacity by several methodologies. Finally, principal component analysis was carried out to visualize relationships between DH and their physicochemical and bioactive properties.

MATERIALS AND METHODS

Materials and chemicals

A pea protein isolate with the commercial name Propulse produced by NutriPea (Portage la Prairie, Manitoba, Canada) was acquired from Dutch Protein and Service (Tiel, Netherlands). Protein content of the isolate was 82.97 g/100 g of dry weight determined by the Kjeldahl method according to AOAC International standard no. 977.02-1977 [AOAC, 1990]. Trypsin (EC 3.4.21.4) with a supplier-declared activity of 1.120 BAEE U/mg was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Its activity, as determined by the Anson [1938] method using hemoglobin as the substrate, was 1.34 AU/g.

2,4,6-Trinitrosulfonic acid (TNBS), L-leucine, 8-anilino-1-naphthalenesulfonic acid (ANS), *cis*-parinaric acid (CPA), *n*-decane, butylhydroxytoluene (BHT), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, linoleic acid, Tween 40, fluorescein, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich. Kits for determination of the antioxidant capacity of water-soluble and lipid-soluble compounds in the photochemiluminescence assay (PCL-ACW and PCL-ACL, respectively) and standards for size-exclusion chromatography were obtained from Analytik Jena (Jena, Germany) and Pharmacia Biotech (Uppsala, Sweden), respectively. Other solvents and reagents were provided by Avantor Performance Materials (Gliwice, Poland).

Production of pea protein hydrolysates at different degrees of hydrolysis

A vessel containing the pea protein isolate (30 g) suspended in 290 mL of distilled water was placed in a thermostatic water bath heated to 50°C [Karamać *et al.*, 2002]. After adjusting the pH to 8.0 using 1 M NaOH solution, 10 mL of a trypsin solution in water (33.5 mg/mL) was added, which corresponded to an enzyme to substrate ratio (E/S) of 15 mAU/g isolate. During 120 min of hydrolysis, the mixture was stirred, and pH was maintained constant by adding 1 M NaOH solution from the burette. Based on the NaOH solution used, after 5, 10 min and then at 10-min intervals up to 120 min, the degree of hydrolysis (DH) was calculated using Equation (1) developed for the pH-stat method by Adler-Nissen [1986]:

$$DH\ (\%) = \frac{B \times M_B}{\alpha \times MP \times h_{tot}} \times 100 \quad (1)$$

where: B is the amount of base used during hydrolysis (mL), M_B is the molarity of a base solution, α is the average degree of dissociation of $-NH_2$ groups, MP is the mass of protein used for hydrolysis (g), and h_{tot} is the total number of peptide bonds in the substrate (meqv Leu-NH_2/g protein).

The value of 1/α was determined experimentally based on the relationship between 1/α and the average pK for the α-amino groups liberated during hydrolysis described by Equation (2):

$$\frac{1}{\alpha} = 1 + 10^{pK-pH} \quad (2)$$

Using Equation (3), the pK was calculated after carrying out hydrolysis at two pH values (pH_1=7.5 and pH_2=8.0) and determining the regression coefficients (b_1 and b_2, respectively) for the linear relationships between the amount of base used during hydrolysis and the number of released α-amino groups. The latter were quantified by the method with TNBS, in which absorbance of the reaction mixture was measured at 340 nm using a DU 7500 Beckman spectrophotometer (Beckman Instruments, Brea, CA, USA), and the results were calculated based on the calibration curve plotted for L-leucine [Panasiuk *et al.*, 1998].

$$pK = pH_2 + \log(b_1 - b_2) - \log(10^{pH_2 - pH_1} \times b_2 - b_1) \quad (3)$$

The total number of peptide bonds in the pea protein isolate was determined with TNBS method [Panasiuk *et al.*, 1998] after acid hydrolysis of 0.5 g of the isolate in 10 mL of 6 M HCl at 105°C for 12 h.

The hydrolysis was performed in triplicate and, based on mean DH in each hydrolysis time point, hydrolysis curve was plotted. To produce the hydrolysates, a series of hydrolyses were carried out under the same conditions of initial protein concentration of 10% (*w/w*), E/S of 15 mAU/ g, pH of 8.0, and temperature of 50°C. They were stopped after the time estimated based on the hydrolysis curve, necessary to produce hydrolysates at the DHs of 2%, 5%, 8%, and 12%. The enzyme was inactivated by heating the suspension at 75°C for 15 min. Then, the hydrolysates were neutralized with 1 M HCl solution and freeze-dried using Lyph Lock 6 system (Labconco, Kansas City, MO, USA).

Determination of molecular weight distribution of the pea protein isolate and hydrolysates

The pea protein isolate and hydrolysates were subjected to size-exclusion chromatography analyses using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech) with a Superdex 75 HR 10/30 column (a separation range of 3–70 kDa) or a Superdex Peptide HR 10/30 column (a separation range of 0.1–7.0 kDa); both from Pharmacia Biotech. The mobile phase was 0.1 M phosphate buffer, pH 7.0, and the flow rate was 0.5 mL/min for the former column and 0.7 mL/min for the latter. UV detection was used at a wavelength of 214 nm. Samples (25 mg) were dissolved in an eluent, centrifuged at 14,000×*g* for 10 min, filtered through a 0.22 µm nylon membrane filter, and injected onto the column (200 µL). The MWs of proteins, polypeptides, and peptides in the samples were estimated based on standards. The Superdex 75 column was calibrated using ribonuclease A, chymotrypsin, ovalbumin, and porcine albumin with MWs of 13.7, 25.0, 43.0, and 67.0 kDa, respectively. The Superdex Peptide column was calibrated using aprotinin with a MW of 6.5 kDa, and synthetic peptides with MWs of 0.89, 2.22, and 8.00 Da. From the chromatograms, areas under curves for MW ranges of >53, 29–53, 9–29, and <9 kDa (Superdex 75 column) and >9, 8–9, 7–8, 4–7, 2–4, and <2 kDa (Superdex Peptide column) were calculated, and relative content of molecules within the defined MW range was expressed as the percentage of total area. Data were processed using FPLC Director software, version 1.03 (Pharmacia Biotech).

Determination of surface hydrophobicity of the pea protein isolate and hydrolysates

The surface hydrophobicity of the pea protein isolate and hydrolysates was determined using fluorimetric assays with ANS and CPA [Avramenko *et al.*, 2013; Kato & Nakai, 1980]. The isolate and hydrolysates (40 mg) were dissolved in 100 mL of 0.01 M phosphate buffer, pH 7.0, and then diluted to obtain finally eight solutions with concentrations of 5–40 mg/100 mL. In the ANS assay, 2 mL of each solution was mixed with 10 µL of 8.0 mM ANS solution in 0.01 M phosphate buffer, pH 7.0. Fluorescence intensity was measured against a blank without ANS at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The measurements were performed using an SFM-25 spectrofluorometer (Kontron Instruments, Ismaning, Germany), which was calibrated with an ANS solution in methanol (at the same concentration as in the test samples). In the CPA assay, 10 µL of 3.6 mM CPA solution in absolute ethanol with an equimolar amount of BHT was added to 2 mL of the sample solution. The fluorescence intensity was measured at an excitation and emission wavelengths of 325 and 420 nm, respectively, and a CPA solution in *n*-decane was used for calibration of an SFM 25 spectrofluorometer. The surface hydrophobicity was expressed as the initial slope of the curves plotted between fluorescence intensity and protein concentration.

Determination of antioxidant capacity of the pea protein isolate and hydrolysates

ABTS radical cation scavenging capacity

In the ABTS assay, $ABTS^{\bullet+}$ was generated, and a stock solution was diluted exactly as in the original method [Re *et al.*, 1999]. Then, 2 mL of the diluted $ABTS^{\bullet+}$ solution was vigorously mixed with 20 µL of the isolate or a hydrolysate solution obtained by dissolving it in 0.1 M sodium phosphate buffer (pH 7.0) to a concentration of 20 mg/mL. The reaction mixture was incubated at 30°C for 6 min, and the absorbance was measured at a wavelength of 734 nm. The standard curve for Trolox was plotted (0.2–2.0 µmol/mL, r=0.999), and results were expressed as mmol of Trolox equivalent *per* g of isolate or hydrolysate.

Oxygen radical absorbance capacity

Oxygen radical absorbance capacity ($ORAC_{FL}$) of the pea protein isolate and hydrolysates was determined according to the procedure described previously by Dávalos *et al.* [2004]. Aliquots of 20 µL of the sample solution in 75 mM phosphate buffer (pH 7.4) were pipetted into a 96-well microplate, and 120 µL of a fluorescein solution in the same buffer (70 nM, final concentration in the reaction mixture) were added. After vigorously

mixing, the microplate was heated at 37°C for 15 min, and then 60 µL of an AAPH solution (12 mM, final concertation) were added to the reaction mixture, which was further incubated at 37°C. Fluorescence measurements were performed at 1-min intervals for 80 min at excitation and emission wavelengths of 485 nm and 520 nm, respectively, using a Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany). Blank samples with 75 mM phosphate buffer (pH 7.4) instead of the isolate/hydrolysate solution were processed in parallel, as well as samples with Trolox solutions (final concertation in the range of 1–8 µM) as a reference, to plot the standard curve. The raw data were calculated exactly as in the previous study [Dávalos *et al.*, 2004], and $ORAC_{FL}$ values were expressed as mmol of Trolox equivalent *per* g of isolate or hydrolysate.

Antioxidant capacity in the photochemiluminescence assays

The antioxidant capacity of water-soluble compounds (ACW) and lipid-soluble compounds (ACL) in the photochemiluminescence (PCL) assays, in which antioxidants scavenge superoxide anion radicals ($O_2^{\bullet-}$) generated from luminol, was determined according to the protocol provided by the manufacturer of the PCL-ACW and PCL-ACL kits, using a Photochem device (Analytik Jena). Briefly, for PCL-ACW determination, the isolate or hydrolysate dissolved in water (10 mg/mL) was mixed with the buffer solution (pH 10.5) and luminol solution. The lag time of luminescence was measured and compared to that of the blank. Reaction mixtures with L-ascorbic acid (10–150 µM) were used to plot a standard curve. Results were expressed as µmol of L-ascorbic acid equivalent *per* g of isolate or hydrolysate. For PCL-ACL evaluation, the solution of isolate or hydrolysate in methanol (10 mg/mL) was properly diluted with methanol and vortexed with luminol and buffer solution. The reaction was carried out for 3 min during which the luminescence signal was measured. The integral under the signal curve was calculated, and results were expressed as µmol of Trolox equivalent *per* g of isolate or hydrolysate based on the standard curve plotted for Trolox (50–300 µM).

Inhibition of β-carotene-linoleic acid emulsion oxidation

The inhibition of β-carotene-linoleic acid emulsion oxidation by the isolate and hydrolysates was determined according to Karamać [2009], with slight modifications. To prepare the emulsion, the emulsifier (Tween 40, 400 mg) and linoleic acid (40 mg) were vortexed with a solution of β-carotene (5 mg) in chloroform (5 mL), then the chloroform was removed under nitrogen, and methanol (10 mL) and water (80 mL) were added to the residue successively. The isolate and hydrolysates were dissolved in a 1% (*w/v*) sodium dodecyl sulfate (SDS) solution (25 mg/mL), and portions of 200 µL were mixed with 2.5 mL of the emulsion. Oxidation was performed at 50°C in dark, and absorbance was read at 470 nm for 120 min at 15-min intervals. In parallel, emulsion with a BHT solution (200 µL, 0.5 mg/mL) and control with an SDS solution instead of antioxidants were incubated. For each time point, the percentage of non-oxidized β-carotene was calculated, and then time-dependent curves were plotted.

Statistical analysis

Three batches of pea protein hydrolysates at a defined DH were prepared. Each analysis was performed in triplicate. Results were expressed as mean and standard deviation. One-way analysis of variance with Tukey's post hoc test was performed using GraphPad Prism software (version 6.04, GraphPad Software, Boston, MA, USA) to show significant differences ($p<0.05$) between the isolate and hydrolysates at the different DHs. Moreover, data were subjected to principal component analysis using Statistica 14.1.0.4 software (Cloud Software Group, Inc., Palo Alto, CA, USA).

RESULTS AND DISCUSION

Degree of hydrolysis

The hydrolysis curve of the pea protein isolate (Propulse) with trypsin is shown in **Figure 1**. The reaction rate was very high for the first 15 min, then it gradually slowed down, and from 100 min the DH increased insignificantly. In summary, trypsin hydrolyzed pea proteins at 50°C, an E/S of 15 mAU/g for 120 min to a DH of 12.84%. As our previous study showed, these conditions were optimal for processing Propulse with this enzyme; DH at temperatures of 35–45°C reached lower values than at 50°C, and an E/S of 15–35 mAU/g had no significant effect on DH after 60 min of the hydrolysis [Karamać *et al.*, 2002]. The final DH was similar to that reported by Asen & Aluko [2022] for a pea protein concentrate hydrolyzed with trypsin (~12%) and even higher than the values shown by García Arteaga *et al.* [2020, 2022], who hydrolyzed a pea protein isolate (6.86–7.59%). In turn, Sareen *et al.* [2023] and Konieczny *et al.* [2020b] obtained tryptic hydrolysates of a pea protein isolate and pea flour with enriched protein content, respectively, at a DH of 10%. Generally, the use of trypsin allows obtaining pea protein hydrolysates at relatively low DH, most likely due to its high substrate specificity. This serine endopeptidase only cleaves peptide bonds at the carboxyl side of basic amino acid, including arginine and lysine [Vajda & Szabó, 1976]. As shown in a previous study, pancreatin and Alcalase, both with broad specificity, hydrolyzed a pea protein concentrate to a 5-fold higher DH than trypsin, whereas the use of chymotrypsin, pepsin, and Flavourzyme allowed for 2.5–3.5-fold higher DH [Asen & Aluko, 2022]. Trypsin was found to be less effective

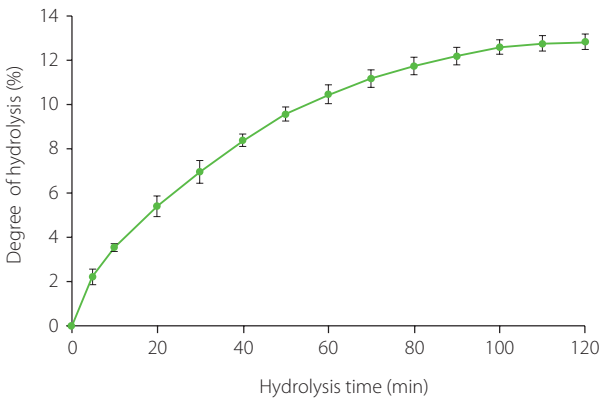

Figure 1. Kinetics of hydrolysis of pea protein isolate with trypsin. Results are shown as mean and standard deviation (n=3).

in hydrolyzing a pea protein isolate than the bacterial alkaline endopeptidases such as Savinase, Alcalase, and Esperase [García Arteaga *et al.*, 2020]. Zhao *et al.* [2025] hydrolyzed pea proteins with papain, Protamex, Alcalase, Neutrase, acid protease, and PaproA and reported the DH in the range of 12.80–18.89%. Another reason for the relatively low DH of tryptic hydrolysis of the pea protein isolate obtained in this study and confirming previous literature findings may be the presence of protease inhibitors in the substrate. Konieczny *et al.* [2020b] reported that the trypsin inhibitor activity of untreated air-classified pea protein enriched flour was 38.35 TIU/mg and this value decreased in tryptic hydrolysates to 11.00–11.95 TIU/mg, regardless of DH. Sareen *et al.* [2023] presumed that the high trypsin inhibitor activity of pea protein isolate prevented the production of tryptic hydrolysate at a DH higher than 10%, which was possible for faba bean protein isolate (DH was twice as high). It should also be noted that methodological aspects may determine the DH value. Determination of DH by the pH-stat method and methods based on spectrophotometric evaluation of $-NH_2$ groups released during hydrolysis, generally, yields lower results than DH estimated based on the nitrogen content in the hydrolysate (after precipitation of unhydrolyzed protein with a trichloroacetic acid solution) relative to the nitrogen content in the substrate [Wróblewska & Karamać, 2003]. This is likely why the DH of the tryptic hydrolysate of protein isolated from pea seed waste determined by the latter method reached as much as 28.7% [Mahgoub *et al.*, 2025].

Molecular weight distribution

Due to the broad range of MWs of proteins, polypeptides, and peptides in the pea protein isolate and hydrolysates, their distributions were determined using two size exclusion chromatography columns, Superdex 75 and Superdex Peptide, differing in the range of separated MWs (3–70 and 0.1–7.0 kDa, respectively). FPLC chromatograms of the pea protein isolate and hydrolysates at different DHs on these two columns are shown in **Figures 2** and **3**, respectively, and the relative content of molecules with defined MWs is summarized in **Tables 1** and 2, respectively. The separation of the pea protein isolate on a Superdex 75 column showed one dominant peak with a retention time of 16.8 min and only small peaks with slightly greater retention times to 23.1 min (**Figure 2**). Proteins with MWs higher than 53 kDa were responsible for the main peak. The other peaks corresponded to MWs of 29–53 kDa. The relative abundance of these two fractions in the pea protein isolate was 59.61% and 21.51%, respectively (**Table 1**). Pea globulins such as legumin (453 kDa), vicilin (138 kDa), and convicilin (248 kDa), as well as legumin and convicilin subunits, all with MWs higher than 60 kDa [Sarigiannidou *et al.*, 2022; Yang *et al.*, 2021], were likely eluted in the first peak. In turn, MWs of 29–53 kDa could be attributed to fractions of various globulins, especially vicilin subunits, but also to pea albumins and trypsin inhibitors [Klost & Drusch, 2019; Sarigiannidou *et al.*, 2022; Yang *et al.*, 2021]. As expected, the relative content of these proteins successively decreased in the hydrolysates at increasing DH, with the share of the fraction with MWs >53 kDa decreasing much faster than

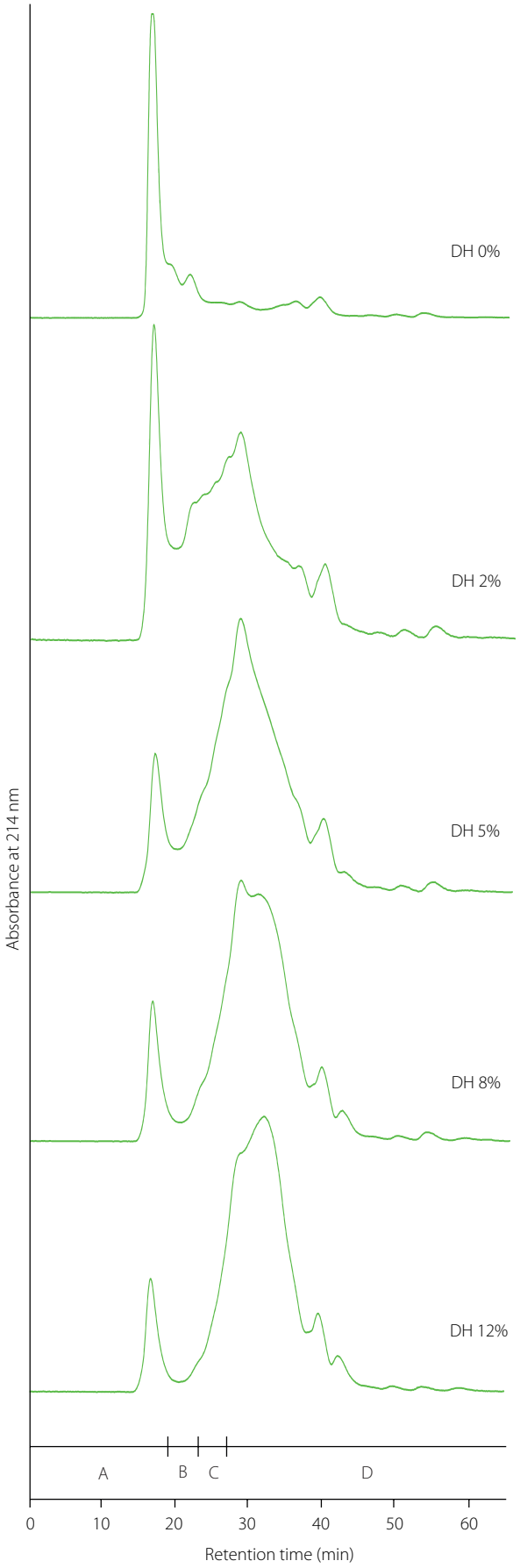

Figure 2. Size-exclusion chromatography separation of pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%) using a Superdex 75 column. Letters A, B, C, and D above x-axis indicate molecular weight ranges of >53, 29–53, 9–29, and <9 kDa, respectively.

that of the fraction with MWs of 29–53 kDa (in the hydrolysate at DH of 5%, the share of the former was only 9.36%). However, it should be noted that even the hydrolysate at the highest DH still possessed proteins with MWs greater than 53 kDa. This finding was consistent with the literature data for tryptic hydrolysates of pea proteins [Sarigiannidou *et al.*, 2022; Yang *et al.*, 2021]. The resistance of pea legumin-T (260 kDa) to hydrolysis by trypsin has already been reported [Schwenke *et al.*, 2001].

Hydrolysis products with MWs of 9–29 kDa constituted approximately 19–20% in the hydrolysates at the DH of 2% and 5%, and significantly ($p<0.05$) less in those at the DH of 8% (14.78%) and 12% (11.37%) (**Table 1**). The contribution of products with MWs <9 kDa increased much more during hydrolysis, from 43.25% (DH 2%) to 80.05% (DH 12%). Among these products, the fraction with MWs in the range of 8–9 kDa dominated in the hydrolysate at a DH of 2% and accounted for 27.52% (**Figure 3**, **Table 2**). The hydrolysate at a DH of 5% contained mainly products with MWs of 7–8 kDa (24.83%), although fractions with MWs of 8–9 kDa and 4–7 kDa were also abundant (22.57% and 23.11%, respectively). In the hydrolysate at a DH of 8%, the main fraction comprised products with MWs of 4–7 kDa (33.54%), followed by those with MWs of 7–8 kDa (24.29%). The hydrolysate at a DH of 12% also contained peptides from these two fractions as dominant, with the contribution of peptides with MWs of 4–7 kDa being significantly ($p<0.05$) higher than in the hydrolysate at a DH of 8%. Although the fraction with MWs of 2–4 kDa in hydrolysates increased significantly ($p<0.05$) with the DH, its contribution was not high and ranged from 6.73% (DH 2%) to 11.74% (DH 12%). Peptides with MWs <2 kDa also occurred at a low level of 7.50–9.42%, and their share did not differ significantly ($p\geq0.05$) in the hydrolysates at the DH of 5–12%. In summary, the hydrolysates contained polypeptides and peptides with a very broad range of MWs, varying depending on the DH, and the contribution of those with MW below 2 kDa was relatively low. The broad range of molecular weights of the products released from pea proteins by trypsin is consistent with literature data [Klost & Drusch, 2019; Sarigiannidou *et al.*, 2022]. By size-exclusion chromatography, Sarigiannidou *et al.* [2022] showed that limited hydrolysis of a pea protein isolate with trypsin (DH 4%) yielded mainly products with MWs between 2 and 30 kDa. In turn, Sareen *et al.* [2023], using capillary gel electrophoresis, found that although the fraction with MWs below 15 kDa predominated in tryptic pea protein hydrolysates at the DHs of 5% and 10%, products with MWs of 15–30 Da also accounted for a considerable portion, particularly in the hydrolysate at the lower DH. Moreover, Awosika & Aluko [2019] demonstrated that the yield of fractions of tryptic hydrolysates of a pea protein concentrate with MW >10 kDa was higher, and that of fractions with peptides (1–3 and 3–5 kDa) was lower compared to the hydrolysates obtained using other alkaline proteases, *i.e.*, Alcalase and chymotrypsin.

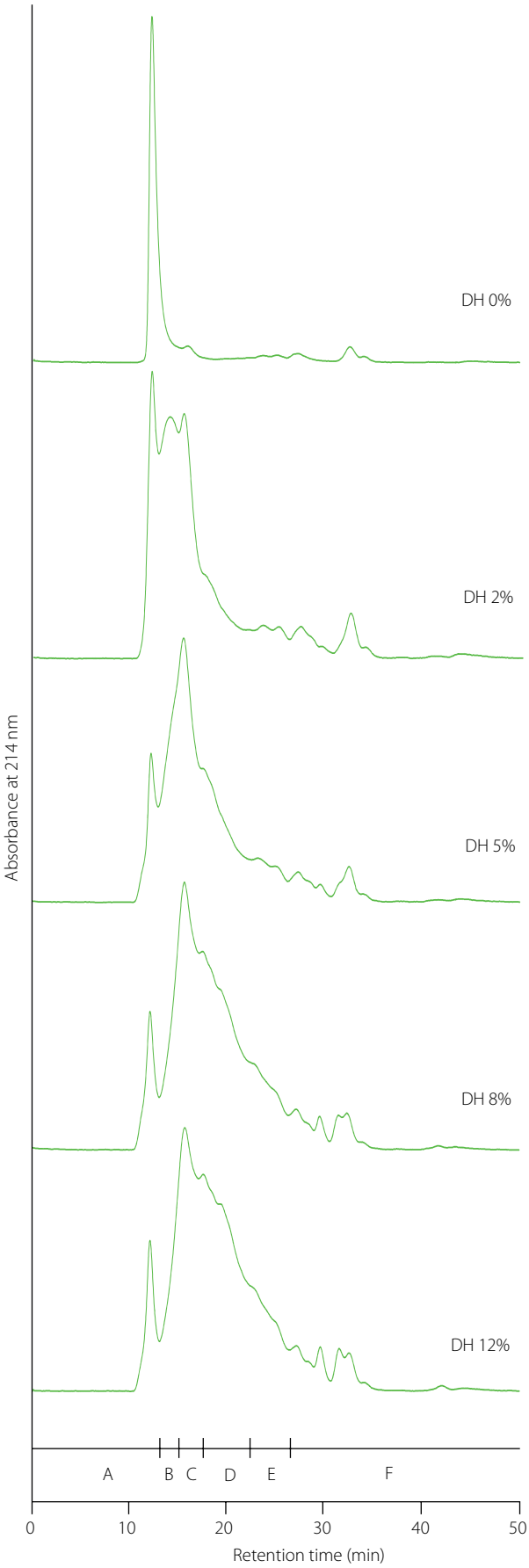

Figure 3. Size-exclusion chromatography separation of pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%) using a Superdex Peptide column. Letters A, B, C, D, E, and F above x-axis indicate molecular weight ranges of >9, 8–9, 7–8, 4–7, 2–4, <2 kDa, respectively.

Surface hydrophobicity

The ANS and CPA assays were performed to determine surface hydrophobicity of the pea protein isolate and its tryptic

Table 1. Relative content (%) of proteins and polypeptides with defined molecular weight ranges in pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%) determined by size-exclusion chromatography using a Superdex 75 column.

DH (%)	>53 kDa	29–53 kDa	9–29 kDa	<9 kDa
0	59.61±0.93^{a}	21.51±0.31^{a}	6.18±0.15^{d}	12.70±0.27^{e}
2	19.74±0.19^{b}	17.62±0.25^{b}	19.39±0.36^{a}	43.25±0.31^{d}
5	9.36±0.44^{c}	9.65±0.09^{c}	19.90±0.18^{a}	61.09±0.37^{c}
8	8.81±0.17^{c}	4.73±0.11^{d}	14.78±0.21^{b}	71.68±0.83^{b}
12	7.24±0.09^{d}	2.34±0.16^{e}	11.37±0.16^{c}	80.05±0.42^{a}

Results are shown as mean ± standard deviation (n=3). Different superscript letters in a column indicate significant differences (p<0.05). DH, degree of hydrolysis.

Table 2. Relative content (%) of polypeptides and peptides with defined molecular weight ranges in pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%) determined by size-exclusion chromatography using a Superdex Peptide column.

DH (%)	>9 kDa	8–9 kDa	7–8 kDa	4–7 kDa	2–4 kDa	<2 kDa
0	68.34±0.75^{a}	14.15±0.53^{c}	5.71±0.19^{d}	3.13±0.12^{e}	3.20±0.13^{e}	5.47±0.17^{c}
2	22.61±0.38^{b}	27.52±0.94^{a}	20.71±0.99^{c}	14.93±0.41^{d}	6.73±0.25^{d}	7.50±0.31^{b}
5	12.67±0.09^{c}	22.57±0.31^{b}	24.83±0.23^{a}	23.11±0.04^{c}	8.00±0.04^{c}	8.76±0.40^{a}
8	8.64±0.19^{d}	14.07±0.23^{c}	24.29±0.25^{ab}	33.54±0.32^{b}	10.74±0.15^{b}	8.72±0.57^{a}
12	7.74±0.31^{d}	11.55±0.41^{d}	23.11±0.23^{b}	36.42±0.19^{a}	11.74±0.61^{a}	9.42±0.35^{a}

Results are shown as mean ± standard deviation (n=3). Different superscript letters in a column indicate significant differences (p<0.05). DH, degree of hydrolysis.

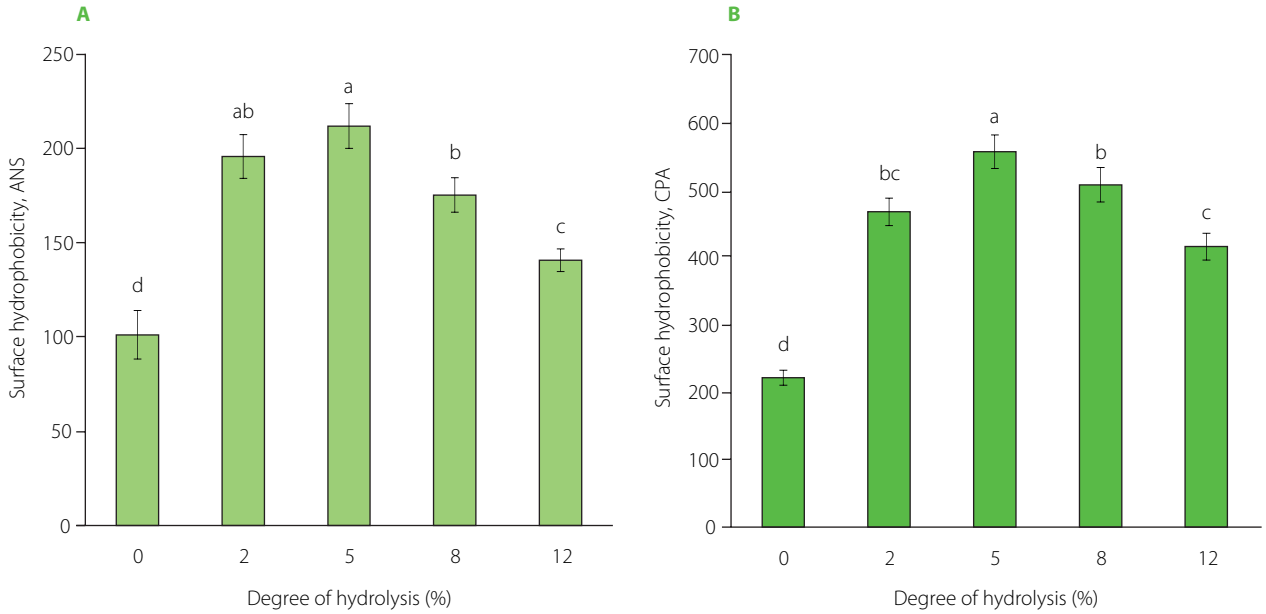

Figure 4. Surface hydrophobicity of pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%) determined by the assay with 8-anilino-1-naphthalenesulfonic acid, ANS (**A**) and *cis*-parinaric acid, CPA (**B**). Different letters above bars indicate significant differences (p<0.05).

hydrolysates. The results are shown in **Figure 4**. In both assays, the pea protein isolate showed the lowest surface hydrophobicity, 101 and 222, respectively. These values initially increased with increasing DH, and after reaching the maximum, ultimately declined. However, even the lowest values of 141 (ANS assay) and 416 (CPA assay) for the hydrolysate at a DH of 12% were significantly (p<0.05) higher than those determined for the pea protein isolate. The highest surface hydrophobicity of 557 was achieved at a DH of 5% in the CPA assay. In the ANS assay, the highest values of 196–212 were found for hydrolysates at

the DH of 2% and 5%, which did not differ significantly from each other ($p \geq 0.05$). In previous studies, the surface hydrophobicity of the pea protein isolate in this assay was within the range of 59.69–115.1 [Sareen *et al.*, 2023; Shay *et al.*, 2023; Tang *et al.*, 2023]. Its initial increase following treatment with trypsin likely resulted from the unfolding of the protein structure and exposure of hydrophobic sites originally located within the molecule [Jung *et al.*, 2005; Tang *et al.*, 2023]. Avramenko *et al.* [2013] postulated that this protein structure unfolding and resulting increase in hydrophobicity may be due not only to hydrolysis but also to substrate heating, and that it is difficult to estimate the contribution of both processes. Peng *et al.* [2016] and Sareen *et al.* [2023] analyzed pea protein isolates before and after heating (85–90°C, 30 min) and reported up to 2-fold higher hydrophobicity of the processed proteins. Since in our study the isolate was not preheated, it seems that heating during hydrolysis could affect the surface hydrophobicity at the initial stage of the process. The reduction in surface hydrophobicity following further hydrolysis may be linked to the cleavage of hydrophobic bonds within the hydrophobic regions and their breakdown, or to the increased hydrophobic interactions between these regions, leading to protein aggregation, confining most hydrophobic bonds to the interior of the aggregates and thus reducing the number or area of hydrophobic groups on the surface [Jung *et al.*, 2005; Shuai *et al.*, 2022; Xu *et al.*, 2016]. A similar trend in the hydrophobicity of a trypsin-hydrolyzed pea protein isolate was observed by Shuai *et al.* [2022], although they determined its maximum value at a lower DH of 2%. In turn, Sareen *et al.* [2023] reported an increase in the surface hydrophobicity of the tryptic hydrolysates compared to the pea protein isolate, but differences between hydrolysates at the different DHs (5% and 10%) were not observed, while Konieczny *et al.* [2020a] found a continuous increase for trypsin-hydrolyzed pea flour at DH up to 10%. It should be noted, however, that changes in surface hydrophobicity following enzymatic hydrolysis depend not only on DH, but also on the type of enzyme and protein. During hydrolysis of pea protein with Alcalase, Flavourzyme, and Neutrase, no initial increase was observed, and in fact, a decrease was found [Shay *et al.*, 2023; Shuai *et al.*, 2022]. Also, the use of trypsin for hydrolysis of proteins other than pea proteins resulted in a different trend in changes in surface hydrophobicity, *e.g.*, a successive increase with increasing DH was reported for a faba bean protein isolate [Sareen *et al.*, 2023], and an initial decrease for a lentil protein isolate [Avramenko *et al.*, 2013].

The surface hydrophobicity of the isolate and the individual hydrolysates determined by the ANS assay was lower than that shown by the CPA assay (**Figure 4**). The reason may be the specificity of the fluorescent probes. Although the reagents of both assays are anionic probes, ANS has an aromatic structure and CPA contains an aliphatic hydrocarbon chain; therefore, their binding sites on the protein may be different and, thus, may affect the measured protein hydrophobicity value [Alizadeh-Pasdar & Li-Chan, 2000]. Our study results indicated that the protein binding sites interacted more readily with aliphatic CPA than with aromatic ANS under assay conditions (pH 7.0). Alizadeh-Pasdar & Li-Chan [2000], who compared the surface hydrophobicity of a few proteins measured in both assays, showed that native β-lactoglobulin had higher hydrophobicity in the CPA assay than in the ANS assay (both performed at pH 7.0), but native and heated bovine serum albumin showed higher values in the ANS than in the CPA assay. Noteworthy is, however, that these differences varied depending on pH, and the authors concluded that the hydrophobicity was influenced not only by the structure of the fluorescent probe but also by electrostatic interactions in the measurements.

Antioxidant capacity

The antioxidant capacity of the pea protein isolate and hydrolysates at different DHs, evaluated as $ABTS^{\bullet+}$ scavenging capacity, oxygen radical absorbance capacity, and antioxidant capacity of water-soluble and lipid-soluble compounds in the PCL assays, is shown in **Table 3**. Moreover, the ability of the isolate and hydrolysates to inhibit the oxidation of β-carotene-linoleic acid model emulsion was determined, and results are presented in **Figure 5**. The $ABTS^{\bullet+}$ scavenging capacity ranged from 0.023 to 0.111 mmol Trolox/g. A slightly broader range of values was obtained for $ORAC_{FL}$ – from 0.044 to 0.320 mmol Trolox/g. In both assays, the pea protein isolate showed the lowest ($p<0.05$) antioxidant capacity, which gradually increased after trypsin

Table 3. Antioxidant capacity of pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%).

DH (%)	ABTS assay (mmol Trolox/g)	$ORAC_{FL}$ (mmol Trolox/g)	PCL-ACW (μmol L-ascorbic acid/g)	PCL-ACL (μmol Trolox/g)
0	0.023 ± 0.001^{d}	0.044 ± 0.004^{e}	0.934 ± 0.004^{a}	1.99 ± 0.21^{c}
2	0.049 ± 0.002^{c}	0.085 ± 0.003^{d}	0.942 ± 0.010^{a}	3.04 ± 0.26^{b}
5	0.090 ± 0.011^{b}	0.171 ± 0.007^{c}	0.937 ± 0.007^{a}	3.68 ± 0.10^{a}
8	0.109 ± 0.000^{a}	0.246 ± 0.003^{b}	0.927 ± 0.005^{a}	3.32 ± 0.09^{ab}
12	$0.111\pm.0001^{a}$	0.320 ± 0.026^{a}	0.936 ± 0.027^{a}	3.18 ± 0.19^{b}

Results are shown as mean ± standard deviation ($n=3$). Different superscript letters in a column indicate significant differences ($p<0.05$). DH, degree of hydrolysis; ABTS assay, assay with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; $ORAC_{FL}$, oxygen radical absorbance capacity; PCL-ACW, antioxidant capacity of water-soluble compounds in the photochemiluminescence assay; PCL-ACL, antioxidant capacity of lipid-soluble compounds in the photochemiluminescence assay.

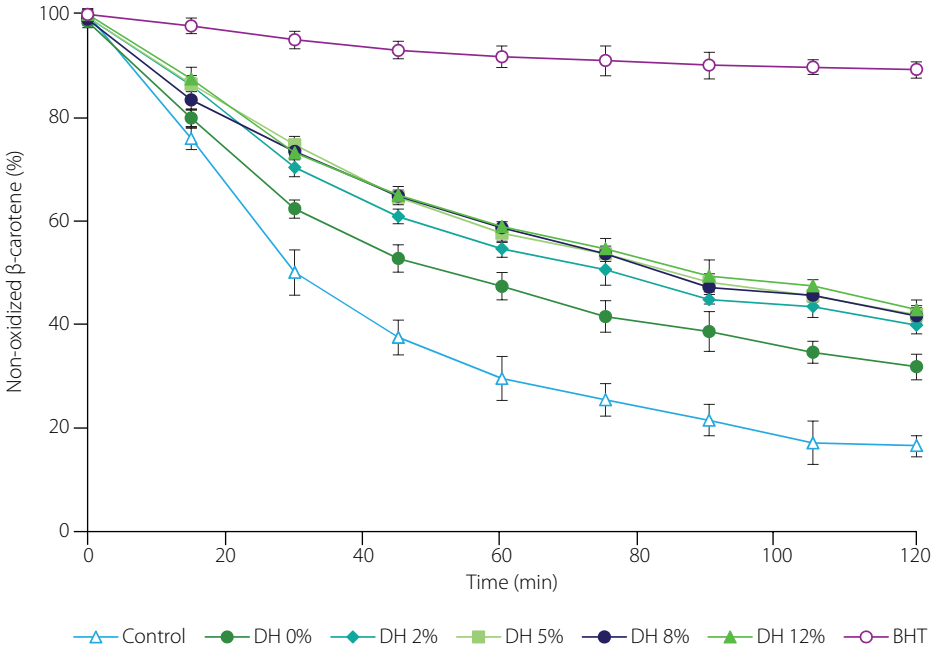

Figure 5. Inhibition of the β-carotene-linoleic acid emulsion oxidation by pea protein isolate (degree of hydrolysis, DH, 0%), its tryptic hydrolysates (DH 2–12%), and butylhydroxytoluene (BHT); Control, emulsion without antioxidants.

treatment with increasing DH. An increase in the antiradical capacity of legume protein hydrolysates with DH in ABTS and $ORAC_{FL}$ assays has been reported in the literature [Gómez *et al.*, 2021; Karamać *et al.*, 2014]. This phenomenon has been linked with an increase in the number of low MW peptides as protein hydrolysis progresses [Irankunda *et al.*, 2025]. However, there are also reports of a higher $ABTS^{\bullet+}$ scavenging capacity and $ORAC_{FL}$ of hydrolysates containing products with intermediate MWs or a broad spectrum of MWs compared to the hydrolysates rich in small-size peptides [Suarez *et al.*, 2021; Sulewska *et al.*, 2022]. Therefore, such relationships should be considered for hydrolyses obtained in defined enzyme-substrate systems. The result of the ABTS assay achieved for the pea protein isolate in our study was similar to those reported by Žilić *et al.* [2012] (24.98–33.84 mmol Trolox/kg of dry weight), although, unlike hydrolysis with trypsin, the cited authors did not find an improvement in $ABTS^{\bullet+}$ scavenging capacity after 15 and 30 min of papain treatment. In turn, the $ORAC_{FL}$ of the hydrolysate at the highest DH in our study was lower than the values of 537.84 and 502–704.15 µmol Trolox/g obtained when *Bacillus licheniformis* LBA 46 proteases and Alcalase, respectively, were used to release peptides from pea proteins [Aguilar *et al.*, 2020; Girgih *et al.*, 2015].

The antioxidant capacity of water-soluble compounds of the pea protein isolate and its hydrolysates in the PCL assay did not differ significantly ($p \geq 0.05$) (**Table 3**). No significant ($p \geq 0.05$) differences were also found between the hydrolysates at different DHs (0.927–0.942 µmol L-ascorbic acid/g). This lack of differences may be due to the specificity of the PCL-ACW assay. Our previous study on the antioxidant capacity of lentil flour hydrolysates prepared with pancreatin also showed no significant differences in PCL-ACW depending on DH [Sulewska *et al.*, 2022]. In turn, the PCL-ACL of the pea protein isolate (1.99 µmol Trolox/g), was significantly ($p<0.05$) lower than that of hydrolysates, among which the highest values were found for those at the DH of 5% (3.68 µmol Trolox/g) and DH of 8% (3.32 µmol Trolox/g) (**Table 3**). Thus, the pattern of changes in PCL-ACL with DH differed from the trend of changes in $ABTS^{\bullet+}$ scavenging capacity and $ORAC_{FL}$, which may be due to different properties of the hydrolysis products that influence their ability to scavenge $O_2^{\bullet-}$ in the PCL-ACL assay compared to other radicals in the other assays. Literature data have shown that, in addition to the MWs of the peptides, their hydrophobicity also plays an important role in their ability to scavenge some radicals [Noman *et al.*, 2022; Pownall *et al.*, 2010]. Pownall *et al.* [2010], who hydrolyzed a pea protein isolate with Thermolysin, reported that more hydrophobic fractions had higher $O_2^{\bullet-}$ scavenging activity, but the presence of hydrophobic amino acids did not contribute to the reducing power of the peptides.

Model emulsion oxidation was inhibited by both the pea protein isolate and hydrolysates, but again, the proteins were less effective than the trypsin-treated products (**Figure 5**). A significantly lower percentage of non-oxidized carotene was determined for the emulsion with the protein isolate from 30 to 120 min of oxidation. Among the hydrolysates, the one at the lowest DH showed a slightly lower ability to inhibit β-carotene-linoleic acid emulsion than the others in the middle of oxidation, but after 120 min, no significant ($p \geq 0.05$) differences were observed between any of them. Trigui *et al.* [2021] also showed lower ability of a black cumin seed protein isolate in inhibiting oxidation of a model emulsion compared to its hydrolysates prepared with Savinase at increasing DH. On the other hand, Jamdar *et al.* [2010] reported higher antioxidant activity of peanut protein hydrolysates obtained using Alcalase at low DH (up to 10%), but lower activity during emulsion oxidation for hydrolysates at DH of 30% and 40% compared to protein isolate.

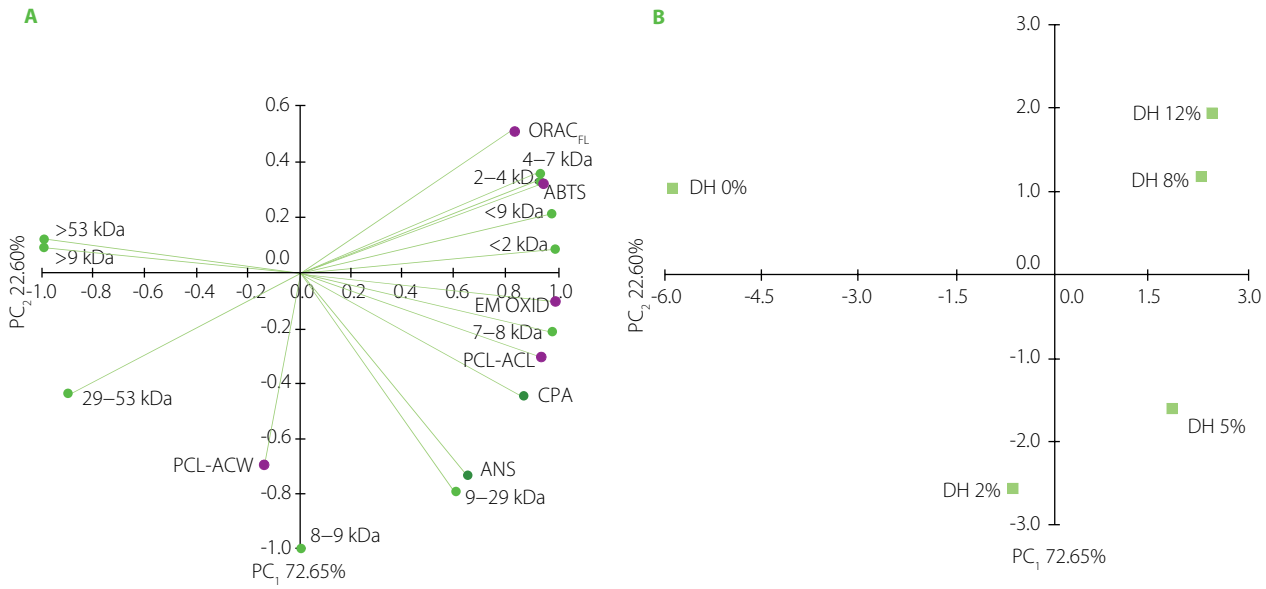

Figure 6. Plots of principal component analysis with (**A**) distribution of the variables including surface hydrophobicity (measured in the assay with 8-anilino-1--naphthalenesulfonic acid, ANS; and *cis*-parinaric acid, CPA), relative content of fractions with different molecular weight (MW) and antioxidant capacity (determined as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging capacity, ABTS; oxygen radical absorbance capacity, ORAC$_{FL}$; antioxidant capacity of water-soluble and lipid-soluble compounds in the photochemiluminescence assay, PCL-ACW and PCL-ACL, respectively; and inhibition of β-carotene-linoleic acid emulsion oxidation, EM OXID), and (**B**) distribution of pea protein isolate (degree of hydrolysis, DH, 0%) and its tryptic hydrolysates (DH 2–12%).

Relationship between physicochemical properties and antioxidant capacity

A data set of physicochemical parameters and antioxidant capacity of the pea protein isolate and its hydrolysates was subjected to PCA to uncover any patterns between the characteristics. The PCA plots are shown in **Figure 6**. The two first principal components (PC$_1$ and PC$_2$) explained 95.25% of the total variance. The isolate was clearly distinguished from all hydrolysates (**Figure 6B**). Furthermore, there was discrimination along PC$_2$ between hydrolysates at higher DHs (8% and 12%) and hydrolysates at lower ones (2% and 5%). As expected, the former were associated with fractions with low MWs (<2 kDa, 2–4 kDa and 4–7 kDa) (**Figure 6A**). These fractions were closely related to antioxidant capacity measured by ABTS and ORAC$_{FL}$ assays, confirming our assumption that peptide size primarily affects ORAC$_{FL}$ and ABTS$^{\bullet+}$ scavenging capacity of tryptic pea protein hydrolysates. In turn, fractions with higher MWs (7–8 kDa and 9–29 kDa) were clustered with surface hydrophobicity. These variables were also closely correlated with PCL-ACL and antioxidant capacity in β-carotene-linoleic acid emulsion oxidation. This observation confirmed the literature data on the influence of hydrophobicity of protein-derived antioxidants on their ability to scavenge $O_2^{\bullet-}$ [Pownall *et al.*, 2010]. In our study, the mentioned variables were particularly associated with the hydrolysate at a DH of 5% (**Figure 6B**).

CONCLUSIONS

Trypsin treatment of the pea protein isolate resulted in a relatively low degree of hydrolysis. However, even this limited hydrolysis effectively improved antioxidant properties, and resulted in the higher antioxidant capacity of the hydrolysates compared to the pea protein isolate. With increasing DH, antioxidant capacity of the hydrolysates increased, but only in assays carried out in the aqueous environment (ABTS and ORAC$_{FL}$ assays). In this case, the antioxidant capacity was affected by the decreasing molecular weights of the released polypeptides and peptides; however, the percentage content of the low MW fraction (<2 kDa) was small even in the hydrolysate at the highest DH. In turn, the surface hydrophobicity of pea protein hydrolysates, to a greater extent than their MW distribution, determined the antioxidant capacity in the PLC-ACL assay and during the oxidation of β-carotene-linoleic acid emulsion. Because the hydrolysate with low DH (5%) was characterized by the maximum surface hydrophobicity, such DH should be considered optimal for antioxidant capacity of tryptic pea protein hydrolysates in lipophilic environment.

Overall, the hydrolysis of pea proteins with trypsin can be considered as a viable means to obtain products with improved antioxidant properties, and the control of the degree of hydrolysis allows for the optimization of these properties under various conditions. The decline in surface hydrophobicity beyond a certain degree of hydrolysis needs to be further investigated to understand its implications for future applications of tryptic pea protein hydrolysates in food systems or nutraceuticals.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Assessment of Physicochemical and Biochemical Characteristics of Oak (*Quercus* spp.), Garland Thorn (*Paliurus spina-christi* Mill.), and Sunflower (*Helianthus annuus* L.) Honeys

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This study aimed to evaluate the physicochemical properties, total phenolic and flavonoid content, and antioxidant activity of oak (*Quercus* spp.), garland thorn (*Paliurus spina-christi* Mill.), and sunflower (*Helianthus annuus* L.) honey samples produced in Kirklareli, Thrace region of Northwest Türkiye. According to the results, contents of total soluble solids varied between 81.20–85.07°Brix, and pH values ranged from 3.41–5.39. The higher electrical conductivity was found in oak honey samples (0.83–1.09 µS/cm) compared to blossom honey types. Optical rotation changed between −1.42 and +0.14 depending on the honey type. The hydroxymethylfurfural (HMF) content was in the range of 0.77–4.51 mg/kg, indicating that the honeys were natural (unprocessed). The highest proline content was found in the oak honey group (884.3 mg/kg) and the lowest value was in the sunflower honey group (568.5 mg/kg). In addition, the diastase activity of sunflower, garland thorn, and oak honeys was observed to vary between 12.26–18.72, 14.00–23.95, and 13.53–24.58, respectively. The oak honey samples exhibited the darkest color and possessed the highest total phenolic content, consequently demonstrating the greatest antioxidant activity in the ABTS and DPPH assays. Among the blossom honey types, the garland thorn honey group showed higher antioxidant activity. In conclusion, although there were similarities in some physicochemical parameters of the samples, certain differences were observed among the honey types.

Keywords: blossom honey, honeydew honey, honey quality, phenolic content

INTRODUCTION

Honey is a unique natural food product with nutritional and bioactive properties produced by honeybees (*Apis mellifera* L.) actively collecting nectar from flowering plants. The quality of this fascinating product is highly affected by the bee species, geographical location, and available nectar source, as well as processing temperature, type of packaging, storage conditions, and climatic changes [Da Silva *et al.*, 2020; Piepiórka-Stepuk *et al.*, 2025; Shamsudin *et al.*, 2019; Smetanska *et al.*, 2021]. In addition to being a significant source of energy, honey is also used as an ingredient in various food products due to its sweetness, color, aroma, and viscosity properties, and as a medicine due to its biological activities coupled with health-promoting properties [Al-Kafaween *et al.*, 2023; Babbar *et al.*, 2022]. Honey is generally classified into two types; blossom honey and honeydew honey. Sunflower honey, chestnut honey, linden honey, and thyme

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honey can be given as examples of blossom honeys, on the other hand, in the recent past, honeydew honeys such as oak, cedar, and pine honeys have been highly sought after by consumers compared to blossom honeys due to their proven higher nutritional values as well as antibacterial and antioxidant activities [Pita-Calvo & Vázquez, 2017].

The Thrace region, which includes the northwestern part of Türkiye, provides a very favorable environment for beekeeping thanks to its plant diversity [Kekeçoğlu *et al.*, 2021]. At this point, the northwestern part of Türkiye serves as a primary agricultural center, contributing more than 50% of the country's total sunflower (*Helianthus annuus* L.) production [Unakıtan & Aydın, 2018], and with the use of bee colonies in terms of oilseed yield, it provides a high nectar intake and efficient honey production [Abbasi *et al*., 2021]. Sunflower honey has high pollen and glucose contents that make it unique in terms of nutritional value and brilliant sweetening properties [Manolova *et al.*, 2021]. Another unique blossom honey specific to this region is produced by the flowering of the plant called garland thorn or Christ's thorn (*Paliurus spina-christi* Miller), belonging to the *Rhamnaceae* family. In the region, the plant blooms between the end of May and July depending on weather conditions, and honeybees collect the nectar from these flowers. This specific honey varies in color from yellow to light brown, is slightly bitter, crystallizes very quickly [Kenjerić *et al.*, 2008], and is also known as spring honey in the region due to its harvest period. Unlike blossom honeys, oak honey belonging to the honeydew honeys, is a valuable type of honey that exhibits differences in terms of its characteristic features such as being very dark in color, rich in phenolic compounds, and possessing a distinctive aroma [Seijo *et al*., 2019]. Oak honey is a natural product made by bees from honeydew secretions released by the oak trees (*Quercus frainetto* Ten. and *Quercus robur*). This particular kind of honey is sourced from Thrace, northwest Anatolia, and the Istranca Mountains, a region distinguished by its abundant oak forests [Kolayli *et al.*, 2018]. Considering the production amounts, local sources report that the annual oak honey production varies between 100 and 300 tons and that of garland thorn honey varies between 150 and 200 tons [MinAF, 2025], attributable to the region's rich oak forests source and diverse flora. In addition, an average of 8,000 tons of sunflower honey is produced annually across Türkiye [Şahin, 2021].

Today, the growing global demand for honey, coupled with its high value, has made the quality and authenticity of products a critical concern. In this context, the systematic analyses of honey on a country- and region-specific basis is crucial for enriching the current literature and creating a comprehensive database of quality parameters for various honey varieties. In that framework, this study investigated the quality properties of oak (*Quercus* spp.), garland thorn (*Paliurus spina-christi* Mill.), and sunflower (*Helianthus annuus* L.) honeys, sourced from the rich plant diversity of the Thrace region. By conducting various analyses and comparing the results, it was aimed to establish a more comprehensive dataset for these specific honey types.

MATERIALS AND METHODS

Materials

A total of 15 honey samples were analyzed in the study, including 5 samples each of sunflower honey (SUN1–SUN5), garland thorn honey (GL1–GL5), and oak honey (OAK1–OAK5) harvested in 2023, and provided in 750-g glass jars from local beekeepers operating in various districts of Kirklareli province located in the northwestern part of Türkiye. The detailed locations of the places where the honey samples were taken are given in **Figure 1**. The samples were stored at room temperature

Figure 1. Detailed locations of the places where the honeys were obtained from local beekeepers operating in various districts of the Kirklareli province. GL, garland thorn honey; SUN, sunflower honey; OAK, oak honey. (The base map was created by the authors using Visme Inc. drawing software).

(22.5±2.5°C) in the dark until analyzed. All chemicals used were of analytical grade and purchased from Sigma-Aldrich, Burlington, MA, USA.

Physicochemical analyses of honey samples

The total soluble solids (TSS) of the honey samples were measured at ambient temperature using a digital refractometer (Hanna HI 96801, Hanna Instruments, Smithfield, RI, USA), and the results were expressed in °Brix (°Bx). The correction factor of 0.00023/°C was used to adjust the readings for a standard temperature of 20°C [AOAC, 2005].

The pH of honey was measured using a pH meter (Hanna HI 2211, Hanna Instruments). To this end, a honey solution was prepared by dissolving 10 g of the sample in 75 mL of distilled water.

Free acidity (FA), ash content, electrical conductivity (EC), optical rotation, hydroxymethylfurfural (HMF) content, diastase activity, and proline content of the honey samples were determined using the harmonized methods of the International Honey Commission (IHC) [Bogdanov *et al.*, 2009]. To determine FA, the aqueous solution of honey at a concentration of 10 g/75 mL of water was titrated with 0.1 M NaOH to reach pH 8.30, and the titrant volume was used to calculate the FA value expressed in meq/kg. The ash content was determined by gravimetry after ashing 2–5 g of honey samples in the muffle furnace (Wisd/DHFHP-03, Seul, Korea) at 600°C for 6 h. The results were expressed in g/100 g of honey. The EC (µS/cm) was measured using a conductivity meter (Jenco/3173 Cond, San Diego, CA, USA) in a 20% (*w/v*) honey solution in distilled water. A Krüss P3000 polarimeter (Krüss Scientific Instruments, Hamburg, Germany) was used to measure the optical rotation of purified aqueous honey solutions. Briefly, a sample of 12 g of honey was dissolved in distilled water, and Carrez I reagent was added, followed by Carrez II reagent in the amount of 10 mL each. The volume of the solution was adjusted to 100 mL, and next the solution was left for 24 h. The mixture was then filtered, and the filtrate was thermostated at 20°C. A polarimeter tube was filled with the thermostated solution, and the rotation angle (α) was read on the polarimeter.

The HMF content was determined spectrophotometrically using the Winkler method [Bogdanov *et al.*, 2009]. Briefly, 10 g of honey was initially diluted in 20 mL of distilled water. To this solution, 1 mL each of Carrez I and Carrez II reagents was incorporated, and the volume was brought up to 50 mL with distilled water. The solution was filtered, and the first 10 mL of the filtrate was discarded to ensure the clarity. For the measurement, two separate test tubes were prepared, each containing 2 mL of the filtered sample solution and 5.0 mL of a *p*-toluidine solution. The blank tube received 1 mL of distilled water, while the sample tube received 1 mL of a 0.5% barbituric acid solution. The absorbance was immediately measured against the blank at 550 nm using a spectrophotometer (UV/Vis-1800, Shimadzu, Kyoto, Japan). The results were subsequently calculated using the formula established for the analysis (Equation 1) and reported as mg/kg of honey:

$$\text{HMF content} = \frac{A \times 192}{W} \times 10 \quad (1)$$

where: A is the absorbance of the sample against the blank, 192 is the factor for dilution and extinction coefficient, and W is the weight of honey (g).

The diastase activity was determined according to the following procedure: honey (10 g) was dissolved in 15 mL of distilled water. To this solution, 5 mL of acetate buffer (pH 5.3) and 3 mL of an NaCl solution (2.9%, *w/v*) were added, and the volume was completed to 50 mL with distilled water. The samples were incubated in a water bath (Wisebath, WB-22, Daihan, Seul, Korea) at 40°C for 15 min. Then, 5 mL of a 2% (*w/v*) starch solution (pre-warmed to 40°C) were added to 10 mL of a sample solution, and a stopwatch was started immediately. The necessary dilution volume was first standardized by calibrating the starch solution. This volume of distilled water ensures that the starch-iodine blank produces a defined initial absorbance (~0.760 at 660 nm) for the test. At fixed time intervals (*e.g.*, every 5 min), a 0.5 mL aliquot was quickly withdrawn from the reaction mixture and mixed with 5 mL of a dilute iodine solution and the calibrated volume of distilled water. The analysis proceeded until the absorbance measured at 660 nm (UV/Vis-1800, Shimadzu) dropped below 0.235. The absorbance values were plotted against the corresponding reaction times in minutes to derive a linear regression equation. The time (t_x) required for the reaction mixture to reach an absorbance of 0.235 was calculated using this equation, and the diastase number was subsequently determined by the formula of $300/t_x$ [Bogdanov *et al.*, 2009].

To determine the proline content, 5-g portions of honey samples were first dissolved in 100 mL of distilled water. Then, 0.5 mL of the designated solution (deionized water for the blank, proline standard solution, and diluted honey sample) was added to three test tubes separately. This was followed by the sequential addition of 1 mL of formic acid and 1 mL of a 3% ninhydrin solution. The capped test tubes, after mixing the solution, were placed in a boiling water bath for 15 min and then transferred to a water bath at 70°C for 10 min. Subsequently, 5 mL of the 2-propanol/water mixture (1:1, *v/v*) were added to each test tube, after cooling to ambient temperature for 45 min. The absorbance was measured at 510 nm (UV/Vis-1800, Shimadzu), and proline content in mg/kg of honey was calculated according to Equation (2) [Bogdanov *et al.*, 2009]:

$$\text{Proline content} = \frac{A_S}{A_P} \times \frac{W_P}{W_S} \times 80 \quad (2)$$

where: A_S is the absorbance of the solution with sample, A_P is the absorbance of the solution with proline, W_P is the weight (mg) of proline used for the standard solution, W_S is the weight of honey (g), and 80 is the dilution factor.

Prior to viscosity analysis, honey samples (225 mL) were adjusted to 25°C. Then, their viscosity was measured using a B One-Plus viscometer (Lamy Rheology Instrument, Lyon, France) with an RV4 spindle at 50 rpm for 20 s. The results were recorded as Pa×s.

The fructose and glucose contents of honey samples were determined by the method based on the Association of Official Analytical Chemists (AOAC) standard [AOAC, 2005] using a high-performance liquid chromatography (HPLC) system (LC-2060C 3D, Shimadzu, Kyoto, Japan) with a refractometric detector (RID-20A). A Zorbax-carbohydrate column (4.6×150 mm, 5µm, Agilent, Santa Clara, CA, USA) was used for sugar separation, and the mobile phase consisting of acetonitrile and ultrapure water (83:17, *v/v*) was fed to the system at a flow rate of 1.00 mL/min. The column temperature was set at 40°C, and the injection volume was 10 µL. For the preparation of honey sample, 5 g of the sample were weighed and dissolved in 50 mL of ultrapure water in a volumetric flask and passed through a millipore 0.45 µm polyvinylidene fluoride (PVDF) filter before being injected. Then, the aliquots of the individual standards were injected, and their retention times were used to identify the peaks corresponding to glucose and fructose in the sample separation. Quantification was done according to an external standard method, and glucose and fructose contents were expressed in g/100 g of honey. Additionally, the fructose-to-glucose ratio (F/G) was calculated.

The color parameters of the samples were determined using a color measurement equipment (Chromameter CR-400 Konica Minolta, Tokyo, Japan). Prior to measurements, the instrument was calibrated with a white standard plate, and the *L** (brightness), *a** (redness-greenness), and *b** (yellowness-blueness) values of the samples were recorded. Then, the chroma (C) and hue angle (h°) of the samples were calculated using Equation (3) and Equation (4), respectively [McLellan *et al.*, 1995]:

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$h° = \text{arctg}\,(b^*/a^*) \quad (4)$$

Preparation of honey extracts

The extraction process was carried out by modifying the method specified by Uçar *et al.* [2023]. First, 4 g of honey were weighed and mixed with 16 mL of 80% (*v/v*) methanol. Then, the samples were kept in a shaking water bath at 25°C for 24 h. At the end of the period, the samples were filtered using a millipore 0.45 µm PVDF filter. The process was carried out in three repetitions for each honey, and each extract was transferred to amber glass bottles and stored at −18°C for further analyses.

Total phenolic content and total flavonoid content analyses

The total phenolic content (TPC) analysis was performed by the colorimetric method of Singleton *et al.* [1999] using the Folin-Ciocalteu reagent with certain modifications. The prepared extract (200 µL) was first pipetted into a test tube. This was followed by the addition of 1 mL of a 0.2 N Folin-Ciocalteu reagent and 1 mL of saturated sodium carbonate (75 g/L). After brief mixing, the reaction was allowed to proceed for 3 min. Then, the final volume was brought up to 10 mL with distilled water. Tubes were left to stand for 90 min in the dark, and absorbance readings were taken at 725 nm (UV/Vis-1800, Shimadzu) against the blank solution. In the meantime, the absorbance values of gallic acid (as a standard) solutions were also determined under the same conditions, and a linear calibration curve was obtained. The total phenolic content of the samples was calculated from the equation of the resulting standard curve, and the results were expressed as mg gallic acid equivalents (GAE)/kg honey sample taking into account the applied dilution factors.

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method according to Shraim *et al.* [2021] with some modifications. Initially, 4 mL of distilled water were added to the test tubes, followed by the pipetting of 1 mL of the extract and 1 mL of distilled water for the blank. Subsequently, 300 µL of 1 M $NaNO_2$ were added to the tubes, and mixtures were then vortexed and allowed to stand for 3 min. Following this, 300 µL of $AlCl_3$ (10%, *w/v*) were added, the mixtures were re-vortexed, and allowed to stand for an additional 3 min. Finally, 2 mL of 1 M NaOH and 2.4 mL of distilled water were added to all tubes to reach a final volume of 10 mL, and the solutions were vortexed. Upon completion of the procedure, the tubes were incubated in the dark for 40 min. At the end of the incubation period, the absorbance values were read against blank at 420 nm using a spectrophotometer (UV/Vis-1800, Shimadzu). The results were expressed as mg catechin equivalents (CE)/kg honey sample, using a catechin calibration curve and taking into account the applied dilution factors.

Antioxidant activity analyses

The method suggested by Nagai *et al.* [2004] was used with a slight modification for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity determination. The previously prepared sample extract (200 µL) was mixed with 200 µL of a 1.0 mM DPPH solution, and then 1,600 µL of methanol was added. For the control, 1,800 µL of pure methanol were added to 200 µL of a 1.0 mM DPPH solution, and the mixture was kept in the dark for 30 min. At the end of the period, the absorbance of the mixtures was read at 517 nm on a spectrophotometer (UV/Vis-1800, Shimadzu). The % inhibition values were calculated using the control and sample absorbances. A calibration curve consisting of Trolox concentrations against % inhibition values was also prepared, and the DPPH radical scavenging activity of the samples was expressed as µmol Trolox/100 g honey, taking into account the dilution rates.

The method of Re *et al.* [1999] was used to determine the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity of the honey samples. First, 7 mM ABTS and 2.45 mM of $K_2S_2O_8$ solutions were mixed at the ratio of 1:1 (*v/v*), and the mixture was left in the dark at ambient temperature for 16 h. Then, the solution with generated $ABTS^{\bullet+}$ was diluted with methanol to an absorbance of 0.700±0.01 at 734 nm to obtain the working solution. For the spectrophotometric assay, 100 µL of the prepared extracts or pure methanol as control and 2 mL of the $ABTS^{\bullet+}$ working solution were mixed, and the solutions were incubated at 30°C for 6 min. The absorbance value of the samples was measured against pure methanol

at 734 nm in a spectrophotometer (UV/Vis-1800, Shimadzu), and the results were recorded as µmol Trolox/100 g honey using a calibration curve consisting of Trolox concentrations *vs.* % inhibition values considering the dilution rates.

Statistical analysis

Windows-based SPSS 17.0.1 (SPSS Inc., Chicago, Illinois, USA) statistical package program was used for statistical evaluation of the data obtained from the analyses. One-way analysis of variance (ANOVA) was applied to the results, and differences between group means were explained using Duncan test at $p<0.05$ level. The bivariate Pearson correlation test (one-tailed) was used to evaluate whether there was a correlation between the results. All measurements were conducted in triplicate, and the results were given as mean values with standard deviation.

RESULTS AND DISCUSSION

Physicochemical parameters of honey

Selected physicochemical properties of the honey samples are provided in **Table 1**. The results showed that the TSS values ranged from 81.20°Bx to 85.07°Bx. The lowest TSS value was determined in OAK5 and the highest in GL samples (GL2, GL3, GL4) ($p<0.05$). The TSS value is linked to the sugar content of honey, which consists mainly of glucose and fructose [Shamsudin *et al.*, 2019]. Thus, the differences in this parameter can be attributed to the different amounts of sugars present in the honeys. Habib *et al.* [2014] found that the TSS values of 16 honeys obtained from arid and non-arid regions ranged between 79.0 and 84.1°Bx, and these values were similar to the current findings. The obtained results also resemble the values obtained for Brazilian honeys (80.89–83.57°Bx) [Da Silva *et al.*, 2017] and Italian honeys (80.0–83.8°Bx) [Conti, 2000]. However, lower values were reported for Indian honeys (76.2–80.4°Bx) by Saxena *et al.* [2010], which may be due to harvesting processes, beekeeping techniques, and also geographical and botanical origin of honey.

The pH values of the samples ranged between 3.41 and 5.39, with the highest pH value being found in the GL4 sample and the lowest pH value being found in the SUN1 sample ($p<0.05$) (**Table 1**). Briefly, GL and OAK samples showed similar pH values, while significantly lower values were observed in SUN samples ($p<0.05$). All honeys are acidic in nature due to the presence of organic acids, and their pH values generally change between 3.5 and 5.5. This acidic environment provided by organic acids prevents microbial growth and thus affects the stability and shelf life of honeys [Khataybeh *et al.*, 2023]. The FA values of the investigated honeys ranged from 17.88 to 49.15 meq/kg. In general, OAK honey samples showed significantly higher FA than the other two sample groups ($p<0.05$) except SUN1 sample, while GL honeys had the lowest values ($p<0.05$). The pH and FA indicate honey quality; however, pH does not directly reflect

Table 1. Physicochemical parameters of sunflower (SUN), garland thorn (GL), and oak (OAK) honeys.

Honey	TSS (°Bx)	pH	FA (meq/kg)	Ash (g/100 g)	EC (µS/cm)	Optical rotation	Viscosity (Pa×s)
SUN1	82.73 ± 0.42^{FGc}	3.41 ± 0.02^{Kd}	48.17 ± 0.56^{ABa}	0.17 ± 0.02^{FGb}	0.44 ± 0.01^{Hb}	-1.42 ± 0.01^{Ld}	18.31 ± 0.14^{Fd}
SUN2	83.47 ± 0.30^{DEb}	3.63 ± 0.01^{Jc}	32.29 ± 0.54^{Eb}	0.12 ± 0.01^{Gc}	0.39 ± 0.00^{Ic}	-1.42 ± 0.01^{Ld}	30.71 ± 0.37^{Ba}
SUN3	83.53 ± 0.11^{CDEb}	3.69 ± 0.02^{Ib}	30.32 ± 1.43^{Fc}	0.22 ± 0.00^{EFa}	0.45 ± 0.01^{Hb}	-1.30 ± 0.01^{Jb}	19.87 ± 0.45^{Ec}
SUN4	84.07 ± 0.11^{BCDa}	3.72 ± 0.03^{HIb}	27.88 ± 0.03^{Gd}	0.11 ± 0.02^{Gc}	0.44 ± 0.01^{Hb}	-1.40 ± 0.02^{Kc}	26.88 ± 0.50^{Db}
SUN5	84.00 ± 0.00^{BCDa}	3.77 ± 0.01^{Ha}	30.31 ± 0.44^{Fc}	0.18 ± 0.04^{FGab}	0.44 ± 0.01^{Hb}	-1.13 ± 0.00^{Ia}	17.10 ± 0.18^{Ge}
GL1	84.20 ± 0.20^{BCb}	4.65 ± 0.03^{Cc}	26.15 ± 0.44^{Ha}	0.38 ± 0.17^{Ca}	0.60 ± 0.00^{Gc}	-0.49 ± 0.00^{Gd}	32.45 ± 0.33^{Aa}
GL2	85.07 ± 0.11^{Aa}	4.74 ± 0.07^{Bb}	22.26 ± 0.51^{Ib}	0.29 ± 0.01^{DEa}	0.59 ± 0.00^{Gc}	-0.33 ± 0.01^{Eb}	29.14 ± 0.16^{Cb}
GL3	84.93 ± 0.11^{Aa}	4.45 ± 0.01^{Fd}	27.17 ± 0.48^{GHa}	0.34 ± 0.00^{CDa}	0.64 ± 0.01^{Fb}	-0.44 ± 0.01^{Fc}	19.05 ± 0.09^{EFc}
GL4	84.88 ± 0.16^{Aa}	5.39 ± 0.01^{Aa}	17.88 ± 0.94^{Jc}	0.32 ± 0.03^{CDa}	0.74 ± 0.04^{Ea}	-0.02 ± 0.01^{Ba}	16.94 ± 0.08^{Gd}
GL5	82.67 ± 0.30^{FGc}	4.32 ± 0.03^{Ge}	22.77 ± 0.83^{Ib}	0.29 ± 0.05^{DEa}	0.45 ± 0.10^{Hd}	-0.62 ± 0.01^{He}	9.10 ± 0.02^{Je}
OAK1	82.80 ± 0.35^{FGbc}	4.59 ± 0.03^{Da}	49.15 ± 0.42^{Aa}	0.61 ± 0.01^{Aa}	1.02 ± 0.00^{Bb}	0.17 ± 0.01^{BCb}	14.24 ± 0.45^{Hb}
OAK2	82.27 ± 0.90^{Gc}	4.47 ± 0.04^{EFbc}	46.92 ± 0.32^{Bb}	0.53 ± 0.02^{ABb}	0.92 ± 0.01^{Cc}	-0.44 ± 0.01^{Fd}	27.22 ± 1.96^{Da}
OAK3	83.27 ± 0.50^{EFab}	4.51 ± 0.01^{Eb}	41.34 ± 1.45^{Dd}	0.37 ± 0.02^{CDd}	0.83 ± 0.01^{Dd}	-0.14 ± 0.01^{Dc}	9.81 ± 0.20^{Ic}
OAK4	84.13 ± 0.11^{BCa}	4.56 ± 0.03^{Da}	44.81 ± 0.87^{Cc}	0.49 ± 0.00^{Bc}	1.09 ± 0.01^{Aa}	0.14 ± 0.01^{Aa}	8.75 ± 0.01^{Kc}
OAK5	81.20 ± 0.20^{Hd}	4.44 ± 0.01^{Fc}	45.50 ± 0.94^{Cbc}	0.48 ± 0.00^{Bc}	1.00 ± 0.01^{Bb}	0.01 ± 0.00^{Cb}	5.59 ± 0.16^{Ld}

Data represent mean values ± standard deviation. There is no significant difference between the results for all samples shown with the same superscript capital letter (A–K) in the same column ($p\geq0.05$). There is no significant difference between the results shown with the same superscript lowercase letter (a–e) in the samples of the same honey type in the column ($p\geq0.05$). TSS, total soluble solids; FA, free acidity; EC, electrical conductivity.

the acidity because various minerals and salts act as buffers [De-Melo *et al*., 2018] and explains the results found for the OAK samples in this study. The FA of honey depends on the presence of organic acids, especially gluconic acid, and their corresponding lactones, usually coming from nectar or bees' secretions [Truzzi *et al.*, 2014a]. Considering the limit value, the free acidity of commercial honey should not exceed 50 meq/kg [Bogdanov *et al.*, 1999], and the current results indicated that all honeys were of good quality and did not undergo undesirable fermentation. Choi & Nam [2020] reported that the free acidities of honeydew, chestnut, multifloral, and acacia honeys were 31.2, 23.1, 19.8, and 13.5 meq/kg, respectively. Similarly, in the study of Truzzi *et al.* [2014a], honeydew samples showed significantly higher FA (38.0 meq/kg) than sunflower (19.4 meq/kg) and acacia honeys (10.9 meq/kg).

The EC of the samples ranged between 0.39 and 1.09 µS/cm (**Table 1**). According to the Council of the European Union, honey with an EC value of >0.80 is classified as honeydew honey, and honey with an EC value of <0.80 is considered as a blossom or a blossom-honeydew honey mixture [Council EU, 2001]. In this regard, while the highest values were detected in OAK honey samples, interestingly, the ECs of GL samples were found to be significantly higher than SUN samples (p<0.05), even though both were blossom honeys. This can be assigned to the significantly higher ash content of the GL samples (p<0.05) since a higher ash content generally points to higher EC values. Similar findings were reported in the studies of Ouchemoukh *et al.* [2007] and Saxena *et al.* [2010], who found that the EC of honey samples increased with an increase in the ash content and determined higher EC values in honeydew honeys compared to blossom honeys.

Considering the optical rotation, the values changed between −1.42 and +0.17, depending on the type of honey (**Table 1**). SUN and GL samples showed negative values which was due to the dominance of fructose with negative optical rotation ($[\alpha]_D^{20}$=−92.4°) over the glucose ($[\alpha]_D^{20}$=+52.7°). Honey can rotate the polarization plane of polarized light, and the optical rotation is mainly related to the types and concentrations of the sugars present in honey [Pita-Calvo & Vázquez, 2017]. In general, blossom honeys are levorotatory, and honeydew, mixed, or adulterated honeys are dextrorotatory [Bogdanov *et al.*, 1999]. Manolova *et al.* [2021] reported that the 27 sunflower honey samples showed a negative optical rotation; but the mean value (−17.23) was lower than that of the samples examined in this study. Kenjerić *et al.* [2008] found the mean optical rotation value of 18 GL honeys as −6.71 with minimum and maximum values of −12.59 and −3.45, respectively. Despite the fact that OAK samples also had higher amounts of fructose compared to glucose (**Table 2**), OAK1, OAK4, and OAK5 samples showed positive values. This can be attributed to the presence of melezitose ($[\alpha]_D^{20}$=+88.2°) and erlose ($[\alpha]_D^{20}$=+121.8°) in OAK samples, which contribute to the dextrorotatory property [Pita-Calvo & Vázquez, 2017]. In this regard, Kolayli *et al.* [2018] reported that 8 out of 20 oak honeys showed positive optical rotation values.

The viscosity of honey samples ranged from 5.59 to 32.45 Pa×s (**Table 1**). There was a significant difference between the samples (p<0.05), and the highest and lowest viscosity values were found in GL1 and OAK5 samples, respectively. Viscosity is a critical parameter as it directly governs the rate and extent of crystallization. Hence, it is of great importance in the handling, processing, and storage of honey [Piepiórka-Stepuk *et al*., 2025]. Scripcă & Amariei [2021] found lower viscosity for sunflower honeys with values changing between 7.02 and 7.87 Pa×s. According to Uçurum *et al.* [2023], the lowest and highest viscosity for 373 pine honeydew honeys were found as 0.75 and 16.20 mPa×s, respectively. Moreover, the authors stated that the mean viscosity values showed significant differences depending on the harvest year and geographical origin. Therefore, the varying viscosity reported in the literature may be due to the maturity of honeys at collection, their chemical composition, and their geographical and botanical origins.

The sugar and HMF contents, diastase activity, and proline content of the honey samples are provided in **Table 2**. The glucose and fructose contents ranged from 26.94–39.58 g/100 g and 29.80–38.47 g/100 g, respectively. SUN honey samples had the highest glucose content (p<0.05), while GL and OAK honey samples had comparable amounts. Considering the fructose content of the samples, it was determined that the highest values were again detected in the SUN honey group (p<0.05), but some samples of GL honey group (GL1, GL2, and GL3) also had values close to the peak value. However, the samples belonging to the OAK honey group were categorized as honeys with the lowest fructose content (29.80–32.81 g/100 g). Fructose content of honey samples was higher than glucose content, except for two SUN honey samples (SUN2 and SUN3). This finding was consistent with observations made in previous studies [Baloš *et al.*, 2023; Kolayli *et al.*, 2018]. Rodríguez-Flores *et al.* [2019] reported that the average glucose content of oak honeys collected from the north-western region of Spain was 26.8 g/100 g, and fructose content was 35.7 g/100 g. In a different study, Malkoç *et al.* [2019] reported that the content of fructose in garland thorn honey was between 30.32 and 38.70 g/100 g, that of glucose was between 23.17 and 30.50 g/100 g, and the fructose-to-glucose ratio (F/G) reached 1.18–1.46. Regarding the same region where the honeys were collected in the current study, it was determined that the glucose content of the oak honeys examined by Ucurum *et al.* [2024] was in the range of 21.29–33.49 g/100 g, fructose content was 29.34–41.86 g/100 g, and F/G was between 1.23 and 1.85.

Considering the HMF content (**Table 2**), it varied between 0.77 and 4.51 mg/kg in all samples, with the lowest value determined in the OAK3 sample (0.77 mg/kg). The HMF content is a crucial indicator of honey quality, as reviewed by Shapla *et al.* [2018]. The authors noted that HMF is typically absent in fresh honey but accumulates significantly during processing, maturation, or when the honey is stored at high temperatures. Therefore, a high HMF content signals overheating or unfavorable storage conditions [Shapla *et al*., 2018]. In this regard, the Codex Alimentarius commission has determined the maximum limit for

Table 2. Glucose, fructose, hydroxymethylfurfural (HMF), and proline contents, and diastase activity of sunflower (SUN), garland thorn (GL), and oak (OAK) honeys.

Honey	Glucose (g/100 g)	Fructose (g/100 g)	F/G	HMF (mg/kg)	Diastase activity	Proline (mg/kg)
SUN1	31.93±2.54^{Cc}	37.15±3.16^{ABCa}	1.16±0.00^{ABCa}	4.22±0.09^{Aa}	18.34±1.65^{CDa}	790.4±24.5^{Ba}
SUN2	38.18±0.54^{Aa}	36.45±2.46^{ABCa}	0.95±0.01^{EFcd}	4.51±0.48^{Aa}	12.26±0.02^{Hc}	598.4±0.0^{Hc}
SUN3	39.58±1.13^{Aa}	35.56±1.52^{ABCDa}	0.90±0.01^{Fd}	1.92±0.28^{CDEb}	17.01±1.18^{DEab}	690.1±8.5^{Eb}
SUN4	37.91±1.12^{Aa}	38.47±1.10^{Aa}	1.01±0.00^{DEbc}	1.39±0.52^{DEb}	15.77±0.19^{EFb}	568.5±10.7^{Id}
SUN5	35.01±0.99^{Bb}	37.21±0.42^{ABCa}	1.06±0.02^{CDEb}	1.92±0.00^{CDEb}	18.72±1.44^{BCDa}	779.7±6.4^{Ba}
GL1	31.79±0.65^{Ca}	37.31±1.67^{ABa}	1.17±0.07^{ABCa}	1.30±0.62^{DEc}	14.00±0.12^{FGHd}	691.2±13.5^{Eb}
GL2	28.89±1.04^{DEFc}	35.82±0.33^{ABCa}	1.24±0.07^{Aa}	2.35±0.14^{BCDb}	20.43±0.55^{Bb}	715.5±7.2^{Da}
GL3	30.32±0.33^{CDEb}	36.69±1.58^{ABCa}	1.21±0.06^{ABa}	2.88±0.48^{BCab}	15.47±0.33^{EFGcd}	653.5±15.3^{Fc}
GL4	28.27±0.82^{Fc}	34.28±1.73^{CDEa}	1.24±0.07^{Aa}	2.74±0.24^{BCab}	23.95±1.46^{Aa}	640.9±0.9^{Fcd}
GL5	28.15±0.21^{Fc}	35.07±1.86^{BCDa}	1.23±0.11^{Aa}	3.41±0.34^{ABa}	16.88±0.96^{DEc}	624.7±9.9^{Gd}
OAK1	26.95±1.21^{Fc}	30.99±1.68^{FGab}	1.15±0.11^{ABCab}	4.22±0.01^{Aa}	17.29±1.46^{DEc}	787.2±12.8^{Bb}
OAK2	30.51±0.52^{CDa}	32.80±0.77^{DEFa}	1.07±0.00^{CDb}	2.02±1.92^{CDEbc}	24.58±0.21^{Aa}	737.0±3.4^{Cc}
OAK3	26.94±1.08^{Fc}	32.81±0.62^{DEFa}	1.22±0.05^{ABa}	0.77±0.01^{Ec}	20.33±0.27^{BCb}	884.3±2.6^{Aa}
OAK4	28.74±0.77^{DEFb}	29.80±0.65^{Gb}	1.04±0.00^{DEb}	2.30±1.34^{BCDabc}	22.71±2.74^{Aab}	698.7±4.3^{Ed}
OAK5	28.52±0.64^{EFbc}	31.72±0.41^{EFGa}	1.11±0.01^{BCDab}	2.83±0.05^{BCab}	13.53±0.75^{GHd}	742.1±3.4^{Cc}

Data represent mean values ± standard deviation. There is no significant difference between the results for all samples shown with the same superscript capital letter (A–H) in the same column ($p \geq 0.05$). There is no significant difference between the results shown with the same superscript lowercase letter (a–d) in the samples of the same honey type in the column ($p \geq 0.05$). F/G, fructose-to-glucose ratio.

HMF in honey at 40 mg/kg (80 mg/kg for honeys originating from tropical regions) [Codex Alimentarius, 2001]. Accordingly, it can be concluded that the HMF content of all honey samples investigated in the current study was well below this limit value, indicating the freshness of the samples and no heat treatment by producers. According to a study by Apaydın [2022] aimed to ascertain the quality characteristics of honey samples from the Thrace Region, the HMF content of sunflower honey samples ranged from 1.45 to 31.50 mg/kg, whereas those of garland thorn and oak honey samples were 0.16 to 0.30 mg/kg and 1.65 to 4.10 mg/kg, respectively.

The diastase activity of SUN, GL, and OAK honeys was in the range of 12.26–18.72, 14.00-23.95, and 13.53-24.58, respectively (**Table 2**). The values varied within the honey samples of the same group ($p<0.05$), and the highest values were found in GL4 (23.95), OAK2 (24.58), and OAK4 (22.71) samples, while the lowest value was detected in the SUN2 sample (12.26) ($p<0.05$). According to the International Honey Commission's Honey Quality and International Regulatory Standards, the number of diastase activity should be above 8 [Bogdanov, 2009]. Within this perspective, the current results showed additional support for the naturalness of honey samples. In other studies, the diastase activity range of sunflower honey was determined to be between 9.00 and 20.80 [Manolova *et al.*, 2021], while the average diastase activity of oak honey was determined to be 24.4 [Rodríguez-Flores *et al.*, 2015], which coincide with the current study values.

Considering the proline content, the acceptance threshold for genuine honey is set at a minimum of 180 mg/kg in regulatory testing facilities [Bogdanov *et al.*, 1999]. In this regard, the highest proline content was 884.3 mg/kg in the OAK3 sample, and the lowest value was 568.5 mg/kg in the SUN4 sample (**Table 2**). Manolova *et al.* [2021] found that the proline content of sunflower honey samples ranged from 218.5–679.5 mg/kg. Furthermore, it was reported that the proline content of sunflower honey was 647 mg/kg [Truzzi *et al.*, 2014b], garland thorn honey had 720.15 mg/kg [Malkoç *et al.*, 2019] and content in oak honey ranged from 434.09–1242.05 mg/kg [Kolayli *et al.*, 2018], which were comparable with the current values.

Color parameters of honey

The results of color analysis are provided in **Table 3**, and the general appearance of the samples in a transparent glass jar is presented in **Figure 2**. The L^* values of all honeys varied between 33.65 and 76.77, a^* values varied between −6.74 and 24.01, and b^* values varied between 22.90 and 59.92. Chroma and hue angle for honey color ranged from 28.34 to 60.17 and from 48.98 to 102.5, respectively. Accordingly, the highest L^* value

Table 3. Color parameters of sunflower (SUN), garland thorn (GL), and oak (OAK) honeys.

Honey	*L**	*a**	*b**	C	h°
SUN1	64.95±1.01^{CDEb}	−3.33±0.58^{Eb}	58.61±1.05^{Aab}	58.70±1.08^{ABab}	93.24±0.52^{BCc}
SUN2	54.46±2.69^{Fc}	−0.45±1.44^{Da}	48.91±3.00^{CDd}	48.92±3.02^{Dd}	90.45±1.61^{Cd}
SUN3	67.99±2.11^{BCDab}	−5.13±0.83^{FGHc}	57.18±0.81^{ABb}	57.42±0.85^{Bb}	95.12±0.79^{Bb}
SUN4	69.17±2.81^{Ba}	−6.74±1.09^{Hd}	53.39±1.63^{BCc}	53.82±1.51^{Cc}	97.22±1.33^{Ba}
SUN5	70.28±2.53^{Ba}	−5.37±1.19^{FGHcd}	59.92±0.91^{Aa}	60.17±0.83^{Aa}	95.13±1.19^{Bb}
GL1	68.53±1.79^{BCb}	−4.37±0.48^{EFGbc}	37.35±10.27^{Ec}	37.61±1.51^{Ec}	96.71±0.98^{Bb}
GL2	66.78±3.68^{BCDEbc}	−3.67±1.32^{EFb}	39.94±1.72^{Ec}	40.13±1.61^{Ec}	95.31±2.06^{Bb}
GL3	64.49±3.28^{DEbc}	0.49±2.16^{Da}	59.13±0.95^{Aa}	59.17±0.89^{ABa}	89.50±2.14^{Cc}
GL4	63.63±3.23^{Ec}	−0.50±1.66^{Da}	48.78±0.65^{CDc}	48.81±0.63^{Db}	90.60±1.97^{Cc}
GL5	76.77±3.47^{Aa}	−5.98±0.62^{GHc}	27.67±3.71^{FGd}	28.34±3.51^{Gd}	102.5±2.7^{Aa}
OAK1	37.76±3.41^{Gb}	24.01±0.92^{Aa}	28.53±5.65^{Fb}	38.66±3.20^{Eb}	49.38±6.56^{Eb}
OAK2	38.60±3.32^{Gb}	20.90±0.96^{Bb}	27.39±6.12^{FGb}	33.04±2.91^{Fcd}	51.94±6.97^{Eb}
OAK3	53.20±3.27^{Fa}	13.60±2.24^{Cc}	47.41±3.30^{Da}	49.39±2.76^{Da}	73.87±3.31^{Da}
OAK4	35.97±1.10^{GHbc}	22.64±0.42^{Aa}	26.07±1.68^{FGb}	34.55±1.00^{Fc}	48.98±2.35^{Eb}
OAK5	33.65±2.86^{Hc}	19.86±1.23^{Bb}	22.90±0.77^{Hb}	30.34±0.71^{Gd}	49.09±2.40^{Eb}

Data represent mean values ± standard deviation. There is no significant difference between the results for all samples shown with the same superscript capital letter (A–H) in the same column ($p \geq 0.05$). There is no significant difference between the results shown with the same superscript lowercase letter (a–d) in the samples of the same honey type in the column ($p \geq 0.05$). *L**, brightness; *a**, redness–greenness; *b**, yellowness–blueness; C, chroma; h°, hue angle.

Figure 2. Appearance of honeys examined within the scope of the study. SUN, sunflower honey; GL, garland thorn honey; OAK, oak honey.

was recorded in the GL group (76.77 for GL5), and the highest *a** values in the OAK group (24.01–22.64 for OAK1 and OAK4). The highest *b** value was found in the GL group (59.92 for SUN5), although the values determined for SUN1, SUN3, and GL3 did not differ significantly ($p \geq 0.05$) from that determined for SUN5. Several samples had high and statistically similar C value (SUN1, SUN5, and GL3). Among the honey samples of the GL group, there was one with the highest h° value (GL5, 102.5). Oroian & Ropciuc [2017] determined that the C and h° values of sunflower honey were higher than of other honeys, and results of the present study were similar. In a study examining the chemical contaminant and physicochemical properties of various honey samples, contrary to the current results, the *L**, *a**, *b**, C, and h° values of sunflower honey were 37.48, 2.22 12.32, 1.38, and 47.84, respectively [Scripcă & Amariei, 2021]. In another study, the *L** values of garland thorn honeys varied between 17.91 and 62.38, *a** values varied between 14.92 and 39.20, and *b** values varied between 24.07 and 92.02 [Malkoç *et al.*, 2019], and these values were similar to the *L** and *b** values of the GL honeys analyzed in our study, but *a** values were higher. In the study of Kolayli *et al.* [2018], the *L**, *a**, and *b** values of oak honey samples were in the range of 15.11–42.13, 27.94–37.38, and 25.73–70.20, respectively. In a study performed on oak honeys collected in Spain, the *L** values of the samples were between 19.59 and 54.57, *a** values between 7.82 and 30.29, *b** values between 8.39 and 27.52, C values between 11.87 and 41.27, and h° values between 42.92 and 73.80 [Jara-Palacios *et al.*, 2019]. These results obtained in different studies show that there may be differences in the color values of the samples due to the techniques applied in the color analysis method together with the type of honey and the region it was sourced from. In fact, the color of honey is primarily determined by its composition, which includes key colorful components like phenolics, pigments, vitamins, and minerals [Becerril-Sánchez *et al.*, 2021]. In this regard, the current results contributed that the OAK honeys were darker than blossom honey samples due to their higher phenolic contents (**Table 4**) and had quite different color characteristics, as seen in **Figure 2**. On the other hand, it was stated that the EC value was also related to the color of the honey; a darker color indicated higher EC [Vĩjan *et al.*, 2023], which was consistent with the current study results provided in **Table 1**, especially for the OAK honey samples.

Total phenolic content, total flavonoid content, and antioxidant activity of honey

The results regarding the phytochemical content and antioxidant activity of honey samples, including TPC, TFC, and DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity, are provided in **Table 4**. The TPC

Table 4. Phytochemical characteristics of sunflower (SUN), garland thorn (GL), and oak (OAK) honeys.

Honey	TPC (mg GAE/kg)	TFC (mg CE/kg)	DPPH (µmol Trolox/100 g)	ABTS (µmol Trolox/100 g)
SUN1	394.5 ± 4.1^{Mc}	189.1 ± 5.0^{KLbc}	51.3 ± 1.1^{Hc}	87.9 ± 7.2^{Eb}
SUN2	327.9 ± 3.6^{Oe}	152.1 ± 27.8^{Lc}	47.2 ± 0.1^{Id}	92.7 ± 8.0^{Eb}
SUN3	414.8 ± 5.5^{Lb}	221.2 ± 24.7^{Jb}	61.4 ± 0.6^{Fa}	112.1 ± 2.0^{Ea}
SUN4	374.3 ± 3.6^{Nd}	177.7 ± 26.4^{KLc}	50.3 ± 0.3^{HIc}	102.6 ± 3.0^{Ea}
SUN5	445.7 ± 3.6^{Ka}	294.4 ± 36.9^{Ia}	57.6 ± 0.9^{Gb}	106.1 ± 3.4^{Ea}
GL1	548.1 ± 14.4^{Hc}	373.0 ± 27.0^{FGbc}	52.3 ± 1.2^{He}	95.9 ± 4.8^{Eb}
GL2	527.9 ± 3.6^{Id}	340.2 ± 8.5^{GHcd}	57.1 ± 0.4^{Gd}	116.4 ± 24.2^{Eb}
GL3	623.1 ± 4.1^{Gb}	408.1 ± 19.9^{Fb}	86.4 ± 1.4^{Da}	165.1 ± 3.4^{Da}
GL4	670.7 ± 9.5^{Fa}	492.0 ± 47.5^{Ea}	79.0 ± 0.1^{Eb}	157.0 ± 6.0^{Da}
GL5	492.1 ± 7.1^{Je}	320.6 ± 9.2^{HId}	62.8 ± 1.1^{Fc}	113.5 ± 20.8^{Eb}
OAK1	$1,362.4\pm30.4^{Aa}$	945.0 ± 17.3^{Aa}	192.2 ± 0.7^{Aa}	712.2 ± 27.0^{Aa}
OAK2	$1,167.1\pm6.2^{Cc}$	781.9 ± 42.1^{Cb}	150.9 ± 0.5^{Bb}	606.9 ± 5.6^{Bb}
OAK3	$1,102.9\pm7.0^{Dd}$	664.6 ± 45.9^{Dc}	153.1 ± 6.0^{Bb}	545.1 ± 36.6^{Cc}
OAK4	$1,052.9\pm6.2^{Ee}$	642.6 ± 30.2^{Dc}	151.2 ± 3.6^{Bb}	559.2 ± 34.7^{Cbc}
OAK5	$1,231.4\pm3.6^{Bb}$	826.6 ± 24.9^{Bb}	144.7 ± 1.4^{Cc}	573.2 ± 8.6^{Cbc}

Data represent mean values ± standard deviation. There is no significant difference between the results for all samples shown with the same superscript capital letter (A–M) in the same column ($p \geq 0.05$). There is no significant difference between the results shown with the same superscript lowercase letter (a–e) in the samples of the same honey type in the column ($p \geq 0.05$). TPC, total phenolic content; TFC, total flavonoid content; DPPH, DPPH$^{\bullet}$ scavenging activity; ABTS, ABTS$^{\bullet+}$ scavenging activity; GAE, gallic acid equivalent; CE, catechin equivalent.

and TFC of all honeys ranged from 327.9 to 1362.4 mg GAE/kg honey and from 152.1 to 945.0 mg CE/kg honey, respectively. The OAK honey group exhibited the highest values for TPC and TFC, while the light-colored SUN honey group demonstrated the lowest values ($p<0.05$). In comparison with lighter varieties, darker honeys generally demonstrate enhanced levels of both total phenolic compounds and mineral micronutrients [Sant'ana *et al.*, 2014], and the current study results confirm exactly this fact. The TPC and TFC are abundant plant-derived metabolites recognized as primary determinants of overall antioxidant activity of samples [Muflihah *et al.*, 2021]. Within this respect, considering the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, the results determined for all honey samples varied between 47.2 and 192.2 µmol Trolox/100 g honey and between 87.9 and 712.2 µmol Trolox/100 g honey, respectively, with the highest values found again in the OAK honey samples, and only a small difference noted between the results within the samples in the other two honey groups. These results are parallel to those found in the literature and show that oak honey is a rich source of phenolic compounds. For instance, Can *et al.* [2015] determined that among the honey samples examined, oak honey had higher TPC (120.04 mg GAE/100 g) than other samples such as chestnut, heather, lavender, and acacia honey. Similarly, in a study performed by Tananaki *et al.* [2024] on various honey samples, it was concluded that oak honey (203.7 mg GAE/100 g) shared the top spot with Manuka honey (179.5 mg GAE/100 g) in terms of TPC, and also contained approximately three times higher TPC than citrus honey and twice as much as thyme honey. On the other hand, it can be concluded that the samples of the other two honey groups examined in the current research also had significant TPC being higher compared to various blossom honeys, such as acacia and clover [Can *et al.*, 2015]. In the meantime, the TFC values also showed similar trends with that of TPC values. In this context, according to the Pearson correlation performed between the TPC and TFC of all honey samples, a highly significant positive correlation ($r=0.989$, $p<0.01$, one-tailed) was determined, indicating that flavonoids are the major phenolics of the samples.

Considering $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, the strongest antioxidant properties of oak honey have also been recognized by other authors [Kolayli *et al.*, 2018; Smetanska *et al.*, 2021]. It was also reported that TPC and/or TFC highly correlated with the antioxidant capacity of honeys [Becerril-Sánchez *et al.*, 2021]. On the contrary, some studies showed that the antioxidant capacity might not only depend on phenolic compounds but also on organic acids, amino acids and proteins, and in this case, the correlation might be low or negative [Shamsudin *et al.*, 2019]. At this point, in the current study, very high positive correlations ($r_{TPC/DPPH}=0.980$, $r_{TFC/DPPH}=0.954$, $r_{TPC/ABTS}=0.979$, $r_{TFC/ABTS}=0.946$, $p<0.01$, one-tailed) were determined between the TPC, TFC and the results of DPPH and ABTS assays of all honey samples. In turn, as seen in **Table 4**, ABTS assay results were higher than DPPH assay results. The difference observed might be due to the different antioxidant compounds in each sample, which react uniquely with the radicals used. It is common knowledge that each method has its own chemistry and execution, and accordingly, it can be concluded from the current study results that the ABTS radical cations, which enable the determination of the antioxidant characteristics of both hydrophilic and lipophilic compounds [Munteanu & Apetrei, 2021], respond more sensitively to the phytochemicals in the extracts of honey samples. In the meantime, while this study provides a comprehensive quality assessment based on various physicochemical and biochemical parameters, the inclusion of advanced chromatographic analyses in such studies could further enhance the scientific content. Therefore, it is crucial to include such analyses in future studies, as they provide a more precise quantitative profile of specific phenolic acids and flavonoids, providing deeper insights into the unique characteristics of these regional honeys.

CONCLUSIONS

This study investigated the diverse quality characteristics of three honey varieties – oak, garland thorn, and sunflower – sourced from the Kirklareli province in Türkiye. All samples were acceptable according to international regulatory standards. However, there were significant differences in the general physicochemical properties and biochemical characteristics of the samples. In particular, HMF and proline contents and diastase activity reflected the naturalness of the honeys and showed no signs of heat treatment. Oak honey samples were significantly darker in color and had a much higher content of phenolic compounds and, consequently, superior antioxidant activity compared to the blossom honeys. This finding underscored the importance of honeydew honeys, like oak honey, as a rich source of health-promoting phytochemicals. Furthermore, the detailed analysis of garland thorn honey's characteristics, along with its unique chemical profile, was a key contribution to the very limited literature available on its properties. By providing a comparative analysis of these three honey varieties, this study not only enriches the academic literature on honey quality but also serves as a crucial data source for beekeepers, researchers, and regulatory bodies, addressing a notable gap in the existing data for garland thorn and oak honeys. However, to confirm these findings on a broader scale, it is of great importance to expand the location and include larger sample sizes in future studies.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests to disclose.

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Influence of Cellulase Pretreatment on the Quality Characteristics, Total Phenolic Content, and Antioxidant Capacity of Hydraulic Press-Extracted Grapeseed Oil

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Grape seeds, a by-product of winemaking, are a valuable source of oil, rich in essential fatty acids and bioactive compounds. This study evaluated the influence of cellulase pretreatment on the hydraulic press extraction of oil from *Vitis labrusca* × *Vitis vinifera* grape seeds. Grape seed powder (GSP) was treated with cellulase at enzyme-to-substrate ratios of 0.5 and 2 g/100 g for 4 h and 18 h. The extracted oils were analyzed for yield, fatty acid composition, total phenolic content, antioxidant capacity, and physicochemical properties. Enzymatic pretreatment significantly enhanced oil recovery compared to the untreated control, with extraction efficiency increasing from 58.21% to 95.05% and oil yield from 6.07 to 9.89 g/100 g GSP. The total phenolic content in oils obtained with cellulase pretreatment ranged from 148.7 to 279.2 mg GAE/kg and was higher compared to 127.5 mg GAE/kg determined in the control oil. Cellulase pretreatment also increased the antioxidant capacity of the oils, particularly in the oil treated at the enzyme-to-substrate ratio of 0.5 g/100 g for 18 h, which showed the highest values in the ABTS and DPPH assays (1.81 and 0.128 mmol TE/kg oil, respectively). The oils maintained a low moisture content (0.47–0.48 g/100 g), a moderate acidity index (2.20–2.89 mg KOH/g), acceptable peroxide values (7.24–11.96 meq O_2/kg), and stable density (0.92 g/mL). The fatty acid profile remained unchanged, dominated by linoleic acid (69.8% of total fatty acids). Overall, cellulase pretreatment effectively increased oil yield and improved the chemical and functional quality of grape seed oil without compromising its physicochemical stability.

Keywords: antiradical activity, enzymatic treatment, physicochemical characteristics, *Vitis labrusca* × *Vitis vinifera*

ABBREVIATIONS

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); CEL, cellulase; DM, dry matter; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FFA, free fatty acids; GAE, gallic acid equivalent; GSP, grape seed powder; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; RI, refractive index; SFAs, saturated fatty acids; SI, saponification index; TE, Trolox equivalent

INTRODUCTION

Grape (*Vitis vinifera* L.), extensively cultivated worldwide, serves various purposes. According to the International Organisation

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of Vine and Wine [OIV, 2024], about 57% of global grape production is used for winemaking, 36% for fresh consumption, and roughly 7% for dried grapes. Fresh grapes consist mainly of water (~83%), followed by carbohydrates (~66%), dietary fiber (~14%), proteins (~3%), and minimal lipids (~1%), reflecting their high energy density and nutritional significance [Kalili *et al.*, 2023]. In the wine-making process, 1 kg of crushed grapes yields ~0.2 kg of grape marc [Yang et al., 2021], composed mainly of skins (40%), seeds (30%), and stems (30%), and amounts to more than 9 million tons of agro-industrial waste generated annually on a global scale [Tociu et al., 2021]. The increasing volume of this biomass positions it among the most significant organic residues from the food industry, posing an environmental management challenge but also affording a valuable opportunity for circular bioeconomy initiatives [Palma *et al*., 2025].

In Peru, the Ica region alone produced approximately 430,000 tons of grapes in 2022, representing almost half (47%) of the national output [Rivera Chávez *et al*., 2025]. Within this context, the pisco industry, an emblematic segment of Peruvian agro-industry, achieves yields ranging from 1.8 to 30 t/ha and requires between 5.06 and 7.48 kg of grapes to produce 1 L of pisco with 42% (*v/v*) alcohol content. From 2017 to 2022, the country's average annual pisco production reached about 6.5 million liters [Palma *et al.*, 2025], which highlights the scale of grape processing and, consequently, the generation of large quantities of by-products such as grape pomace and seeds.

From a sustainability perspective, the valorization of winery waste has become a strategic priority, aligning with global goals for waste minimization and "zero waste" production [Tociu *et al*., 2021]. Notably, grape seeds represent a high-value fraction of the pomace due to their oil content (8–20%). This lipid fraction is characterized by a high proportion of polyunsaturated fatty acids (PUFAs), particularly linoleic acid (67–80% of total fatty acids), and monounsaturated fatty acids (MUFAs), especially oleic acid (15%–18% of total fatty acids) [Gitea *et al*., 2023]. Furthermore, grape seeds are composed of 5–8% of phenolic compounds, and approximately two-thirds of the total phenolic content is distributed within the seed coat, while between 20% and 60% exists in an insoluble form, covalently bound to the structural matrix of the cell wall [Saykova *et al.*, 2018]. Additionally, phenolic compounds, known for their plant-based antioxidant properties, have been linked to the antioxidant and enzyme inhibitory activities of oils, alongside tocopherols and polyunsaturated fatty acids [Özyurt *et al.*, 2021]. Depending on their structure, phenolic compounds can be simple phenols, phenolic acids, flavonoids, xanthones, stilbenes, and lignans [Oluwole *et al*., 2022].

Oil extraction from oleaginous seeds has increasingly benefited from the use of hydrolytic enzymes, which have proven to be effective in enhancing both yield and process efficiency. Among these, cellulases facilitate the release of oleosomes by weakening the plant cell wall through the breakdown of cellulose microfibrils [Vovk *et al*., 2023]. The cellulase complex, comprising endoglucanases, exoglucanases, and β-glucosidases, targets β-1,4-glycosidic bonds within cellulose chains to facilitate their breakdown [Łubek-Nguyen *et al*., 2022]. In this context, although the combined use of enzymes typically results in a more comprehensive disruption of cellular structures, the application of a single enzyme targets only one structural component, such as cellulose, which may lead to incomplete oleosome membrane rupture and require greater mechanical energy input [Vovk *et al*., 2023]. Some studies have opted for the individual application of enzymes such as cellulase in moringa seed oil extraction [Fernández *et al*., 2018]. Additionally, enzymes such as cellulase or pepsin have been applied individually in sesame oil extraction due to the incompatibility of their optimal activity conditions. This strategy reflects a technical adaptation aimed at balancing enzymatic efficiency with the operational feasibility of the process [Zaheri Abdehvand *et al*., 2025].

One approach to improving grapeseed oil extraction includes utilizing protease and/or cellulase enzymes combined with a screw extrusion press. Proteases break down proteins to facilitate oil release, whereas cellulases decompose cellulose in cell walls to boost efficiency [Sun *et al.*, 2022]. An enzymatic combination of pectinase and cellulase in a 3:1 (*w/w*) ratio was applied to by-products from the Chilean wine industry [Tobar *et al*., 2005]. In a different approach, pectin lyase, a specific commercial enzyme, was exclusively used for the pretreatment of grape seeds obtained from winemaking residues in Romania [Tociu *et al*., 2021]. In view of the above, this study aimed to evaluate the quality and antioxidant capacity of grapeseed oils obtained through hydraulic pressing with cellulase pretreatments, using different enzyme-to-substrate ratios and enzymatic treatment times. The oil properties were compared to those of grape seed oil pressed without enzyme support to show the effect of cellulase pretreatment.

MATERIALS AND METHODS

Plant material

The grape seeds (red and white Borgoña varieties, *Vitis labrusca* × *V. vinifera*), obtained as by-products from the winemaking process at Zapata Winery (Lunahuaná District, Cañete Province, Lima, Peru), were kindly provided in the framework of the research project "Use of residues of fruit processing: grapes (*V. vinifera*) and passion fruit (*Passiflora edulis*)" by the Universidad Nacional Agraria "La Molina" (Lima, Peru).

Extraction of grapeseed oil with and without enzymatic pretreatment

The procedure for obtaining grapeseed oil with and without enzymatic pretreatment is illustrated in **Figure 1**. The seeds were cleaned, washed, and dried to a moisture content of 9 g/100 g at 40°C using a tray dryer (local manufacture, Lima, Peru). The dried seeds were ground in a hand mill (Corona, Manizales, Colombia), and the resulting powder was sieved through a 35-mesh (0.5 mm) stainless steel sieve. Before oil extraction, the grapeseed powder was heated at 80°C for 20 min to inactivate endogenous enzymes, coagulate proteins, and promote oil droplet

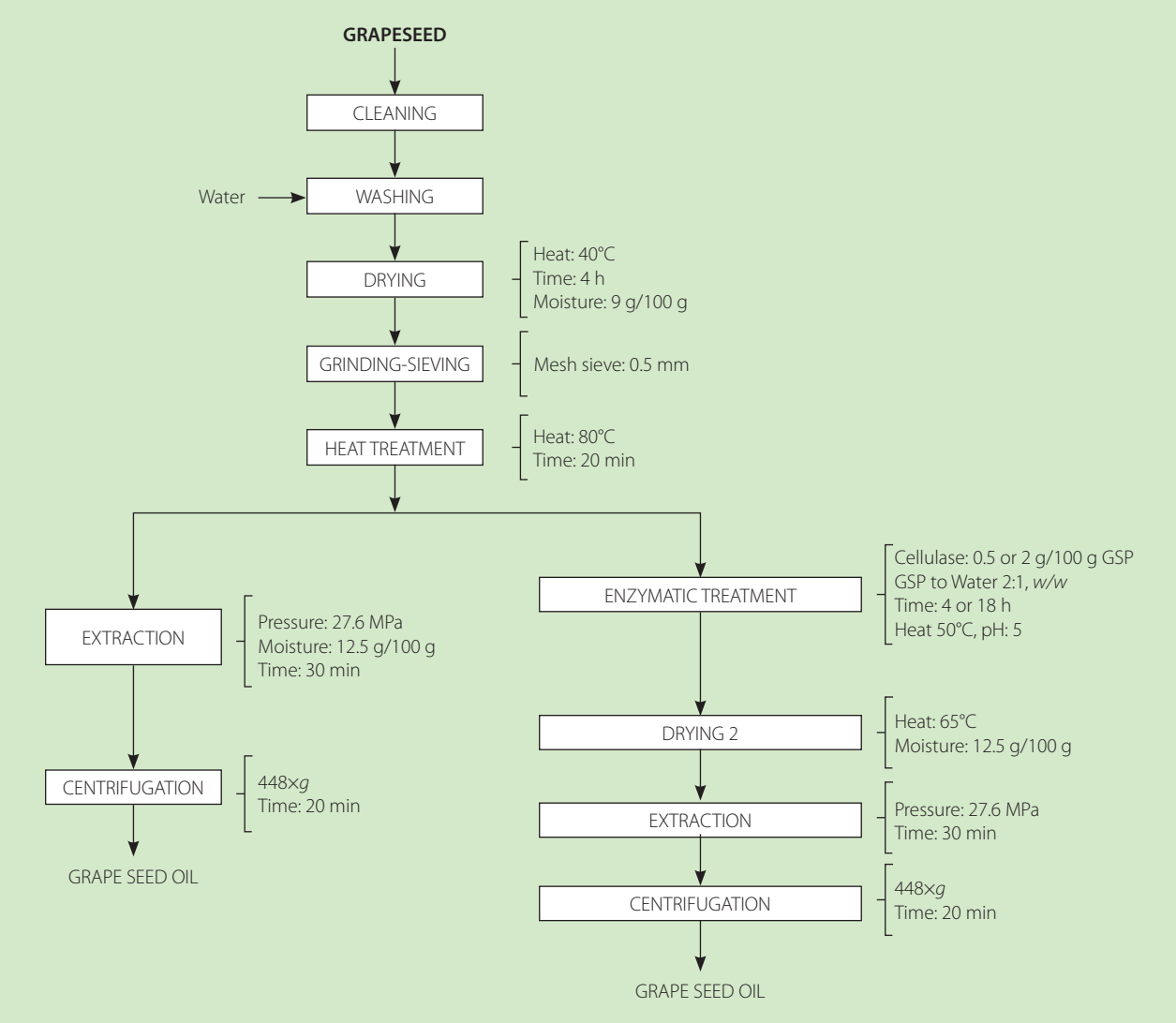

Figure 1. Process flow for obtaining grapeseed oil with and without cellulase treatment. GSP, grape seed powder.

coalescence [Santoso *et al.,* 2014]. Oil extraction was performed on 350 g of powder with a moisture content of 12.5 g/100 g using a manual hydraulic press (maximum capacity 48 MPa; local manufacture, Peru), applying a pressure of 27.6 MPa for 30 min at room temperature. The extracted oil was centrifuged at 448×*g* for 20 min using a centrifuge (Hettich EBA 206, Tuttlingen, Germany) to remove residual solids.

The grape seed powder (GSP) previously conditioned at 80°C for 20 min was used for enzymatic pretreatment. The thermally-treated powder was dispersed in distilled water (2:1, *w/w*) by vortexing and treated with cellulase (CEL) from *Aspergillus niger* (Sigma-Aldrich, Waltham, MA, USA; cellulase activity ≥0.3). The enzymatic hydrolysis was conducted in a climatic chamber (Memmert, Schwabach, Germany) at 50°C and pH 5. Two enzyme-to-substrate ratios (0.5 or 2 g CEL/100 g grape seed powder, GSP) and two incubation times (4 or 18 h) were evaluated resulting in four treatments: T1 (0.5 g CEL/100 g GSP, 4 h), T2 (0.5 g CEL/100 g GSP, 18 h), T3 (2 g CEL/100 g GSP, 4 h), and T4 (2 g CEL/100 g GSP, 18 h). After incubation treatment, the enzymatically-treated GSP was dried at 65°C to a moisture content of 12.5 g/100 g using a laboratory oven (Model 4-1411, Instru, Lima, Peru). This drying stage ensured the inactivation of residual enzymatic activity and stabilized the substrate for subsequent hydraulic press oil extraction.

Oil yield was calculated as the ratio between the mass of oil extracted (MOI) and the initial dry matter (DM) of seeds (DMS) using Equation (1), while extraction efficiency was determined relative to the total oil content (TOC) obtained by ether extraction using Equation (2):

$$\text{Oil yield (g/100 g DM)} = (\text{MOI/DMS}) \times 100 \quad (1)$$

$$\text{Extraction efficiency (\%, } w/w\text{)} = (\text{MOI/TOC}) \times 100 \quad (2)$$

Physical and chemical analyses

The oils obtained from the control (T0) and the four enzymatic pretreatments (T1–T4) were analyzed for moisture content (g/100 g oil) [AOAC, 2007; method 926.12], free fatty acid content (FFA, g oleic acid/100 g oil) [AOAC, 2007; method 940.28], and peroxide value (PV, meq O_2/kg oil) [AOAC, 2007; method

965.33]. The acidity index (AI) was derived from the free fatty acid measurement using the conversion AI=1.99 × FFA and expressed in mg KOH/g oil. Additionally, iodine index (g I_2/100 g oil), refractive index (RI, dimensionless), density (g/mL), saponification index (SI, mg KOH/g oil), unsaponifiable matter (g/kg oil), and *p*-anisidine value (dimensionless) were determined according to AOAC methods 920.159, 921.08, 920.212 [2007], 920.160 [1990], 933.08 [1998], and AOCS Cd 18-90 [1990], respectively.

The total phenolic content (TPC) was determined using the Singleton *et al.* [1999] colorimetric method with a Folin–Ciocalteu reagent with slight modifications according to Günç Ergönül & Aksoylu Özbek [2018]. Approximately 15 g of oil was dissolved in 20 mL of *n*-hexane and extracted three times with a MeOH/H_2O (40:60, *v/v*) mixture to a final extract volume of 50 mL. Then, 1 mL of the extract was mixed with 5 mL of Folin–Ciocalteu reagent (Merck, Darmstadt, Germany) and 20 mL of Na_2CO_3 solution (20%), and the volume was adjusted to 100 mL with distilled water. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 725 nm using a UV–Vis spectrophotometer (PerkinElmer, Shelton, CT, USA). The results were expressed as mg of gallic acid equivalents (GAE) *per* kg of oil (mg GAE/kg oil).

The antioxidant capacity of the oils was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, according to the original methods described by Brand-Williams *et al.* [1995] and Re *et al.* [1999], respectively, with modifications based on Samaniego Sánchez *et al.* [2007]. The methanolic extract obtained from the oil sample was used for the DPPH assay. Two grams of the oil were mixed with 1 mL of *n*-hexane and 2 mL of anhydrous methanol. The mixture was vortexed for 2 min and centrifuged at 700×*g* for 5 min to separate the lipid and methanolic phases. The methanolic phase was re-extracted once under the same conditions, and this extract was used for analysis. For the assay, 400 µL of the methanolic extract was mixed with 3 mL of a 0.1 mM $DPPH^{\bullet}$ solution. The mixture was shaken and kept in the dark at room temperature for 60 min until equilibrium was reached, and the absorbance was measured at 517 nm. For the ABTS assay, 100 µL of the oil sample (diluted 1:4, *v/v*, in *n*-hexane) was mixed with 2 mL of the $ABTS^{\bullet+}$ solution. The ABTS radical cation was generated from a 7 mM ABTS stock solution reacted with 140 mM potassium persulfate, and the mixture was allowed to stand in the dark for 12 h before use. After 30 min of reaction, the absorbance was measured at 734 nm. Results from both assays were expressed as mmol Trolox equivalents *per* kg of grape seed oil (mmol TE/kg oil).

Two oil samples were analyzed for their fatty acid composition following the method of Prévot & Mordret [1976], with minor modifications. One sample was the untreated control (T0), and the other was obtained under the selected enzyme pretreatment and reaction time (T2). Approximately 50 mg of the oil were converted to fatty acid methyl esters (FAMEs) and analyzed using a Perkin Elmer Autosystem XL gas chromatograph with a flame ionization detector (FID) (Perkin Elmer, Waltham, MA, USA) equipped with a Supelco SP-2560 capillary column (100 m × 0.25 mm, 0.20 µm; Sigma-Aldrich, St. Louis, MO, USA). The injector and detector temperatures were 250°C and 270°C, respectively, with helium as the carrier gas (1.0 mL/min) and an injection volume of 2 µL. Fatty acids were identified using a certified FAME standard mix (Supelco, 37 components), and results were expressed as relative percentages of total fatty acids.

Statistical analysis

The analysis results were presented as the mean and standard deviation (SD), with each analysis performed in triplicate. A one-way analysis of variance (ANOVA) was applied to identify statistically significant differences ($p<0.05$) between treatments, followed by Tukey's multiple comparison test for *post hoc* analysis. All statistical analyses were performed using Statgraphics Centurion version 16 (Stat Point Technologies, Inc., VA, USA). In addition, Pearson's correlation analysis and principal component analysis (PCA) were conducted using RStudio software (v. 2023.09.1, Boston, MA, USA) with the FactoMineR, factoextra, and corrplot packages to evaluate associations among the studied parameters and visualize sample groupings.

RESULTS AND DISCUSSION

Extraction of grapeseed oil

The total fat content of grape seeds, determined by ether extraction, was 10.4 g/100 g DM. The results of grapeseed oil extraction without (T0) and with enzymatic pretreatment (T1–T4) are shown in **Table 1**. The enzymatic pretreatment improved the oil yield and extraction efficiency compared to the traditional approach. Treatments T2, T3, and T4 were particularly effective, yielding 9.89, 9.83, and 9.59 g/100 g DM and achieving extraction efficiencies of 95.05%, 94.42%, and 92.11% (*w/w*), respectively. According to Carmona-Jiménez *et al.* [2022], oil yields from grape pomace were 3.81 to 6.71 g /100 g for white varieties and 8.89 to 9.73 g/100 g for red ones. For seeds, the cited authors obtained oil yields varying between 14.74 and 22.52 g/100 g for white and 12.46 and 15.89 g/100 g for red varieties, and these values were higher than those determined in our study for grape seeds of cv. *Vitis labrusca* × *V. vinifera*. Nevertheless, the positive effect of cellulase pretreatment was consistent with literature data; Tobar *et al.* [2005] obtained a 55% (*w/w*) extraction efficiency from grape seeds after using a 2% (*w/w*) solution of Ultrazym-Celluclast enzymes (enzyme-to-substrate ratio of 3:1) for 9 h, compared to a 30% (*w/w*) extraction rate of the enzyme-less control.

Researchers also noticed an increase in the oil extraction rate from other seeds after enzymatic treatments. For example, Anwar *et al.* [2013] reported values for flax (*Linum usitatissimum* L.) ranging from 35% to 38% (*w/w*) compared to 33% (*w/w*) of the control. Fernández *et al.* [2018] achieved a 28.4% (*w/w*) yield in moringa oil extraction through an enzymatic treatment, which involved 24 h hydrolysis using 2% (*w/w*) hemicellulase, surpassing the 25% (*w/w*) yield obtained in the control group. In contrast, Candan & Arslan [2021], who investigated the effects of an enzyme preparation with cellulolytic, peptinolytic, and hemicellulolytic activity on grapeseed oil extraction, found that the use of 1 g of enzyme *per* 100 g of seeds did not increase

Table 1. Oil yield, extraction rate, and physicochemical characteristics of oils obtained from untreated (T0) and cellulase-treated (T1–T4) grape seed powder (GSP) of cv. *Vitis labrusca* × *Vitis vinifera*.

Parameter	T0	T1	T2	T3	T4
Oil yield (g/100 g DM)	6.07±0.31^{c}	8.59±0.86^{b}	9.89±0.67^{a}	9.83±0.52^{a}	9.59±0.05^{ab}
Extraction efficiency (%, *w/w*)	58.21±0.29^{c}	82.63±0.79^{b}	95.05±0.61^{a}	94.42±0.47^{a}	92.11±0.04^{a}
Moisture content (g/100 g oil)	0.47±0.27^{a}	0.48±0.25^{a}	0.48±0.27^{a}	0.48±0.25^{a}	0.48±0.27^{a}
Free fatty acid content (g/100 g oil)	1.05±0.05^{d}	1.59±0.04^{a}	1.26±0.03^{c}	1.30±0.03^{c}	1.45±0.03^{b}
Acidity index (mg KOH/g oil)	2.20±0.02^{b}	2.89±0.60^{a}	2.50±0.03^{ab}	2.64±0.05^{ab}	2.78±0.04^{a}
Peroxide value (meq O_2/kg oil)	7.24±0.06^{d}	11.96±0.60^{a}	9.93±0.14^{c}	10.90±0.51^{b}	11.24±0.60^{ab}
Refractive index (25°C)	1.48±0.00^{a}	–	1.48±0.00^{a}	–	–
Iodine index (g I_2/100 g oil)	133.6±7.79^{a}	–	131.7±6.19^{a}	–	
Density (20°C, g/mL)	0.92±0.00^{a}	–	0.92±0.00^{a}	–	–
Saponification index (mg KOH/g oil)	187.04±0.00^{a}	–	152.20±4.65^{b}	–	–
Unsaponifiable matter (g/kg oil)	0.76±0.00^{a}	–	0.67±0.00^{b}	–	–
p-Anisidine value	1.83±0.58^{a}	–	1.71±0.29^{a}	–	–

Values are expressed as mean ± standard deviation (n=3). Different letters indicate significant differences (p<0.05) among samples within the row. DM, dry matter; T0, control (without enzymatic pretreatment); T1, 0.5 g cellulase (CEL)/100 g GSP for 4 h; T2, 0.5 g CEL/100 g GSP for 18 h; T3, 2 g CEL/100 g GSP for 4 h; T4, 2 g CEL/100 g GSP for 18 h.

the oil yield significantly. Consistent with these findings, Tociu *et al*. [2021] reported that comparable oil extraction yields could be obtained when using a single enzyme, instead of multi-enzyme cocktails, and that this strategy could substantially reduce processing costs while maintaining similar extraction performance.

Physicochemical characteristics

The physicochemical characteristics of treatments T0–T4 are presented in **Table 1**. The enzyme-treated oils had a higher FFA content (1.26–1.59 g /100 g oil) than the enzyme-less control (1.05 g /100 g oil). According to the Codex Alimentarius Commission's CXS210-1999 Standard for Named Vegetable Oils [Codex Alimentarius, 1999], the acceptable upper limits of FFA are 0.3% oleic acid for refined oils and up to 2% for most cold-pressed and virgin oils, indicating that the values obtained in this study remain within the acceptable quality range. Moreover, oils containing less than 5% FFA are generally classified as edible, since excessive free fatty acids accelerate oxidative degradation and rancidity during storage [Lamani *et al*., 2021]. A similar increase in FFA content following enzymatic pretreatment has been reported for flaxseed, apricot, and grapeseed oils, where the moisture introduced with the enzyme carrier solution promoted triglyceride hydrolysis [Candan & Arslan, 2021]. Furthermore, the activation of endogenous lipases during seed grinding was suggested to contribute to FFA formation [Candan & Arslan, 2021].

The grapeseed oils exhibited acidity indices ranging from 2.20 to 2.89 mg KOH/g oil, being slightly higher in the enzymatically-treated samples (T1–T4) than in the untreated control (T0) (**Table 1**). However, all values remained below the maximum limit (4.0 mg KOH/g oil) established by the Codex Alimentarius standard CXS 210-1999 [Codex Alimentarius, 1999]. These results were slightly higher than those reported by de Menezes *et al.* [2023] for grape seed oils from the Ives and Cabernet Sauvignon varieties extracted by hydraulic press or Soxhlet, which ranged from 1.87 to 2.09 mg KOH/g oil.

The peroxide value of the oils ranged from 7.24 mEq O_2/kg in T0 to 11.96 mEq O_2/kg in T1, with intermediate values observed in T2–T4 (9.93–11.24 mEq O_2/kg) (**Table 1**). This increase suggests a moderate intensification of oxidative reactions following enzymatic pretreatment, possibly linked to the release of prooxidant phenolics or trace metal ions during cell wall disruption [de Menezes *et al*., 2023]. Comparable behavior was observed by Candan & Arslan [2021], who reported that increased moisture during enzymatic treatments facilitated primary oxidation reactions and resulted in higher peroxide values, particularly in grape seed and sesame oils. Notably, the peroxide value of T2 (9.93 mEq O_2/kg) was comparable to that reported by de Menezes *et al.* [2023] for the Ives variety (9.98 mEq O_2/kg), indicating similar oxidative behavior in hydraulically pressed oils. Despite this increase, all values remained below the Codex Alimentarius [1999] limit for virgin oils (15 mEq O_2/kg), confirming that enzymatic pretreatment did not compromise oxidative stability. The slight rise in peroxide levels, which reflects the accumulation of primary oxidation products, could be associated with triglyceride hydrolysis induced by cellulase, releasing free fatty acids that accelerate lipid peroxidation, particularly in oils

rich in polyunsaturated fatty acids, which are inherently more susceptible to oxidative degradation [Ravagli *et al*., 2025].

The moisture content of the oils remained consistently low (0.47–0.48 g/100 g oil) (**Table 1**), a desirable characteristic that limits hydrolytic reactions and minimizes oxidative susceptibility during storage. Similar findings were reported by de Menezes *et al.* [2023], who obtained oils with <1% moisture after drying grape seeds at 40°C using pressing, Soxhlet, and ultrasound extraction methods. According to the International Olive Council [IOC, 2019], the recommended moisture content should be below 0.20% for virgin olive oil and 1.50% for crude pomace oil, confirming that the values observed in this study fall within acceptable quality limits.

In a subsequent stage, only T0 and T2 were analyzed for complementary quality parameters, including iodine value, saponification index (SI), unsaponifiable matter, *p*-anisidine value, refractive index (RI), and density. The iodine value measures the degree of unsaturation in oils and fats, providing an indirect indicator of oxidative susceptibility, with higher values indicating greater unsaturation [Hagos *et al.*, 2023]. In this study, T0 and T2 showed iodine values of 133.6 and 131.7 I_2/100 g oil (**Table 1**), respectively, both within the range established by the Codex Alimentarius [1999] for grapeseed oil (128–150 I_2/100 g).

The saponification index (SI) provides important information about the molecular characteristics of oils. When the SI of an oil is below 190 mg KOH/g, it indicates the presence of high-molecular-weight triglycerides, which are typically esterified with polyunsaturated fatty acids such as linoleic and linolenic acids [Lamani *et al*., 2021]. The SIs for T0 and T2 were slightly below the Codex range for grape seed oil (188–194 mg KOH/g oil), whereas the unsaponifiable fraction remained below 20 g/kg, in agreement with the standard [Codex Alimentarius, 1999]. These findings align with those reported by de Menezes *et al.* [2023] for grapeseed oils and by Fernández *et al.* [2018] for moringa oils obtained with hemicellulase pretreatment.

The *p*-anisidine value is a reliable indicator of secondary oxidation in vegetable oils, as it quantifies non-volatile aldehydes resulting from the decomposition of primary oxidation products. Specifically, it measures reactive aldehydic compounds, such as 2-alkenals and 2,4-dienals, which are produced during the advanced stages of lipid oxidation. Elevated *p*-anisidine values indicate pronounced oxidative degradation and a consequent decline in oil quality. For edible oils, values should remain below 10, whereas those under 2 are considered indicative of high-quality oils [Gharby *et al*., 2025]. In this study, the *p*-anisidine values were 1.83 for T0 and 1.71 for T2, showing no significant differences ($p \geq 0.05$). This behavior agrees with findings reported by Candan & Arslan [2021], who observed that enzymatic treatment did not significantly affect *p*-anisidine values in grape seeds and flaxseed oils, despite variations in primary oxidation indices. Fernández *et al.* [2018] reported comparable results for oils from enzymatically-treated moringa seeds, supporting the observation that this process does not substantially influence this oxidation index.

The refractive index of T0 and T2 was 1.48 at 25°C, slightly higher but consistent with the mean RI of conventional edible oils such as corn, soybean, olive, and sunflower (~1.47) [Gunstone, 2008]. Regarding density, the oils with and without enzymatic pretreatment (T1–T4) exhibited values within the range established for seed oils (0.920–0.926 g/mL at 20°C) according to the Codex Alimentarius [1999]. These results indicate that the enzymatic treatment did not adversely affect the physicochemical integrity or overall quality of the oils.

Phenolic content and antioxidant capacity

Phenolic compounds, owing to their hydrophilic nature, exhibit low solubility in the oil phase, and only a small fraction is transferred from the solid matrix during oil extraction [Saykova *et al*., 2018]. Consequently, most phenolics remain in the seed cake after pressing [Vidal et al., 2022]. Therefore, processing strategies that promote cell wall disruption and facilitate the migration of phenolic compounds into the oil phase, such as enzyme-assisted pretreatments, can substantially enhance their recovery [Brienza *et al*., 2025]. Enzymatic treatments applied during oil extraction enable the hydrolysis of cell wall polysaccharides, releasing bound phenolics and enhancing the bioactive profile of the resulting oil [Zaheri Abdehvand *et al*., 2025].

Grapeseed oils pretreated with cellulase exhibited significantly higher total phenolic content (TPC), showing an increase of 14% to 119% compared to the control (**Table 2**). The TPC of the oils ranged from 127.5 to 279.2 mg GAE/kg, values comparable to those reported by Rombaut *et al*. [2015] for grape seeds from Distillerie Jean Goyard in France (48–153 mg GAE/kg) and by Konuskan *et al.* [2019] for several varieties, including Sauvignon Blanc (102.55 mg GAE/kg), Syrah (148.21 mg GAE/kg), Merlot (151.51 mg GAE/kg), Sangiovese (177.31 mg GAE/kg), and Cabernet Sauvignon (182.41 mg GAE/kg). Kapcsándi *et al.* [2021] reported values around 240 mg GAE/kg for Pinot Noir, whereas Mollica *et al.* [2021] documented markedly higher TPC

Table 2. Total phenolic content and antioxidant capacity in ABTS and DPPH assays of oils obtained from untreated (T0) and cellulase-treated (T1–T4) grape seed powder (GSP) of cv. *Vitis labrusca* × *Vitis vinifera*.

Oil	Total phenolic content (mg GAE/kg oil)	Antioxidant capacity (mmol TE/kg oil)	
		ABTS assay	DPPH assay
T0	127.5±6.33^{e}	1.60±0.13^{e}	0.053±0.003^{e}
T1	148.5±6.33^{d}	1.71±0.02^{d}	0.094±0.001^{d}
T2	279.2±5.23^{a}	1.81±0.04^{a}	0.128±0.002^{a}
T3	253.2±7.68^{b}	1.79±0.04^{b}	0.112±0.001^{b}
T4	179.5±6.65^{c}	1.72±0.02^{c}	0.110±0.003^{c}

Values are expressed as mean ± standard deviation (n=3). Different letters indicate significant differences (p<0.05) among samples within the column. GAE, gallic acid equivalent; TE, Trolox equivalent; T0, control (without enzymatic pretreatment); T1, 0.5 g cellulase (CEL)/100 g GSP, 4 h; T2, 0.5 g CEL/100 g GSP, 18 h; T3, 2 g CEL/100 g GSP, 4 h; T4, 2 g CEL/100 g GSP, 18 h.

(12,030 mg GAE/kg) in Montepulciano grape seeds. These variations can be attributed to intrinsic and extrinsic factors, including grape cultivar, environmental conditions, agronomic practices, and extraction parameters, as extensively discussed by Vidal *et al.* [2022], who emphasized the influence of post-harvest conditions and enzymatic pretreatment on phenolic transfer efficiency.

Comparable patterns have been reported in other oilseeds subjected to enzymatic treatments. Teixeira *et al.* [2013] found significantly higher extraction rates of phenolic compounds (51%) and carotenes (153%) using tannase and cellulase plus pectinase, respectively, in palm oil. Similarly, Anwar *et al.* [2013] reported that oil pressed from flaxseeds treated with Viscozyme L, Kemzyme, and Feedzyme (2% *w/w*; 6 h; 40°C; 50% moisture) contained more total phenolics (8.61–10.5 mg GAE/100 g) than the oil from the control seeds (6.21 mg GAE/100 g). Özyurt *et al.* [2021] found that enzymatic extraction with Alcalase 2.4 L increased the TPC (3.30 mg GAE/kg) compared to cold pressing (2.97 mg GAE/kg) in oil of tomato seeds, although with a lower oil yield (9.66% *vs*. 12.80%, *w/w*), emphasizing the enzyme's role in enhancing bioactive compound release.

The TPC values obtained in this study were also higher than those achieved through other extraction methods applied to grapeseed oil. Ubaid & Saini [2024] evaluated several extraction techniques and reported 117.54 mg GAE/kg (cold-pressed), 109.77 mg GAE/kg (hexane extraction), 127.18 mg GAE/kg (supercritical CO_2 extraction), 132.01 mg GAE/kg (supercritical CO_2 + 10% ethanol extraction), and 123.53 mg GAE/kg (*p*-cymene-assisted extraction). The higher values obtained in the present study indicate that cellulase pretreatment, conducted under mild and solvent-free conditions, represents an effective green strategy for enhancing phenolic recovery in vegetable oils.

The effectiveness of enzyme-assisted pretreatments largely depends on operational parameters such as enzyme concentration, incubation time, temperature, and pH, which determine the extent of cell wall hydrolysis and the subsequent release of phenolics [Tociu *et al*., 2021]. The results of this study revealed that the interaction between cellulase-to-substrate ratio and hydrolysis duration was decisive. Treatment T2 (0.5 g CEL/100 g GSP; 18 h; 50°C; pH 5) yielded the highest TPC (279.2 mg GAE/kg oil), indicating that a moderate enzyme-to-substrate ratio and a controlled hydrolysis period favor efficient cell wall disintegration and gradual phenolic diffusion. Conversely, increasing the enzyme load to 2 g CEL/100 g GSP (T3–T4) did not yield further improvement, possibly due to enzyme saturation or diffusional limitations within the solid matrix. Numerous studies have corroborated the influence of enzyme concentration and incubation time on extraction efficiency. In grape seeds, Sun *et al.* [2022] demonstrated that enzyme-to-substrate ratio was the most significant factor, while hydrolysis time (2–4 h) had no notable effect within that range. Conversely, Zaheri Abdehvand *et al.* [2025] found that the application of cellulase (3%, *w/v*, pH 7) or pepsin (2%, *w/v*, pH 2) for 6 h at 40°C in sesame seeds significantly enhanced both TPC and antioxidant capacity, nonetheless, cellulase (oil yield: 25.85%) exhibited superior efficiency in extracting oil, pigments, and proteins, whereas pepsin (oil yield: 21.83%) was more effective in promoting carbohydrate release and modestly enhancing antioxidant capacity. Both enzymatic methods demonstrated sustainability and efficiency, positioning them as promising alternatives to conventional solvent-based extraction techniques. Liu *et al.* [2016] reported that hydrolysis periods up to 18 h under mild conditions improved wall disintegration and phenolic diffusion, while Tociu *et al.* [2021] identified an optimal duration of 24 h using pectin lyase in grape seeds.

Overall, the results demonstrate that cellulase pretreatment for 18 h enables effective hydrolytic action on the grape seed matrix, facilitating the migration of phenolics into the oil phase under environmentally sustainable conditions. Considering that phenolic compounds contribute significantly to the antioxidant potential of vegetable oils, their antioxidant capacity was subsequently evaluated.

The antioxidant capacity of the five grape seed oils is presented in **Table 2**. Oils from enzymatically-treated seed powders demonstrated significantly higher antioxidant capacities than the control in both assays. T2 reached the highest values, with 1.81 mmol TE/kg (ABTS assay) and 0.128 mmol TE/kg (DPPH assay). It should be noted that antiradical activity against $ABTS^{\bullet+}$ was evaluated directly in the oil (lipophilic matrix), whereas $DPPH^{\bullet}$ scavenging activity was assessed after extraction with a polar solvent; consequently, the ABTS assay response likely reflects the contribution of lipophilic antioxidants (*e.g.*, tocopherols and carotenoids), explaining the higher apparent capacity in this assay compared with DPPH, in line with prior observations in oil systems [Anwar *et al*., 2013].

Comparable findings have been reported in other vegetable oils. Zaheri Abdehvand *et al.* [2025] observed a significant increase in antioxidant capacity of sesame oils obtained by cellulase and pepsin-assisted extraction relative to the control; notably, the cellulase-treated oil showed the highest DPPH radical inhibition (~95%), followed by pepsin (~93%), while the control reached ~85%, underscoring the positive effect of enzyme-assisted extraction on antioxidant performance. This enhancement has been attributed to the enzymatic release of bioactive constituents, including phenolics, tocopherols, carotenoids, and chlorophylls, which collectively contribute to greater oxidative stability [Latif *et al*., 2008]. Additionally, the enzymatic hydrolysis of phenolic compounds and the reduction of polyphenol–polysaccharide complexation further enhance the antioxidant potential of the extracted oil [Latif *et al*., 2008; Teixeira *et al*., 2013].

Evidence from previous studies indicates that enzyme type, its concentration, and incubation time significantly affected not only the oil extraction yield, but also phenolic recovery and antioxidant capacity of the oils [Anwar *et al*., 2013]. The three enzymes (Viscozyme L, Feedzyme, and Kemzyme) employed for the enzymatic pretreatment of flaxseed oil extraction yielded different extraction rates (38%, 35.2%, and 36.5%, respectively). However, despite the relatively high oil yields, the use of Kemzyme resulted in the lowest total phenolic content and antioxidant capacity,

suggesting that higher extraction efficiency does not necessarily correspond to improved bioactive potential [Anwar *et al*., 2013]. Moreover, enzymatic pretreatment with pectinases, proteases, and cellulases significantly improved the TPC in pomegranate seed oil, thereby enhancing its antioxidant capacity [Kaseke *et al*., 2021]. The results demonstrate that the enzymatic treatment enhances the release of antioxidant compounds, leading to improved antioxidant potential and probably oxidative stability of grapeseed oil.

Fatty acid composition

The fatty acid composition of the grapeseed oil extracted without and with cellulase pretreatment at 0.5 g CEL/100 g GSP for 18 h (T2) revealed a consistent profile (**Table 3**). Both oils were rich in PUFAs (>70% of total fatty acids), whereas saturated fatty acids (SFAs) accounted for <12% of total fatty acids. The major fatty acids identified, in a descending order of content, were linoleic, oleic, palmitic, stearic, and linolenic acids. Linoleic acid was the predominant fatty acid, accounting for 69.8% (T0) and 69.8% (T2) of total fatty acids, confirming the high PUFA character typically attributed to grapeseed oil. Notably, the stearic acid content increased from 4.25% in the untreated oil (T0) to 4.63% in T2. This result aligned with findings of Gitea *et al.* [2023], who reported that stearic acid levels in grapeseed oil obtained by different methods ranged from 2.75% to 5.32% of total fatty acids. Similarly, Kapcsándi *et al*. [2021] determined a notable content of stearic acid in grape seed oils, ranging from 3.42% to 9.93% of total fatty acids, further confirming the variability depending on plant cultivar. Candan & Arslan [2021] also observed a slight increase in stearic acid content (from 4.32% to 4.33%) when applying a cellulolytic–pectinolytic enzyme complex and attributed this behavior to moisture-mediated hydrolysis and native lipase activity rather than to enzymatic modification of fatty acid biosynthesis. This interpretation is consistent with findings from a study by Zaheri Abdehvand *et al.* [2025], who confirmed that cellulase primarily enhanced oil release by disrupting cell wall polysaccharides without altering triglyceride structure or fatty acid composition.

The fatty acid profile of the grape seed oil was consistent with compositions reported for grapeseed oils extracted using different methods and cultivars. Carmona-Jiménez *et al.* [2022] reported grapeseed oil compositions from five grape varieties containing linoleic acid (66.0%–69.0% of total fatty acids), oleic acid (17.0%–20.0% of total fatty acids), palmitic acid (8.20%––9.40% of total fatty acids), and stearic acid (3.70%–5.20% of total fatty acids). Odabaşioğlu [2023] analyzed grape seed oils from 16 grape genotypes obtained by Soxhlet extraction, identifying 13 to 15 fatty acids. The linoleic acid content ranged from 56.13% to 69.36% of total fatty acids, and the oleic acid content ranged from 15.99% to 30.97% of total fatty acids. Similarly, Di Stefano *et al.* [2021] reported linoleic acid levels of 67.2–71.1% and stearic acid levels of 3.26–4.33% in cold-pressed grapeseed oils. Likewise, de Menezes *et al.* [2023] observed linoleic acid contents of 65.25–68.76% of total fatty acids in oils obtained *via* pressing, Soxhlet, and ultrasounds. Taken together, these results confirm that the oils obtained in this study fall within the compositional variability expected for grape seed oil and meet the ranges established by the Codex Alimentarius [1999] (C16:0: 5.5–11.0%; C18:0: 3.0–6.5%; C18:1: 12.0–28%; C18:2: 58.0–78%; C18:3: <1%).

Finally, the preservation of high PUFA levels (70.3% in both T0 and T2) alongside stable U/S ratios (unsaturated/saturated fatty acids) supports the observation of Candan & Arslan [2021] that enzyme pretreatment may increase oil recovery efficiency without compromising lipid nutritional quality. In this case, the use of a single cellulase enzyme allowed achieving results comparable to those obtained with multi-enzyme complexes, indicating that more complex enzymatic systems are not necessary to maintain or enhance the fatty acid composition of grapeseed oil.

Table 3. Fatty acid composition (% total fatty acids) of oils obtained from untreated (T0) and cellulase (CEL)-treated grape seed powder (GSP) (T2, 0.5 g CEL/100 g GSP, 18 h) of cv. *Vitis labrusca* × *Vitis vinifera*.

Fatty acid	T0	T2
Palmitic acid (C16:0)	7.98±0.16^{a}	7.72±0.15^{a}
Stearic acid (C18:0)	4.25±0.03^{b}	4.63±0.15^{a}
Oleic acid (C18:1*n*9)	17.5±0.07^{a}	17.4±0.06^{a}
Linoleic acid (C18:2*n*6)	69.8±0.05^{a}	69.8±0.04^{a}
Linolenic acid (C18:3*n*3)	0.48±0.01^{a}	0.50±0.01^{a}
Total SFAs	11.4±0.13^{a}	11.0±0.12^{b}
Total MUFAs	18.4±0.08^{a}	18.0±0.06^{b}
Total PUFAs	70.3±0.06^{a}	70.3±0.05^{a}

Values are expressed as mean ± standard deviation (n=3). Different letters indicate significant differences (p<0.05) among samples within the row. SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

Relationship between oil characteristics

Pearson's correlation analysis revealed strong associations among oil extraction yield, oil quality parameters, total phenolic content, and antioxidant capacity (**Table 4**). Yield exhibited very strong positive correlations with antioxidant capacity in DPPH and ABTS assays (r=0.908 and r=0.852, respectively) and a strong correlation with TPC (r=0.756) and peroxide value (r=0.752). These results indicate that greater extraction efficiency was accompanied by higher recovery of phenolic compounds and antioxidant capacity, likely due to the enhanced release of bound phenolics and bioactive molecules facilitated by enzymatic cell wall degradation [Kaseke *et al*., 2021]. Results of DPPH and ABTS assays showed a very strong positive correlation (r=0.937, p<0.001), confirming that both assays provide consistent assessments of antioxidant capacity in grapeseed oil. Total phenolic content also exhibited strong positive correlations with ABTS and DPPH assay results (r=0.877, p<0.01), underscoring the predominant contribution of phenolic compounds to the antioxidant behavior of the samples. These relationships align with the well-established link

Table 4. Coefficients of linear correlations among oil yield, physicochemical parameters, total phenolic content, and antioxidant capacity (in ABTS and DPPH assays) of grape seed oils obtained from cv. *Vitis labrusca* × *Vitis vinifera* seed powders without and with enzymatic pretreatment in different conditions.

Parameter	Yield	Moisture	Free fatty acids	Acidity index	Peroxide value	Total phenolic content	ABTS assay
Moisture	0.249	–	–	–	–	–	–
Free fatty acids	0.539	0.157	–	–	–	–	–
Acidity index	0.616	0.359	0.753**	–	–	–	–
Peroxide value	0.752**	0.176	0.933***	0.794**	–	–	–
Total phenolic content	0.756**	0.042	–0.048	0.084	0.248	–	–
ABTS assay	0.852**	–0.084	0.315	0.280	0.550	0.877**	–
DPPH assay	0.908***	–0.016	0.393	0.366	0.613	0.877**	0.937***

* Correlation significant at $p<0.05$. ** Correlation significant at $p<0.01$. *** Correlation significant at $p<0.001$.

between phenolic content and radical scavenging activity, where hydroxyl-rich phenolics efficiently donate hydrogen atoms or electrons to neutralize free radicals [Vucane *et al*., 2024].

Among oil quality parameters, free fatty acids showed a very strong positive correlation with the peroxide value ($r=0.933$, $p<0.001$) and a strong correlation with the acidity index ($r=0.753$, $p<0.01$), while acidity index also significantly correlated with the peroxide value ($r=0.794$, $p<0.01$) (**Table 4**). These relationships confirm the close association among oxidative stability indicators, as hydrolysis and oxidation processes tend to progress simultaneously, leading to increased levels of free fatty acids and primary oxidation products [Kaseke *et al*., 2021; Symoniuk *et al*., 2022].

In contrast, the correlations of TPC and antioxidant capacity with oil quality parameters were weak, indicating that phenolic content and oxidative indicators evolve independently under the studied conditions. This trend suggests that, although phenolic compounds contribute to the oil's antioxidant defense, their content is not the main determinant of immediate oxidation status. Similar observations were reported by Symoniuk *et al.* [2022] and Kaseke *et al.* [2021], who found that the relationship between phenolic compounds and primary oxidation indices in vegetable oils may be limited due to differences in compound polarity, solubility, and reaction kinetics within the lipid matrix.

Principal component analysis

PCA was conducted using yield, moisture content, free fatty acid content, acidity index, peroxide value, total phenolic content, and results of ABTS and DPPH assays across treatments (T0–T4). The first two principal components explained 82.9% of the total variance (PC1: 58.2%, PC2: 24.7%), effectively summarizing most of the data variability. As shown in the biplot (**Figure 2**), yield, total phenolic content, and ABTS and DPPH results exhibited high loadings on PC1, forming a cluster associated with extraction efficiency and antioxidant capacity. In contrast, free fatty acids, acidity index, and peroxide value grouped in the opposite direction, reflecting indicators related to oil degradation. Moisture content, which contributed primarily to PC2, remained isolated from these clusters, indicating a secondary and independent effect on the dataset. These results highlight that PC1 differentiated treatments by functional enrichment, while PC2 captured compositional differences, consistent with previous studies on enzyme-assisted extraction of plant oils [Kaseke *et al*., 2021].

The distribution of treatments further reflected these relationships. Treatments T2 and T3 were positioned close to the vectors representing oil yield, total phenolic content, and antioxidant capacity (DPPH/ABTS), indicating that cellulase-assisted pretreatment enhanced both extraction efficiency and the release of bioactive phenolic compounds. In contrast, T0, which did not receive enzymatic treatment, appeared on the negative side of PC1, reflecting lower extraction efficiency and minimal release of functional compounds.

Along PC2, T1 projected toward the positive axis, associated with an increased free fatty acid content and acidity index. Meanwhile, T1 and T4 were located closer to the moisture vector, suggesting milder enzymatic effects and a less pronounced improvement in functional parameters compared to T2 and T3. Although the enzymatically-treated oils (T1–T4) showed slightly higher acidity and peroxide values than the control, all samples remained within the quality limits established by the Codex Alimentarius [1999], confirming compliance with international standards. Overall, the biplot indicates that treatments promoting greater phenolic release (particularly T2 and T3) were associated with higher extraction efficiency and antioxidant capacity, whereas treatments with milder enzymatic effects (T1 and T4) showed more moderate responses.

The PCA configuration, in agreement with the correlation analysis, revealed an inverse association between oil oxidation indicators (free fatty acid content, acidity index, peroxide value) and functional parameters (TPC and antioxidant capacity). This finding suggests that the higher content of phenolics may have

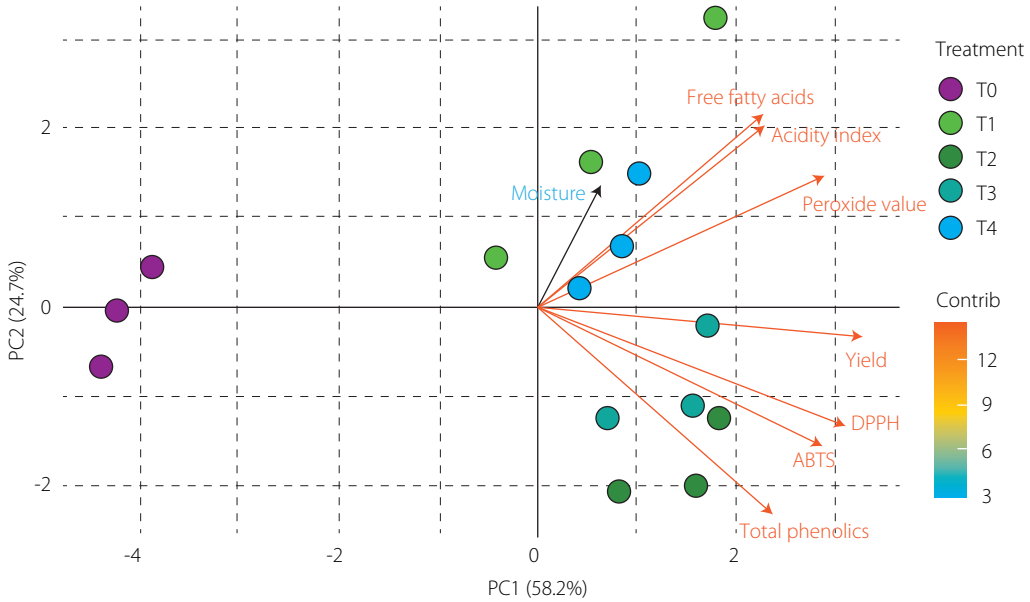

Figure 2. Biplot of principal component analysis (PCA) of grapeseed oils (*Vitis labrusca* × *Vitis vinifera*) obtained by hydraulic pressing without (T0) and with cellulase pretreatments (T1–T4), showing relationships among oil yield, physicochemical parameters, total phenolic content, and antioxidant capacity in ABTS and DPPH assays. The biplot illustrates both the loading patterns of the variables and the distribution of treatments along PC1 and PC2.

mitigated oxidative degradation during enzymatic extraction by stabilizing unsaturated lipids and interrupting radical chain reactions. Similar trends have been reported by Vardakas *et al.* [2024], who observed that cellulase pretreatment enhanced the release of bound phenolic compounds, thus improving antioxidant performance and oxidative resilience. Moreover, Kaseke *et al.* [2021] highlighted that enzyme-assisted systems strengthened the interdependence between phenolic enrichment and oxidative stability, confirming the protective role of phenolics in maintaining oil quality.

CONCLUSIONS

This study demonstrated that the enzymatic pretreatment of grape seeds with cellulase enhanced both oil extraction efficiency and the enrichment of phenolic compounds and antioxidant capacity, while maintaining physicochemical properties within acceptable quality standards.

Correlation and principal component analyses confirmed a strong positive relationship between extraction yield and bioactive compound content, suggesting that enzymatic activity promoted the release of functional metabolites during pressing. Among the evaluated treatments, T2 showed a distinctive performance, combining higher extraction efficiency with greater functional enrichment.

Overall, cellulase pretreatment improved both the extraction efficiency and functional properties of grapeseed oil. The conditions of enzymatic pretreatment significantly determined the TPC and antioxidant capacity of the oil; a lower enzyme concentration for a longer time of action (T2) proved to be the best combination among the treatments used. In conclusion, enzymatic pretreatment represents a viable and sustainable technological strategy for the valorization of grape seed by-products, supporting the development of functional vegetable oils and contributing to a more circular use of agro-industrial residues.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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Nutritional Enhancement of Milk Tablets Using Avocado (*Persea Americana* Mill.) Pulp Powder

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Freeze-dried avocado pulp with maltodextrin was incorporated into milk tablets at substitution levels of 15%, 25%, and 35% (*w/w*) for milk powder. The tablets' physicochemical characteristics and consumer acceptance were then assessed. The powder formulations exhibited fair flowability, except for the formulation with the highest avocado content, which showed poor flow properties. Increasing the proportion of avocado pulp powder reduced tablet hardness and density, while increasing their friability, resulting in their lower mechanical strength and durability. Higher avocado pulp powder levels significantly increased moisture, total lipid, crude fiber, and ash contents. Tablets with 35% (*w/w*) substitution of milk powder with avocado pulp powder exhibited the highest total phenolic content (1.91 µg GAE/g) and antioxidant capacity (3.84, 1.38, and 9.24 µmol TE/g in DPPH, ABTS, and FRAP assays, respectively). Additionally, microbial analysis revealed significantly higher counts of total microbes, and molds and yeasts with increased avocado powder fortification; however, all values remained within the acceptable limits established by the Thai Industrial Standards Institute. Sensory evaluation indicated that the milk tablets with 15% and 25% (*w/w*) substitution with avocado powder were the most acceptable with no significant difference between them. These findings highlight the potential of avocado pulp powder as a functional food ingredient particularly in milk tablet confections.

Keywords: antioxidant capacity, freeze-dried avocado powder, milk powder substitution, sensory evaluation

INTRODUCTION

Avocado (*Persea Americana* Mill.), a subtropical fruit native to Mexico, has recently gained popularity as a cultivated crop in northern Thailand. *Per* 100 g of fresh pulp, avocado provides approximately 15.40 g of lipids, 8.64 g of carbohydrates, 6.80 g of dietary fiber (about 70% insoluble and 30% soluble), and 1.96 g of proteins, making it a valuable energy source [Ford *et al.,* 2023; Krumreich *et al.,* 2018]. Avocado is particularly rich in monounsaturated fatty acids (approximately 9.80–12.37 g/100 g fresh pulp), along with polyunsaturated (about 1.82–4.46 g/100 g fresh pulp), and saturated fatty acids (around 2.13–3.18 g/100 g fresh pulp) [Ford *et al.,* 2023; USDA, 2011]. Besides being high in lipids with monounsaturated fatty acids, avocado pulp contains a variety of essential nutrients and bioactive compounds, including minerals (potassium, sodium, magnesium), vitamins (A, C, B_6 and K, folate, niacin, pantothenic acid, riboflavin, choline and tocopherols), carotenoids (mainly lutein and/or zeaxanthin), phytosterols, phenolic compounds, organic acids and acetogenins as well as exhibits antioxidant, anti-inflammatory, anticancer, and antimicrobial properties, all of which contribute to its broad potential health benefits [Dreher & Davenport, 2013; Ford *et al.,* 2023; Krumreich *et al.*, 2018]. Due to its beneficial lipid

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composition, avocado consumption can help lower total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels, while increasing high-density lipoprotein (HDL) cholesterol, thereby supporting a healthier blood lipid profile [Mahmassani *et al.*, 2018]. Thus, freeze-dried avocado shows promise as an ingredient for replacing fat or flour, while also enhancing the fiber content and nutritional value of food products [Cortés Rodríguez *et al.*, 2019; Moolwong *et al.*, 2023].

Milk tablets are compact, chewable forms of milk powder offering a convenient, portable, and shelf-stable source of essential nutrients such as calcium, protein, and vitamins. However, despite their convenience, traditional milk tables are high in sugar posing health risks such as diabetes, obesity, and high blood pressure when consumed excessively [Endy *et al.,* 2024]. Consuming milk tablets enriched with high levels of dietary fiber and phytochemical from fruits and vegetables significantly contributes to a healthy diet. Therefore, developing healthier milk tablets by partially replacing milk powder with avocado pulp powder could help reduce the risk of chronic diseases and enhance nutritional values in small doses, working best when used as a supplement or snack especially for children, or individuals with limited access to fresh milk. From a production perspective, the use of avocado pulp powder in milk tablets can improve texture, mouthfeel, and sensory attributes, potentially opening new avenues for value-added dairy and plant-based products. This innovation aligns with industry trends toward clean-label, nutrient-dense, and minimally processed foods, providing manufacturers with opportunities to differentiate their products in a competitive market. Thus, this study aimed to optimize the substitution ratio of milk powder with avocado pulp powder in fortified milk tablets and evaluate its impact on physical properties and sensory acceptability, with the goal of developing a functional, health-promoting milk tablet enriched with dietary fiber and antioxidant phytochemicals.

MATERIALS AND METHODS

Materials and reagents

Avocados (*Persea Americana* Mill.), milk powder, and icing sugar were sourced from a local market in Bangkok, Thailand. Food-grade CAB-O-SIL and magnesium stearate were obtained from Krungthepchemi Co., Ltd., Bangkok, Thailand. The chemicals: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, iron(III) chloride hexahydrate, 2,4,6-tripyridyl-*s*-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and gallic acid were procured from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent was acquired from Fisher Scientific (Pittsburgh, PA, USA). All culture media for total plate count, mold and yeast count and *Escherichia coli* count were purchased from HiMedia Laboratories (Mumbai, India). All other materials utilized were of analytical quality.

Preparation of avocado pulp powder

Freeze-dried avocado pulp powder was prepared following the method outlined by Chuacharoen *et al.* [2021]. Briefly, ripe avocado pulp was blended with added maltodextrin in amounts of 0 g, 10 g, 20 g, and 30 g *per* 100 g of dry matter (d.m.) of pulp and homogenized using a homogenizer (T10 Ultra-turrax, IKA-Labortechnik, Staufen, Germany) at 500 rpm for 5 min. The resulting avocado paste was then frozen in static air at –80°C and freeze-dried in a laboratory-scale freeze dryer (Christ Alpha 1-2LD Plus, GmbH, Osterode am Harz, Germany) at –50°C for 72 h under a pressure of less than 0.11 mbar. The freeze-dried avocado pulp powder was ground using a rotor mill (FRITSCH P-14, Franconia, Germany) and stored in a desiccator until used.

Preparation of avocado-fortified milk tablets

In the fortified milk tablet formulations, 15% (A15), 25% (A25), and 35% (A35) of the milk powder was replaced by weight with avocado pulp powder incorporated with 30% maltodextrin (**Table 1**). A control sample without avocado (A0) was also prepared. Other tablet ingredients included CAB-O-SIL added to improve powder flow, content uniformity, and overall tablet quality, and magnesium stearate, acting primarily as a lubricant and secondarily as a mineral supplement [Binte Abu Baker *et al.,* 2019]. All dried ingredients were sifted using a 0.5 mm sieve and mixed in a dry blender until completely mixed. The obtained dried powder was sampled to test flow rheology. Subsequently, the tableting of mixture powders was performed using a laboratory-scale single-punch tablet machine (Yiao-heng Co., Ltd., Bangkok, Thailand), and the resulting tablets were kept in an aluminum foil bag and stored at –18°C for further analyses.

Table 1. Formulations of control milk tablet (A0) and milk tablets containing different levels of avocado pulp powder (A15–A35).

Ingredient	A0 (control)	A15	A25	A35
Milk powder	70.00	59.50	52.50	45.50
Avocado pulp powder	0.00	10.50	17.50	24.50
Icing sugar	28.00	28.00	28.00	28.00
CAB-O-SIL	1.50	1.50	1.50	1.50
Magnesium stearate	0.50	0.50	0.50	0.50

Data are expressed as g of component *per* 100 g of total ingredients. Formulations refer to substitution of milk powder with avocado pulp powder in a weight ratio of 0% (A0, control), 15% (A15), 25% (A25), and 35% (A35).

Analysis of physical parameters

Before tableting, all powder formulations were assessed for bulk density and tapped density. After tableting, the physical properties of the avocado-fortified milk tablets were examined for the uniformity of weight, thickness, hardness, density, and friability. Bulk and tapped densities were determined using a tap density tester (Electrolab India Pvt. Ltd., Mumbai, India). In summary, 100 g of the powder mixture were weighed and poured into a 250 mL measuring cylinder. Bulk density was calculated by dividing the sample weight by its initial volume. The sample was then tapped 200 times to obtain its final volume, which was used to calculate the tapped density by dividing the sample weight by the final volume [Salum *et al.,* 2022]. The calculated bulk density and tapped density values were used to categorize flowability using the Carr's index (Equation 1) and Hausner ratio (Equation 2), respectively.

$$\text{Carr's index (\%)} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100 \quad (1)$$

$$\text{Hausner ratio} = \frac{\text{Tapped density}}{\text{Bulk density}} \quad (2)$$

Twenty tablets from each formulation were individually measured for weight, thickness, and diameter. The tablet weight was measured using a high-precision laboratory balance (Sartorius CP32025, Sartorius AG, Göttingen, Germany). Their thickness and diameter were measured using a vernier caliper. The tablet hardness was measured using a texture analyzer (TA.XT.Plus, Stable Micro System, Godalming, Surrey, UK) in a compression mode (heavy duty platform/confectionery holder; HDP/CH) with a P/6 probe (pre-test speed: 1 mm/sec, test speed: 2 mm/sec, post-test speed: 10 mm/sec). The measurement was repeated 10 times, and the results were recorded as the hardness at the highest force, expressed in N.

For tablet density, six tablets were randomly selected, and their individual weights were recorded using a digital scale. Chloroform was used, and the solution was poured into a graduated cylinder. The volume change of the liquid inside the cylinder was recorded. The density was then calculated as the ratio of its mass to the displaced volume (g/mL), and results were expressed as mean and standard deviation (n=6).

The friability is a key parameter for quality assessment. Twenty tablets were initially dusted using a dusting brush and weighed to record their initial weight. They were then placed in a friabilator (Roche Model PTFR-A, Pharmatest, Germany) and rotated at a speed of 25 rpm for 4 min. Subsequently, the tablets were removed, dedusted, and reweighed to determine their final weights. The friability (%) was calculated based on the weight difference before and after rotation (Equation 3).

$$\text{Friability (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad (3)$$

Color measurements were performed using a MiniScan XE Plus colorimeter (Hunterlab, Reston, VA, USA). The instrument was calibrated prior to measurement using a standard white calibration tile and a black calibration plate according to the device manufacturer's instructions. The L^*, a^*, and b^* values were recorded, where L^* denotes lightness, ranging from 0 (black) to 100 (white). The a^* coordinate represents the green-red axis, with negative values corresponding to green hues and positive values corresponding to red hues. The b^* coordinate corresponds to the blue-yellow axis, where negative values indicate blue hues and positive values indicate yellow hues.

Proximate analysis and water activity measurement

Proximate composition was analyzed according to the standard methods of the Association of Official Analytical Chemists [AOAC, 2019]. Moisture and total solid contents of avocado pulp and milk tablets fortified with avocado pulp powder were determined gravimetrically by drying the sample in a convection oven at 110°C for 2 h (method 968.11). Ash content of avocado-fortified milk tablets was quantified by incinerating the samples at 550°C in a muffle furnace (method 945.38). Crude protein was determined using the Kjeldahl digestion method (method 971.09), while crude fiber was analyzed through sequential acid and alkaline digestion (method 962.09). To determine total lipid content, lipids were extracted using the Soxhlet extraction method with *n*-hexane as the solvent in a Soxtec Foss apparatus (ST 255 Soxtec™, FOSS Analytical Solutions Pty. Ltd., Victoria, Australia) at 120°C for 80 min (method 920.39). Water activity (a_w) was measured using an AquaLab 4TE meter (Meter Group Inc., WA, USA).

Determination of total phenolic content and antioxidant capacity

To determine total phenolic content (TPC) and antioxidant capacity of avocado-fortified milk tablets, the samples were extracted following the method of Pongtongkam *et al.* [2024] with slight modifications. In brief, approximately 0.5 g of avocado milk tablet was extracted using 50 mL of 80% (*v/v*) methanol, followed by mechanical shaking using an incubator (C25KC incubator shaker, New Brunswick Scientific, NJ, USA) at 40 rpm and room temperature for 4 h. The mixture was then centrifuged using an MPW-352R centrifuge (MPW Med. Instruments, Warsaw, Poland) at 1,277×*g* for 30 min. The resulting supernatant was collected and stored at −20°C for further analyses.

Total phenolic content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu reagent by Singleton *et al.* [1999] method with minor modifications. In brief, 20 µL of the extract, gallic acid standard, or blank sample were transferred into separate test tubes and mixed with 1.58 mL of distilled water. Subsequently, 100 µL of Folin–Ciocalteau reagent were added, and the mixture was thoroughly vortexed. Within 8 min, 300 µL of a 7.5% sodium carbonate solution were introduced, and immediately mixed. The samples were then incubated in the dark at room temperature for 30 min. Absorbance was recorded at 765 nm using a UV-Vis spectrophotometer (Spectronic GENESYS2, Thermo Fisher Scientific, Waltham, MA, USA). The TPC was calculated

from a gallic acid calibration curve, with results expressed as µg of gallic acid equivalent *per* g of milk tablet (µg GAE/g).

Antioxidant capacity

The antioxidant capacity of the avocado milk tablets was evaluated using three *in vitro* antioxidant assays – 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and ferric reducing antioxidant power (FRAP).

The DPPH assay was performed according to the method of Brand-Williams *et al.* [1995] with slight modifications. Briefly, 200 µL of the extract was mixed with 2.8 mL of a 100 µM DPPH radical solution prepared in methanol and incubated in the dark for 30 min. A control containing the DPPH radical solution and 80% (*v/v*) methanol was also prepared. The absorbance was measured at 515 nm using a UV-Vis spectrophotometer (Spectronic GENESYS2, Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was plotted using Trolox standard solutions at various concentrations in 80% (*v/v*) methanol. Results were expressed as µmol of Trolox equivalent *per* g of milk tablet (µmol TE/g).

The ABTS assay was carried out following the method of Re *et al.* [1999] with slight modifications. Briefly, the $ABTS^{\bullet+}$ was generated by dissolving 7 mM ABTS in 2.45 mM potassium persulfate, and the mixture was left in the dark at room temperature for 15 h before use. The resulting $ABTS^{\bullet+}$ solution was then diluted with 5 mM sodium phosphate buffer (pH 7.4) to achieve an absorbance of 0.70±0.02 at 734 nm, measured using a UV-Vis spectrophotometer (Spectronic GENESYS2, Thermo Fisher Scientific). Subsequently, 50 µL of each sample were combined with 150 µL of the $ABTS^{\bullet+}$ solution and incubated for 10 min at 25°C. The $ABTS^{\bullet+}$ scavenging activity was quantified using a Trolox calibration curve, with results expressed as µmol of Trolox equivalent *per* g of milk tablet (µmol TE/g).

The FRAP assay was conducted according to the method described by Benzie & Strain [1996] with minor modifications. In brief, 100 µL of the extract were mixed with 300 µL of distilled water and 3 mL of FRAP reagent containing a 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM HCl, acetate buffer (pH 3.6), and ferric chloride solution in a test tube. The mixture was incubated in the dark for 30 min, after which the absorbance of the resulting ferrous tripyridyltriazine colored complex was measured at 593 nm using a UV-Vis spectrophotometer (Spectronic GENESYS2, Thermo Fisher Scientific). The FRAP was determined from a Trolox standard curve, with results expressed as µmol of Trolox equivalent *per* g of milk tablet (µmol TE/g).

Microbiological evaluation

The microbiological quality was evaluated following the method described by Pongtongkam *et al.* [2024] with slight modifications. Twenty-five g of milk tablets were weighed and mixed with 225 mL of a 0.1% peptone solution, followed by blending for 2 min. The mixture was then serially diluted to obtain decimal dilutions ranging from 10^{-1} to 10^{-4}. For total plate count, 1 mL of each dilution was transferred to a sterile Petri dish. Then, molten plate count agar (PCA) was poured over the sample. The plate was gently swirled to mix the sample and agar. All PCA plates were incubated at 35°C for 48 h. Total plate count was computed from colony enumeration. For mold and yeast counts, 1 mL of 10^{-1} dilution was plated by dividing into 3 aliquots (0.3, 0.3, 0.4 mL each) onto 3 separate potato dextrose agar (PDA) plates. The spread plate technique was then performed on each plate. PDA plates were incubated at 25°C for 5 days. Microbial counts were first calculated as colony-forming units (CFU) *per* g of milk tablet (CFU/g) and then converted to $\log_{10}$ CFU/g (log CFU/g) for reporting in the results table. For *E. coli* enumeration, 3 dilutions of 10^{-1}, 10^{-2} and 10^{-3} were used for a 3-tube most probable number (MPN) analysis. To confirm the presence of *E. coli*, all presumptive, confirmed, completed, and IMViC tests were performed. The IMViC series included the indole test (I), methyl red test (M), Voges-Proskauer test (V), and citrate utilization test (C), which together provided characteristic biochemical results indicative of *E. coli*. The number of sample tubes containing *E. coli* was used to consult the MPN table for *E. coli* count (MPN/g). All analyses were performed in triplicate.

Sensory evaluation

A sensory evaluation of the avocado-fortified milk tablet was conducted with 60 untrained panelists due to practical constraints related to participant availability and study logistics. The panel included 27 men and 33 women, aged 18–55 years, representing the target consumer group. Each panelist was presented with one piece of randomly coded samples, labeled with three-digit codes, in a randomized order. Water was provided for cleansing the palate between samples. The sensory attributes were assessed using a 9–point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely). The panelists evaluated the tablets based on appearance, color, aroma, flavor, texture, hardness, and overall acceptability [Karaman *et al.,* 2014; Tobin *et al.,* 2013]. The hardness in the instruction sheet was defined as the force required to bite or compress the tablet, using a scale from 1 (very soft: breaks immediately with light pressure) to 9 (very hard: difficult to bite and requires strong pressure).

Statistical analyses

All experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation. Statistical analyses were performed using SPSS software version 26 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was applied to determine significant differences between mean values, with a significance level set at $p<0.05$.

RESULTS AND DISCUSSION

Avocado pulp powder preparation

Avocado pulp was lyophilized with various maltodextrin contents. The powders produced at 0 and 10 g maltodextrin *per* 100 g d.m. of pulp were not completely dry, exhibiting a sticky and rubbery texture, making it unsuitable for tablet formulation. In contrast, at 20 and 30 g maltodextrin *per* 100 g d.m. of pulp, the powder was completely dry, puffy, and bright

Table 2. Physical properties of avocado pulp powders with the addition of maltodextrin.

Parameter	Maltodextrin level (g/100 g pulp dry matter)	
	20	30
Moisture (g/100 g)	4.53±0.35^{a}	4.30±0.13^{a}
Water activity (a_w)	0.41±0.01^{a}	0.33±0.02^{b}
*L**	78.89±0.24^{b}	82.51±0.14^{a}
*a**	−2.41±0.04^{a}	−2.52±0.06^{b}
*b**	35.00±0.04^{a}	32.48±0.24^{b}

Data are mean ± standard deviation (n=3). Different superscripts (a–b) represent a significant difference within the same row (p<0.05). *L** represents lightness (0=black, 100=white); *a** represents the green (–) to red (+) axis; *b** represents the blue (–) to yellow (+) axis.

green, making it ideal for tablet production. The moisture content of the powders with maltodextrin at 20 and 30 g/100 g pulp d.m. did not differ significantly (p≥0.05), whereas the water activity was different (p<0.05), with a lower value found in the avocado pulp powder with maltodextrin at 30 g/100 g pulp d.m. (**Table 2**). This indicates that the powder at this level was the most suitable for use as an ingredient in tablet formulation. The addition of more maltodextrin resulted also in brighter, greener, and less yellowish avocado pulp powder (**Table 2**). The physical properties of the powder with maltodextrin at 30 g/100 g pulp d.m. were consistent with our previous findings [Chuacharoen *et al.*, 2021]. In general, the standard moisture content required for dried food products is between 4 to 6 g/100 g, and mixtures with high water content affect the physical properties of tablet formation, particularly the texture and hardness.

Characterization of mixture formulations and avocado-fortified milk tablets

The physical properties of the powder mixtures and milk tablets with substitutions of 0%, 15%, 25%, and 35%, *w/w*, milk powder with avocado pulp powder are summarized in **Table 3**. Bulk and tapped densities decreased progressively with increasing avocado content, indicating that higher levels of avocado pulp produced lighter, less dense powders with reduced packing efficiency.

Flowability was evaluated using Carr's index and the Hausner ratio, derived from bulk and tapped densities. Lower Carr's index values (0–15%) and Hausner ratios near 1.0 indicate excellent to good flow, while higher values reflect progressively poorer flow. Values above 38% for Carr's index or above 1.60 for the Hausner ratio represent very poor flow [Lebrun *et al.,* 2012; Reddy *et al.,* 2014].

The avocado-fortified formulations showed Carr's index ranging from 18.0 to 30.8 and Hausner ratio from 1.23 to 1.48 (**Table 3**), indicating generally fair flowability. Only the formulation with the highest avocado pulp powder content (A35) demonstrated poor flowability. Assessing the Hausner ratio is important for understanding how humidity influences powder cohesiveness over shelf-life, as it indicates transitions between free-flowing and cohesive states [Lumay *et al.,* 2012]. Consistent with these indicators, A15 and A25 showed fair flow characteristics, with no significant difference from the control, while A35 exhibited reduced flow.

Table 3. Physical properties of formulation mixtures and milk tablets without (A0, control) and with 15% (A15), 25% (A25), and 35% (A35) substitution by weight of milk powder with avocado pulp powder.

Product	Parameter	A0 (control)	A15	A25	A35
Powder formulation	Bulk density (kg/L)	0.65±0.46^{a}	0.47±0.04^{b}	0.36±0.04^{c}	0.24±0.02^{d}
	Tapped density (kg/L)	0.79±0.02^{a}	0.58±0.02^{b}	0.42±0.02^{c}	0.36±0.04^{c}
	Carr's index (%)	18.0±6.3^{b}	19.3±8.9^{b}	19.5±7.2^{b}	30.8±12.3^{a}
	Hausner ratio	1.23±0.09^{b}	1.25±0.14^{b}	1.25±0.11^{b}	1.48±0.29^{a}
	Flowability	Fair	Fair	Fair	Poor
Tablet	Hardness (N)	47.73±0.67^{a}	38.95±1.55^{a}	33.04±0.73^{b}	25.78±0.78^{c}
	Density (g/mL)	0.50±0.01^{a}	0.44±0.00^{b}	0.43±0.02^{b}	0.38±0.10^{c}
	Friability (%)	7.6±1.5^{c}	12.8±3.5^{b}	10.8±2.6^{b}	20.1±1.9^{a}
	*L**	95.48±0.31^{a}	88.31±0.30^{b}	86.22±0.01^{c}	85.59±0.26^{d}
	*a**	−1.25±0.10^{a}	−3.12±0.03^{b}	−3.29±0.01^{c}	−3.78±0.03^{d}
	*b**	10.32±0.68^{d}	18.76±0.10^{c}	20.75±0.14^{b}	22.22±0.10^{a}

Data are mean ± standard deviation. Different superscripts (a–d) represent a significant difference within the same row (p<0.05). *L** represents lightness (0=black, 100=white); *a** represents the green (–) to red (+) axis; *b** represents the blue (–) to yellow (+) axis.

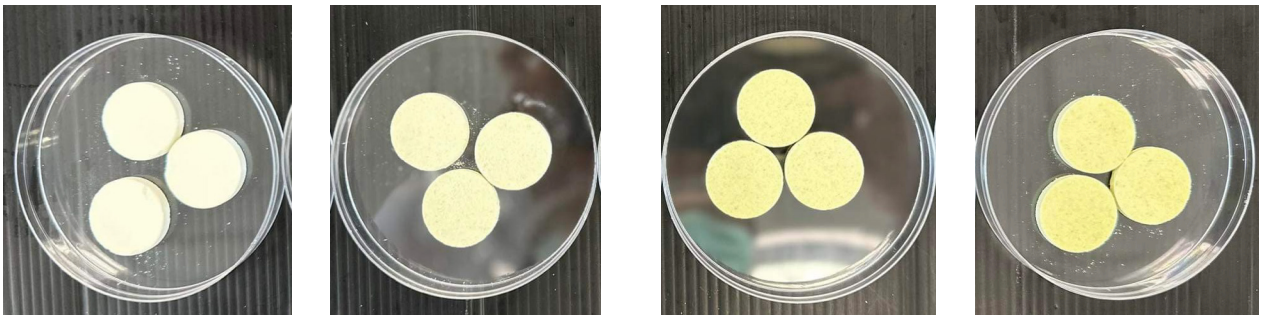

Figure 1. Images of milk tablets without (A0, control) and with 15% (A15), 25% (A25), and 35% (A35) substitution by weight of milk powder with avocado pulp powder.

The obtained tablets had weight, thickness, and diameter ranges of 2.45 to 2.50 g, 3.99 to 4.22 mm, and 5.07 to 5.33 mm, respectively. Weight uniformity is considered a critical physical characteristic, as it is closely related to the content and consistency of the chemical ingredients. The control milk tablets exhibited the highest hardness (47.73 N), which decreased as the proportion of avocado pulp powder in the formulation increased, and hardness of the tablets with 35% (*w/w*) substitution of milk powder with avocado pulp powder was 25.78 N (**Table 3**). Density and friability are important indicators of whether a tablet is suitably formed for consumer acceptability, particularly in terms of chewability. The increased avocado pulp powder content led to a decrease in tablet density, from 0.50 g/mL for control tablets to 0.38 g/mL for A35 sample. This reduction in density was correlated with increased friability (from 7.6% to 20.1%), as lower-density tablets are more prone to breaking. Adding avocado pulp powder (high in lipid and moisture) to milk tablet formulations tends to decrease their hardness and increase friability, mainly because the lipids act as a lubricant, weaken protein-protein bonding, and modify moisture distribution within the compressed structure [Liu, 2023; Chauhan *et al.,* 2020].

The color of a food product plays an important role in visual appeal, which directly influences consumer acceptance [Moolwong *et al.,* 2023]. The appearance and color parameters of milk tablets containing avocado pulp powder are shown in **Figure 1** and **Table 3**, respectively. Incorporating avocado pulp powder resulted in changes in the color of the tablets. Increasing the avocado powder ratio led to a noticeable decrease in brightness with L^* values from 85.59 for A35 sample to 95.48 for control tablets, along with shifts in green hues (decreasing a^* values from −1.25 to −3.78) Moreover, the intensity of the yellow hue (b^* values) increased significantly ($p<0.05$). These changes in yellow and green hues were clearly visible to the naked eye (**Figure 1**).

Moisture content and water activity may affect the texture, compressibility, and microbial stability of the tablets. As shown in **Table 4**, an increase in the content of avocado pulp powder in tablets primarily results in their higher moisture content. Water activity (a_w) of the milk tablets with 35% (*w/w*) substitution was 0.46 and this value was significantly ($p<0.05$) higher than that for control tablets and tablets with other substitution levels (0.43–0.44). The crude protein content of the tablets decreased

Table 4. Proximate composition and water activity of milk tablets without (A0, control) and with 15% (A15), 25% (A25), and 35% (A35) substitution by weight of milk powder with avocado pulp powder.

Parameter	A0 (control)	A15	A25	A35
Water activity	0.43±0.12^{b}	0.44±0.01^{b}	0.44±0.01^{b}	0.46±0.01^{a}
Moisture (g/100 g)	2.80±0.58^{c}	3.01±0.02^{b}	3.28±0.02^{ab}	3.79±0.43^{a}
Crude protein (g/100 g)	1.30±0.08^{a}	1.24±0.01^{b}	1.10±0.01^{c}	1.03±0.02^{d}
Total lipids (g/100 g)	2.45±0.28^{c}	2.57±0.49^{b}	3.53±0.20^{b}	4.21±0.96^{a}
Crude fiber (g/100 g)	0.38±0.06^{c}	0.43±0.12^{c}	0.76±0.05^{b}	1.11±0.06^{a}
Ash (g/100 g)	1.20±0.20^{c}	1.27±0.05^{b}	1.42±0.05^{b}	2.46±0.36^{a}

Data are presented as mean ± standard deviation ($n=3$). Different superscripts (a–d) represent a significant difference within the same row ($p<0.05$).

Table 5. Total phenolic content (TPC), antioxidant capacity, and microbiological properties of milk tablets without (A0, control) and with 15% (A15), 25% (A25), and 35% (A35) substitution by weight of milk powder with avocado pulp powder.

Parameter	A0 (control)	A15	A25	A35
TPC (μg GAE/g)	0.06±0.01^{d}	0.73±0.06^{c}	1.29±0.08^{b}	1.91±0.12^{a}
DPPH$^{\bullet}$ scavenging activity (μmol TE/g)	0.16±0.01^{d}	1.78±0.00^{c}	2.88±0.04^{b}	3.84±0.06^{a}
ABTS$^{\bullet+}$ scavenging activity (μmol TE/g)	0.64±0.06^{d}	0.92±0.07^{c}	1.14±0.00^{b}	1.38±0.07^{a}
FRAP (μmol TE/g)	8.43±0.37^{c}	8.93±0.30^{b}	9.22±0.67^{a}	9.24±0.35^{a}
Total plate count (log CFU/g)	2.83±0.04^{d}	2.91±0.02^{c}	2.96±0.02^{b}	3.00±0.05^{a}
Mold and yeast count (log CFU/g)	<1.00^{d}	1.46±0.15^{c}	1.67±0.19^{b}	1.89±0.14^{a}
Escherichia coli (MPN/g)	<3.00	<3.00	<3.00	<3.00

Data are presented as mean ± standard deviation (n=3). Different superscripts (a–d) represent a significant difference within the same row (p<0.05). CFU, colony forming unit; MPN, most probable number; GAE, gallic acid equivalent; TE, Trolox equivalent; FRAP, ferric reducing antioxidant power.

with an increasing substitution level, because the control's protein was mainly derived from milk powder, which was partially replaced by avocado pulp powder. This finding is consistent with a study on milk ice cream where milk fat was substituted with fresh avocado pulp [Moolwong *et al.,* 2023]. The total lipid content was significantly increased by partially replacing milk powder with avocado pulp powder (p<0.05) (**Table 4**). Increasing lipid content with avocado pulp powder can be considered a healthy choice, as avocado is rich in monounsaturated fatty acids and is recognized as a source of healthy fats [Bes-Rastrollo *et al.,* 2008]. Additionally, the lipid fraction in avocado contains lipid-soluble vitamins and tocopherols, making it a valuable ingredient for enhancing the nutritional quality of fat-containing food products [Green & Wang, 2020]. Naturally, avocado pulp powder contains lipids and moisture-retaining compounds, when adding to milk tablets or other milk products, it changes the texture because the lipid droplets from avocado interact with milk proteins, these interactions can make the products either softer or more compact, depending on lipid and moisture contents retained [Pan *et al.,* 2025].

Fresh avocado pulp is rich in dietary fiber [Slater *et al.,* 1975; Viera *et al.,* 2023]; thus, its incorporation into the tablets led to a significant increase in crude fiber content, with clear differences observed among the various formulations (p<0.05) (**Table 5**). The crude fiber content in the tablets containing avocado at 35% (*w/w*) substitution was three times higher than that of the control. It has been hypothesized that incorporating plant-derived cellulose fibers into food products can enhance their nutritional value. Studies have concluded that enriching foods with dietary fiber is an effective strategy to improve both their nutritional and physiological properties. This observation is consistent with Abraham *et al.* [2025], who reported that fiber-rich plant powders improve nutritional profiles and reduce carbohydrate density in dairy-based snacks. Ash content of the milk tablets increased with higher avocado pulp powder levels, rising from 1.20 g/100 g in the control to 2.46 g/100 g in the A35 formulation (**Table 5**), indicating a higher mineral content in the avocado-fortified tablets. Therefore, avocado milk tablets offer a healthier alternative, providing a higher fiber content compared to standard milk tablet formulation. Incorporating dietary fibers from plants not only alters the physical properties of food but also enhances the bioavailability of nutrients and the phytochemical aspects of the food [Unlu *et al.,* 2005].

■ Total phenolic content and antioxidant capacity of avocado milk tablets

Total phenolic content of the avocado milk tablets was determined using the method with Folin-Ciocalteu reagent, which relies on a redox reaction [Berker *et al.,* 2013]. Antioxidant capacity was evaluated using DPPH, ABTS and FRAP assays. The DPPH assay operates through both single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms, in which antioxidants neutralize the DPPH radical either by donating an electron or a hydrogen atom, respectively. This dual mechanism allows DPPH to detect a broad range of radical-scavenging compounds and is particularly sensitive to lipophilic antioxidants [Yamauchi *et al.,* 2024]. In contrast, ABTS and FRAP assays are more effective for antioxidants with higher polarity, as they primarily measure electron transfer reactions in aqueous systems [Knez *et al.,* 2025; Martysiak-Żurowska & Wenta, 2012]. TPC and antioxidant capacity of the tablets containing avocado pulp powder were significantly higher than those of the control tablets without avocado (p<0.05) (**Table 5**). This was expected, because, as indicated by literature data, avocado pulp powder has high TPC and antioxidant potential in ABTS, DPPH, and FRAP assays [Chuacharoen *et al.,* 2021; Nguyen *et al.,* 2022, 2023]. The TPC of the avocado-incorporated tablets ranged from 0.73 to 1.91 μg GAE/g, increasing with higher avocado pulp powder ratios. The antioxidant capacity of the fortified tablets also significantly (p<0.05) increased with increasing levels of substitution of milk powder with avocado pulp powder and ranged from 1.78 to 3.84 μmol TE/g in DPPH

assay, from 0.92 to 1.38 µmol TE/g in ABTS assay, and from 8.93 to 9.24 µmol TE/g in FRAP assay. The increased total phenolic content and antioxidant capacity observed at higher avocado levels align with the findings of Moolwong *et al*. [2023], who reported concentration-dependent increases in DPPH scavenging activity when avocado powder was incorporated into milk-based functional ice cream. Similarly, studies by Novelina *et al.* [2022], Santos *et al.* [2024], and Siol & Sadowska [2023] reported that avocado pulp and seed flours could significantly enhance the fiber and phenolic content of baked products, thereby improving their antioxidant capacity. These results collectively indicate that the incorporation of fruit-derived bioactive ingredients, such as avocado pulp powder, can effectively enrich the TPC and antioxidant properties of food matrices.

Microbial quality of avocado milk tablets

Microbiological qualities of avocado milk tablets are displayed in **Table 5**. In terms of the total plate count, a continuous rise in microbial numbers was observed in the samples with an increasing content of avocado pulp powder. The tablets with the avocado ratio of 35% (*w/w*) had the highest microbial load of 3.00 log CFU/g ($p<0.05$). However, this level of microbes was within the microbiological specification (6 log CFU/g) set by the Thai Industrial Standards Institute [2007], according to the Thai Community Product Standard 1405/2550. Similarly, the maximum mold and yeast count of 1.89 log CFU/g was detected in the tablets with 35% (*w/w*) substitution with avocado pulp powder ($p<0.05$), which was within the acceptable limit according to the specification (2 log CFU/g). Regarding the *E. coli* count, *E. coli* was absent in all sample tubes analyzed by the MPN method, with results reported as <3 MPN/g, fully meeting the specification (<3 MPN/g). Nevertheless, the presence of some microorganisms in the avocado milk tablets is most likely attributable to the avocado pulp powder quality, as the freeze-drying process used for the avocado is less lethal to microbial cells compared to traditional thermal processing [Bourdoux *et al.,* 2016]. Contamination by aerobic microorganisms, molds, and yeasts can occur during grinding, ingredient sifting and mixing, and tableting, particularly if the equipment or processing environment is not fully sterile. From a microbiological perspective, the low a_w of the avocado milk tablets generally prevents microbial spoilage, provided they are stored in a dry environment.

Sensory scores of avocado milk tablets

Generally, guidelines recommend a minimum of 90 panelists for formal sensory studies; however, smaller sample sizes are commonly used in consumer testing to capture general perceptions of product attributes. This study used 60 untrained panelists because it remained in the product development stage, where a minimum of 60 panelists is considered sufficient for home-use tests employing a 9-point hedonic scale with a 95% confidence interval to assess overall consumer acceptance. Sensory evaluation results for the avocado milk tablets are presented in **Table 6**. The addition of avocado pulp powder influenced the sensory attributes, with overall acceptability decreasing at higher substitution levels due to the characteristic grassy aroma of avocado. Tablets with substitution of 35% (*w/w*) of milk powder with avocado pulp powder received the lowest scores for both odor and taste, with the decline in taste scores being statistically significant ($p<0.05$) compared to A25 formulation. As taste is a key sensory attribute for consumer acceptance, it is notable that no significant difference in taste was observed between the avocado-fortified tablets with 15% and 25% (*w/w*) substitution, suggesting that up to 25% (*w/w*) avocado powder can be incorporated without negatively impacting acceptability. Texture and hardness scores also declined as the avocado pulp powder ratio increased, which correlated with physical measurements obtained from texture analysis. This reduction in sensory scores is associated with the higher fiber and lipid content of avocado pulp powder, which affects the structural integrity of the tablets. A 35% inclusion of avocado pulp powder led to a significant decrease in overall acceptability, indicating that this level exceeds the sensory threshold for consumer preference. The control tablets displayed moderate sensory scores across all attributes but were consistently rated lower than the 15% and 25% formulations for color, texture, hardness, and overall acceptability. This indicates that incorporating avocado pulp

Table 6. Sensory scores of milk tablets without (A0, control) and with 15% (A15), 25% (A25), and 35% (A35) substitution by weight of milk powder with avocado pulp powder.

Attribute	A0 (control)	A15	A25	A35
Color	6.0 ± 1.2^{b}	7.0 ± 1.8^{a}	6.5 ± 1.7^{ab}	5.6 ± 2.0^{b}
Odor	6.3 ± 1.7^{a}	6.4 ± 1.7^{a}	6.3 ± 1.9^{ab}	5.4 ± 2.2^{b}
Taste	6.2 ± 1.1^{a}	6.3 ± 1.8^{a}	6.0 ± 1.8^{a}	4.6 ± 2.4^{b}
Texture	6.1 ± 1.7^{a}	6.3 ± 2.0^{ab}	6.5 ± 1.7^{a}	5.4 ± 2.0^{b}
Hardness	6.0 ± 1.1^{b}	6.4 ± 2.1^{a}	6.4 ± 1.9^{a}	5.3 ± 2.3^{b}
Overall acceptability	6.1 ± 1.1^{ab}	6.5 ± 1.9^{a}	6.3 ± 1.7^{a}	5.0 ± 2.4^{b}

Data are presented as mean ± standard deviation. Different superscripts (a–b) represent a significant difference within the same row ($p<0.05$).

powder, particularly at 15-25% enhanced consumer perception compared to the control.

CONCLUSIONS

This study successfully developed a functional food product – milk tablets enriched with freeze-died avocado pulp powder – to address the growing demand for health-oriented foods fortified with bioactive compounds. Incorporation of avocado pulp powder affected both the nutritional composition and the physical characteristics of the tablets. The avocado-enriched formulations showed a higher crude fiber content than conventional milk tablets, attributable to the insoluble fibers and cell-wall components naturally present in avocado pulp powder. A substitution--dependent increase in total phenolic content and antioxidant capacity was also observed. Among the tested formulations, 15% and 25% (*w/w*) substitution provided the best balance between functional enhancement and sensory acceptability, demonstrating that the bioactive-rich plant ingredient can improve health-promoting properties without compromising consumer acceptance. This suggests that developing avocado milk tablets offers a convenient, shelf-stable way to deliver the combination of nutritional benefits of avocado pulp and milk presenting a promising innovation for the food industry trends towards functional, value-added foods. For consumer perspective, these tablets provide essential nutrients, healthy lipids, and antioxidants in a practical, easy-to-consume form.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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ADDITIONAL INFORMATION

Ethical approval for the involvement of human subjects in this study was granted by Suan Sunandha Rajabhat University Ethics Committee, Certificate number COA. 1-028/2025, Study Code: 68-025-1-2.

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Enhancing Edible Bird's Nest with Water Kefir: A Novel Approach to Boost Antioxidant, Anti-Tyrosinase, and Probiotic Growth-Stimulating Properties for Functional Beverage Development

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Water kefir has been extensively utilized to produce non-dairy drinks that provide significant health advantages. To expand the range of fermented products and cater to consumer preferences, research into new fermentation substrates for water kefir is essential. In this study, we used edible bird's nest (EBN) as a fermentation substrate, focusing on its antioxidant, anti-tyrosinase, and prebiotic properties, along with total phenolic content, protein profile, and sensory characteristics over an 8-day fermentation period. Our results showed that the capacity to scavenge $ABTS^{\bullet+}$ and $^{\bullet}OH$ free radicals, inhibit tyrosinase activity, and promote the growth of *Lactobacillus acidophilus* and *Lactococcus lactis* improved during fermentation, with the most pronounced effects observed at 8 days of fermentation. Additionally, the total phenolic content increased over time, peaking at day 8 with a value of approximately 2,100 µg GAE/mL. SDS-PAGE analysis revealed the emergence of new small proteins and peptides under 10 kDa. Sensory evaluation indicated that EBN water kefir received moderate overall acceptance. Notably, when 10% (*v/v*) rose syrup was incorporated into the final product fermented for 8 days, it was highly appreciated by the panelists. These findings highlight the potential health benefits of EBN water kefir and open avenues for developing a functional beverage from fermented EBN in the future.

Keywords: antioxidant, anti-tyrosinase, edible bird's nest, probiotic growth-stimulating and water kefir

INTRODUCTION

In recent years, especially after the COVID-19 pandemic, consumer preferences have undergone significant changes. While carbonated soft drinks were once the dominant choice, there is now an increasing shift toward refreshing and health-conscious beverages. Water kefir has gained popularity as a functional drink valued for its enjoyable taste – mildly sweet, tangy, and effervescent, and its potential numerous health benefits. These include probiotic qualities, the ability to inhibit harmful bacteria, antioxidant activity, healing support, immune system modulation, digestive assistance, ulcer prevention, liver protection, reduction of blood lipid levels, and regulation of blood sugar [Bozkir *et al.*, 2024; Moretti *et al.*, 2022]. This rising awareness of health advantages has driven the rapid growth of the global water kefir market, which was valued at $1.23 billion in 2019 and is expected to reach $1.84 billion by 2027 [Moretti *et al.*, 2022].

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Water kefir grains contain a complex community of microorganisms, including lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*), acetic acid bacteria (primarily *Acetobacter*), and yeasts (*Saccharomyces*, *Zygosaccharomyces*, and *Brettanomyces*) [Moretti *et al.*, 2022]. The health-promoting effects of water kefir are largely attributed to microorganisms residing within the grains. For instance, *Lentilactobacillus hilgardii*, *Lacticaseibacillus paracasei*, *Liquorilactobacillus satsumensis*, *Lactobacillus helveticus*, *Lentilactobacillus kefiri*, *Saccharomyces paradoxus*, and *Saccharomycodes ludwigii* isolated from water kefir grains exhibit probiotic characteristics [Romero-Luna *et al.*, 2022; Tan *et al.*, 2022b]. These microorganisms have demonstrated antimicrobial activity against intestinal pathogens, strong antioxidant capacity, and viability in simulated gastrointestinal conditions [Romero-Luna *et al.*, 2022; Tan *et al.*, 2022b]. Notably, *Lactobacillus mali* APS1 has shown potential in reducing liver fat accumulation in rats fed a high-fat diet by modulating lipid metabolism and enhancing antioxidant defenses [Chen *et al.*, 2018]. Beyond the beneficial microorganisms, bioactive metabolites generated during fermentation also significantly contribute to the health benefits of water kefir. Lactic, acetic, propionic, and malic acids are renowned for the antimicrobial effects of water kefir [Bozkir *et al.*, 2024]. Soluble exopolysaccharides, including *O*3- and *O*2-branched dextrans and levans, are believed to enhance antimicrobial, antioxidant, and prebiotic properties while phenolic compounds are significant contributors to the antioxidant capacity of the beverage [Azi *et al.*, 2020; Fels *et al.*, 2018].

Traditionally, water kefir is prepared by inoculating grains into sugary water, often supplemented with dried figs and lemon slices [Moretti *et al.*, 2022]. However, a variety of other substrates, such as plant juices or even protein sources, can also be used for fermentation [Alrosan *et al.*, 2023; Corona *et al.*, 2016; Ozcelik *et al.*, 2021]. Using alternative substrates may lead to increased production of bioactive compounds, thereby improving the health benefits of water kefir. A prior research found that water kefir made from coffee cherry showed superior levels of organic acids, phenolics, and amino acids compared to unfermented sample, resulting in higher antioxidant capacity and antibacterial effects on foodborne pathogens like *Staphylococcus aureus* and *Bacillus subtilis* [Chomphoosee *et al.*, 2025]. Similarly, fermenting soy whey with water kefir elevated levels of isoflavone aglycones and phenolic acids, boosting its free radical-scavenging and ferric reducing antioxidant power abilities [Azi *et al.*, 2020]. These studies underscore the potential for exploring diverse substrates to create innovative water kefir products.

Edible bird's nest (EBN) is a highly prized delicacy in China, Malaysia, Indonesia, Thailand, Vietnam, and the Philippines. It is produced from the nests built by swiftlets belonging to the *Aerodramus* and *Collocalia* genera, which are plentiful in Southeast Asian countries [Dai *et al.*, 2021]. Known as one of the most expensive animal-derived products, EBN can cost between $1,000 to $10,000 *per* kilogram, due to its reputed nutritional and medicinal benefits. The primary composition of EBN is proteins, predominantly glycoproteins, accounting for 62–63% of EBN dry weight [Kathan & Weeks, 1969; Marcone, 2005]. Besides proteins, it contains other components such as carbohydrates (25.62–27.26%), minerals (2.1%), and lipids (0.14%–1.28%) [Marcone, 2005]. The glycoproteins include 9% of sialic acid, 7.2% of galactosamine, 5.3% of glucosamine, 16.9% of galactose, and 0.7% of fructose [Kathan & Weeks, 1969]. Traditionally, EBN has been used in folk medicine to help clear phlegm, reduce coughing, combat fatigue, and aid recovery after surgery. Modern scientific studies have highlighted its antioxidant, antiviral, antibacterial, anti-tyrosinase, anti-aging, and immune-boosting properties [Dai *et al.*, 2021].

EBN is conventionally prepared for soup through double boiling; however, this method results in low solubility, limiting its nutritional and therapeutic potential. Alternative techniques such as over-boiling and enzymatic hydrolysis have been explored to address these issues [Wong *et al.*, 2017]. As fermentation with water kefir has been applied to improve the biological properties of foods [Tu *et al.*, 2019], the current study attempts to investigate EBN water kefir, focusing on its antioxidant, anti-tyrosinase, and probiotic growth-promoting properties. Additionally, total phenolic content and protein profile are analyzed to understand how changes in phenolics and proteins influence the biological effects of the fermented product. Our findings aim to develop an effective method for producing a health-promoting beverage and making EBN-based products more affordable and accessible worldwide.

MATERIALS AND METHODS

Preparation of edible bird's nest water kefir

White, house-farmed EBN cups were kindly supplied by Phuoc Tin Development Trading Service Co., Ltd. (Ho Chi Minh city, Vietnam). The EBN slurry was prepared by boiling 0.5 g of EBN in 100 mL of distilled water (0.5%, *w/v*) for 3 h to enhance the solubility of the EBN glycoproteins and thereby facilitate fermentation. To create the starter culture, 15 g of water kefir grains, locally sourced from Ho Chi Minh City, Vietnam, were added to 300 mL of a 10% brown sugar solution and allowed to ferment at 25°C for 3 days as previously described [Alrosan *et al.*, 2023]. After fermentation, 10 mL of the liquid starter culture were mixed with 90 mL of water or 90 mL of EBN slurry (10%, *v/v*) in a glass jar covered with sterilized cheesecloth. Fermented samples were collected every 2 days over an 8-day period, boiled at 100°C for 30 min, then centrifuged at 13,751×*g* for 15 min to separate the supernatants. The boiling and centrifugation steps were performed for all experiments, except for the direct counting of viable microorganisms. The pH of the supernatants was adjusted to 7.0. The EBN water kefir samples collected at day 0, 2, 4, 6, and 8 of fermentation are herein referred to as D0, D2, D4, D6, and D8, respectively. The water kefir controls harvested at day 0, 2, 4, 6, and 8 of fermentation showed similar results across all assays. Therefore, we presented representative data designated as control 1 in the figures for clarity and simplicity. Control 2, boiled EBN without starter culture, was stored at 4°C until all the EBN water kefirs and control 1 were prepared. The preparation of EBN water kefir and controls is summarized in **Figure 1**.

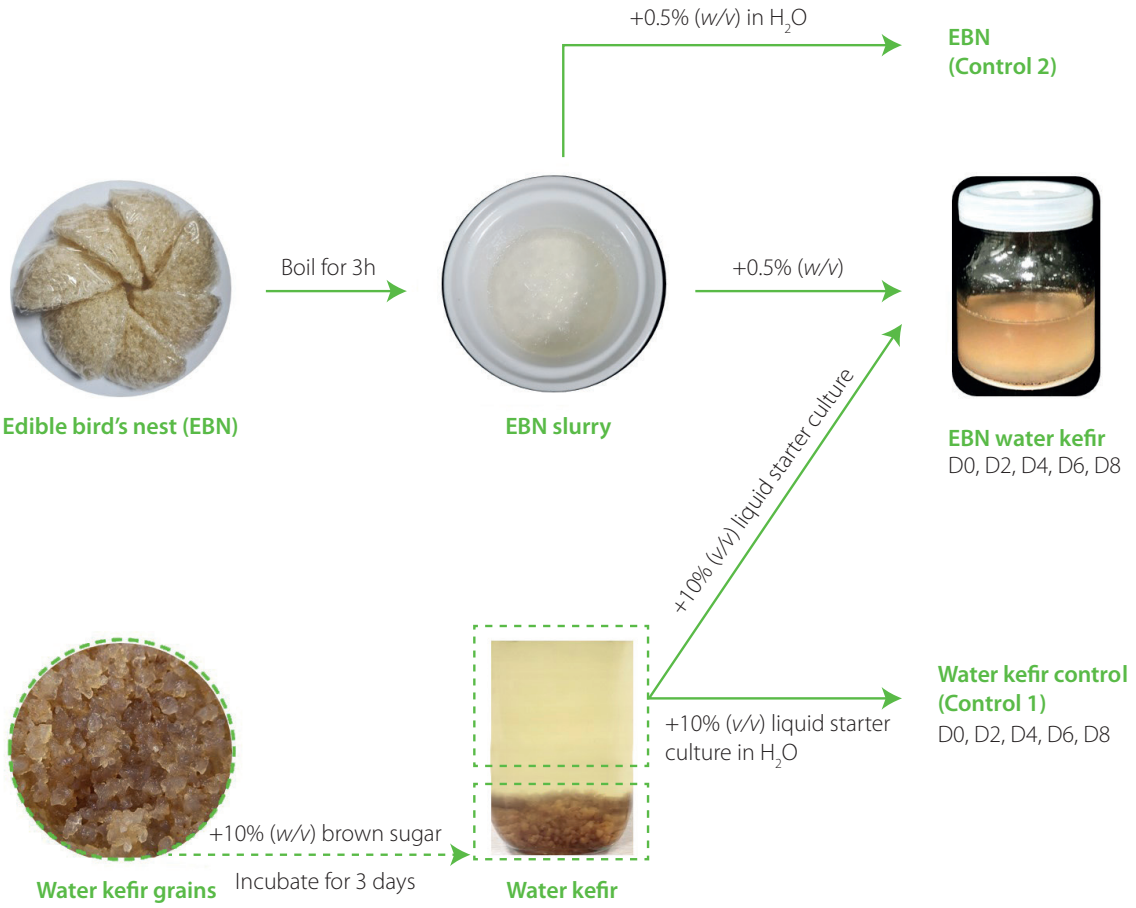

Figure 1. Preparation of edible bird's nest (EBN) water kefir.

Determination of pH and total soluble solid content

The pH values of the control 1 and EBN water kefir samples (D0, D2, D4, D6, and D8) were assessed using a pH meter (Mettler-Toledo International, Inc., Greifensee, Switzerland). The total soluble solid content (°Brix) in the samples were measured with a refractometer (Hana Instruments, Cluj Napoca, Romania).

Determination of microbial growth

The control 1 and EBN water kefir samples (D0, D2, D4, D6, and D8) were diluted in phosphate buffered saline (PBS; Sigma-Aldrich Pte. Ltd., Singapore). The populations of lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts were enumerated by plating the diluted samples on specific agar media: de Man-Rogosa-Sharpe (MRS) agar (Oxoid Ltd., Cheshire, UK) supplemented with 400 mg/L of cycloheximide (Sigma-Aldrich Pte. Ltd.) for LAB; glucose-yeast extract-calcium carbonate (GYC) agar (composed of 40 g/L of glucose, 10 g/L of yeast extract, 30 g/L of $CaCO_3$, and 15 g/L of agar) supplemented with 400 mg/L of cycloheximide for AAB; and potato dextrose (PD) agar (Oxoid Ltd.) for yeasts [Guangsen *et al.*, 2021]. The plates were incubated at 35°C for 2 days for bacterial cultures and at 25°C for 2 days for yeast cultures. Microbial populations were expressed as colony-forming units (CFU) *per* mL of the samples.

Determination of antioxidant activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$)-scavenging capacity was determined following the previously established protocol [Nguyen *et al.*, 2024b]. In brief, EBN water kefirs and control 2 were diluted to different concentrations (0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, and 0.5 mg/mL of the original EBN amount). For the assay, 900 µL of the $ABTS^{\bullet+}$ solution were mixed with 100 µL of each diluted sample and incubated at room temperature for 15 min in the dark. The absorbance was then measured at 734 nm using a microplate reader (VICTOR Nivo 3F, PerkinElmer, Waltham, MA, USA). The capacity of the samples to scavenge $ABTS^{\bullet+}$ radicals was determined using Equation (1):

$$ABTS^{\bullet+}\text{-scavenging capacity (\%)} = 100 - 100 \times (A_2 - A_3)/A_1 \quad (1)$$

where: A_1 is absorbance of the $ABTS^{\bullet+}$ solution with Milli-Q water instead of a sample, A_2 is absorbance of the sample after reacting with $ABTS^{\bullet+}$, and A_3 is absorbance of the sample without $ABTS^{\bullet+}$ (blank).

For the hydroxyl radical ($^{\bullet}OH$)-scavenging assay, 100 µL of diluted EBN water kefirs and control 2 (at concentrations of 0.125, 0.25, 0.5, 1, and 2 mg/mL of the original EBN amount) were incubated with 20 µL of 9 mM salicylic acid for 20 min in a 96-well

plate [Nguyen *et al.*, 2025b]. Afterward, each well received 50 µL of a mixture containing 9 mM ferrous sulfate ($FeSO_4$) and 100 µL of 8 mM hydrogen peroxide (H_2O_2). The absorbance was then recorded at 530 nm using a microplate reader (VICTOR Nivo 3F, PerkinElmer). The •OH-scavenging capacity was determined using Equation (2):

$$\text{•OH-scavenging capacity (\%)} = 100 - 100 \times (A_2 - A_3)/A_1 \quad (2)$$

where: A_1 is absorbance of negative control (Milli-Q water), A_2 is absorbance of the sample after reacting with salicylic acid, and A_3 is absorbance of the sample without salicylic acid.

Determination of anti-tyrosinase activity

The tyrosinase inhibitory assay was conducted following a previously published method [Nguyen *et al.*, 2024a]. In brief, 30 µL of mushroom tyrosinase enzyme (250 U/mL in PBS buffer, pH 6.8) (Sigma-Aldrich Pte. Ltd.) were combined with 100 µL of EBN water kefirs and control 2 at a concentration of 1.25 mg/mL of initial EBN in a 96-well plate. This mixture was then incubated in the dark at 28°C for 10 min. Subsequently, 110 µL of L-tyrosine substrate (0.3 mg/mL in PBS buffer, pH 6.8) (Sigma-Aldrich Pte. Ltd.) were added to each well. After another 10-min incubation at 28°C, the inhibitory activity of tyrosinase was measured by recording absorbance at 480 nm using a microplate reader (VICTOR Nivo 3F, PerkinElmer), to monitor dopachrome formation. The tyrosinase inhibitory capacity (TIC) of the samples was calculated as follows (Equation 3):

$$\text{TIC (\%)} = 100 \times [(A_1 - A_3) - (A_2 - A_4)]/(A_1 - A_3) \quad (3)$$

where: A_1 is absorbance of negative control (Milli-Q water instead of a sample), A_2 is absorbance of sample after reacting with tyrosinase, A_3 is absorbance of the blank for the negative control without L-tyrosine substrate, and A_4 is absorbance of the blank for the sample without L-tyrosine substrate.

Assessment of the growth of *Lactobacillus acidophilus* and *Lactococcus lactis*

The promoting potential of the EBN water kefir samples on the growth of the probiotic *L. acidophilus* ATCC 4356 and two potential probiotic strains isolated from Chinese cabbage (*Brassica rapa* subsp. Pekinensis), *L. lactis* VLC.1 and *L. lactis* VLC.2, was assessed as previously described with some modifications [Nguyen *et al.*, 2025b; Vu-Quang *et al.*, 2024]. Following inoculation into a broth containing 2/3 de Man-Rogosa-Sharpe (MRS) and 1/3 tryptic soy (TS) (Oxoid Ltd.) and incubation at 37°C for 24 h, the bacterial suspension was diluted to approximately 10^6 CFU/mL. A 100 µL aliquot of this suspension was then treated with a mixture containing 40 µL of each sample at a concentration of 1 mg/mL of initial EBN, combined with 60 µL of the 2/3 MRS and 1/3 TS medium. Optical density at 600 nm (OD_{600}) measurements were taken every 2 h over a 24-h incubation period at 37°C to generate bacterial growth curves. After 24 h, probiotic growth was quantified by performing decimal serial dilutions in PBS buffer, followed by plating on 2/3 MRS and 1/3 TS agar plates, and counting the resulting colony-forming units (CFU/mL).

Determination of total phenolic content

The total phenolic content (TPC) in the samples was determined *via* the colorimetric assay with the Folin-Ciocalteu reagent [Singleton *et al.*, 1999]. To begin, 400 µL of each sample of EBN water kefirs and control 2 was mixed with 400 µL of 10% Folin-Ciocalteu reagent (Sigma-Aldrich Pte. Ltd.). Subsequently, 400 µL of 10% sodium carbonate (Na_2CO_3) was added to the mixture, which was then incubated at 40°C for 30 min. The absorbance was measured at 765 nm using a UV spectrophotometer (Genway Biotech, San Diego, CA, USA). Gallic acid (Sigma-Aldrich Pte. Ltd.) was used as the standard to plot a calibration curve. The equation of the calibration curve was $y=0.021x+0.030$ ($R^2=0.9995$). The TPC results were expressed as µg of gallic acid equivalents (GAE) *per* mL.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following a previous protocol [Gallagher, 2006]. A volume of 1 mL of each sample (D0, D2, D4, D6, and D8) was freeze-dried to obtain a powder. These powders were then mixed with 20 µL of SDS-PAGE loading buffer, heated at 90°C for 30 min to denature the proteins, and subsequently loaded onto a 12.5% separating gel. Electrophoresis was conducted using a Bio-Rad Laboratories system (Bio-Rad Laboratories, Hercules, CA, USA). After separation, the gel was stained with Coomassie Brilliant Blue for 2 h and then destained with a solution containing 10% absolute ethanol and 10% acetic acid to visualize the protein bands. A color prestained protein standard (NEB, Ipswich, MA, USA) was used for molecular weight determination.

Sensory evaluation

The training procedure for panelists and the sensory evaluation test were conducted as previously described [Nguyen *et al.*, 2025a]. Samples of original EBN water kefir (D0, D2, D4, D6, and D8) and water kefir (control 1) sweetened with 10% (*v/v*) rose syrup (Monin, *Bourges, France*) were assessed for sensory qualities, including appearance, color, odor, sourness, taste, and overall acceptability, by a panel of 10 trained evaluators (comprising 5 women and 5 men aged 20 to 40 years) at NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam. A 9-point hedonic scale, where 1 indicates "dislike extremely" and 9 indicates "like extremely" was used to measure each sensory attribute. The results provided an initial evaluation of the sensory attributes of EBN water kefirs. Additional studies involving a larger number of participants are needed to obtain more definitive insights into consumer preferences and the overall acceptability of the product.

Statistical analysis

All analyses were performed on samples from three independent fermentations. Statistical comparisons in the assays were conducted using Student's t-test (GraphPad Prism software, version 5.0, Insightful Science LLC, San Diego, CA, USA). A $p \leq 0.05$ was considered indicative of statistical significance.

RESULTS AND DISCUSSION

pH value, total soluble solid content, and microbial growth of edible bird's nest water kefir during fermentation

During the fermentation process, pH values dramatically dropped from 5.0 at the initial time of fermentation (D0) to approximately 2.5 at day 8 of fermentation (D8), indicating robust fermentation activity in EBN water kefir (**Figure 2A**). The observed decrease in pH in our study is consistent with results from other substrates fermented with water kefir, which is due to the microbial production of organic acids such as lactic acid, acetic acid, gluconic acid, citric acid, and butyric acid [Chomphoosee *et al.*, 2025; Lee *et al.*, 2025; Tireki, 2022]. These organic acids play a crucial role in water kefir by imparting a balanced tangy flavor, extending shelf life, and potentially inhibiting harmful gut bacteria through their antimicrobial effects [Lee *et al.*, 2025]. Conversely, in the water kefir control (control 1), where EBN was replaced with water, pH levels did not decrease significantly during fermentation (**Figure 2A**). This is likely due to the limited nutrient availability, which slowed microbial activity and subsequent organic acid production.

In contrast to pH, the total soluble solid content changed minimally over the fermentation period. At the start of fermentation, the content of total soluble solids was already low (approximately 1.3°Brix) because sugar was not added to the medium in our experiment. During fermentation, there was a slight decrease of about 0.2°Brix, reaching 1.1°Brix at day 6 and day 8,

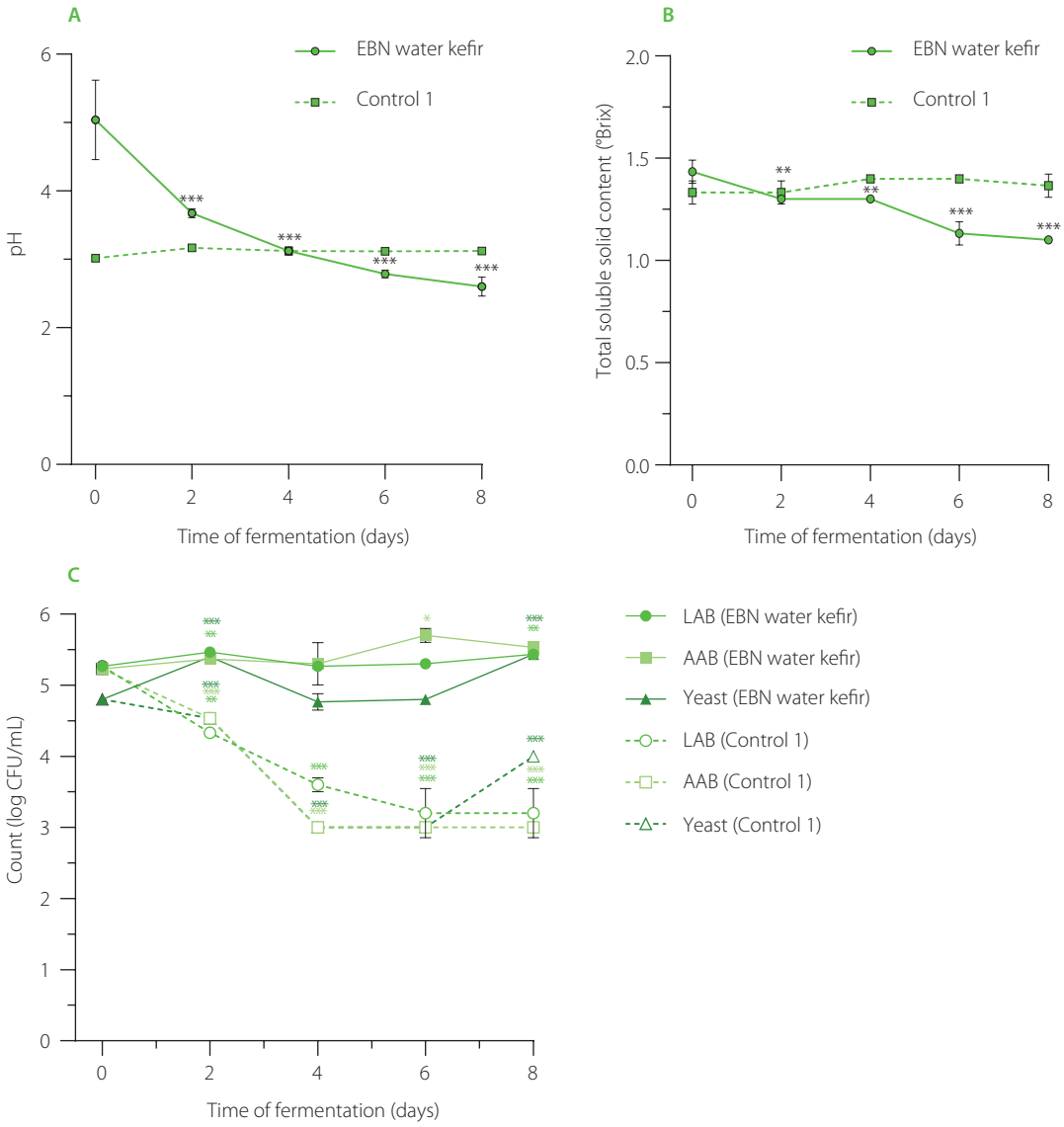

Figure 2. (**A**) pH value, (**B**) total soluble solid content (°Brix), and (**C**) microbial growth of edible bird's nest (EBN) water kefir and control 1 (EBN replaced with water) during fermentation. Each data point is presented as mean ± standard deviation (n=3). Asterisks indicate significant differences between water kefir at the fermentation time point and on day 0 determined by Student's t-test at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). LAB, lactic acid bacteria; AAB, acetic acid bacteria.

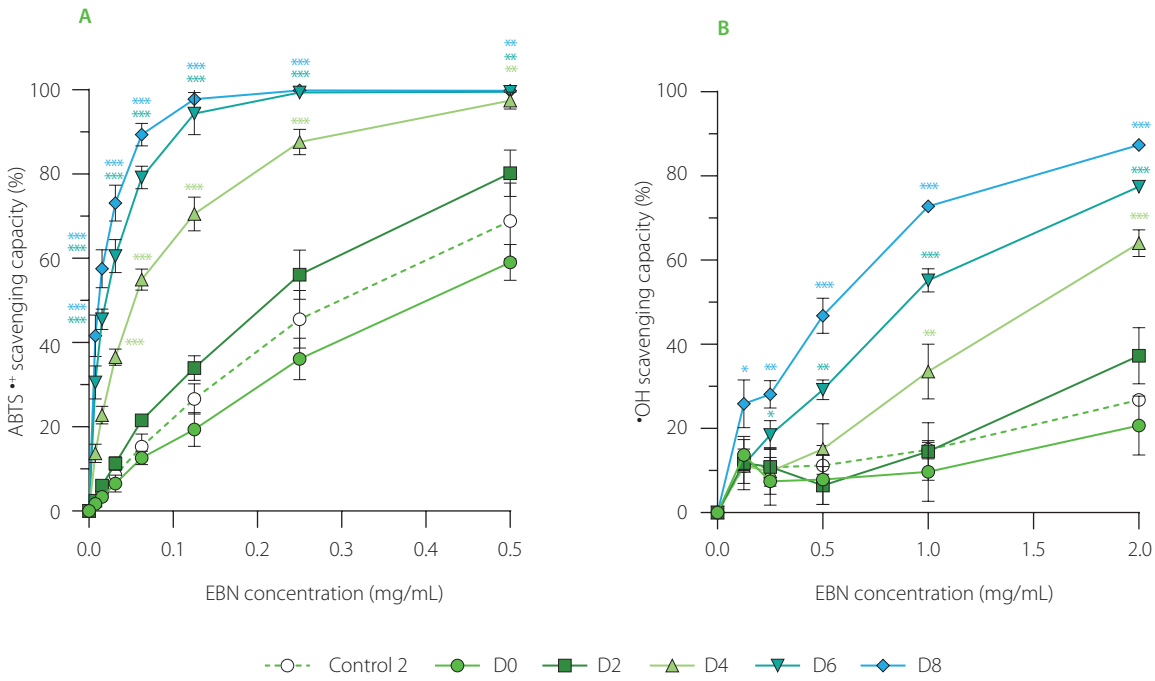

Figure 3. (**A**) ABTS$^{\bullet+}$-scavenging capacity and (**B**) $^{\bullet}$OH-scavenging capacity of edible bird's nest (EBN) water kefir and control 2 (EBN without starter culture) during fermentation. Each data point is presented as mean ± standard deviation (n=3). D0, D2, D4, D6, and D8; EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively. Asterisks indicate significant differences between EBN water kefir and control 2 at each test concentration determined by Student's t-test at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

while no change was observed in the water kefir control sample (**Figure 2B**). The reduction in total soluble solid content in EBN water kefir was consistent with a previous observation, which showed a decrease from 1.5ºBrix to 1.2ºBrix during whey protein fermentation with water kefir [Alrosan *et al.*, 2023]. Catalytic metabolism of the protein components may be a factor contributing to the decrease in total soluble solids in both fermented products. In EBN water kefir, the degradation of amino acids and minerals in EBN during fermentation could also contribute to this reduction [Gong *et al.*, 2025]. The low total soluble solid content in EBN water kefir suggests that it could be beneficial for individuals seeking to reduce their sugar intake.

Regarding microbial growth, the populations of LAB and yeast in EBN water kefir increased after 2 days of fermentation, while AAB rose after 6 days, indicating that EBN provides sufficient nutritional content to support microbial activity (**Figure 2C**). It is plausible that, in a low-sugar medium, yeasts initially degrade glycoproteins in EBN by enzymatically breaking down glycans from the protein core [Hirayama *et al.*, 2019]. The sugars released from these glycans then serve as a carbon source for LAB. Additionally, LAB secrete proteinases to hydrolyze proteins into peptides, which are transported into their cells for further breakdown into amino acids [Raveschot *et al.*, 2018]. Ethanol and lactic acid, produced as secondary metabolites from yeast and LAB fermentation, are utilized by AAB, leading to a delayed increase in AAB populations compared to yeasts and LAB [Yassunaka Hata *et al.*, 2023]. The pattern of microbial growth varies depending on the substrate's composition. For example, in soy whey water kefir, LAB, AAB, and yeasts increased after 2 days and remained relatively stable up to day 4 [Tu *et al.*, 2019]. Conversely, in whey protein water kefir, all microbial populations rose at day 2, but yeast populations declined by day 4 [Alrosan *et al.*, 2023]. Despite these differences, the interactions among microorganisms across all substrates enable coexistence and collective functioning [Tu *et al.*, 2019]. In contrast, in the water kefir control, populations of LAB and AAB decreased over prolonged fermentation, while yeasts initially declined during the first 6 days but showed a slight resurgence at day 8, likely due to their ability to utilize bacterial byproducts as nutrients in a nutrient-deprived environment (**Figure 2C**).

■ Antioxidant capacity of edible bird's nest water kefir

Free radicals, including hydroxyl radical, superoxide anion radical, nitric oxide radical, and peroxynitrite radical, are independently existing molecular species with unpaired electrons. They are unstable and rapidly attack lipids, proteins, and nucleic acids, leading to cell damage and disruption of homeostasis [Lobo *et al.*, 2010]. While free radicals are essential for cell signaling and immune defense, excessive levels can be detrimental, resulting in oxidative stress and contributing to cardiovascular diseases, neurodegenerative disorders, cancer, and inflammatory diseases [Srivastava & Kumar, 2015]. Therefore, antioxidant agents, particularly those derived from natural foods that can scavenge free radicals, have attracted significant attention for their potential in preventing and treating free radical-related health issues. Given that the free-radical scavenging capacity

has been demonstrated in many water kefir products, we aimed to investigate this property in EBN water kefir [Chomphoosee *et al.*, 2025; Gökırmaklı *et al.*, 2025].

Both $ABTS^{\bullet+}$- and $^{\bullet}OH$-scavenging assays indicated that fermenting EBN with water kefir enhanced its antioxidant capacity (**Figure 3**). The EBN control (control 2) and non-fermented control (D0) removed approximately 35%–45% of $ABTS^{\bullet+}$ radicals at a concentration of 0.25 mg/mL of initial EBN, and 10%–15% of $^{\bullet}OH$ radicals at a concentration of 1 mg/mL. In contrast, EBN fermented for 2 (D2), 4 (D4), 6 (D6), and 8 (D8) days showed significant increases in scavenging capacity, with $ABTS^{\bullet+}$ radical inhibition rising to 55%, 85%, and 100%, and $^{\bullet}OH$ radical inhibition to 18%, 35%, 55%, and 75%, respectively. The pattern of increasing free radical-scavenging capacity during fermentation with water kefir was similar to the trends observed in coffee cherry and red beetroot juice water kefirs [Chomphoosee *et al.*, 2025; Wang & Wang, 2023].

The enhancement of the antioxidant capacity of EBN can be attributed to the production or release of phenolic compounds, exopolysaccharides, and bioactive peptides during fermentation with water kefir, as these bioactive substances are recognized as key free-radical scavengers in kefir products [Chomphoosee *et al.*, 2025; Hasheminya & Dehghannya, 2020]. Chomphoosee *et al.* [2025] suggested that the ability of coffee cherry water kefir to inhibit free radicals may be linked to higher levels of phenolic acids, including hydroxycinnamic acids and hydroxybenzoic acids. In a study by Hasheminya & Dehghannya [2020], kefiran, a water-soluble polysaccharide, was capable of inhibiting over 70% of DPPH radicals at a low concentration of 0.8 mg/mL, indicating its effectiveness as a free-radical scavenger. Additionally, bioactive peptides have also been proposed as active contributors to the free radical-scavenging property of milk kefir [Malta *et al.*, 2022]. It would be worthwhile to identify specific bioactive compounds responsible for the antioxidant activity of EBN water kefir in future investigations.

Water kefir intake has shown antioxidant effects in a mouse model. In a study by Kumar *et al.* [2021], mice given 2.5 mL/kg/day of water kefir exhibited no signs of toxicity, behavioral changes, or adverse effects, while displaying elevated levels of superoxide dismutase (SOD), enhanced ferric reducing antioxidant power (FRAP), and decreased nitric oxide (NO) levels in brain and kidney tissues. Similarly, Falsoni *et al.* [2022] found that pretreatment with water kefir provided gastroprotection against HCl/ethanol-induced ulcers in mice by reducing protein oxidation and boosting the activity of antioxidant enzymes such as SOD and catalase. These findings suggest that further *in vivo* research into the antioxidant properties of EBN water kefir could reveal its potential health benefits.

Anti-tyrosinase activity of edible bird's nest water kefir

Tyrosinase is the key enzyme responsible for melanin synthesis, which determines the coloration of skin, hair, and eyes [Qian *et al.*, 2020]. Inhibiting tyrosinase is valuable in the cosmeceutical sector for skin whitening and addressing melanin-related skin issues such as freckles, melasma, age spots, as well as certain skin cancers [Kim & Uyama, 2005]. In addition, tyrosinase inhibitors are relevant for the food industry to prevent enzymatic browning of fruits [Peng *et al.*, 2023]. Although the anti-tyrosinase activity has been documented in various fermented broths, reports on water kefir are scarce [Abd. Razak *et al.*, 2021; Lee *et al.*, 2012]. This led us to investigate this property in EBN water kefir.

Our findings showed that at day 2 of fermentation, the ability of EBN water kefir to suppress tyrosinase was comparable to that of EBN (control 2) and unfermented EBN (D0), reducing activity by about 20% (**Figure 4**). As fermentation time extended beyond day 2, the anti-tyrosinase effect significantly increased, reaching approximately 55% inhibition at day 8. This indicates that fermenting EBN with water kefir enhances its capacity to inhibit tyrosinase.

In fermented products, phenolic compounds are key components contributing to tyrosinase inhibition. The contents of total phenolics and total flavonoids increased from 10.1 mg/mL and 9.7 µg/mL, respectively, in non-fermented red ginseng to 14.3 mg/mL and 133.2 µg/mL in the fermented version [Lee *et al.*, 2012]. Likewise, fermented broken rice after 18 days contained over 130 times more total phenolics than its non-fermented counterpart [Abd. Razak *et al.*, 2021]. These increases correlate with stronger tyrosinase inhibitory activity observed in both fermented red ginseng and broken rice [Abd. Razak *et al.*, 2021; Lee *et al.*, 2012]. Certainly, the potent anti-tyrosinase activity of our EBN water kefir may also result from the production or release of phenolic compounds during fermentation.

Research has shown that tyrosinase inhibitors decrease melanin production by directly interacting with tyrosinase through four different mechanisms: binding to the free enzyme to prevent substrate attachment at the active site (competitive

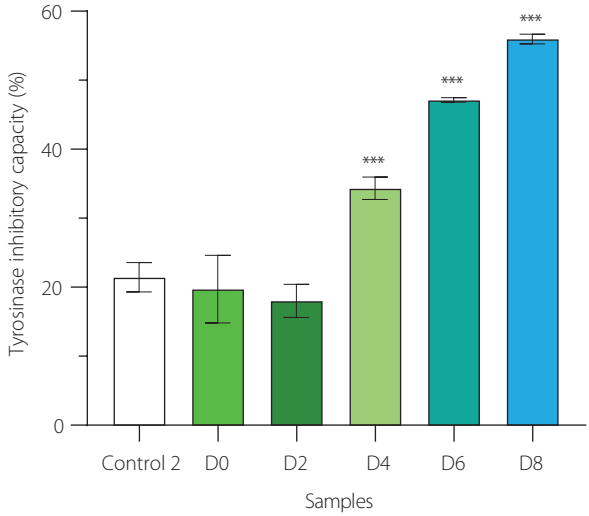

Figure 4. Anti-tyrosinase activity of edible bird's nest (EBN) water kefir and control 2 (EBN without starter culture) during fermentation. Each data bar is presented as mean ± standard deviation (n=3). D0, D2, D4, D6, and D8; EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively. Asterisks indicate significant differences between EBN water kefir and control 2 determined by Student's t-test at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

mechanism); binding exclusively to the enzyme-substrate complex (uncompetitive mechanism); binding to both the free enzyme and the enzyme-substrate complex (mixed mechanism); or binding to the free enzyme and the enzyme-substrate complex with equal affinity (noncompetitive mechanism) [Panzella & Napolitano, 2019]. Additionally, a tyrosinase inhibitor may also lower melanin synthesis by modulating the expression of genes involved in melanogenesis, especially the key regulator microphthalmia-associated transcription factor (MITF) [Yu *et al.*, 2022]. Given that EBN water kefir in our study demonstrates tyrosinase inhibitory activity, it would be valuable to explore its specific mechanism of action on tyrosinase and its influence on the molecular pathways governing melanogenesis in future research.

Effect of edible bird's nest water kefir on the growth of *L. acidophilus* and *L. lactis*

The potential of EBN water kefir to promote the growth of probiotics was evaluated using *L. acidophilus* ATCC 4356, a well-known lactic acid bacterium that supports human gut health and two probiotic potential strains, *L. lactis* VCL.1 and VCL.2, isolated from Chinese cabbage *Brassica rapa* subsp. Pekinensis [Sarikhani *et al.*, 2018; Vu-Quang *et al.*, 2024]. Our experiments revealed notable differences between control groups (water kefir control (control 1), EBN (control 2), water (control 3), and non-fermented EBN (D0)) and fermented EBN at day 2 (D2), 4 (D4), 6 (D6), and 8 (D8) (**Figure 5**). These results suggest that EBN fermentation with water kefir enhances the viability of *L. acidophilus* and *L. lactis*, potentially contributing to improved host

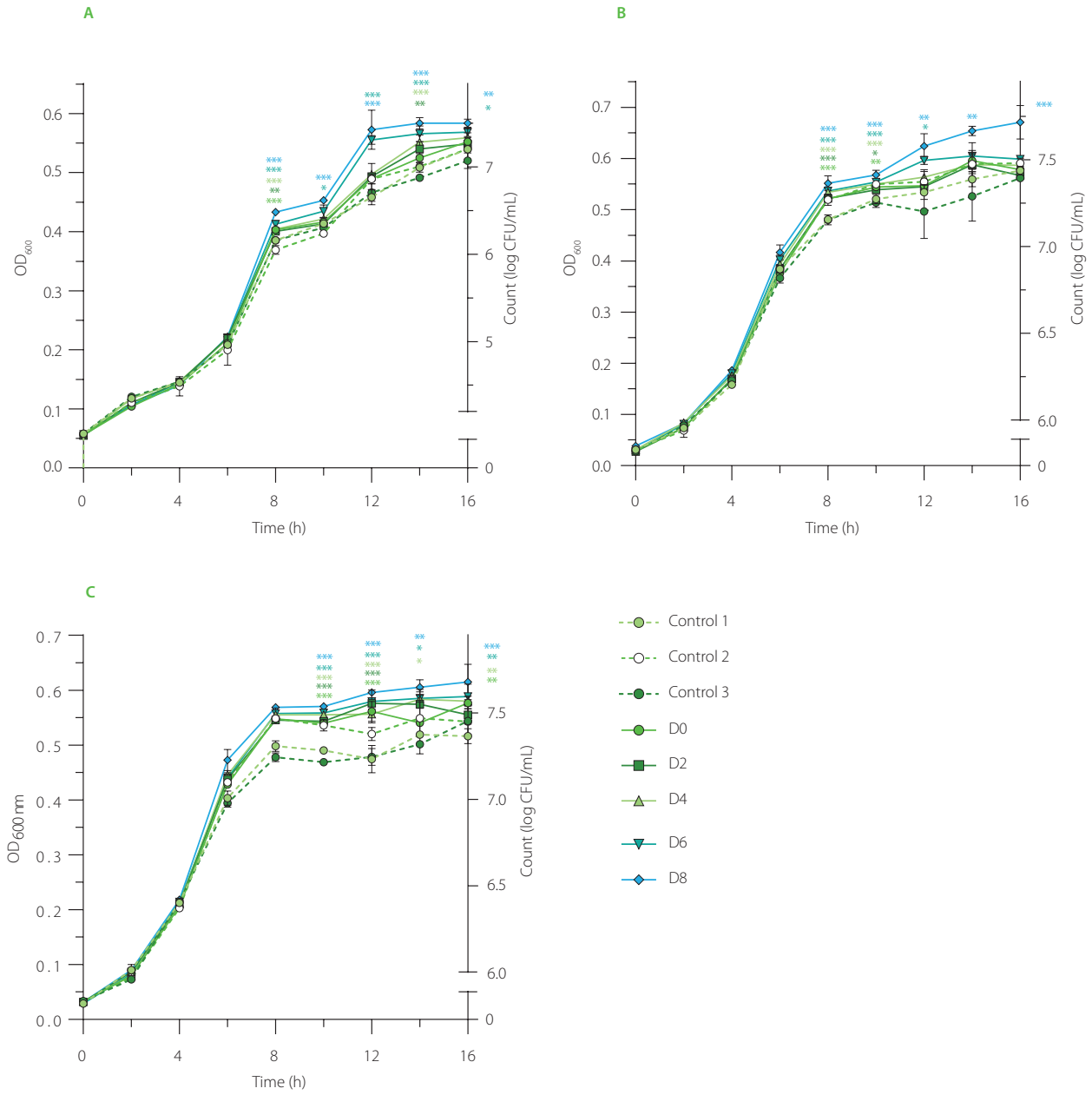

Figure 5. The ability of edible bird's nest (EBN) water kefir to promote the growth of (**A**) *Lactobacillus acidophilus* ATCC 4356, (**B**) *Lactobacillus lactis* VCL.1, and (**C**) *Lactobacillus lactis* VCL.2. Control 1, water kefir control; control 2, EBN without starter culture; control 3, water; D0, D2, D4, D6, and D8, EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively. OD_{600}, optical density at 600 nm. Asterisks indicate significant differences between EBN water kefir and control 3 at each time point determined by Student's t-test at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

health. It is also worth noting that a slight increase in the bacterial growth was detected in the EBN (control 2) sample, indicating that it contains ingredients capable of stimulating the growth of *L. acidophilus*. In EBN, this intrinsic property is likely due to the presence of protein-attached glycan chains, which have been shown to foster the growth of probiotics such as *Bifidobacteria* and *Bacteroides-Prevotella*, while also inhibiting key pathogens like *Clostridium histolyticum* [Babji & Daud, 2021; Daud *et al.*, 2019].

The increased activity of promoting the growth of *L. acidophilus and L. lactis* observed in EBN through water kefir fermentation may be attributed to bioactive compounds released during the process, including exopolysaccharides and phenolics. In a study by Tan *et al.* [2022a], *Lactobacillus satsumensis* strains isolated from water kefir grains were shown to produce α-glucan exopolysaccharides. When hydrolyzed polysaccharides by these strains are combined with kefir probiotics in synbiotic formulations, they can further enhance health benefits by promoting the growth of *Bacteroidetes* and increasing the production of beneficial short-chain fatty acids such as acetate, propionate, and butyrate [Tan *et al.*, 2022a].

Phenolics with prebiotic potential have not yet been identified in water kefir samples; however, their contribution to this property should be taken into account, as they have been indicated in fermented foods [Yang *et al.*, 2023]. In one study, Budryn *et al.* [2019] found that fermentation of legume sprouts with lactic acid bacteria significantly increased the content of isoflavones and the number of lactic acid bacteria while decreasing the number of molds and pathogenic bacteria. In another study by Zhou *et al.* [2021], polyphenols from Fu brick tea, a post-fermented dark tea, were shown to attenuate gut microbiota dysbiosis in high-fat diet-fed rats by improving key intestinal microbes such as *Akkermansia muciniphila*, *Alloprevotella*, *Bacteroides*, and *Faecalibaculum*, as well as reducing intestinal oxidative stress, inflammation, and enhancing the integrity of the intestinal barrier [Zhou *et al.*, 2021]. Overall, our study emphasizes the potential of EBN water kefir to promote the growth of *L. acidophilus* and *L. lactis*, highlighting its promise as a prebiotic candidate for further investigation on human gut microbiota.

Total phenolic content in edible bird's nest water kefir

In our study, EBN (control 2), non-fermented and 2-day fermented EBN exhibited low total phenolic contents (TPC) of approximately 50-200 µg GAE/mL. As fermentation progressed beyond 2 days, the TPC significantly increased to 500; 1,300; and 2,100 µg GAE/mL at day 4, 6, and 8 of fermentation, respectively (**Figure 6**). The TPC levels in EBN water kefir were higher than those in vegetable water kefirs but lower than in coffee cherry water kefir, likely due to differences in the types and amounts of phenolic compounds present in the fermenting substrates [Chomphoosee *et al.*, 2025; Corona *et al.*, 2016]. The upward trend in TPC during fermentation closely mirrored the increases in antioxidant activity, anti-tyrosinase, and prebiotic effects observed in EBN water kefir, suggesting these biological properties may be associated with the phenolic compounds present.

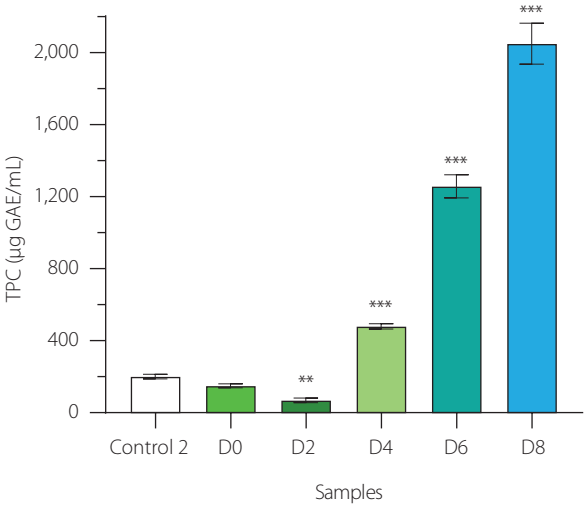

Figure 6. Total phenolic content (TPC) in edible bird's nest (EBN) water kefir and control 2 (EBN without starter culture) during fermentation. Each data bar is shown as mean ± standard deviation (n=3). D0, D2, D4, D6, and D8; EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively. GAE, gallic acid equivalent. Asterisks indicate significant differences between EBN water kefir and control 2 determined by Student's t-test at $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

Higher phenolic levels in fermented foods are often due to enzymatic activity from microorganisms, such as tannases, esterases, phenolic acid decarboxylases, and glycosidases, which facilitate the biotransformation of phenolic compounds into more bioactive molecules [Yang *et al.*, 2023]. For example, in coffee cherry fermentation with water kefir, chlorogenic acid, which is formed by an ester bond between caffeic acid and quinic acid, may be hydrolyzed by microbial cinnamic esterase into caffeic acid and quinic acid. Furthermore, caffeic acid can be converted into ferulic acid *via* the phenolic acid biosynthetic pathway. Since ferulic acid exhibits superior free radical scavenging activity compared to chlorogenic acid, its formation in coffee cherry water kefir could partly explain the enhanced antioxidant capacity of this beverage [Chomphoosee *et al.*, 2025; Yang *et al.*, 2023]. In a similar scenario, during EBN fermentation with water kefir, microbial metabolism of phenolic compounds may lead to increased TPC levels, which likely contribute to the improved antioxidant, anti-tyrosinase, and prebiotic properties of the fermented product.

Protein profile of edible bird's nest water kefir during fermentation

EBN contains a majority of proteins that microorganisms in water kefir grains can metabolize to produce bioactive compounds. Therefore, we examined how the protein composition of EBN changes during fermentation. SDS-PAGE analysis revealed that EBN is rich in proteins with molecular weights exceeding 10 kDa, with those over 34 kDa being predominant (**Figure 7**). After 2 and 4 days of fermentation, a significant reduction in EBN proteins was observed, likely due to microorganisms consuming these proteins as sources of carbon and nitrogen. Notably, smaller molecules with molecular weights less than 10 kDa appeared

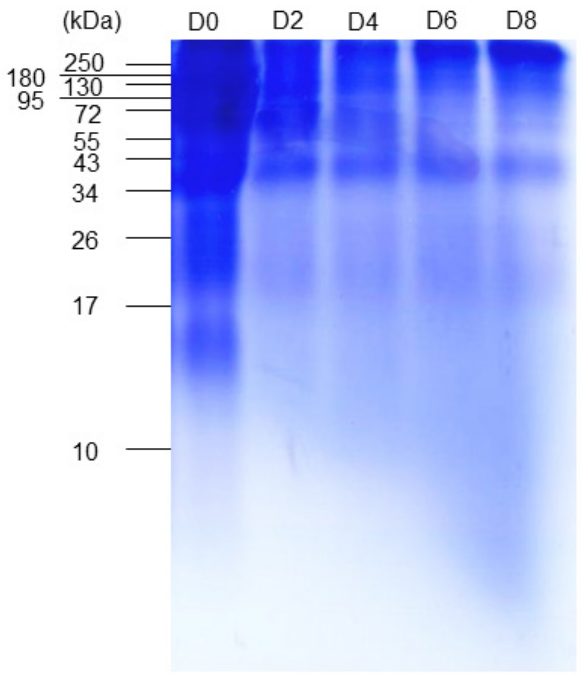

Figure 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing the protein profile of edible bird's nest (EBN) water kefir during fermentation. D0, D2, D4, D6, and D8; EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively.

at day 6 and 8 of fermentation, indicating the formation of small proteins and peptides over extended fermentation periods.

Lactobacillus bacteria require certain amino acids from their environment to grow. To acquire these, they produce cell envelope proteinases (CEPs) that break down proteins into peptides. These peptides are released into the fermentation medium and then transported into the bacteria, where internal peptidases further degrade them into amino acids [Raveschot *et al.*, 2018]. The presence of various *Lactobacillus* species capable of producing bioactive peptides, such as *L. casei*, *L. paracasei*, *L. plantarum*, and *L. kefiranofaciens*, in water kefir grains suggests that these bacteria can hydrolyze EBN proteins and release peptides into the extracellular [Moretti *et al.*, 2022; Raveschot *et al.*, 2018; Silva *et al.*, 2024]. Since bioactive peptides derived from fermented products are known to have antioxidant properties, the generation of peptides during the fermentation of EBN water kefir may contribute to enhancing its antioxidant activity [Raveschot *et al.*, 2018].

Sensory characteristics of edible bird's nest water kefir during fermentation

Sensory evaluation plays a vital role in understanding how consumers perceive a product through their senses. These assessments offer valuable insights for product development, quality assurance, and marketing strategies, ultimately affecting consumer acceptance and purchasing choices [Ruiz-Capillas & Herrero, 2021]. Since EBN water kefir was reported for the first time in our study, we conducted tests on EBN water kefir at various fermentation durations and specifically on the 8-day fermented product sweetened with rose syrup, using a 9-point hedonic scale to evaluate color, odor, sweetness, sourness, taste, appearance, and overall acceptability [Nguyen *et al.*, 2025a].

Among the samples tested, the EBN water kefir fermented for 2 days (D2) received the lowest scores across all attributes, primarily due to its lack of insufficient sourness and sweetness. The samples fermented for 4, 6, and 8 days (D4, D6, and D8) scored around 6.0 for color, sourness, taste, and appearance, indicating a "like slightly" response from tasters. The weakest aspect among these three was sweetness, which was linked to their very low sugar levels (**Figure 2**). To enhance the sensory qualities, especially given that D8 exhibited superior bioactive properties, we added rose syrup to the D8 sample to reach a final concentration of 10%, resulting in a total sugar content of approximately 8.6°Brix. After sweetening, the sample was rated as "like much" across all characteristics (**Figure 8**).

Previous studies have indicated that water kefir produced from various substrates can elicit different consumer preferences. For example, drinks made from pomegranate and rosehip juices were highly favored, while those from cherry, hawthorn, and red plum juices only received general approval [Gökırmaklı *et al.*, 2025; Ozcelik *et al.*, 2021]. In our research, the original EBN water kefir was regarded as moderately acceptable. Due to its higher levels of phenolic compounds, bioactive peptides, and enhanced antioxidant, anti-tyrosinase, and prebiotic properties compared to traditional boiled EBN, it may attract consumers looking for functional beverages with minimal sugar content. When enriched with rose syrup, the EBN water kefir was notably more preferred (**Figure 8**). This suggests promising potential for further development of EBN water kefir into a functional drink that meets the preferences of most consumers.

CONCLUSIONS

Our study is the first to demonstrate a novel EBN water kefir with enhanced abilities to scavenge free radicals, inhibit tyrosinase activity, and promote the growth of *L. acidophilus* ATCC 4356 and *L. lactis* VCL.1 and VCL.2, particularly after 8 days of fermentation. The fermentation process also increased total phenolic content and generated bioactive peptides. Evaluation tests indicate that the EBN water kefir at day 8 of fermentation achieved a "likely like" overall acceptability, while syrup-sweetening can further enhance the sensory attributes of the fermented product to "like very" on the evaluation scale. This underscores the potential of EBN water kefir as a functional beverage rich in health-promoting compounds. Additionally, the research presents a sustainable approach to reducing EBN usage compared to traditional methods while still producing a product with high nutraceutical values. This facilitates the future development of a commercial EBN water kefir beverage that is more affordable and accessible globally.

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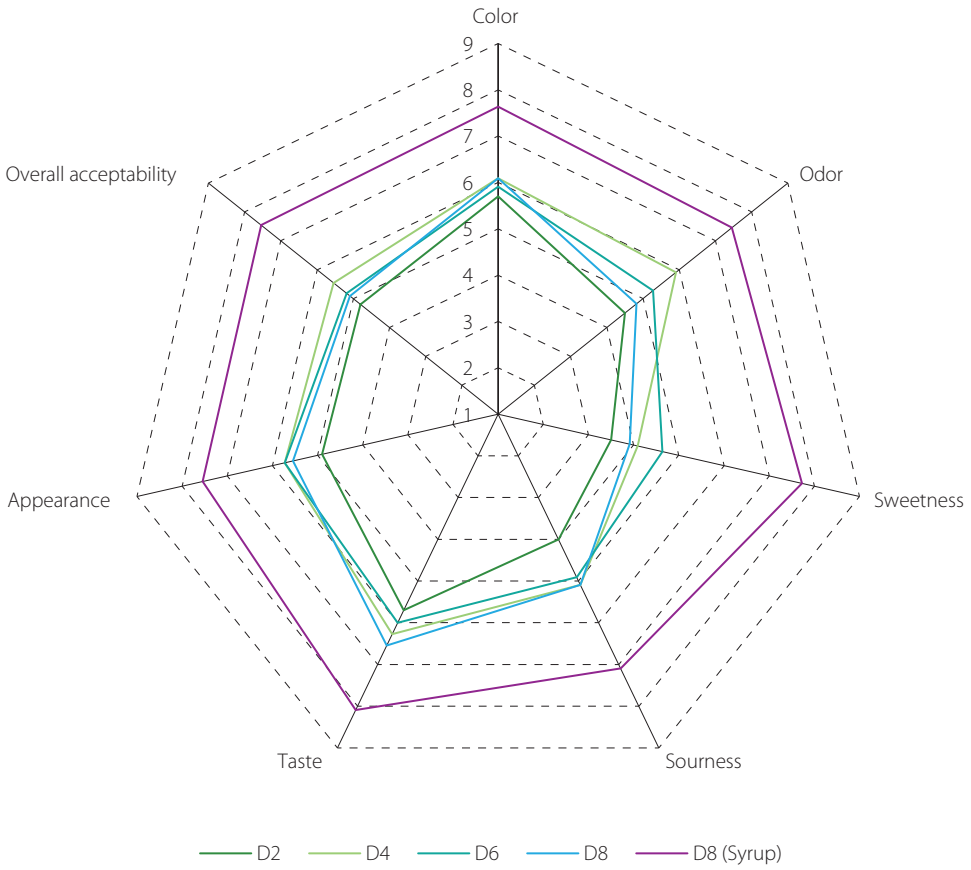

Figure 8. Sensory attributes of original and syrup-sweetened edible bird's nest (EBN) water kefir. D0, D2, D4, D6, and D8; EBN water kefir at day 0, 2, 4, 6, and 8 of fermentation, respectively.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Stevia and Steviol Glycosides in Dairy Products: An Overview on Usage, Microbial Dynamics, and Gut Health Effects – a Review Article

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Stevia rebaudiana (Bertoni), a plant belonging to the Asteraceae family, has long been valued as a natural alternative to sugar. The sweet compounds it contains, known as steviol glycosides, can be up to 450 times sweeter than sucrose. In recent years, stevia has taken a prominent place among natural additives, as rising interest in natural products continues to emphasize plant-based alternatives. This paper focused on the potential benefits of incorporating stevia into dairy products. It provides a general overview of the use of stevia as a sugar substitute, mainly recognized for its zero-calorie sweetening properties. Moreover, it highlights that the whole leaf of *S. rebaudiana* contains additional bioactive compounds, including minerals, vitamins, and antioxidants, which may contribute to its nutritional value. This study also highlights the effects of adding stevia to dairy products, both as a prebiotic that enhances the viability of lactic acid bacteria and as an additive that inhibits the growth of spoilage-causing bacteria such as *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhi, and *Staphylococcus aureus*. While contributing to intestinal well-being by promoting the growth of gut microbiota, the addition of stevia to dairy products improves their sensory properties. In dairy systems, the incorporation of stevia hay into livestock feed may also be explored as a strategy to improve milk yield and quality. Given the growing trend in the consumption of probiotic and prebiotic foods, incorporating functional ingredients such as stevia into dairy products appears increasingly relevant.

Keywords: functional foods, gut microbiota, low-calorie sweeteners, prebiotics, probiotics

ABBREVIATIONS

ADI, acceptable daily intake; ATCC, American Type Culture Collection; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CFU, colony forming units; CGMCC, China General Microbiological Culture Collection Center; CMCC, China Medical Culture Collection; DMAPP, dimethylallyl diphosphate; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; FSE, freeze-dried aqueous stevia extract; FSSAI, Food Safety and Standards Authority of India; GRAS, Generally Recognized As Safe; HT-29/MTX, Human colorectal adenocarcinoma cell line HT-29 adapted to methotrexate; IBD, inflammatory bowel disease; IPP, isopentenyl diphosphate; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MEP, methyl-D-erythritol phosphate; Reb A, rebaudioside A; SCF, Scientific Committee on Foods; SPF BALB/c, specific pathogen free bagg albino laboratory-bred mouse, subline c; TPC, total phenolic content; TI, time intensity; TDS, temporal dominance of sensations; TMA, trimethylamine; VFA, volatile fatty acid; WHO, World Health Organization.

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INTRODUCTION

The World Health Organization (WHO) strongly recommends that both adults and children limit their intake of free sugars to less than 10% of their total daily energy intake [WHO, 2015]. This recommendation aligns with a growing trend among consumers, who have become more aware of their dietary choices and increasingly seek healthier and more functional food products [Bimbo *et al.,* 2017]. The current trend in the food industry emphasizes the development of low-calorie, functional products and the incorporation of natural bio-preservatives, particularly in beverages, confectionery, and dairy items [Miele *et al.,* 2017]. Growing attention is being paid to reducing the sugar content and the use of synthetic additives in food products, largely due to the health concerns associated with synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), as well as artificial sweeteners like aspartame and saccharin [Chen & Hou, 2024; Debras *et al*., 2022, 2023; Mika *et al.,* 2023]. This increasing demand for natural alternatives has highlighted stevia as one of the most promising sweeteners, owing to its dual role as a sugar substitute and as a source of beneficial nutrients, including minerals, vitamins, and antioxidants [Gerdzhikova *et al*., 2018; Leszczyńska *et al.,* 2021; Muanda *et al.,* 2011]. Several studies have reported that stevia extracts exhibit various health-promoting properties [Peteliuk *et al*., 2021], and strong antioxidant activity [Nuryandani *et al.,* 2024]. In addition, stevia displays antibacterial activity, which can inhibit the growth of certain pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [Myint *et al.,* 2023]. Due to the growing trend in the consumption of probiotic and prebiotic products, as well as low-calorie dairy foods [Mahato *et al.,* 2020; Narayana *et al.,* 2022], stevia has emerged as a promising natural sweetener that can replace sugar in sweetened dairy products, with the added benefit of potential bio-preservative properties and a positive impact on intestinal well-being.

WORLDWIDE USE OF STEVIA

In 1941, stevia became known in Britain, where the plant was proposed as a sugar substitute during wartime shortages, however, it failed to gain wide acceptance, in part because of its licorice-like taste [Chesterton & Yang 2016]. The first commercialization of steviol glycosides as a sweetener dates back to 1971 by the Japanese company Morita Kagaku Kogyo. At that time, few people were interested in stevia [Rai & Han, 2022]. In 2007, stevia accounted for 40% of the intense sweeteners market in Japan, with an annual consumption estimated at 200 tonnes [Rajasekaran *et al.,* 2007]. In recent decades, cultivation of *S. rebaudiana* has developed in many countries namely; Paraguay, Brazil, Uruguay, Central America, China, Thailand, and India. The area under stevia cultivation has therefore increased from an estimated 20,000–25,000 ha in 2008 to 67,000–80,000 ha in 2011. Other countries are experimenting with commercial crops such as Canada, New Zealand, Australia and in Europe: Belgium, Germany, England, France, Italy, and Greece [Gautam *et al.,* 2022]. For more than ten years, stevia has been consumed in several countries such as Japan, China, Brazil, Korea, and the United States, and no significant adverse effects associated with its consumption have been reported [EFSA, 2022; Singh *et al.,* 2024].

STEVIA REBAUDIANA (BERTONI) AND STEVIOL GLYCOSIDES

Stevia rebaudiana (Bertoni) is a perennial herbaceous plant of the Asteraceae family, native to Paraguay (South America). Stevia leaves produce diterpene glycosides called steviol glycosides, which are up to 450 times sweeter than sucrose [Peteliuk *et al*., 2021]. The precursor to steviol glycosides is *ent*-13-hydroxykaur-16-en-19-oic acid, more commonly known as steviol (**Figure 1**). Its biosynthesis begins in the chloroplasts from pyruvate and glyceraldehyde-3-phosphate until the formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which ends with the methyl-D-erythritol phosphate (MEP) pathway [Moon *et al.,* 2020]. Steviol is an aglycone from the family of tetracyclic diterpenes. In structure of steviol glycosides, carbohydrate residues can be bounded to the steviol backbone at two positions, at the C13 by an *O*-glycosidic bond and at the C19 by an ester-type bond. The action of glycosyltransferases on the steviol backbone allows the addition of one or more rhamnose or glucose units at each position, resulting in ten main steviol glycosides, including stevioside, rebaudiosides A, B, C, D, E and F, dulcoside A, steviolbioside, and rubusoside, although their derivatives have also been identified [Molina-Calle *et al*., 2017]. These glycosides are present in different proportions in the plant, and their content varies depending on the plant genotype, growing conditions, and harvesting time [Pacifico *et al*., 2017; Dyduch-Siemińska *et al*., 2020]. Stevioside and rebaudioside A are the most abundant steviol glycosides in *S. rebaudiana* leaves, with stevioside generally predominating [Leszczyńska *et al.,* 2021]. In turn, rebaudioside A is recognized as the sweetest compound [Wang *et al*., 2022]. Other rebaudiosides (B–F) and dulcoside A occur in lower amounts [Dyduch-Siemińska *et al*., 2020; Leszczyńska *et al.,* 2021; Pacifico *et al*., 2017].

SAFETY ASPECTS AND LEGISLATION

Over the past 50 years, numerous biological and toxicological studies have been conducted on the steviol compounds in

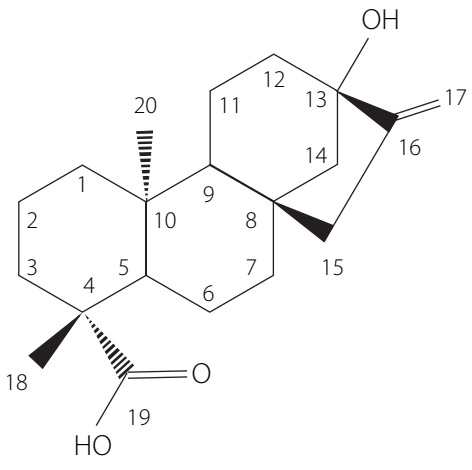

Figure 1. Structure of steviol.

TABLE 1. Maximum doses of steviol glycosides (E960) for use in certain food products [European Commission, 2011].

Product	Maximum quantity (in mg/L or mg/kg depending on the case)
Flavored fermented milk products, including heat-treated	100
Ice cream	200
Spreads made from fruit or vegetables	200
Chewing-gum	3,300
Breakfast cereals	330
Fine bakery products	330
Soups, stews, and broths	40
Sauces	120–175
Flavored drinks	80
Food supplements in solid form	670
Food supplements in liquid form	200

stevia [Brusick, 2008; Peteliuk *et al.,* 2021]. The safety of steviol glycosides has been evaluated over several decades by various regulatory bodies. The European Commission's Scientific Committee on Foods (SCF) first reviewed this natural sweetener in 1985 and again in 1999, noting concerns regarding the absence of standardized purity criteria [SCF, 1999]. Subsequently, the Joint Food and Agriculture Organization and World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) introduced provisional purity specifications in 2004, which were later finalized as permanent standards. In 2008, JECFA set an acceptable daily intake (ADI) of 4 mg *per* kg of body weight *per* day for purified steviol glycosides and confirmed their safe use as sweeteners in various foods and beverages [JECFA, 2008]. **Table 1** summarizes the authorized proportions of steviol glycosides used in some food products.

Steviol glycosides received generally recognized as safe (GRAS) status in 2008 by the US Food and Drug Administration (FDA) [FDA, 2008]. The European Food Safety Authority (EFSA) concluded that steviol glycosides pose no genotoxic or carcinogenic risk and are suitable for use as additives in foods and beverages. In the same assessment, EFSA established an ADI consistent with that set by JECFA, reaffirming the safety of these diterpene glycosides [EFSA, 2010]. In 2011, the European Commission added steviol glycosides as E-960 to the list of authorized sweeteners in the European Union and described the conditions of use. The stevia, whole plant and dried leaves, is then considered as "novel food" [European Commission, 2011]. Thereafter, the FDA, the SCF, and the Food Safety and Standards Authority of India (FSSAI) also recommended using stevia as a sweetener in some food products with restricted ADI [FDA, 2008; FSSAI, 2015; SCF, 1999].

Food manufacturers are then increasingly interested in the development of food products containing stevia, especially since the use of synthetic sugars, such as aspartame, saccharin, neotame, acesulfame-K, and sucralose, has been restricted due to their several health hazards [Chen & Hou, 2024; Debras *et al.*, 2022, 2023].

APPLICATION AND STABILITY OF STEVIOL GLYCOSIDES IN FOOD PRODUCTS

Nowadays, there are many brands of stevia-based products available in the market. Steviol glycosides are generally used as hypocaloric sweeteners and flavor enhancers in a variety of products and beverages, such as tea beverages, carbonated soft drinks, fruit juices and nectars, jams, jelly candy, chewing gum, and dairy products such as ice cream, yoghurts and milkshakes [Schiatti-Sisó *et al.,* 2023]. Owing to their thermostability and non-fermentable properties, these compounds are frequently incorporated into baked and cooked foods [Rai & Han, 2022]. In Japan, stevia has been used for several decades in a variety of foods and beverages, including soft drinks, confectionery, pickled vegetables, and seafood products [Koyama *et al.,* 2003]. Major beverage companies such as The CocaCola Company and PepsiCo have introduced stevia-sweetened products (for example, CocaCola Life and SoBe Lifewater) as part of their portfolio in reducedcalorie beverages [Rai & Han, 2022]. Mogra & Dashora [2009] evaluated the amount of stevia extract, prepared by boiling stevia leaf powder in water, necessary to achieve a sweetness level comparable to that of sugar. They concluded that 1.5 mL of extract *per* 100 mL of liquid equates to the sweetness of 5 g of sugar. The extract was subsequently applied as a sugar substitute in products including milk, coffee, tea, milkshakes, yoghurt, lemon water, and custard. Their results revealed that products containing stevia were more acceptable than the other tested products that contained artificial sweeteners. Besides, the members of the panel gave the highest sensory acceptance scores to products containing stevia (7.67–7.90). These findings indicate the real potential of stevia to be used in a large panel of food products as a sugar substitute delivering similar physical and sensory properties and providing beneficial health effects for consumers.

Steviol glycoside preparations are crystalline, odorless powders that are white or slightly yellowish. They are soluble in water and alcohol. The solubility of stevioside is 1.25 g/L and that of rebaudioside A is 3.5 g/L [Celaya *et al.,* 2016]. They can be easily extracted with aqueous solvents. These compounds are stable in acidic solutions and at pH values ranging from 2 to 10. They do not interact with any other food ingredients and do not cause browning [Jooken *et al.,* 2012; Kroyer, 1999]. Steviol glycosides remain chemically stable for at least one year when stored as a dry powder under ambient conditions [Prakash *et al.,* 2014]. The high thermal stability of steviol glycosides has been demonstrated in various food matrices, including baked goods, where they remain largely intact even at elevated processing temperatures [Jooken *et al.,* 2012]. Unlike sugar, which

caramelizes at approximately 150–160°C, steviol glycosides can withstand temperatures of up to 200°C [Jooken *et al.,* 2012].

STEVIA AND STEVIOL GLYCOSIDES IN DAIRY PRODUCTS

Steviol glycosides extracted from the leaf of *S. rebaudiana* have great potential to be used as a natural sugar substitute in dairy products [Mahato *et al.,* 2020]. According to Tate & Lyle's consumer survey [Lyobumirova, 2022], there is significant potential to attract young consumers with great-tasting, low-calorie formulations using stevia, as many perceive dairy products to be too high in fat and sugar. Among these young consumers, 77% who had reduced their dairy intake reported that they would be willing to increase consumption if healthier dairy options were available. The same survey also revealed that, between 2019 and 2021, 61% of newly launched dairy products containing stevia were yoghurts. Narayana *et al.* [2022] revealed that replacing 100% of sugar is possible with stevia extract for the production of vanilla flavoured set yoghurt, and concluded that among the natural alternative sweeteners, stevia is one of the promising ones. Indeed, it has been reported that adding stevia in the manufacture of dairy products enhances product quality. Muenprasitivej *et al.* [2022] demonstrated that the incorporation of steviol glycosides into ice cream formulations significantly improved sensory properties such as texture, flavor, and overall acceptability, while Akalan *et al.* [2024] showed that instant stevia powder can be successfully applied to yoghurt and other dairy products, contributing to better consistency and taste.

Steviol glycosides are highly stable in dairy products, as confirmed by Jooken *et al.* [2012], who observed no detectable degradation of steviol glycosides in different dairy products (including semi-skimmed milk, whole and skimmed yoghurt, and ice cream) stored under relevant conditions; their recoveries were between 96% and 103%. Similarly, Kim *et al.* [2021] reported recoveries ranging from 83.6% to 104.8% in non-fermented milk and 84.7% to 103.9% in fermented milk after spiking with steviol glycosides. In 2019, de Carvalho *et al.* [2019] conducted a study in which they used freeze-dried aqueous stevia extract (FSE) to produce stevia-fortified yoghurts. The results revealed that throughout the 30 days of cold storage, the addition of FSE to the formulated yoghurts did not affect the survival of the strains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in comparison with the control. Furthermore, the pH, acidity and syneresis were not affected by the addition of FSE in the yoghurt's matrix. The results also showed that during 30 days of storage, stevia-fortified yoghurt samples showed a higher total phenolic content (TPC) and a higher antioxidant activity than the control yoghurt. Moreover, during simulated gastrointestinal conditions, the yoghurt matrix preserved its TPC and antioxidant capacity. Finally, stevia-fortified yoghurt has shown great potential as a dairy functional food, improving both the antioxidant properties and TPC of yoghurt, not only during storage but also in simulated gastrointestinal conditions.

Ribeiro *et al.* [2020] conducted a study in which they used different stevia mix for formulation of high protein plain yoghurt. They concluded that the formulation comprising 55% of stevia 1 (75% rebaudioside A + stevioside), 5% of stevia 2 (95% rebaudioside A), and 40% of stevia 3 (50% rebaudioside A), was the best. This mix presented sensory characteristics similar to those of sucrose and sucralose in the yoghurts and had the lowest mixture development cost. Alizadeh *et al.* [2014] tested several ice cream formulations by replacing part of the sugar with stevia purified to 90%. Their study showed that stevia can successfully serve as a natural alternative to sucrose for producing low-calorie ice cream, without altering the product's physicochemical characteristics or sensory quality. They also highlighted that combining sucrose with stevia improved overall consumer acceptance. Among the tested formulations, the best results were obtained with a recipe containing 13.95 g of sucrose and 20 mg of stevia, incorporated into a standard mixture of 500 mL skimmed milk, 120 g cream powder, 80 g whole milk powder, 1 g emulsifier, and 0.9 g vanilla.

To better understand how sucrose can be replaced or reduced in dairy products, Medel-Marabolí *et al.* [2024] carried out a study assessing the temporal sensory properties of five sweeteners such as sucrose, sucralose, stevia, aspartame, and tagatose both in aqueous solutions and in yoghurt. Using the time intensity (TI) method, panelists evaluated that stevia had a distinct temporal profile compared to the other sweeteners, with a notably longer persistence of sweetness. When tested in yoghurt and evaluated through temporal dominance of sensations (TDS) and temporal acceptability by consumers, stevia (score: 5.28) and aspartame (score: 5.13) received the highest ratings on a 9-point scale, which reflects consumer preference. In solution, stevia was also characterized by the longest perceived sweetness (13 s), along with higher dominance and acceptability over time. Overall, stevia and aspartame emerged as the most preferred options among the sweeteners studied.

Stevia is valued not only as a natural sweetener but also for its nutritional and therapeutic potential. Whole leaves contain amino acids, vitamins, minerals, fiber, and phenolic compounds that provide antioxidant and bioactive benefits [Atteh *et al.,* 2011; Leszczyńska *et al.,* 2021; Muanda *et al.,* 2011; Periche *et al*., 2014]. Thus, consuming the whole leaf allows exploiting the entire potential of its bioactive compounds. Much of this nutritional richness is lost during processing into purified steviol glycosides, which mainly provide sweetness. Nevertheless, refining stevia into these purified compounds shifts the focus from nutritional benefits to technological functionality. They serve mainly a technological role as low-calorie sweeteners, rather than providing notable nutritional value. Nonetheless, the incorporation of stevia or steviol glycosides into foods, such as dairy products, remains promising, as it can improve both the organoleptic qualities and the bioactivity of foods while serving as a natural low-calorie sweetener and antioxidant.

STEVIA EFFECTS ON LACTIC ACID BACTERIA OF DAIRY PRODUCTS

Several studies have highlighted the multifunctional role of *S. rebaudiana* in supporting probiotic development and gut health. Weber & Hekmat [2013] demonstrated that steviol glycosides are suitable sweeteners for probiotic yoghurts, as they do not inhibit the growth of *Lacticaseibacillus* strains. Similarly, Bahnas *et al.* [2019] reported that adding 1% stevia leaf extract to cheese whey or milk permeate-based beverages significantly enhanced the viability of *Lacticaseibacillus paracasei*, an effect attributed to the presence of phytochemicals and minerals. In accordance with these findings, stevioside and stevia leaf extract were found to enhance lactic acid production in *Lacticaseibacillus casei*, *Levilactobacillus brevis*, and *Lactiplantibacillus plantarum,* according to Davoodi *et al.* [2016]. Tested at 0.5%, 1.0%, and 2.0% (*w/v*), a stevia leaf extract showed a content-dependent effect, while stevioside maintained a relatively consistent impact across all levels.

Beyond the leaves, other parts of the plant, particularly the roots, also hold promise: they contain fructans, recognized as functional prebiotic ingredients that stimulate the growth of beneficial bacteria such as *Bifidobacterium* and *Lacticaseibacillus* [Sanches Lopes *et al.,* 2016]. Thus, although root extracts are not used commercially as sweeteners, they highlight the broader nutritional and functional potential of the plant beyond its sweetening properties.

Kim *et al.* [2023] evaluated how supplementing fermented milk with stevia extract influences both bacterial growth and product quality. They tested different levels of stevia extract and observed that higher levels (0.5%, *w/v*) accelerated fermentation by stimulating the proliferation of beneficial lactic acid bacteria, particularly *S. thermophilus*, *Lacticaseibacillus acidophilus*, and *Bifidobacterium longum*. Stevia extract supplementation also resulted in an increase in the titratable acidity, total solid content, viscosity and water-holding capacity. In line with this, Ozcan *et al.* [2017] conducted a study in which a 10% commercial stevia extract was used during fermentation, resulting in improved survival of *L. casei* in both basal medium and fermented milk. Similarly, Dong *et al.* [2021] demonstrated that adding 12% (*v/v*) stevia extract during fermentation reduced the fermentation time (from 12 h to 9 h) and enhanced the viability of *L. plantarum* (8.72 log CFU/mL) under simulated gastric digestion. Ozdemir & Ozcan [2020] suggested that fermented milks containing steviol glycosides may serve as promising functional dairy products by effectively delivering probiotic bacteria.

Alizadeh [2021] explored the use of steviol glycosides for the development of probiotic mango nectar enriched with *L. plantarum*. Different formulations were then prepared by combining the probiotic strain (*L. plantarum*, 10^6 CFU/mL) with varying amounts of inulin (0%, 4%, and 8%, *w/w*) as a prebiotic texturizer, and stevia (0%, 2%, and 4%, *w/w*) as a natural low-calorie sweetener. The findings indicated that the formulation containing 4% (*w/w*) stevia and 4% (*w/w*) inulin not only enhanced the survival of *L. plantarum* over a 45-day storage period but also offered the most favorable physicochemical characteristics and sensory qualities.

In 2014, Kunová *et al.* [2014] explored the potential prebiotic role of steviol glycosides (2 g/L) on various *Bifidobacterium* and *Limosilactobacillus* strains. While some strains, such as *B. bifidum*, *B. breve*, *B. adolescentis*, and *L. mucosae*, showed a slight increase in growth, the overall capacity of these bacteria to metabolize steviol glycosides remained very limited, as the compounds are not fermentable. The authors concluded that a prebiotic effect could not be demonstrated, as their analysis focused exclusively on bacterial growth without considering potential metabolic outputs [Kunová *et al*., 2014]. More in-depth analysis of the bacterial metabolome would make it possible to detect more subtle functional modifications. In addition, only 10 bacterial genera are tested in this study, which does not reflect the true diversity of the gut microbiota. Another study conducted by Li *et al.* [2014] investigated the impact of Rebaudioside A (Reb A) on the growth of two probiotic strains: *B. longum* ATCC 15707 and *L. plantarum* ACCC 11095. The results showed that Reb A had no significant effect on the growth of *B. longum* ATCC 15707, whereas it significantly enhanced the growth of *L. plantarum* ACCC 11095, particularly at concentrations of 0.5% and 1%. Ozcan & Eroglu [2023] investigated the prebiotic effects of steviol glycosides on *B. animalis* subsp. *lactis* for dietetic dairy products. They used *in vitro* fermentation assay with basal medium (non-carbohydrate containing Man, Rogosa and Sharpe agar) supplemented with different concentration of steviol glycosides (from 0.025% to 1%, *w/v*) and inulin at 1%. The combination of 0.025% steviol glycosides + 1% inulin showed the highest prebiotic activity, enhancing bacterial viability and short-chain fatty acid production. While steviol glycosides alone also supported growth, the best results were seen with the combination, suggesting a synergistic effect. The study concludes that stevia, particularly with inulin, can be used as a functional sugar substitute and prebiotic for modulating gut microbiota. Rosa *et al.* [2021] emphasized that enriching dairy products with prebiotics can provide several health benefits, such as the improvement of intestinal well-being, anti-diabetic, and anti-hypertensive properties. They also noted that prebiotics may enhance product quality by influencing physicochemical, microbiological, and sensory properties. Nevertheless, the effectiveness of these benefits largely depends on selecting the right type and concentration of prebiotic compounds.

To classify a food ingredient as a prebiotic it must fulfil specific criteria. It has to be resistant to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption, it must be fermentable by intestinal microbiota; and selectively stimulate the growth and/or activity of those intestinal bacteria that contribute to health and well-being [Roberfroid, 2007]. Steviol glycosides meet some of these criteria, however more in-depth studies are necessary to confirm or refute their prebiotic effect. Moreover, most of the cited studies are performed *in vitro* or in animal models. Randomized clinical trials in humans are required to validate the effects on the gut microbiota

and digestive physiology before drawing definitive conclusions about the potential benefits of stevia.

STEVIA EFFECTS ON BACTERIA THAT CAUSE SPOILAGE OF DAIRY PRODUCTS

Several researchers have highlighted the antimicrobial activity of stevia crude and purified extracts against a wide range of pathogenic bacteria and fungi [Chen *et al*., 2024; Jayaraman *et al.,* 2008; Myint *et al.,* 2023]. Chai *et al.* [2024] demonstrated that extracts of *S. rebaudiana* leaves fermented with *L. plantarum* exhibited strong antimicrobial activity against food-borne pathogens, including *E. coli* and *S. aureus*. Interestingly, these fermented extracts displayed a selective action, as they did not significantly inhibit beneficial bacteria such as *Lacticaseibacillus* spp. and *Bifidobacterium* spp., thereby underscoring their potential as natural food preservatives. This antibacterial effect was mainly attributed to secondary metabolites produced during fermentation through microbial deglycosylation and enzymatic decomposition of stevia compounds, since the unfermented aqueous extract exhibited little to no antibacterial activity. Jayaraman *et al.* [2008] provided evidence of the antibacterial action of stevia leaf extracts at 50 mg/mL. The acetone extract showed the strongest activity, producing inhibition zones of 19 mm against *S. aureus* and 18 mm against *B. subtilis*, indicating greater effectiveness against Gram-positive than Gram-negative bacteria. The ethyl acetate extract also inhibited *V. cholerae* with an 18 mm zone, whereas no activity was detected for the aqueous extract. In a subsequent study, Puri & Sharma [2011] demonstrated that purified stevioside solution at 100 µg/mL inhibited the growth of several bacterial species, including *B. subtilis*, *B. cereus*, *Klebsiella pneumoniae*, *P. aeruginosa* and *S. enterica* ser. Typhi. In particular, a clear inhibition zone of 12 mm was observed against *B. cereus*, a spoilage microorganism frequently associated with milk and dairy products, highlighting the possible use of stevioside to enhance food shelf life.

Li *et al.* [2014] examined how Reb A influences the growth of common foodborne pathogens. The study included two Gram-negative strains (*E. coli* O157:H7 and *S. enterica* ser. Typhimurium ATCC 13311) and two Gram-positive strains (*S. aureus* CGMCC 26001 and *Listeria monocytogenes* CMCC 54007). Their findings revealed that Reb A at 0.5% (*w/v*) significantly reduced the growth of *S. aureus* ($p<0.05$), with an even stronger effect ($p<0.01$) observed at 1.0% (*w/v*). By contrast, only minor inhibitory effects were detected against *E. coli*, *S. enterica* ser. Typhimurium, and *L. monocytogenes*. Chen *et al.* [2024] found that an extract from stevia containing chlorogenic acid isomers (notably isochlorogenic acid C) exhibited strong antibacterial activity against multiple strains of *E. coli*. The minimum inhibitory concentration was 2 mg/mL, and the bactericidal concentration was 8 mg/mL for some strains. The mechanism included damage to cell wall and membrane permeability, leakage of intracellular proteins and potassium ions, and loss of outer membrane integrity.

Based on the above results, it can be concluded that stevia extracts could be used as food additives in dairy products, as they inhibit the growth of pathogens and enhance the growth of probiotic bacteria.

METABOLISM OF STEVIOL GLYCOSIDES BY GUT MICROBIOTA AND MICROBIAL ENZYMES

The human gut microbiota contributes significantly to the host's health by regulating metabolism and cellular immune response. *Bacillota* and *Bacteroidota* represent 90% of the dominant phyla in the intestinal flora [Rinninella *et al.,* 2019]. However, the composition and function of the gut microbiota can be positively or negatively influenced by diet [Kasti *et al.,* 2022; Wen & Duffy, 2017]. In this context, with the increased use of stevia as a sugar substitute, several studies have investigated the effect of its consumption on intestinal well-being [Becker *et al.,* 2020; Singh *et al.,* 2024].

The metabolism of stevia presented in **Figure 2** depends on gut microbiota and microbial enzymes that break down the steviol glycosides into steviol [Becker *et al.,* 2020; Kasti *et al.,* 2022]. Salivary and gastric juice enzymes, such as pancreatic α-amylase, pepsin, and pancreatin, cannot degrade steviol glycosides, these compounds pass intact through the upper gastrointestinal tract, where they are hydrolyzed by intestinal bacteria enzymes [Renwick & Tarka, 2008]. Among the bacterial group found in gastrointestinal tracts of humans and animals, *Bacteroidota* are primarily responsible for the hydrolysis of stevioside and rebaudioside A to steviol, whereas other bacteria, such as *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Escherichia* (coliforms), and *Enterococcus*, were unable to hydrolyze and use steviol glycosides as a usable substrate [Kasti *et al.,* 2022; Renwick & Tarka, 2008]. After degradation, steviol is absorbed *via* the portal vein and then reaches the liver, where it is metabolized to steviol glucuronide, which elicits great detoxification effects on the liver [Li *et al.,* 2014]. Then, it is excreted in the urine [Kasti *et al.,* 2022; Renwick & Tarka, 2008].

EFFECT OF STEVIA CONSUMPTION ON INTESTINAL MICROBIAL DIVERSITY

Total species diversity is determined by two indicators: the first one is α-diversity (the average species diversity in a particular area or habitat), and the second one is β-diversity (the diversity of species between two habitats or the measure of similarity or dissimilarity of two regions). Several studies have focused on the effect of stevia consumption on intestinal microbial diversity [Li *et al.,* 2014; Kwok *et al*., 2024; Mahalak *et al.,* 2020]. Some of these studies showed that stevia consumption has led to higher α-diversity [Li *et al.,* 2014; Mahalak *et al.,* 2020; Nettleton *et al.,* 2020]. Whereas, others indicated that there are no changes in α-diversity [Nettleton *et al.,* 2019; Wang et *al.,* 2018; Yu *et al.,* 2020]. In turn, some studies demonstrated that stevia consumption did not significantly change β-diversity [Mahalak *et al.,* 2020; Nettleton *et al.,* 2019, 2020]. Even if stevia consumption may affect the colonic microenvironment, it seems that this effect depends on the amount and the frequency of intake. Li *et al.* [2014] investigated the dose-dependent effects of rebaudioside A in an *in vivo* model using SPF BALB/c mice, administered

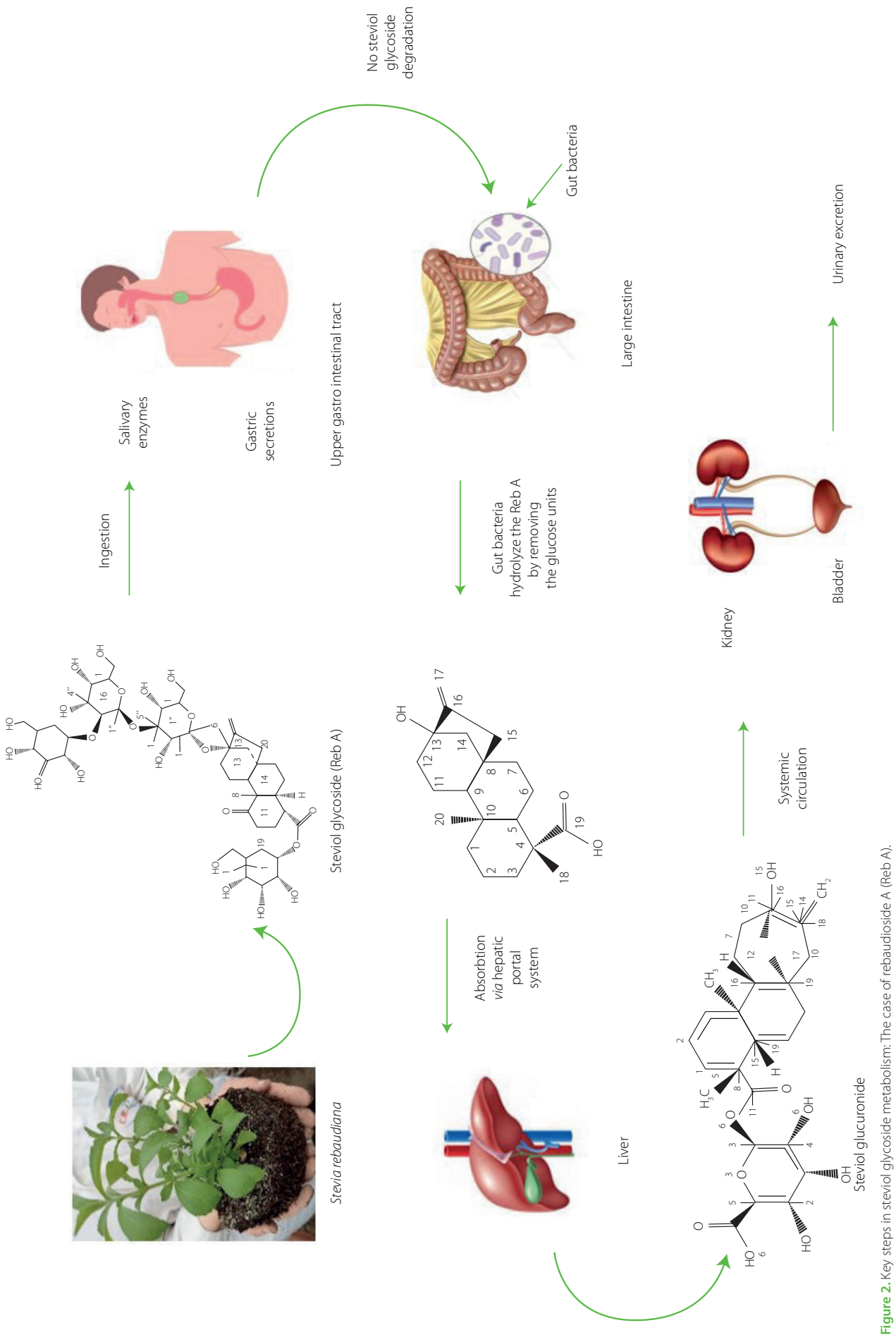

Figure 2. Key steps in steviol glycoside metabolism: The case of rebaudioside A (Reb A).

at low (0.5 mg/mL) and high (5.0 mg/mL) concentrations. They found that the two concentrations did not alter the growth of enterobacteria and lactobacilli and did not affect the microbial diversity, however it could have modified the number of some bacterial genera. This study reflects some methodological limitations. The analyzed samples were relatively small (*n*=15 mice randomly divided into three groups) and from a single breeding stock, thereby restricting the extent to which the findings can be generalized. Moreover, the statistical significance of the observed changes in some bacterial genera was not reported. Studies with larger and more varied samples would be necessary to confirm or refute these effects.

Mahalak *et al.* [2020] conducted a study in which they compared the changes that occur in the gut microbiota in the feces of a healthy donor after exposure to steviol glycosides and erythritol. The findings indicated that steviol glycosides significantly increased the growth of gut bacteria. Despite this, the overall structure of the gut microbiota remained unchanged, with the *Bacteroidaceae* family being predominant, followed by *Lachnospiraceae*, *Fusobacteriaceae*, and *Ruminococcaceae*. Furthermore, other research reported that when mixed fecal bacteria from volunteers were exposed to stevioside, there was a slight inhibition of anaerobic bacteria, whereas rebaudioside A caused mild inhibition of aerobic bacteria, particularly coliforms [Gardana *et al*., 2003]. Further long-term studies are needed with appropriated doses and adequate subject sizes to evaluate the real effects of stevia consumption on gut microbiota.

Kim *et al.* [2023] investigated the effect of stevia leaf extracts on fermented milk and the health effect of this product on human colon cells. They demonstrated that stevia extract-supplemented fermented milk enhanced mucin glycoprotein production in human colon epithelial cells (HT-29/MTX). Mucin contributes to the formation of a viscoelastic gel within the mucus layer, serving as a protective barrier for the gastrointestinal tract against certain conditions, such as bacterial infections and inflammatory bowel disease (IBD) [Corfield, 2015]. Recently, Ma *et al.* [2023] demonstrated that the leaf extract of *S. rebaudiana* fermented with *Pediococcus pentosaceus* LY45 repressed trimethylamine (TMA) production. Oxidation product of TMA – trimethylamine *N*-oxide (TMAO), is recognized as a risk marker of cardiometabolic and hepatic diseases, as well as other chronic diseases [Lynch & Pedersen, 2016]. These findings suggest that fermenting stevia extracts with lactic acid bacteria could be a promising approach for generating specific metabolites that may help modulate and restore the gut microbiota.

Zhao *et al.* [2018] reported that dietary supplementation with stevia residue extracts in mice improved impaired glucose regulation and helped restore intestinal balance. The intervention was associated with modulation of gut microbiota composition, indicating that bioactive compounds from *S. rebaudiana* can influence metabolic health while supporting beneficial intestinal bacteria. These findings support the potential role of stevia extracts in maintaining gut microbial homeostasis and preventing metabolic disturbances.

STEVIA IN DAIRY ANIMALS' FEEDING

In addition to studies on stevia-supplemented dairy products, many authors have investigated the effects of its feeding in dairy livestock. They demonstrated that stevia or its extracts may enhance productive performance and milk quality [Gerdzhikova *et al.,* 2018]. Stevia can be used in animal feeding as both an additive and hay substitute, depending on the specific application and the type of animal being fed. Stevia hay can be used as a substitute for traditional hay in animal feeding. It provides high levels of metabolizable energy *per* kg of dry matter; 2,894 kcal in leaves and 2,052 kcal in stems and blossoms [Gerdzhikova *et al.,* 2018]. It is also rich in proteins, amino acids, minerals and fibers, mainly in the stems. The metabolizable energy and fiber content of stevia stem suggest its usage oriented to ruminants rather than monogastric animals [Atteh *et al.,* 2008, 2011]. Stevia extract, specifically stevioside, can be used as a feed additive to increase feed intake and/or growth rate in animals [Montero *et al.,* 2016].

Stevia has been studied for its potential benefits in dairy species; sheep, goats and cows, but there is a limited research on its specific effects on milk quality. The introduction of stevia straw, which is a by-product of the stevia sugar crop, has been studied in sheep, as it is rich in nutrients and active compounds, making it a good feed material. It has been demonstrated that adding 1% (*w/w*) of stevia stalk to sheep rations enhanced rumen fermentation capacity, as indicated by a decrease in pH value and an increase in ammonia nitrogen concentration [Zhang *et al.,* 2023]. In goats, Han *et al.* [2019] showed that the supplementation of basal diet with stevioside did significantly affect feed intake, ruminal fermentation and digestion, and improved blood metabolites, indicating potential benefits for goat health. These studies, did not directly measure the effects of stevia on milk quality, but it is possible that the improved rumen fermentation and nutrient utilization could indirectly contribute to better milk quality [Jiang *et al.,* 2022]. In dairy cows, the introduction of stevia hay into a diet for Holstein lactating cows to partially substitute alfalfa hay improved rumen fermentation, as demonstrated by an increase in volatile fatty acid (VFA) content, nitrogen utilization, and lactation performance [Jiang *et al.,* 2022]. The study showed that diets supplemented with 6% and 12% (*w/w*) of stevia hay caused the most significant improvements in rumen fermentation and milk performance with a higher yield and fat content. Stevia may also positively affect animal products' quality by providing antioxidants and functional nutrients.

In summary, stevia used in feeding dairy animals has many benefits and may be considered in ration formulation as it improves the feed intake, rumen fermentation, nitrogen utilization, and lactation performance. However, more research is needed to fully understand the optimal doses, the duration of supplementation and its long-term effects to improve milk quality.

CONCLUSIONS

The growing consumer demand for sugar reduction in foods has prompted the dairy industry to explore natural sweetener substitutes like stevia to reduce sugar content in their products. This review highlights the promising potential of stevia

and its glycosides as sugar replacers in dairy products. Based on the above studies, fortification of dairy products with stevia can provide excellent sensory properties to the product that can promote the intestinal well-being. Stevia can act as a sweetening agent with zero calories, as an enhancer of probiotic bacteria, and as a natural bio-preservative inhibiting the growth of pathogens. However, further research is recommended to determine optimal stevia doses for specific dairy applications and to better understand its long-term impacts on human health through clinical trials. *In vitro* and animal studies compiled in this review demonstrate stevia's ability to enhance growth and viability of probiotic bacteria in dairy matrices. However, elucidating the precise prebiotic mechanisms of action *via* human trials and gut microbiota studies could further validate stevia's role as a functional ingredient. In addition to glycosides, stevia also offers other phytochemicals, such as flavonoids, phenolic acids, as well as fatty acids, proteins, vitamins, and minerals that contribute to the antioxidant potential and health attributes of stevia-fortified dairy products. However, controlled studies in humans are required to substantiate these functional benefits. Research is also needed to evaluate the effects of incorporating stevia extracts or stevia by-products in dairy animals' feeding on subsequent milk yield and quality.

While long-term stevia consumption appears safe based on existing toxicological data, future research should continue assessing potential allergenicity concerns as usage increases globally. Comprehensive safety evaluation will enable greater consumer confidence in this natural sweetener. In summary, the unique nutritional and functional characteristics of stevia present opportunities for innovative dairy product development aligned with consumer preferences for natural, low-sugar, and health-promoting foods. Harnessing stevia's multi-faceted potential through strategic research and application in dairy industry could accelerate the growth of this ingredient in the functional foods market.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests to disclose.

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Food Technology:
- Innovative technology of food development including biotechnological and microbiological aspects
- Effects of processing on food composition and nutritional value

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- Bioactive constituents of foods
- Chemistry relating to major and minor components of food
- Analytical methods

Food Quality and Functionality:
- Sensory methodologies
- Functional properties of food
- Food physics
- Quality, storage and safety of food

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- Announcements of congresses
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