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Preparation of lentil and quinoa protein complexes through protein–protein interactions and water kefir–assisted fermentation to improve protein quality and functionality

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Introduction: Industrial applications of lentil (LP) and quinoa (QP) proteins are limited due to their relatively poor water solubility. In this study, a combination of protein–protein interaction (PPI) and fermentation was used to improve the functionality and nutritional value of LP by conjugating them with QP. The reaction conditions between LP and QP for producing these conjugates were established.

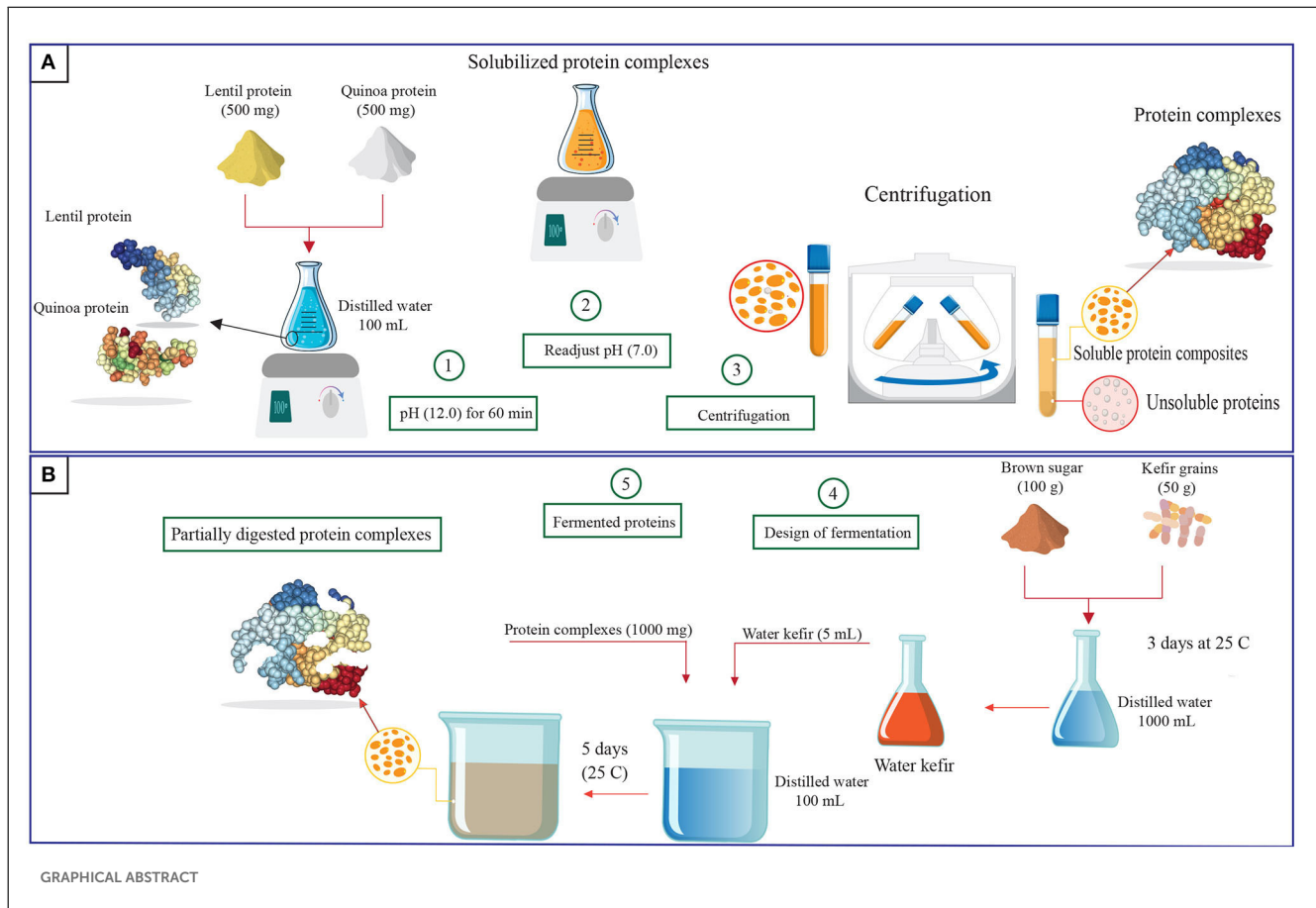
Methods: The ratio of LP to QP was equal (50:50), and complexation was carried out at 25°C for 60 min. Fermentation of the solubilized LP–QP complexes (1%, w/v) for 5 days at 25°C with water kefir (5%, v/v) was carried out to enhance the protein quality and functionality of the LP–QP complexes.

Results: The combined technique significantly enhanced protein digestibility, decreased the proportion of α -helices in the protein structure in favor of random coil components, and improved the phenolic content of the LP–QP complexes. Digestibility increased to 87%, up from 76% for unfermented LP–QP. Moreover, the LP–QP complexes produced using the combined technique generated a highly nutritional protein with a reduced saponin content.

Conclusion: This research revealed that a combination of PPI and water kefir fermentation significantly enhances the nutritional and functional quality of LP, creating new opportunities for leveraging the growing popularity of plant-based proteins into high-value industrial applications.

KEYWORDS

lentil protein, quinoa protein, protein digestibility, water kefir fermentation, phenolic compounds



Highlights

- Lentil and quinoa protein complexes were prepared using a dual treatment approach.
- A combined dual treatment (pH cycling and water kefir fermentation) was proposed.
- The resulting protein complexes can be excellent alternatives to animal-based proteins.
- Fermentation plays an important role in the modification of nutrient and non-nutrient compounds.

1. Introduction

Plant-based proteins have gained steady popularity recently due to concerns about environmental, ethical, and health issues associated with animal-based proteins such as milk, eggs, and meat. Lentil (*Lens culinaris*) is the most common leguminous plant in the world and is extensively employed as a commercial source of food components (Jarpa-Parra et al., 2014). Lentil proteins (LP) are some of the promising plant-based proteins due to their low cost, low allergenicity, high environmental sustainability, and high nutritional value (Alrosan et al., 2022a). Another protein, the quinoa protein (QP), found in a pseudocereal from the Amaranthaceae family, is considered a trend in food applications due to its high nutritional value since QP contains all essential amino acids

and is deemed non-allergenic (Lingiardi et al., 2022). However, several barriers exist to using LP as a primary protein source in diets, such as low protein digestibility (Alrosan et al., 2021) and low water solubility (Alrosan et al., 2022b). In addition, our understanding of the relationships between the basis of protein quality and the functional performance of LP and QP remains limited.

To address these issues, scientists have aimed to improve the functionality of plant proteins using different techniques involving enzymatic, chemical, and physical processes. Many researchers have successfully enhanced the functionality of plant proteins using protein-protein interactions (PPI), including the solubility of rice proteins (Wang et al., 2019) and LP (Alrosan et al., 2021, 2022b) and the emulsifying properties of wheat gluten proteins (He et al., 2020). These results stem from the altered secondary and tertiary protein structures caused by structural interactions and the molecular forces governing PPI, such as hydrogen bonds, hydrophobic interaction, and electrostatic interaction. A complex series of alterations to these molecular forces, which are initiated by the structural interactions between molecules on the surface of the proteins, lead to the modification of the protein higher order structure and secondary structure components (α -helix, β -turn, β -sheet, and random coil).

Furthermore, fermentation has been shown to significantly enhance the nutritional value and protein quality of plant proteins. For example, fermentation improved the digestibility of LP (Alrosan et al., 2021), reduced the levels of anti-nutritive

factors present with soya-whey proteins (Alrosan et al., 2023), and enhanced the digestibility of pea proteins (Çabuk et al., 2018). Some fermenting microorganisms and their enzymes can degrade the ester linkages among carbohydrates, proteins, and naturally occurring non-nutrients such as phenolic compounds (Alrosan et al., 2022a). Among the various types of fermentation, water kefir is considered an excellent source of lactic acid bacteria (*Leuconostoc mesenteroides* ssp., *Lactocaseibacillus casei*, and *Lentilactobacillus hilgardii*), acetic acid bacteria (e.g., *Acetobacter lovaniensis*, *Acetobacter tropicalis*, and *Gluconobacter liquefaciens*), and yeasts (e.g., *Saccharomyces cerevisiae*) (Lynch et al., 2021).

Dual-treatment techniques, i.e., the combination of two different treatments, such as dehydration, enzyme hydrolysis, vacuum microwave, and ultrasonic treatments, have been reported to enhance the functional properties of plant-based proteins (Yan et al., 2021; Yen and Pratap-Singh, 2021). Zhao et al. (2022) showed that the combination of high-pressure treatment and ultrasound-assisted Maillard reaction increased the water solubility of pea protein isolates by over 80%. The objective of the present study was 2-fold: (i) to prepare a protein complex made of crude lentil proteins (CLP) and quinoa protein concentrate (QPC) using a combination of PPI (pH cycling technique) and water kefir fermentation and (ii) to investigate how this dual treatment may improve the protein quality and functionality of LP to boost its industrial applications as an alternative to animal-based proteins. The fermented LP-QP complexes were assessed for protein digestibility, protein secondary structure, phenolic profile, and total saponin content. Microorganism counts and pH were monitored to confirm the growth of yeasts, lactic acid, and acetic acid bacteria during fermentation.

2. Materials and methods

Kefir grains, lentil seeds (*Lens culinaris*), quinoa seeds (*Chenopodium quinoa*), and brown sugar (one batch each) were purchased from a local food market in Malaysia. All the chemicals and reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA), including pepsin, pancreatin, Folin-Ciocalteu reagent, fructose, glucose, sucrose, dextrose sorbitol mannitol (DSM) agar, potato dextrose agar, de Man, Rogosa and Sharpe (MRS) agar, oleonic acid, phenolic compound standards, and HPLC grade solvents. The thermosensitive samples were transferred to the lab in a temperature-controlled container and stored at 4°C.

2.1. Preparation of crude lentil protein

CLP was prepared in accordance with the technique described by Jarpa-Parra et al. (2014). Several batches were prepared, then pooled and mixed before further use. Lentil seeds were washed twice and left at room temperature at 25°C for 1 day to reduce the saponin content (Abugoch et al., 2008). A rotating mill (Retsch, ZM 300, Haan, Germany) was used to grind the lentil seeds into fine flour with a particle size of <0.5 mm. The resulting flour was collected in plastic bags that were sealed and stored at 4°C

until protein extraction by sequential precipitation. Lentil flour and distilled water were combined at a ratio of 1:10 (w/v). The slurry was stirred using a digital magnetic stirrer (JoanLab, SH-4, Fristaden Lab, NV, USA) and its pH was adjusted to pH 9.5 using NaOH (0.1 M). Stirring (1,000 rpm) was continued for 2 h at 40°C with pH monitoring every 30 min, followed by centrifugation (Kubota, S700TR, Tokyo, Japan) at 8,500 ×g for 15 min at 23°C. The supernatant was obtained, acidified to pH 4.2 with HCl (0.1 M), then kept overnight before centrifugation at 1,590 ×g for 30 min. The precipitate was collected and lyophilized (Büchi, R-220, Flawil, Switzerland). The crude protein content of the lyophilized CLP (61.78% ± 2.10) was determined using the AOAC Method 930.29, N × 6.25 (AOAC, 2012). The lyophilisate was ground, placed in plastic bags, sealed, and stored at 4°C until complexation with QPC.

2.2. Preparation of quinoa protein concentrate

QPC was prepared as previously described (Alrosan et al., 2023). Several batches were prepared, then pooled and mixed before use. In brief, the quinoa seeds were washed multiple times with distilled water until no visible foam remained on the water. The seeds were dried (50°C) and ground using a ZM 300 rotating mill. Defatted quinoa flour was prepared by mixing the flour with hexane at a ratio of 10:1 (w/v) with continuous stirring for 24 h using an SH-4 digital magnetic stirrer, followed by air-drying at 21°C. To extract the proteins, distilled water was added to the defatted quinoa flour at a ratio of 10:1 (v/w) and the pH of the suspension was adjusted to pH 9.0 using 0.1 M NaOH with continuous stirring (SH-4 digital magnetic stirrer) at 40°C. The suspension was then centrifuged at 9,000 ×g for 15 min, the supernatant was collected, acidified to 5.0 using HCl (0.1 M), and kept overnight to allow the protein to precipitate before centrifugation at 9,000 ×g for 20 min. The precipitate was collected and lyophilized. The crude protein content (N × 6.25) of the lyophilized QPC (82.21% ± 1.43) was determined according to the AOAC Method 930.29 (AOAC, 2012). The lyophilisate was pulverized before being sealed in plastic bags and stored at 4°C until complexation with CLP.

2.3. Protein–protein interaction complexation

Complexation of CLP and QPC was performed as reported by Alrosan et al. (2022b). Equal amounts of CLP and QPC were mixed in distilled water at 1% (v/w), and the pH of the mixture was adjusted to pH 12 with NaOH (0.05 M). The protein dispersion was stirred at 1,000 rpm with a magnetic stirrer at room temperature (25°C). After 1 h, the pH of the dispersion was readjusted to 7.0 with HCl (0.05 M), followed by centrifugation at 7,000 ×g for 10 min. The supernatant was collected and lyophilized. The lyophilisate was pulverized, sealed in plastic bags, and stored at 4°C until fermentation with water kefir.

2.4. Water kefir fermentation of LP-QP complexes

Water kefir was produced by blending the kefir grains with brown sugar in distilled water at a ratio of 5:10:100 (w/w/v), respectively. The mixture was incubated for 3 days at 25°C in a chamber incubator (Corepoint, PRF122WWW/0CAD, Summerville, SC, USA) with shaking every 12 h to keep the kefir grains viable. At the end of the incubation period, a sterile sieve was utilized to separate the kefir grains. The filtrate (water kefir) was collected for fermentation. Water kefir fermentation of LP-QP complexes was carried out in triplicate. For each replicate, the samples were prepared by mixing the lyophilized LP-QP complexes with water kefir in distilled water at a ratio of (1:5:100, w/v/v), respectively, and incubated for 5 days at 25°C (Corepoint chamber incubator, PRF122WWW/0CAD) with shaking every 12 h. Aliquots were collected every 24 ± 1 h for further analyses (Section 2.5) which were performed in triplicate, except for microbial counts which were determined in duplicate. Samples collected on Day 0, immediately before fermentation, were labeled as unfermented LP-QP complexes.

2.5. Characterization of the fermented and unfermented LP-QP complexes

2.5.1. pH, total soluble solids, and total saponin content

The pH and total soluble solids (TSS, expressed as °Brix) of the unfermented and fermented samples were measured daily with a pH meter (MRC Laboratory-Instruments, INE-I500T, Essex, UK) and a digital refractometer (Rudolph Research Analytical, J257, Hackettstown, NJ, USA), respectively. Total saponin content was determined by absorbance using the perchloric acid-vanillin-glacial acetic acid method, as previously reported (Alrosan et al., 2023), and expressed as mg oleanolic acid equivalents (OAE) per 100 g.

2.5.2. Microbiological counts

Lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast counts in both unfermented and fermented LP-QP samples were determined using the spread plate method. The microbial analyses were conducted in duplicate every 24 h throughout fermentation. Each sample was serially diluted and then plated by distributing 1 mL of each serial dilution in duplicate over the surface of the appropriate growth media. Yeasts, AAB, and LAB were plated on potato dextrose agar, DSM agar, and MRS agar, respectively.

2.5.3. Protein digestibility

The protein digestibility of the LP-QP complexes was determined as described by Almeida et al. (2015). Protein samples (0.25 g) were mixed in HCl (15 mL, 0.1 M) and pepsin (1.5 mg/mL), followed by incubation in a water bath (Munro, WBH-100, Essex, UK) at 37°C. After 180 min, 7.5 mL of 0.5 M NaOH containing 0.005 M sodium azide was added to the mixtures to prevent microbial growth. Then, 10 mg of pancreatin and

10 mL of 0.2 M phosphate buffer (pH 8.0) were added and mixed, and the suspensions were incubated at 37°C for 24 h (Corepoint chamber incubator) before centrifugation at $10,000 \times g$ for 20 min. The nitrogen content of the samples and the collected supernatants was determined using the Kjeldahl method (AOAC, 2012). Protein digestibility, expressed in %, was calculated according to Equation (1).

$$\text{Protein digestibility (\%)} = [(N_S - N_B)/N_T] \times 100\% \quad (1)$$

where N_T and N_S are the total nitrogen content of the LP-QP complexes before and after centrifugation, respectively, and N_B is the nitrogen content in the supernatant of the blank.

2.5.4. Determination of sugars

Glucose, fructose, and sucrose concentrations in the suspensions of fermented and unfermented LP-QP complexes were determined by HPLC (Alrosan et al., 2021). The samples (1 mL) were diluted 2× with deionized water, vortexed for 10 min, and centrifuged at $15,000 \times g$ for 10 min. The supernatants were collected and filtered (0.45 μm Whatman membrane) before injection (20 μL) in an HPLC system equipped with a refractive index detector (Agilent, 1200 Series, East Brunswick, NJ, USA) and a Cosmosil Sugar-D HPLC column (4.6 mm × 250 mm) (Nacalai Tesque Inc., Kyoto, Japan) to separate the sugars at 40°C at a flow rate of 1.2 mL/min. The mobile phase was composed of water and acetonitrile in a 25:75 (v/v) ratio. The calibration curves of fructose, glucose, and sucrose were used to quantify each sugar in the samples.

2.5.5. Determination of total phenolic content and phenolic profile

The total phenolic content (TPC) of LP-QP complexes was measured using the Folin-Ciocalteu method as previously reported (Alrosan et al., 2021). Protein samples (100 μL) were combined with distilled water (8.4 mL) and Folin-Ciocalteu reagent (500 μL) in 50-mL test tubes followed by vortexing for 4 min. Sodium carbonate (1 mL, 5%) was added, and the mixtures were thoroughly mixed by vortexing and then stored in a dark environment for 1 h before reading the absorbance at 725 nm (Shimadzu UV-Vis spectrophotometer, UV-3600, Kyoto, Japan). TPC was expressed in mg gallic acid equivalents (GAE) per 100 g.

The individual phenolic compounds (caffeic acid, catechin, chlorogenic acid, epicatechin, ferulic acid, gallic acid, quercetin, rutin, sinapic acid, and syringic acid) in LP-QP complexes were quantified by HPLC according to Liu et al. (2021). In brief, 1 mL of the protein dispersions and 8 mL of methanol were mixed by vortexing before being subjected to ultrasonication at 35°C for 3 min or until the residues turned colorless. The supernatants were collected and filtered (0.45 μm) before being measured using an Agilent 1200 Series HPLC system equipped with a UV detector (Agilent, NJ, USA) and a Plus C18 column (4.6 × 250 mm). The injection volume and flow rate were set at 40 μL and 0.7 mL/min, respectively. The absorbance was read at 272 nm for caffeic acid,

catechin, chlorogenic acid, epicatechin, ferulic acid, gallic acid, quercetin, and rutin, or 254 nm for sinapic acid and syringic acid.

2.5.6. Protein secondary structure components

The secondary structural components of the LP-QP complexes were quantified using Fourier transform infrared (FTIR) as described by Alrosan et al. (2021). Pre-weighed LP-QP lyophilisate (60 mg) was carefully inserted in the FTIR spectrometer (Malvern, MultiTect™, Burlington, Canada) for scanning. The standardized data from the amide I area were subjected to a baseline correction. The analyzed FTIR spectra were measured between 1,600 and 1,700 cm^{-1} at an interval of 4 cm^{-1} . The percentages of protein secondary components, specifically α -helix, β -turn, β -sheet, and random coil, were used to express the results (Zheng et al., 2017).

2.6. Statistical analyses

Data are reported as means of three independent replications unless otherwise indicated. Statistical analyses were conducted with SPSS software version 23.0 (IBM, Chicago, IL, USA). Duncan's multiple range test and one-way analysis of variance (ANOVA) were performed to identify statistically significant differences ($P < 0.05$) between the means.

3. Results and discussion

3.1. pH, TSS, and microbiological counts

As shown in Table 1, the pH of the LP-QP dispersions decreased significantly ($P < 0.05$) during fermentation with water kefir, from 6.61 initially to 3.73 on Day 5, which can be attributed to the release of organic acids, including lactic acid by LAB. The pH of the protein dispersions decreased rapidly over the first 24 h, reaching 3.90 on Day 2, which probably coincided with the exponential growth phase of the bacteria found in water kefir. Lynch et al. (2021) reported that LAB present in the water kefir significantly reduce the pH during the fermentation of protein substrates, while also breaking down proteins into smaller molecules, including short- and medium-sized peptides and amino acids. Similarly, Azi et al. (2020) and Alrosan et al. (2021) reported that LAB (*Lactobacillus* genus) led to increased acidity during water kefir fermentation. In agreement with our findings, the pH of water kefir is often below 4.0 after fermentation due to the production of both lactic and acetic acids (Laureys et al., 2019; Jia et al., 2021). Thus, the possibility of unfavorable microbial activity is limited (Fessard et al., 2017; Alrosan et al., 2022b). Protein degradation into short peptides, amino acids, and NH_4^+ also plays a role in lowering the pH (Tepari et al., 2020).

Similarly, the TSS of the fermented LP-QP dispersions were significantly decreased on Day 2, from 1.60 to 1.10 °Brix, and were further reduced until the final stage of the fermentation reached 0.90 °Brix on Day 5 (Table 1). These findings are consistent with the utilization of TSS (e.g., sugars and amino acids) by the fermenting microorganisms to support their growth. Indeed, the activity of bacterial enzymes is one of the main reasons for the drop in

TSS during fermentation. Similarly, the TSS of fermented soy-whey proteins dropped from 9.20 to 4.43 °Brix during water kefir fermentation (Tu et al., 2019), while a reduction from 1.88 to 1.45 °Brix was found after water kefir fermentation of soymilk proteins (Dos Santos et al., 2019).

LAB (e.g., *Liquorilactobacillus hordei*, *Lactocaseibacillus casei*, *Lentilactobacillus parafarraginis*, and *Liquorilactobacillus satsumensis*), AAB (e.g., *Acetobacter lovaniensis*, *Acetobacter fabarum*, *Acetobacter okenawensis*, *Gluconacetobacter liquefaciens*, and *Acetobacter orientalis*), and yeasts (e.g., *Saccharomyces cerevisiae*, *Dekkera bruxellensis*, *Candida ethanolica*, and *Saccharomyces cerevisiae*) are considered the primary microbial constituents of water kefir (Lynch et al., 2021; Alrosan et al., 2022c). These microorganisms and their enzymes were likely responsible for the modifications in the fermented LP-QP complexes and other nutrient and non-nutrient profiles that resulted from fermentation. On Day 2, AAB and yeasts multiplied to over 7.2 log CFU/mL from <6 log CFU/mL on Day 0. In comparison, LAB reached 6.8 log CFU/mL on Day 2 (Figure 1). The LP-QP complexes and the sugar composition of water kefir are good sources of nutrients to sustain the growth of these fermenting microorganisms and the production of their enzymes. In water kefir multiculture fermentation, yeasts initiate the fermentation process and produce enzymes that play a key role in hydrolyzing simple sugars, including fructose, glucose, and sucrose, as well as in producing ethanol (Lynch et al., 2021). The primary sources of carbon sustaining the development of LAB are glucose and fructose, while ethanol is the primary substrate supporting AAB growth. The microbial diversity of water kefir and the synergic action of AAB, LAB, and yeasts enable the production of distinctively flavored and health-promoting beverages that are a popular alternative to sugary drinks (Gulitz et al., 2011).

3.2. Protein digestibility

Water kefir fermentation significantly improved the digestibility of the LP-QP complexes from 76.43% before fermentation to 85.31% on Day 3 and then to 87.25% on Day 5 (Table 1). The fermenting microorganisms in water kefir produce a variety of enzymes, including proline-specific peptidases, exopeptidase, and intracellular peptidases, which can contribute to the breakdown of large protein assemblies into oligopeptides and then smaller peptides and amino acids (Tepari et al., 2020). Moreover, the linkages (e.g., amide, ether, and ester bonds) between nutrients (e.g., proteins, lipids, and carbohydrates) and non-nutrient components such as phenolic compounds, tannins, and saponins are also susceptible to cleavage by microbial enzymes (Chandra-Hioe et al., 2016; Emkani et al., 2022; Alrosan et al., 2023). Protein unfolding as the pH decreases promotes access of the enzymes to their cleavage sites. As illustrated in Figure 2, these enzymatic reactions decrease the molecular weight of the protein assemblies and modify the biological and physicochemical properties of fermented proteins (Siracusa, 2019; Alrosan et al., 2022c).

Increased *in vitro* digestibility of the fermented LP-QP complexes is consistent with the wide variety of fermenting microorganisms found in water kefir (Rodrigues et al., 2016)

TABLE 1 Changes in the pH, total soluble solids (TSS, °Brix), protein digestibility (%), total saponin content (TSC, mg OAE/100 g), and sugar profile (g/L) of unfermented (day 0) LP-QP complexes and water kefir fermented LP-QP complexes (days 1–5).

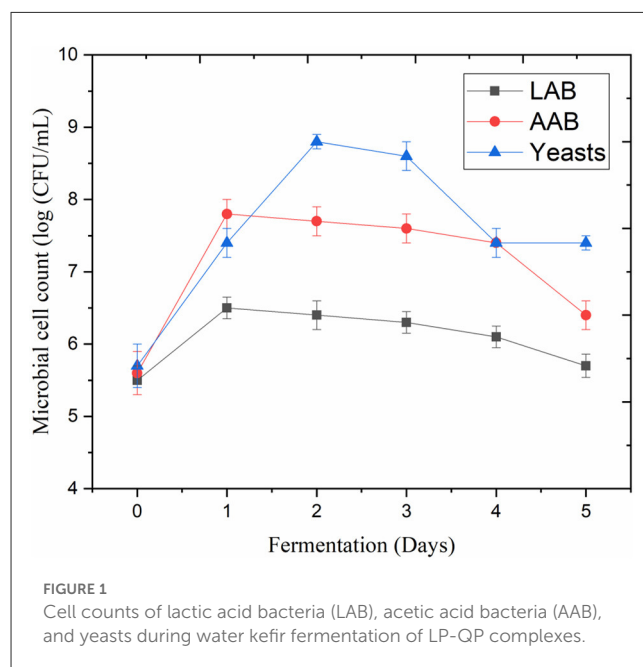
Parameters	Fermentation period (days)						P-value
	0	1	2	3	4	5	
pH	6.61 ± 0.08 ^a	4.17 ± 0.02 ^b	3.90 ± 0.01 ^c	3.84 ± 0.01 ^d	3.81 ± 0.00 ^d	3.73 ± 0.02 ^e	P < 0.05
TSS	1.60 ± 0.00 ^a	1.10 ± 0.00 ^b	1.06 ± 0.05 ^b	1.00 ± 0.00 ^c	0.96 ± 0.05 ^c	0.90 ± 0.00 ^d	P < 0.05
Protein digestibility	76.43 ± 1.92 ^c	78.40 ± 1.89 ^c	84.17 ± 2.14 ^b	85.31 ± 1.00 ^{ab}	86.23 ± 0.89 ^{ab}	87.25 ± 1.17 ^a	P < 0.05
TSC	74.52 ± 0.35 ^a	72.96 ± 1.64 ^a	66.63 ± 1.06 ^b	63.30 ± 1.05 ^c	61.93 ± 1.11 ^{cd}	60.50 ± 0.60 ^d	P < 0.05
Sugars							
Fructose	0.49 ± 0.01 ^c	2.07 ± 0.03 ^a	0.94 ± 0.01 ^b	0.41 ± 0.01 ^d	0.17 ± 0.00 ^e	0.05 ± 0.01 ^f	P < 0.05
Glucose	ND	1.15 ± 0.01 ^b	2.54 ± 0.02 ^a	1.06 ± 0.01 ^c	0.50 ± 0.01 ^d	0.22 ± 0.01 ^a	P < 0.05
Sucrose	4.85 ± 0.09 ^a	0.88 ± 0.01 ^b	0.11 ± 0.00 ^c	0.05 ± 0.00 ^{bc}	ND	ND	P < 0.05

Data are means ± standard deviation (n = 3). Values with different superscripts within the same row are statistically significant from each other (P < 0.05). LP-QP, lentil proteins-quinoa proteins (1:1 ratio); ND, not detected; OAE, oleanolic acid equivalents.

and with earlier findings. Aguirre et al. (2008, 2014) showed that LAB (*Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus helveticus*, *Limosilactobacillus reuteri*, *Lapidilactobacillus dextrinicus*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Limosilactobacillus fermentum*, and *Lacticaseibacillus* genus) and their enzymes can degrade the 7S β-conglycinin and 11S glycinin fractions of soy proteins. Improved protein digestibility from 70.5 to 77.2% was reported after faba bean and chickpea flour fermentation with *Lactobacillus* genus and *Streptococcus bulgaricus* from lyophilized yogurt cultures (Chandra-Hioe et al., 2016). *Lactobacillus* fermentation was also found to enhance the digestibility of amaranth and pea proteins (Çabuk et al., 2018; Ayala-Niño et al., 2019) as well as ovomucoid and ovalbumin (Jia et al., 2021). Recently, Alrosan et al. (2023) reported an increase in the digestibility of casein-LP complexes prepared by PPI and water kefir fermentation, from 79 to 86%.

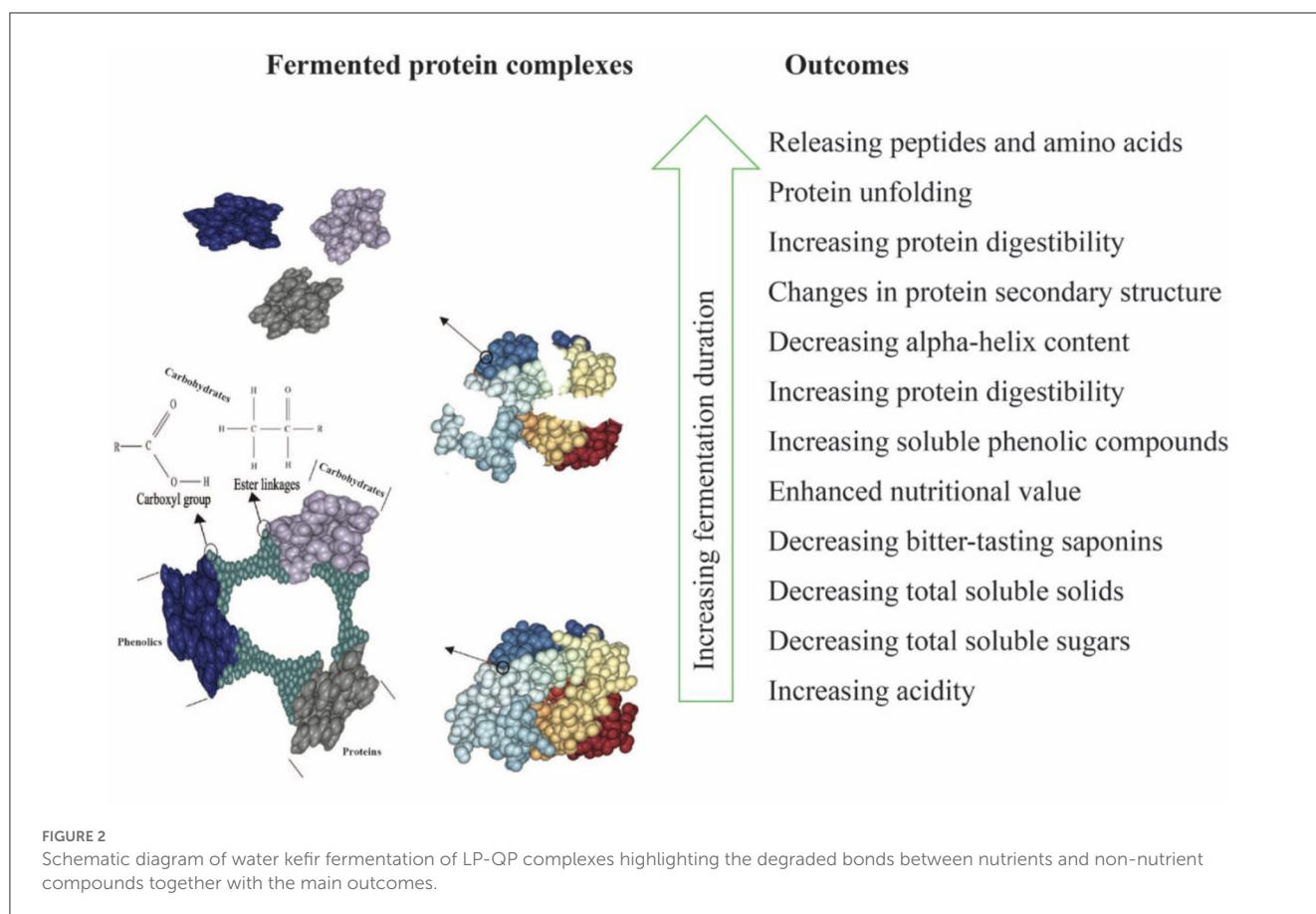
3.3. Protein secondary structure

The secondary structure components of the LP-QP complexes were assessed by FTIR. As shown in Table 2, all the secondary structure elements were present in appreciable proportions in the unfermented complexes, specifically β-turns (39.50%), β-sheets (33.58%), random coil (RC, 14.77%), and α-helices (12.13%). The contents of β-sheets and β-turns were not significantly modified during water kefir fermentation. This finding is consistent with improved protein digestibility because increased β-sheet content has been associated with impaired protein digestibility (Carbonaro et al., 2012; Bai et al., 2016). In contrast, the α-helix and RC contents of the unfermented and fermented LP-QP complexes differed significantly (P < 0.05), and the α-helix to β-sheet ratio dropped from 36.12 (Day 0) to 26.37 (Days 2 and 5). Positive correlations between the amount of α-helix, RC, and the α-helix to β-sheet ratio and the *in vitro* digestibility of feed proteins have been described in the literature (Peng et al., 2014; Bai et al., 2016). Thus, our findings of improved digestibility of fermented LP-QP complexes could be due to increased RC content



(from 14.77% on Day 0 to 19.46% on Day 5) and unchanged β-sheet content. It is possible that the decreased content of α-helix and α-helix to β-sheet ratio may have been moderate enough so that no net reduction in protein digestibility was detected. Another plausible explanation is that the associations between the amount of these secondary structures and protein digestibility may be influenced by the food/feed matrix and composition, and/or by the experimental conditions used to determine protein digestibility. In line with our findings, Samadi and Yu (2011) showed that *in situ* protein digestibility and α-helix to β-sheet ratio varied in opposite directions after heat treatment of soybean seeds. Similar findings were reported with flaxseeds (Doiron et al., 2009).

While water solubility of the LP-QP complexes was not assessed in the present study, it is possible that the reduction in α-helix



content and α -helix to β -sheet ratio achieved after fermentation might have improved the water solubility of these complexes. According to Alrosan et al. (2023), increasing the proportion of casein in LP-casein complexes significantly increased their water solubility from 68.6 to 91.4%. They further showed that subsequent fermentation with water kefir reduced the α -helix to β -sheet ratio and increased protein digestibility from 79.5 to 86.8%. Future studies will be needed to establish the water solubility and other important functional properties of LP-QP complexes.

Such structural changes could be explained by concomitant acidification and enzymatic action. The acidification caused by the production of lactic and acetic acids weakens the ionic interactions that stabilize the protein's secondary and tertiary structures, which promotes protein unfolding and changes in protein structures (Meinlschmidt et al., 2016). According to Wang et al. (2021), LAB and their enzymes are essential in fermented food applications and contribute to the hydrolysis of both sugars and proteins in plant proteins. LAB can secrete extracellular enzymes and hydrolyze carbohydrates and proteins into smaller fragments. Most sugar- and polysaccharide-hydrolyzing bacterial strains, such as *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, and *Lactiplantibacillus plantarum* (Gänzle and Zheng, 2019; Wang et al., 2021), can produce amylase to degrade starch into dextrin and ultimately glucose. Similarly, protein hydrolysis by LAB involves several steps, including protein breakdown, peptide transport and degradation, and amino acid catabolism (Kunji et al., 1996; Christensen et al., 1999). This

process is initiated by cell envelope proteinases that hydrolyze proteins into oligopeptides, which are in turn converted into tri- and dipeptides and amino acids by the action of various peptidases enzymes, such as tri- and dipeptidases, aminopeptidases, endopeptidases, and proline-specific peptidases (Vesanto et al., 1996).

Our findings provide evidence of rearranging secondary structure components (α -helix and RC) of LP-QP complexes induced by fermentation. The α -helices content was significantly reduced ($P < 0.05$) to 7.63% at the end of water kefir fermentation, while RC increased to 19.46% (Table 2). The helix-coil conformational transition could explain these observations because the magnitude of change in α -helix and RC proportions was similar in absolute value (ca. -4 and $+4$ points, respectively), while the proportions of other structural elements remained stable. Similarly, fermentation with *Aspergillus ficuum* was found to alter the secondary protein structure of soy protein and to decrease the α -helix content from 19.16 to 14.48% after 24 h (Yasar et al., 2020). In Yasar et al.'s (2020) study, the α -helix component was completely degraded after 48 h, leading to a significant increase in RC and β -turn contents. Other types of fermentation were shown to impact these structural components. Alrosan et al. (2023) evidenced changes in the α -helix and RC contents of casein-LP complexes upon water kefir fermentation, while Yakubu et al. (2022) reported variations in β -sheets (55–48%), RC (12–15%), and β -turns (34–29%) after alkaline fermentation of locust bean flour.

TABLE 2 Changes in the percentage of secondary structure components [β -sheet, random coil (RC), α -helix, and β -turn] of unfermented LP-QP complexes (day 0) and water-kefir fermented LP-QP complexes (days 1–5) based on FTIR measurements.

Secondary protein components	Peak (1/cm)	Fermentation period (days)						P-value
		0	1	2	3	4	5	
β-sheet								
	1,614.42	13.24	12.56	12.65	11.66	11.07	10.15	
	1,622.13	11.06	11.23	11.55	10.48	10.36	9.64	
	1,633.71	9.28	9.64	10.11	9.70	9.53	9.02	
β -sheet (Σ)		33.58 ^a	33.43 ^a	34.31 ^a	31.83 ^a	30.95 ^a	28.81 ^a	$P > 0.05$
RC (Σ)	1,645.28	14.77 ^f	15.54 ^e	16.22 ^d	17.66 ^c	18.39 ^b	19.46 ^a	$P < 0.05$
α -helix (Σ)	1,654.07	12.13 ^a	11.23 ^b	9.04 ^c	7.82 ^d	7.70 ^e	7.63 ^f	$P < 0.05$
β-turn								
	1,668.43	8.31	8.41	8.68	8.94	9.14	9.14	
	1,681.93	15.78	15.88	16.02	16.87	16.97	17.01	
	1,693.50	15.42	15.49	15.72	16.86	16.83	17.93	
β -turn (Σ)		39.50 ^a	39.78 ^a	40.41 ^a	42.67 ^a	42.94 ^a	44.08 ^a	$P > 0.05$
Ratio ¹		36.12	33.59	26.37	24.59	24.89	26.50	

Data are means ($n = 3$). Values with different superscripts within the same row are statistically significant from each other ($P < 0.05$).

LP-QP, lentil proteins-quinoa proteins (1:1 ratio).

¹Ratio of α -helix/ β -sheet.

3.4. Soluble sugars

As shown in Table 1, the concentrations of fructose, glucose, and sucrose evolved rapidly throughout water kefir fermentation. Sucrose concentration decreased from 4.85 g/L on Day 0 to 0.11 g/L on Day 2 and then to non-detectable levels on Days 4 and 5. In contrast, fructose and glucose concentrations peaked on Day 1 (2.07 g/L) and Day 2 (2.54 g/L), respectively, and steadily decreased subsequently. These findings are consistent with the rapid growth of LAB, AAB, and yeasts and the corresponding pH decline that occurred within the first 48 h of fermentation (Figure 1; Table 1). They concur with existing findings (Martínez-Torres et al., 2017; Tu et al., 2019; Alrosan et al., 2023). In Alrosan et al.'s (2023) study, fructose and glucose levels rose within the first 48 h of water kefir fermentation of casein-LP complexes, followed by a steady diminution of all soluble sugars until the end of fermentation.

Kefir yeasts not only metabolize these soluble sugars for growth and enzymatic activities, but they also release glucose and fructose that the commensal kefir microorganisms can use. Moreover, they play a crucial role by releasing peptides and amino acids to the LAB (e.g., *Lentilactobacillus hilgardii*) (Leroi and Pidoux, 1993; Rodriguez et al., 2022). Previous studies by Stadie et al. (2013) and Xu et al. (2019) have shown mutually beneficial outcomes between LAB and yeasts in water kefir, including enhanced microbial growth when LAB (e.g., *Liquorilactobacillus hordeii* and *Liquorilactobacillus nagelii*) and yeasts (e.g., *Saccharomyces cerevisiae* and *Zygorulasporea florentina*) are co-cultivated. Overall, our findings suggest that the water kefir fermentation process can be optimized to produce LP-QP complexes with low sugar content and desirable pH by adjusting the fermentation conditions and establishing adequate proportions of yeasts and LAB similar to those shown in

Figure 1. Further studies would be required to optimize this fermentation process.

3.5. Phenolic compounds

Plant-based proteins are associated with a wide range of health-promoting compounds, including phenolic compounds (Oomah et al., 2011). Phenolic compounds can form covalent bonds with proteins and other nutrients (Santos-Zea et al., 2018), which could influence protein digestibility and functional properties. In the present study, the TPC of the LP-QP complexes significantly increased after water kefir fermentation, from 364 mg GAE/100 g on Day 0 to 492 and 409 mg GAE/100 g on Days 2 and 5, respectively (Table 3). The main phenolic compounds identified were catechin, chlorogenic acid, epicatechin, quercetin, rutin, caffeic acid, ferulic acid, gallic acid, sinapic acid, and syringic acid (Table 3). The most abundant phenolics in the unfermented complexes were epicatechin, chlorogenic acid, and syringic acid (96.5, 48.8, and 29.5 mg/100 g, respectively). After fermentation for 5 days, catechin also reached a high level (31.5 mg/100 g). The levels of the individual phenolic compounds peaked on Days 2 or 3. A 2-fold increase was evidenced for catechin (Day 2), caffeic acid, gallic acid, and sinapic acid (Day 3). Other phenolics, including chlorogenic acid, epicatechin, and rutin, markedly increased on Day 2 or 3 (from 48.83 to 65.97, 96.49 to 134.69, and 7.39 to 12.05 mg/100 g, respectively).

The increased phenolic content of fermented LP-QP complexes could be due to the activities of the fermenting microorganisms found in water kefir, which may produce enzymes such as phenolic acid esterases and tannases that cleave the ester bonds that connect phenolic compounds to other compounds, most

TABLE 3 Changes in total phenolic content (TPC, mg GAE/100 g) and individual phenolic compounds (mg/100 g) of unfermented LP-QP complexes (day 0) and water kefir fermented LP-QP complexes (days 1–5).

	Fermentation period (days)						P-value
	0	1	2	3	4	5	
TPC	364.61 ± 3.58 ^f	379.54 ± 2.73 ^e	492.36 ± 12.18 ^a	477.44 ± 3.58 ^b	449.98 ± 4.50 ^c	409.38 ± 1.79 ^d	P < 0.05
Phenolic compounds							
Catechin	18.95 ± 0.27 ^d	35.27 ± 0.31 ^b	36.36 ± 0.44 ^a	34.79 ± 0.12 ^b	31.71 ± 0.14 ^c	31.47 ± 1.29 ^c	P < 0.05
Chlorogenic acid	48.83 ± 0.64 ^f	54.17 ± 0.39 ^e	65.97 ± 0.36 ^a	59.11 ± 0.55 ^b	56.88 ± 0.44 ^c	55.98 ± 0.37 ^d	P < 0.05
Epicatechin	96.49 ± 0.21 ^c	110.91 ± 0.34 ^b	134.69 ± 0.90 ^a	111.46 ± 0.82 ^b	109.55 ± 3.49 ^b	107.61 ± 4.26 ^b	P < 0.05
Quercetin	12.54 ± 0.65 ^d	14.26 ± 0.37 ^c	16.88 ± 0.47 ^a	17.47 ± 0.67 ^a	15.57 ± 0.31 ^b	6.69 ± 0.21 ^e	P < 0.05
Rutin	7.39 ± 0.32 ^e	8.15 ± 0.16 ^d	9.39 ± 0.25 ^b	12.05 ± 0.35 ^a	9.10 ± 0.37 ^{bc}	8.58 ± 0.40 ^{cd}	P < 0.05
Caffeic acid	5.44 ± 0.24 ^d	7.94 ± 0.09 ^c	9.51 ± 0.40 ^b	10.44 ± 0.18 ^a	9.34 ± 0.30 ^b	9.16 ± 0.24 ^b	P < 0.05
Ferulic acid	13.19 ± 13.19 ^d	15.19 ± 0.23 ^c	16.82 ± 0.76 ^b	18.50 ± 0.65 ^a	15.66 ± 0.86 ^c	13.93 ± 0.67 ^d	P < 0.05
Gallic acid	14.04 ± 0.64 ^d	19.46 ± 0.41 ^c	22.91 ± 0.38 ^b	29.39 ± 3.01 ^a	18.40 ± 0.60 ^{cd}	16.77 ± 0.07 ^d	P < 0.05
Sinapic acid	8.78 ± 8.78 ^e	13.81 ± 0.86 ^b	15.22 ± 0.98 ^a	15.71 ± 0.46 ^a	11.40 ± 0.41 ^c	10.06 ± 0.29 ^d	P < 0.05
Syringic acid	29.51 ± 0.78 ^c	30.66 ± 0.31 ^{bc}	33.47 ± 0.48 ^a	32.52 ± 0.27 ^{ab}	32.11 ± 1.95 ^{ab}	30.91 ± 0.98 ^{bc}	P < 0.05
Phenolic compounds (Σ)	255.16 ^c	309.82 ^c	361.23 ^a	341.45 ^b	309.72 ^c	291.19 ^d	P < 0.05

Data are means ± standard deviation ($n = 3$). Values with different superscripts within the same row are statistically significant from each other ($P < 0.05$). GAE, gallic acid equivalents; LP-QP, lentil proteins-quinoa proteins (1:1 ratio).

notably proteins (via hydroxyl groups) and carbohydrates (via carboxylic groups) (Bhanja et al., 2009; Dordević et al., 2010; Adebo and Medina-Meza, 2020; Shahidi and Dissanayaka, 2023), as illustrated in Figure 2. Such enzymatic action may not only increase the amounts of free phenolic compounds but may also release smaller or otherwise modified phenolic-derived metabolites with distinct biological activities (Liyana-Pathirana and Shahidi, 2006; Knockaert et al., 2012; Santos et al., 2018). Phenolic compounds and proteins also form complexes via non-covalent interactions through hydrophobic, electrostatic, van der Waals forces, and hydrogen bonding (Shahidi and Dissanayaka, 2023), which may be weakened or disrupted during fermentation as a result of the changing pH and ionic strength. Our findings are supported by earlier reports pointing to a rise in the phenolic compounds during the fermentation of plant-based proteins (Lai et al., 2013; Gunenc et al., 2017; Alrosan et al., 2023). Aiello et al. (2020) concluded that fermentation with water kefir is an effective strategy for enhancing the phenolic content and antioxidant capacity of plant-based materials.

We further found that after Days 2 or 3 of water kefir fermentation, the level of phenolic compounds started to decrease ($P < 0.05$). However, for most individual phenolics, except ferulic acid, gallic acid, and syringic acid, the levels achieved on Day 5 remained significantly greater than those in the unfermented complexes. This gradual reduction may be attributed to the hydrolysis of some phenolic compounds by some fermenting microorganisms, including *Lactobacillus* spp. (Adebo and Medina-Meza, 2020), which could result in the production of smaller catabolites. Further studies are needed to clarify the mechanisms underlying this phenomenon and to assess the bioactive properties of the resulting compounds.

3.6. Total saponin content

Saponins are a diverse group of bioactive phytochemicals containing a carbohydrate moiety linked to a triterpenoid or steroid aglycone (Zhang et al., 2018). Many pharmacological properties have been reported for these compounds, including hypocholesterolemic, hypoglycemic, anti-inflammatory, and immunostimulant activities (Marrelli et al., 2016). However, saponins in cereals and legumes can confer a bitter taste resulting in reduced consumer acceptance (Han et al., 2018). According to Suárez-Estrella et al. (2021), high amounts of saponins (e.g., 860 mg/100 g in quinoa sprouts) can lead to poor consumer acceptability. The total saponin content of extracts from LP and QP was found to range from 0.19 to 10.63 g/100 g and 0.26 to 5.51 g/100 g, respectively, depending on the extraction solvent (del Hierro et al., 2018). The amount of saponins interacting with these proteins depends on the raw materials (e.g., variety, source, and origin) and on the preparation and extraction methods, especially solvent polarity which strongly influences the extraction and recovery yield.

We showed that the unfermented LP-QP complexes contained a relatively high amount of TSC (74.52 mg OAE/100 g) (Table 1). Upon fermentation by water kefir, TSC declined significantly ($P < 0.05$) to 66.63 mg OAE/100 g on Day 2 and ultimately to 60.50 mg OAE/100 g on Day 5. This drop may be attributed to weaker hydrogen bonding and electrostatic or hydrophobic interactions between saponins and proteins (Li et al., 2023) and/or to saponin hydrolysis by microbial enzymes during fermentation (Dajanta et al., 2011). Decreasing TSC is consistent with previous findings obtained with casein-LP complexes subjected to water kefir fermentation (Alrosan et al., 2023). Other studies

indicated that fermentation with *Aspergillus oryzae*, *Rhizopus stolonifer*, and *Bacillus* can reduce saponin and phytate levels due to the action of their extracellular enzymes (Nout, 2009; Tsuji et al., 2015). Lactic fermentation by LAB was found to decrease the TSC in soymilk from 115.02 to 60.99 mg saponin equivalent/g (Lai et al., 2013). The reduced TSC contents achieved at the end of the fermentation could reduce the perceived bitterness while preserving the health benefits associated with dietary saponins.

4. Conclusion

This study provides a novel combination strategy for improving the functionality of LP and QP by forming soluble LP-QP complexes produced by protein-protein interactions, possibly including other nutritious compounds, and further modified by fermentation with water kefir. This combined (dual) treatment improved the quality of the fermented LP-QP complexes. The significant enhancement of protein digestibility *in vitro* may reflect the unique protein configurations and composition achieved through complexation and fermentation. Modified α -helix and random coil contents were evidenced over the course of fermentation, which were attributed to the activity of water kefir fermenting microorganisms and their enzymes. These improvements may lead to increased biological value and more widespread utilization of LP and QP, which could have important implications for overall health and nutrition. The increased phenolic content is also noteworthy, suggesting that these health-promoting compounds become more readily accessible upon fermentation with water kefir. Concomitantly, the reduced saponin content of the protein complexes could translate into reduced bitterness and thus greater acceptability of this novel multifunctional ingredient. Further research is needed to assess the sensory and functional properties of the fermented LP-QP complexes, their bioactive peptide content, and their bioavailability *in vivo*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

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Author contributions

MAI collected test data and drafted the manuscript. MAI and T-CT designed the study, interpreted the results, and revised the manuscript. CCT contributed in conceptualization, visualization, interpreting the results, resources, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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