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Methods in Food Chemistry and Food Science Technology

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Methods in food chemistry and food science technology

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Editorial: Methods in food chemistry and food science technology

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Editorial on the Research Topic
Methods in food chemistry and food science technology

This Research Topic is aimed to highlight the latest experimental techniques and methods used to investigate fundamental questions in Food Chemistry/Food Science Technology research. This Topic includes technologies and up-to-date methods which help advance science. It highlights recent advances in food chemistry and improves the knowledge of bioactive components of food from a nutritional and sensorial point of view. In this special e-collection, 10 papers covering the above-mentioned aspects have been included.

By 2050, it has been estimated that the world's population will exceed 10 billion people, which will lead to a deterioration in the global food security. To avoid aggravating this problem, dietary changes to reduce the intake of animal calories and increase consumption of sustainable, nutrient-rich, and calorie-efficient products have been recommended. The nutrient-dense grain legumes are one of the most promising alternative proteins which could exert a key role in preventing global food security and environmental sustainability. Among legumes, lupin seeds have an exceptional nutritional profile, with a high protein and dietary fiber content, making them an ideal candidate to help meet the world's growing demand for supplemental sources of protein. The major storage proteins of lupin seeds, termed α-, β-, δ- and γ-conglutins, exert both nutritional and anti-nutritional properties, thus variations in the abundance of these protein families may influence the nutritional and physiological properties of different lupin varieties. Therefore, the study by Tahmasian et al. aimed to explore the conglutin protein profiles of 46 domestic and wild narrow-leaved lupin genotypes. These authors applied discovery proteomics to identify 16 known conglutin subfamilies and high-performance liquid chromatography–multiple reaction monitoring-mass spectrometry (HPLC–MRM-MS) to evaluate their abundance in the different genotypes. These authors reported large variability in the β- and δ-conglutin content among lupin varieties and identified potential hypoallergenic genotypes in several domestic cultivars. These findings will allow the development of lupin varieties with higher levels of bioactive proteins and lower allergen content, which will be of great relevance to lupin-allergic consumers and food industries.

Purple speckled kidney bean, commonly known as *Phaseolus vulgaris* L., is one of the most important edible legumes because of its high content of proteins, complex carbohydrates, and essential vitamins and minerals. In addition of its nutritional role, proteins from this legume play an important role as source of bioactive peptides. These

peptides are inactive within the source protein but can exert biological activities once released by enzymatic hydrolysis, gastrointestinal digestion and/or fermentative processes. In the study of Li et al., a protein hydrolyzate of purple speckled kidney bean with pepsin and trypsin was used as the raw material to isolate, by using a macroporous resin under optimized conditions, and to purify, by reversed-phase high-performance liquid chromatography (RP-HPLC), three radical scavenging peptides which sequences were FLVDRI, FLVAPDD, and KDRVISEL. The high specificity and biocompatibility of bioactive peptides released from plant, animal, and marine protein sources make them a suitable alternative to pharmacological therapies for promoting health and treating chronic diseases of high incidence and mortality in our society. Although their promising role as food preservatives, ingredients for functional foods and nutraceuticals has been already recognized, application of bioactive peptides in the clinical and food sectors is still very limited due to some disadvantages such as toxicity, bitterness, instability, and susceptibility to enzymatic degradation in the gastrointestinal tract. Thus, it is needed to employ different strategies to enhance the bioactivity and bioavailability of bioactive peptides, and to increase the production yield from natural sources. The review by Majura et al. focuses on the existing evidence on bioactive peptides and the strategies that have been implemented to overcome their limitations and to promote the development and commercialization of peptides-based ingredients with practical applications.

Despite their high nutritional value, their key role as source of bioactive peptides, and their techno-functional and rheological properties, some food proteins can contain immunogenic peptides responsible for allergenic response in humans. Thus, gluten is a complex mixture of water insoluble proteins rich in glutamine and proline, and markedly resistant to the action of gastrointestinal enzymes. This resistance favors the release in the gut lumen of large gluten proteins-derived peptides with potential to activate inflammatory responses in celiac disease patients. The review of Mamone et al. shows the state of art of analytical and functional methods currently used to evaluate the immunogenicity potential of gluten proteins from different cereal sources, raw seed flours and complex food products. The analytical design for assessing the content and profile of gluten-immunogenic peptides is based on the harmonized oral-gastrointestinal INFOGEST protocol followed by extensive characterization of residual gluten peptides by proteomic (coupled to tandem mass spectrometry, HPLC-MS/MS) and immunochemical ELISA analyses. The functional studies described in the review are based on the evaluation of the immunostimulatory ability of gluten peptides on gut mucosa T cells or peripheral blood cells collected from celiac disease patients after being subjected to a short oral gluten challenge.

In addition to their high content of essential amino acids, mung bean proteins have been reported to exert many health benefits, such as enhancing glycolipid metabolism, preventing and managing non-alcoholic fatty liver disease, and modulating activity of antioxidant enzymes. These proteins are commonly used for producing protein beverages, and emulsion stabilizers. However, their use in staple foods such as noodles is limited because of the absence of gluten. The addition of other legume proteins, such as peas and soybean proteins, to noodles significantly enhances their taste, palatability, and cooking characteristics. Thus, to broaden the use of mung bean proteins in noodles, Diao et al. applied a hydrothermal modification method based on gel property indicators such as gel strength, water absorption capacity, and disulfide bond content to improve the gel properties of these proteins. The efficacy of the treatment was assayed adding the modified proteins to noodles, thus demonstrating their positive effects on the wheat dough properties and noodle quality. These results support the incorporation of new proteins into staple foods.

Tribology is the science of the interaction of moving surfaces, considering the lubrication and friction of the materials. In recent years, this discipline has been applied to mimic the friction between foods and the oral cavity to understand the sensory attributes of selected food products. In the exhaustive review of Corvera-Paredes et al., the authors showed the application of soft tribology to dairy products perception and explained the important role of saliva in oral processing and interaction with dairy proteins. Sensory attributes such as astringency, smoothness and creaminess were explained based on tribology experiments and it was observed that milk proteins would produce a sensory perception of astringency by salivary interaction. In contrast, dairy fat would diminish the sensation of astringency in the oral cavity, reducing the coefficient of friction and increasing the smoothness and creaminess of whole dairy products. The study of Tian et al. also highlighted the importance of composition on the sensory quality of steamed breads. These authors evaluated three varieties of rice (long-grained rice, polished round-grained rice and black rice) mixed with different flours, and determined the effect that protein, fat, several microelements and total dietary fiber exerted on steamed bread quality. The addition of rice flour significantly increased the arginine content of the mixtures and, when the amount of rice flour exceeded 15%, the elasticity and sensory score of steamed breads were gradually reduced. Although the acceptability of this type of dough needs further investigation, this work is an excellent starting point to use rice flour as a promising ingredient to improve the nutritional quality of foods, and the results would be of interest to the steamed bread and noodle industry.

On the other hand, the current Research Topic also addresses the need for simple, fast and cost-effective methods to quantitatively determine food composition. In the study of Hueso et al., the most common methods used for total protein quantification were compared in milk samples of different species and ultrafiltration products. The ultrafiltration process is widely used in the dairy industry to concentrate whey proteins, resulting in a high total solids retentate and a permeate, and its monitoring by quantifying total protein content is critical. In this research, the Kjeldahl reference method for total protein determination, which is time-consuming and requires specific instrumentation, was compared to the bicinchoninic acid assay (BCA), the detergent compatible Bradford assay and the Dumas method. It was observed that the BCA assay was significantly affected by the composition of the food matrix and its use for protein quantification in dairy samples should be avoided. In contrast, the Bradford assay and the Dumas method provided accurate results. Taking into consideration that the Dumas method is destructive and requires specific instrumentation, these authors recommend the

Bradford assay to determine the total protein content in milk and ultrafiltration products. This assay provided fast and accurate results, with a small amount of sample and without the need for specific equipment, and was not influenced by the amount of non-protein nitrogen in the food sample.

Another interesting improvement of the methodology commonly used for fat extraction is the one proposed by Watanabe et al. in dairy samples. These authors found that lipid recovery from liquid infant formulas and human breast milks was lower than expected when the Röse-Gottlieb reference method was applied. The yield of neutral lipids was not improved neither by increasing the time and temperature of sample heating with ammonia water before solvent extraction, nor by increasing the volume of extraction solvent per volume of sample, or by using chloroform or dichloromethane as extraction solvents. In this study, the use of solid phase extraction cartridges with silica gels successfully improved the extraction yield of neutral lipids (∼10%) in both liquid infant formula and ruminant milk. It was suggested that silica gel may help to disrupt the emulsion of the samples and improve lipid recovery. Furthermore, this approach was even more effective in extracting triglycerides from human milk, where lipid recovery was increased by >25%.

Finally, the research of Wang et al. investigated the effects of supplementation with uridine in a sow-piglets model on reproductive performance and amino acid metabolism. Uridine is the precursor of uridine monophosphate, the most abundant nucleotide in sow milk, and it is of vital importance to maintain basic cellular functions in animal tissues. The addition of uridine in the diet of sows during their second trimester of gestation significantly reduced the average number of stillborn piglets per litter, with higher average piglet weight at birth. Besides, the free amino acids profile in sow serum, newborn piglet serum and colostrum was modified by maternal uridine supplementation. The expression of amino acids transporter and the viability of pTr2 cells was also affected. This novel study showed that the addition of uridine would alter the growth of placental tissue by increasing the viability of pTr2 cells, and the amino acid metabolism in sows and their newborn piglets.

Author contributions

BH-L and PG-C contributed equally to the writing of the manuscript and approved the submitted version.

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Evaluation of the Major Seed Storage Proteins, the Conglutins, Across Genetically Diverse Narrow-Leafed Lupin Varieties

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Lupin seeds have an excellent nutritional profile, including a high proportion of protein and dietary fiber. These qualities make lupin seeds an ideal candidate to help meet the growing global demand for complementary sources of protein. Of consequence to this application, there are nutritional and antinutritional properties assigned to the major lupin seed storage proteins—referred to as α-, β-, δ- and γ-conglutins The variation in the abundance of these protein families can impact the nutritional and bioactive properties of different lupin varieties. Hence, exploring the conglutin protein profiles across a diverse range of lupin varieties will yield knowledge that can facilitate the selection of superior genotypes for food applications or lupin crop improvement. To support this knowledge generation, discovery proteomics was applied for the identification of the 16 known conglutin subfamilies from 46 domestic and wild narrow-leafed lupin (NLL) genotypes. Consequently, the diversity of abundance of these proteins was evaluated using liquid chromatography–multiple reaction monitoring-mass spectrometry (LC–MRM-MS). This comparative study revealed a larger variability for the β- and δ-conglutin content across the lines under study. The absence/lower abundance of the β2- to β6-conglutin subfamilies in a subset of the domesticated cultivars led to substantially lower overall levels of the allergenic β-conglutin content in these NLLs, for which the elevation of the other conglutin families were observed. The diversity of the conglutin profiles revealed through this study—and the identification of potential hypoallergenic genotypes—will have great significance for lupin allergic consumers, food manufactures as well as grain breeders through the future development of lupin varieties with higher levels of desirable bioactive proteins and lower allergen content.

Keywords: narrow-leafed lupin, *Lupinus angustifolius*, plant-based protein, conglutin, legume, proteomics, LC-MRM-MS

Abbreviations: LC-MS, Liquid chromatography-mass spectrometry; LC-MRM-MS, Liquid chromatography-multiple reaction monitoring-mass spectrometry; NLL, Narrow-leafed lupin; DTT, dithiothreitol; PCA, Principal component analysis; HCA, Hierarchical cluster analysis; GO, Gene ontology; IDA, Information dependent acquisition.

INTRODUCTION

Interest in plant-based protein sources is on the rise (1) with growth driven by a rapidly increasing population, consumer demand and a groundswell for planetary health (2). The nutrient-dense grain legumes, also known as pulses, are one of the most promising plant-based complementary sources of protein, which could have a major contribution in enhancing global food security and environmental sustainability (3). Among the legumes, the lupin seeds stand out, owing to their favorable nutritional profiles associated to the remarkably high levels of quality protein and dietary fiber, as well as the nutraceutical benefits, including obesity-, type 2 diabetes-, and cardiovascular disease prevention (4).

Lupins belong to the diverse *Lupinus* genus of the Fabaceae family, which have been cultivated for thousands of years. Currently, narrow-leafed lupin (NLL, *Lupinus angustifolius)*, white lupin (*Lupinus albus*), pearl lupin (*Lupinus mutabilis*) and yellow lupin (*Lupinus luteus*) are the most cultivated species of lupin and are mainly grown as green manure in rotation with cereal crops or used as stockfeed. Despite their vast potential as a plant-based protein source, this ancient legume is under-utilized as a food ingredient; however, with the increasing demand in nutritious plant-based protein sources, the lupin market has the potential to expand beyond animal feed applications. NLL, also known as Australian sweet lupin, is one of the most recently domesticated crops (5). Genetic diversity studies targeting the NLL germplasm have resulted in the identification of a wide genetic and adaptive diversity, which can be accessed for improving lupin crops (6). In contrast, measurements of variation in the proteome of NLL germplasm have been hitherto underexplored.

The conglutin seed storage proteins are the most abundant protein class in NLL seeds, which not only serve as a nutrient reservoir for the germinating seed but also impact the nutritional quality of lupin seeds as a protein source for humans and livestock. These proteins have been classified into four major families: α-, β-, γ-, and δ- conglutins, which comprise three, seven, two, and four subfamilies, respectively (7). Distinct nutritional and nutraceutical properties have been attributed to each conglutin family; for example, the γ-conglutins are known to have blood glucose lowering effects (8), whilst β-conglutin proteins—which have demonstrated anti-inflammatory and antioxidant activities—are reported as the major proteins responsible for lupin allergy (9). Hence, variation in abundance of these proteins across different lupin varieties can impact their nutritional quality and bioactive effects. In fact, comparative evaluations have identified substantial variations in the functional properties (10), health benefits (11), allergenicity potential (12, 13) and digestibility (14) across different lupin cultivars. The relationships of the conglutin profiles with these traits are yet to be explored. The previous effort to study the NLL intraspecific proteome diversity have included a qualitative evaluation of the Australian domesticated cultivars using the polymorphism in the protein mass peak profiles (15), as well as the qualitative assessment of the four major conglutin families across a limited set of NLL cultivars (16). Still, there remains an opportunity to expand current knowledge regarding conglutin abundance in a wider variety of domesticated cultivars and diverse wild accessions to unravel the natural proteome diversity in lupin seeds and exploit this knowledge for crop improvement.

In this study, the diversity of the conglutin profiles across a panel of 46 genetically diverse NLL genotypes (including 16 domesticated and 30 wild accession) were assessed through a combination of discovery and targeted proteomics measurements. The detected conglutin peptides were used to evaluate the conservation of the conglutin protein sequences across the analyzed NLLs. The differentiation and quantitative study of the 16 known conglutin proteins across these genotypes were achieved through monitoring a set of marker peptides deemed representative of each conglutin subfamily. The knowledge gained from this work will facilitate progress in the identification of genotypes containing higher levels of desirable nutritional, and lower content of antinutritional, conglutins. The resulting varieties can be expected to produce seeds tailored for heath and food purposes or be incorporated in NLL breeding programs to further enhance the quality of the lupin grain as a food ingredient.

MATERIALS AND METHODS

Plant Material

Overall, 46 NLL accessions were included in this study. This panel included: (a) 30 genetically diverse wild accessions, representing the natural geographic range of the species throughout the Mediterranean Basin (17, 18); and (b) 16 fully domesticated and semi-domesticated NLL accessions which comprise of 11 cultivars released throughout the four different phases of Australian lupin breeding program, two breeding lines (Australian and Belarusian) as well as three Polish cultivars. The seed material for these genotypes were obtained from the Australian Grains Genebank (Horsham, VIC, Australia), CSIRO Agriculture and Food (Floreat, WA, Australia) and Australian Grain Technologies (Northam, WA, Australia). The identifiers used for these accessions throughout the manuscript, as well as the information on their country of origin, domesticated/wild status and supplier are summarized in **Supplementary Table 1**. All the seed samples were inspected to exclude foreign contamination and ground into a fine powder using a mixer mill (model MM400 Retsch, Germany).

Chemicals, Enzymes, and Solvents

All the reagents and chemicals used were of analytical grade. Tris-hydroxymethyl aminomethane hydrochloride (Tris-HCl), dithiothreitol (DTT), iodoacetamide (IAM), n-hexane, ammonium bicarbonate, acetonitrile (ACN), and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Bayswater, VIC, Australia). Urea and formic acid were acquired from ChemSupply (Gillman, SA, Australia). Sequencing grade trypsin and protease inhibitor cocktail was purchased from Promega (Alexandria, NSW, Australia). BCA Protein Assay Kit Reducing Agent Compatible was obtained from Thermo Fisher Scientific

(Scoresby, VIC, Australia). The HPLC-grade water (18 MΩ cm) was prepared using an Arium® pro ultrapure water system (Sartorius, Gottingen, Germany).

Sample Defatting

For lipid removal from the lupin samples, 200 μL of *n*-hexane solvent was added to micro-tubes containing ∼20 mg of lupin flour and vortexed until thoroughly combined. The samples were further mixed using a thermoshaker (model GRA13687717, Thermo Fisher Scientific, Scoresby, VIC, Australia) at 1,000 rpm for 20 min. This was followed by centrifugation (10 min) at 20,800 × g (with all steps repeated three times). Subsequently, the samples were air dried in a fume hood and stored at 3°C for protein extraction (which was conducted within 24 h).

Protein Extraction and Protein Estimation

A recently optimized urea-based protocol was used for the efficient extraction of the proteins from lupin seeds (19). Briefly, 800 μL of urea buffer (8 M urea, 2% (w/v) DTT, 1% (v/v) protease inhibitor cocktail in 0.1 M Tris-HCl; pH 8.2) was added to 20 mg of defatted lupin varieties (n = 3). The mixture was vigorously vortexed and incubated in a thermoshaker (600 rpm) at RT for 45 min. The solutions were centrifuged for 15 min at 20,800 × g and aliquots of the supernatant were collected for subsequent analysis. Protein estimations were conducted using a BCA Protein Assay Kit (Pierce™, Thermo Fisher Scientific) according to the manufacturer's protocol.

Tryptic Digestion

The tryptic peptides were generated using the filter-aided sample preparation (FASP) procedure as described previously (20) with minor modifications. In brief, 25 μL of the protein extracts were transferred onto 10 kDa molecular weight cut-off (MWCO) filters (Millipore, Bayswater, VIC, Australia) and washed twice using 100 μL of urea buffer (8 M urea in 0.1 M Tris-HCl, pH 8.2) with centrifugation (20,800 × g, 10 min). After which 100 μL of 25 mM iodoacetamide (in 8 M urea in 1 M Tris-HCl) was added for cysteine alkylation and the samples incubated in the dark at RT for 20 min. The excess iodoacetamide was then removed through two consecutive washes (100 μL of urea buffer) and centrifugation (20,800 × g, 10 min) steps. This was followed by buffer exchange with two 100 μL volumes of 100 mM ammonium bicarbonate (pH = 8.4) and centrifugation (20,800 × g, 10 min). Subsequently, 125 μL of 0.02 μg/μL sequencing grade trypsin (in 50 mM ammonium bicarbonate) was added to each filter and the samples were incubated overnight at 37°C. The digested peptides were collected in fresh centrifuge tubes with centrifugation at 20,800 × g for 15 min. The filters were washed with 200 μL 0.1% formic acid and the combined filtrates were evaporated to dryness in a vacuum centrifuge.

Global Proteome Measurement

The tryptic peptides were resuspended in 50 μL of 0.1% formic acid and the pooled samples of biological replicates were subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS), using an Ekspert nanoLC415 (Eksigent, Dublin, CA, United States) coupled to a TripleTOF 6600 MS (SCIEX, Redwood City, CA, United States) system. The detailed LC–MS acquisition method parameters were described precisely by Colgrave et al. (21). In summary, the peptide samples (6 μL) were desalted on a polar C18 ProteCol trap column (Trajan; 3 μm Particle Size x 300 Å Pore Size, 10 mm x 300 μm ID) with 0.1% formic acid for 5 min at a flow rate of 10 μL/min, then separated at flow rate of 5 μL/min on a ChromXP C18 (3 μm, 120 Å, 150 mm × 0.3 mm) column (30°C) by applying the following gradient: 5–45% B over period of 40 min, 45–90% B in 5 min, 5 min hold at 90% B, return to 5% B over 1 min and 11 min re-equilibration. Solvent A consisted of aqueous 5% DMSO and 0.1% formic acid, whilst solvent B consisted of aqueous 5% DMSO, 90% acetonitrile, and 0.1% formic acid.

The eluent from HPLC was introduced to the DuoSpray ion source of the mass spectrometer. The ion spray voltage floating was set to 5,500 V; the curtain gas to 30 psi; ion source gas 1 and 2–18 and 20 psi, respectively; and the heated interface was set at 100°C. The data was acquired in information dependent acquisition (IDA) mode, where the MS1 scan range was defined between 350 and 1,250 m/z using an accumulation time of 0.25 s. The 30 most intense precursors meeting the selection criteria of charge state between 2 and 5 and intensity greater than 150 were selected for further fragmentation (MS/MS). MS2 spectra were acquired over the mass range of 100–1,800 m/z with 0.05 s accumulation time. For optimum peptide fragmentation the manufacturer's rolling collision energy (CE) and a collision energy spread (CES) of 5 V was applied. The dynamic exclusion was enabled to exclude precursor ions after two occurrences within a 15 s interval and a mass tolerance of 100 ppm (peaks within 4 Da of the precursor m/z were excluded).

Lupin-Specific Database Creation

The *de novo* transcriptome assemblies of Tanjil, Unicrop and P27255 released by CSIRO (22), were retrieved from the Lupin Genome Portal.[1] Open reading frames with a minimum of 150 nucleotide length cut-off value were predicted from the RNA-seq data. These sequences were translated and functionally annotated using Pfam domain analysis within the CLC Main Workbench v21.0.4 (23), which applies the hmmsearch algorithm (from the HMMER3 package version 3.1b1) for domain identification in proteins. These sequences were appended to the UniProt *Lupinus* protein sequence entries (32,833 sequences downloaded on 21/04/2020), CSIRO NLL protein database (see text footnote 1), Biognosys iRT pseudo-protein sequence as well as the common repository of adventitious proteins (cRAP). Subsequently, the sequences with 100% identity were excluded from the final database (63,046 sequences).

Prediction of Potential Conglutin Sequences, Phylogenetic Analysis, and Epitope Mapping

To identify the potential conglutin isoforms within the database, the 16 reported NLL conglutin sequences (referred to as reference

[1] www.lupinexpress.org

conglutins throughout the manuscript) were searched against the lupin database, using the BLASTp algorithm in CLC Main Workbench v21.0.4. For discrimination of the conglutin subtypes the sequences with minimum 75% sequence identity with reference conglutins were subjected to homology searching (BLASTp) in NCBI, the resulted conglutin hits were additionally confirmed by the evaluation of the distinctive structural features such as the conserved Pfam domains and cysteine patterns. The known and predicted conglutin sequences were aligned using the Clustal W algorithm (24) and the corresponding phylogenetic tree for each conglutin family was constructed within the CLC Main Workbench v21.0.4. For identification of the potential allergenic regions in conglutin sequences, the list of the known epitopes was retrieved from the Immune Epitope Database and Analysis Resource website[2] and used as an input for a motif search (100% sequence identity) within the CLC Main Workbench v21.0.4.

Protein Identification

ProteinPilot 5.0.3 software (SCIEX) with the Paragon and ProGroup algorithms was used to search the raw MS (IDA) data against the *in silico* tryptic digests of the custom-built lupin database (25). The search effort was set as "Thorough ID" with iodoacetamide as cysteine alkylation agent and trypsin as digestion enzyme; TripleTOF 6600 was selected for the instrument type and biological modifications were enabled for ID focus. The SCIEX FDR analysis tool was used for estimation of false discovery rate (FDR) (26). A database search combining all IDA files was conducted to attain a complete list of proteins identified from all the lupin varieties; the reported identification yields represent data at a 1% global FDR cut-off. The protein identifications were robustly aligned across the group files using the SCIEX Protein Alignment Template v3.002p.

Conglutin-Derived Peptide Mapping

A custom R script was used to filter the conglutin-derived tryptic peptides meeting a 1% FDR threshold with no unusual modifications (allowing carbamidomethyl Cys, oxidation of Met, and pyroglutamination of N-terminal Gln) from the FDR reports. These peptides were then aligned across the NLL samples by means of an in-house Python script and classified into six groups based on their identification frequency, as follow: identified across (a) 1–5; (b) 6–15; (c) 16–25; (d) 26–35; (e) 35–40 and (f) 41–46 NLL accessions. Using the motif search tool in the CLC Main Workbench 21.0.4 software, the peptide sequences were mapped to the reference conglutin protein sequences using 100% sequence identity, color-coded based on the groups above and evaluated for defining the conserved/variable regions of these proteins among the study genotypes.

Targeted Assay Development and Peptide Specificity Analysis

The conglutin proteins identified at 1% FDR from the combined database searching were imported into Skyline software (v19.1.0.193) and subjected to *in silico* digestion. The ProteinPilot group files from the discovery proteomics data collected from lupin samples previously in our laboratory (19) and the combined search from the present study were used to build a BiblioSpec library containing peptides with a confidence value of > 0.95 (1% FDR). The library matched tryptic peptides (allowing one missed cleavage) between 8 to 30 amino acids in length with no variable modifications retained for the preliminary analyses (278 peptides). The spectral library was employed to determine the optimal multiple reaction monitoring (MRM) transitions for each peptide (six transition per peptide) and this unscheduled method was used to acquire data using a pool of all replicates. The results from these analyses were used to schedule retention times and refine the peptide and MRM transition list (minimum three transitions per peptide). The final targeted method included 760 transitions and 250 peptides (including modified forms), representing 21 proteins (**Supplementary Data Sheet 2**).

For discerning the unique peptides for each conglutin protein, the specificity of the MRM peptides was investigated (on 2021/06/02) using the peptide match tool within the Protein Information Resource website, where the Tanjil database (accessed directly from UniProt) was set as the background proteome (27).

Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry

The experimental samples were divided into two batches. To allow the post-acquisition removal of the systematic non-biological variance between the batches, the injection order of the samples was randomized. A QC sample composed of 1 μg/μL BSA tryptic digestion spiked with iRT peptides (1:10) was analyzed prior to the batch and periodically throughout the batch and was used to monitor the instrument performance and the batch effect.

The chromatographic separation of the tryptic peptides (4 μL) was achieved on an Exion AD UHPLC system (SCIEX), and the quantitative acquisition was performed with a 6500 + QTRAP mass spectrometer (SCIEX). The MRM data was acquired in positive ion mode with the ion source temperature and the ion spray voltage—floating set at 500°C and 5,500 V, respectively. The MRM transitions were scheduled to be monitored within 60 s of their expected retention time ($\pm$ 30 s) and the cycle time was set at 0.6 s. Skyline software (v19.1.0.193) was utilized for peak integration (28), wherein all the MRM peaks were inspected manually to ensure correct peak detection, signal-to-noise (S/N) > 5, and accurate integration.

Peptide Data Processing and Statistical Analysis

The peptide abundance data was exported from Skyline and the batch effect was corrected by using the "Remove batch effect" function within the *Limma* R package using the batch information and the sample injection order as the covariate (29). The technical variation across the triplicates of each genotype was assessed by examining the coefficient of variation (CV) for

[2]https://www.iedb.org/

each peptide. In total, 82 MRM peptides specific to the conglutin subfamilies (which exhibited the highest signal intensity and the lowest technical variability) were selected for the evaluation of the conglutin protein profiles across the NLL genotypes.

The heatmap of the quantitative peptide data (log10 transformed) was generated in the Morpheus analysis software[3] (Broad Institute, Cambridge MA, United States) where the one minus Pearson correlation metric was selected for conducting the unsupervised hierarchical cluster analysis (HCA). The multivariate relationships of the observations were assessed through principal component analysis (PCA) of the peptide level data (log10 transformed) within the SIMCA software (Sartorius Stedim Biotech, v15.0). For multiple comparisons of the experimental groups one-way ANOVA followed by Dunnett's test was applied in GraphPad Prism v8 and the graphs comparing the conglutin profiles were generated using a custom R script.

RESULTS AND DISCUSSION

Lupin conglutins are the major seed storage proteins which are known to have many health-promoting attributes, for example blood glucose lowering (8) and enhanced satiety (30) effects. In spite of these attributes conglutin proteins have also shown some undesirable allergenic properties. In the present study, the qualitative and quantitative diversity of the conglutin proteins were evaluated across a wide range of domesticated and wild NLL accessions from 14 countries (Algeria, Australia, Belarus, Cyprus, France, Greece, Israel, Italy, Morocco, Poland, Portugal, Spain, Syria, and Turkey) using LC–MRM-MS. Screening the available *Lupinus angustifolius* germplasm will provide insight into the intraspecific proteome diversity and lead to identification of varieties that contain higher levels of preferential and lower amount of detrimental conglutins. The outcome of which can benefit lupin breeding programs in the development of varieties with an improved protein complement.

Discovery of Potential Conglutin Sequences Within the Database

The lupin database used in this study was constructed using the proteomic, transcriptomic, and genomic resources available for different lupin varieties; consequently, it may include many inter-cultivar conglutin protein variants. Using bioinformatic techniques all the putative conglutin sequences present in the database were determined. To this end, the sequences with > 75% sequence identity with the 16 known NLL conglutin proteins were defined, and this repertoire was further constricted by evaluating the presence of discriminative structural features (such as conserved Pfam domains and cysteine patterns) in these sequences. This resulted in the identification of an additional 33 potential conglutin isoforms within the database, belonging to the four major α-, β-, δ-, and γ-conglutin families. The α-conglutins were recognizable by the presence of the conserved patterns of CX_nC and two cupin_1 (PF00190) domains (between 36–201 and 405–552 residues) in their sequences (**Figure 1A**). The structural characteristics considered for distinguishment of the β-conglutins included the presence of two cupin_1 (PF00190) domains (between 180–330 and 390–551 residues) and lack of cysteine residues (**Figure 1B**), which were found with a specific pattern in other members of vicilin-like proteins (31). Moreover, two semi-conserved motifs (HYX_nR and $QQDEQEX_nYX_nLS$) were noted in the globular domain of these proteins. The δ-conglutin proteins were distinct due to the existence of the conserved patterns of $CX_nCX_nCCX_nCXCX_nCX_nCX_nC$ which is generally a characteristic of the prolamin superfamily proteins (32). Furthermore, a tryp_alpha_amyl domain (PF00234) was found in the δ2 protein structure (**Figure 1C**). Finally, the xylanase inhibitor N- and C-terminal domains (TAXi-N; PF14543 and TAXi-C; PF14541) between 62–237 and 269–430 residues were the main structural characteristic of the γ-conglutins where the cystine rich N-terminal region included conserved CX_nC repeated patterns (**Figure 1D**).

Protein and Peptide Identification

The IDA data for each NLL variety was searched against the lupin specific database. This resulted in variable protein and peptide identification yields at 1% FDR (between 1,418–1,994 proteins and 6,508–10,113 peptides) from these lines, the highest and the lowest protein identification yields were observed for D1 and W28 accessions, respectively (**Supplementary Table 2**). In addition to the individual searches a combined database search was conducted on the discovery proteomics datasets encompassing all the 46 NLLs, which led to the identification of 3,534 proteins and 20,371 peptides (1% FDR). The comparative qualitative analysis of the detected proteins across the analyzed lines, indicated that ∼14% of the identified proteome was commonly detectable across all the 46 NLL genotypes, whilst ∼5% the identifications were unique to an individual NLL variety. The substantially higher (>43%) identification yield achieved through the combined search compared to the individual searches, as well as the low proportion of the commonly identified proteome affirms a high level of proteome diversity among the analyzed lines. The evaluation of the aligned discovery metrics for the representation of the predicted conglutin proteins revealed identification evidence for the characterization of four α-, twelve β-, three δ-, and two γ-conglutin proteins based on unique peptide evidence. There are three α-conglutins known from NLL. The extra α-conglutin identification refers to a transcriptome translated sequence from *L. angustifolius* cultivar Unicrop which shares high sequence identity (96.1%) with α2-conglutin reference sequence (UniProt ID: F5B8V7). Based on the phylogenetic relationships of these proteins (**Supplementary Figure 1A**), this is likely to be a cultivar specific variant of the α2 isoform. Whereas the additional four β-conglutin hits (present in the UniProt NLL proteome database) exhibited a separate clustering from the known β-conglutins in the phylogenetic tree (**Supplementary Figure 1B**). However, the presence of other conglutin subfamilies in NLL has been suggested to be unlikely based on the in-depth cv Tanjil transcriptomic and draft genome sequence analyses (7). Furthermore, the identification of the δ1-conglutin protein was unachievable, which can be explained by the high sequence identity (> 98%) of this protein with the

[3]https://software.broadinstitute.org/morpheus/

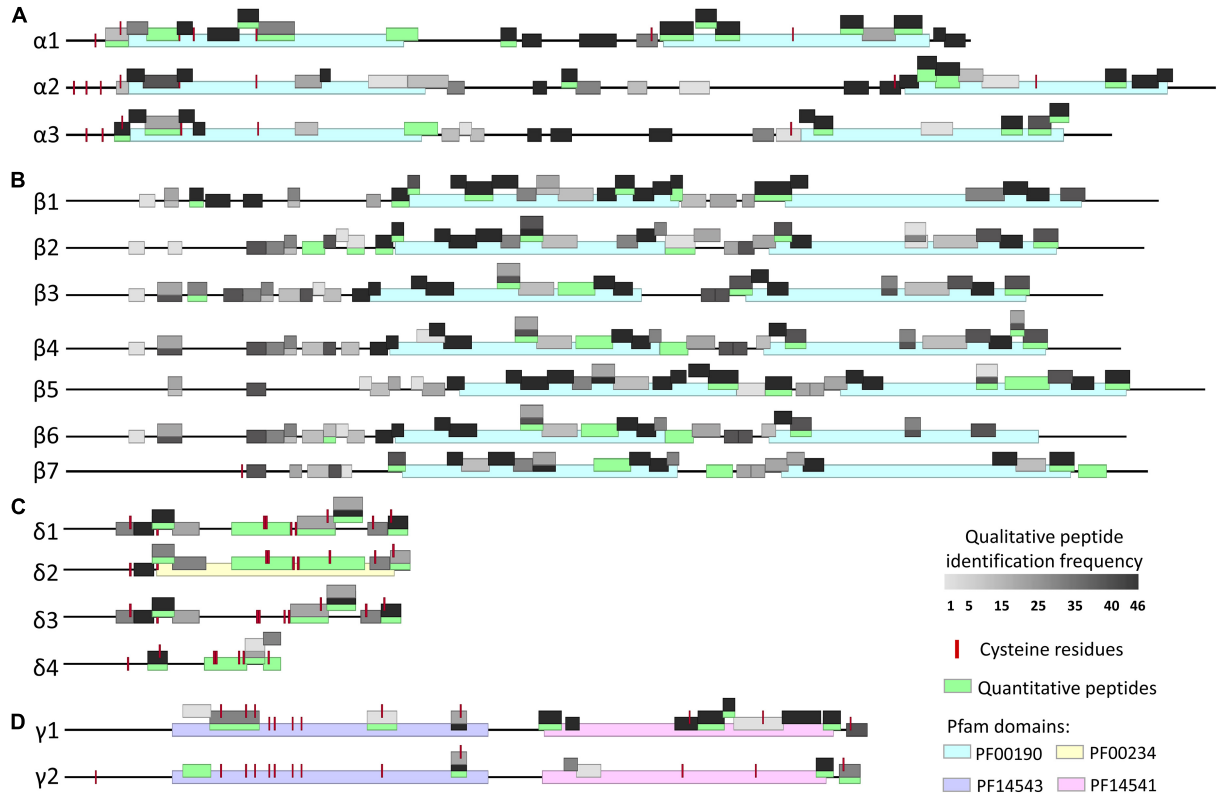

FIGURE 1 | Schematic representation of the main structural findings and conserved regions in **(A)** α-, **(B)** β-, **(C)** δ-, and **(D)** γ-conglutin sequences, as well as the marker peptides used in LC-MRM-MS measurement of these proteins. The Pfam domains are highlighted in blue (Cupin_1), yellow (Tryp_alpha_amyl), purple (TAXi_N), and pink (TAXi_C). The red lines show the position of cysteine residues, and the green blocks are representative of MRM marker peptides (quantitative peptides). The conglutin peptides identified by IDA (qualitative peptides) are depicted by gray blocks, wherein the darker color indicates sequence regions commonly identified across the analyzed lines (white blocks, 1–5 cultivars; black blocks, 40–46 cultivars).

δ3 isoform. The 21 conglutin sequences identified based on unique peptide evidence were selected for targeted quantitative method development.

Evaluation of Conglutin Protein Sequence Conservation Across the Analyzed Narrow-Leafed Lupin Lines

The alignment of the detected conglutin peptides across the samples was used to investigate the conglutin protein sequence conservation between the 46 NLL genotypes under study. In total, 244 conglutin peptides were detected within the samples of which 86 peptides (∼35%) were commonly identified across 41–46 NLL varieties, whilst 42 (∼17%) peptides were only detected in 1 to 5 NLL lines. The highest number of conglutin peptides (166) were identified from the Syrian W10 accession, followed by the Italian W1 (163 conglutin peptides) accession and the domesticated Australian D7 and D13 (162 conglutin peptides) cultivars. All conglutin peptides detected through the discovery proteomics (fully tryptic and containing no unusual modifications) and the MRM quantitative peptides were mapped onto the 16 reference conglutin sequences (**Figures 1A–D**). The highly conserved α- and β-conglutin peptides (identified in more than 40 lines) were mainly found on the two β-barrel structures of these sequences, which correspond to the cupin_1 domain regions in these proteins (**Figures 1A,B**). Notably, for β-conglutins, the least conserved peptides (present in less than 5 accessions) were mostly mapped on the first 200 N-terminal amnio acid residues of these sequences. This region—consisting of 8–10 α-helices—is known as the mobile N-terminal arm, which has been also shown to embrace the largest structural variability of the seven NLL β-conglutins (33). It has been suggested that the putative functional differences of the β-conglutin isoforms can be related to these structural differences (9). In the γ1-conglutin the peptide sequences that were identifiable across more than 40 lines were found within the 269–430 residues that correlates with the TAXi-C domain position within this protein (**Figure 1D**). Few peptides with high conservations were also identifiable within the δ2 to δ4 and γ2 sequences (**Figures 1C,D**).

Selection of Marker Peptides for Each Conglutin Subfamilies

The conglutin-derived peptides identified by the combined database searching were selected for MRM-MS relative quantitation of these proteins. The peptides for each conglutin subfamily were selected using the following criteria: (1) specificity

of the peptide sequence to the target protein (evaluated against Tanjil reference proteome); (2) fully tryptic; (3) identified with ≥ 95% confidence; (4) restricted modifications to oxidation (M), carbamidomethyl (C), and pyro-Glu (N-terminal Gln); and (5) a signal to noise ratio (S/N) > 5. For the accurate quantitation of the target proteins, the peptide list was further filtered to retain maker peptides with the highest peak signal intensity and the lowest technical variability for each conglutin protein; a total of 22, 37, 13, and 10 marker peptides were selected for evaluation of the α-, β-, δ-, and γ-conglutin proteins across 46 NLL genotypes. On the occasions where the measurement of an individual protein was unachievable (because of high sequence similarity and a lack of unique peptides), the quantified relative abundance of the peptides was allocated to a protein group. The list of marker peptides selected for the conglutin protein subclasses, their corresponding technical variance and the average peak areas are represented in **Supplementary Table 3**. Typically, technical variation related to sample preparation and analysis of < 13% was observed for the marker peptides (with the exclusion of five peptides).

Multivariate Analysis of the Relative Abundance Data

Unsupervised principal component analysis (PCA) was applied to the peptide level abundance data (log10 scaled), to probe the relationships of the analyzed NLLs in terms of the conglutin protein contents. The PCA score plot revealed vivid differences in the conglutin profiles of the 46 lines (**Figure 2A**). The first and second principal components (PC1 and PC2, respectively) together explained ∼57.4% of the variability of the data, where PC1 (39.9%) effectively summarized the variance primarily related to the D1–D6 cultivars, and PC2 (17.5%) represented the differences mainly driven from W13-W17, W19-W23, W28, W31, and D15 accessions.

Evaluation of the loading vectors determined that the peptides assigned to the β 2-, β 3-, and β4,6- subfamilies with negative correlation in PC1, together with the δ2 and δ4 peptides—which correlated positively in PC2—led the separation across the accessions. Although distinct differences were observed across the domesticated cultivars based on their conglutin contents, no clear grouping was revealed based on the European or Australian origin of these NLLs. Previous genetic diversity studies provided concordant evidence regarding the close relationships of the Australian and European domesticated populations, which suggests shared ancestry or exchange of breeding materials (34).

The heatmap of the log10 transformed quantitative peptide data and the corresponding unsupervised hierarchical cluster analysis (HCA) revealed that the NLL lines fall into two major clusters based on the distribution of the conglutin peptides (**Figure 2B**). Therein, the stratification of the six domesticated lines (D1–D6) from the remaining accessions was clearly replicated. The Australian and Polish cultivars in this group demonstrated distinct conglutin protein profiles compared to the other 40 NLL accessions, the pedigree information available for the Australian NLL cultivars in this cluster (35) suggests a distant relationship among the D1, D4, D5, and D6 cultivars (relatively closer for D1 and D4). This indicates that the pedigree records solely are unable to explain the distinctiveness of the conglutin profiles of this cluster.

Within the second major cluster the Australian D7–D12 as well as the Polish D14 cultivated varieties, exhibited close grouping with a set of wild accessions mainly from the Iberian Peninsula (Spain and Portugal) and of North African (Morocco and Algeria) origins. The similarity of the conglutin profiles of these accessions can be ascribed to the western Mediterranean origin of the cultivated NLL germplasm (34). Contrarily, the more recent Australian D15 cultivar, which includes new wild ecotypes in its pedigree (36), appeared in a separate sub-group clustered with wild NLLs from Southern Europe (Greece and Cyprus), Iberian Peninsula (Portugal, Spain) and North Africa (Morocco). Moreover, within this major cluster the Belarussian D16 breeding line displayed a separate clustering from the other domesticated cultivars, the previous comparative diversity study of the available NLL varieties and breeding lines identified the Belarusian domesticated accessions genetically discrete, this was concluded to be a consequence of the complex wild crosses used in the Belarusian breeding program (34). Lastly, among the wild accessions under study, the W27–W30 accessions displayed the most distinct conglutin profiles.

Comparative Quantitative Evaluation of the Conglutin Families Across the Analyzed Narrow-Leafed Lupin Accessions

Distinctive biological functions and nutritional properties have been ascribed to the four major conglutin families. The comparative quantitative studies of these proteins across the NLL resources available, in addition to its significance for crop improvement, can also provide insight into the differing nutritional and health beneficial properties of the varieties available. Herein, the diversity of the major seed storage proteins across 46 NLLs was evaluated through comparing the abundances of the specific peptides monitored for each conglutin subfamily. To facilitate the comparison of different peptide abundance the raw peak peptide areas are converted into a percentage relative to the average peak area for all 46 NLL accessions and presented in a series of line graphs (**Figures 3A–D**). The overall levels of conglutin families were assessed using the summed peak area of the peptides belonging to each family, which was also normalized to the average percentage of total α-, β-, δ- and γ-conglutin contents across the analyzed lines (**Supplementary Figures 3A–D**). These analyses showed major differences in the abundance of the conglutin seed storage proteins. Results and discussion for each family follow below.

Alpha-Conglutin Protein Family

The α-conglutin family, also known as legumin-like or 11S globulin family, is the second most abundant protein class in lupin seeds and comprises three unique protein subfamilies, among which the α1 protein is more divergent in amino acid sequence (< 40% sequence identity) in comparison to the α2 and α3 proteins, which are closely related (∼70% identity).

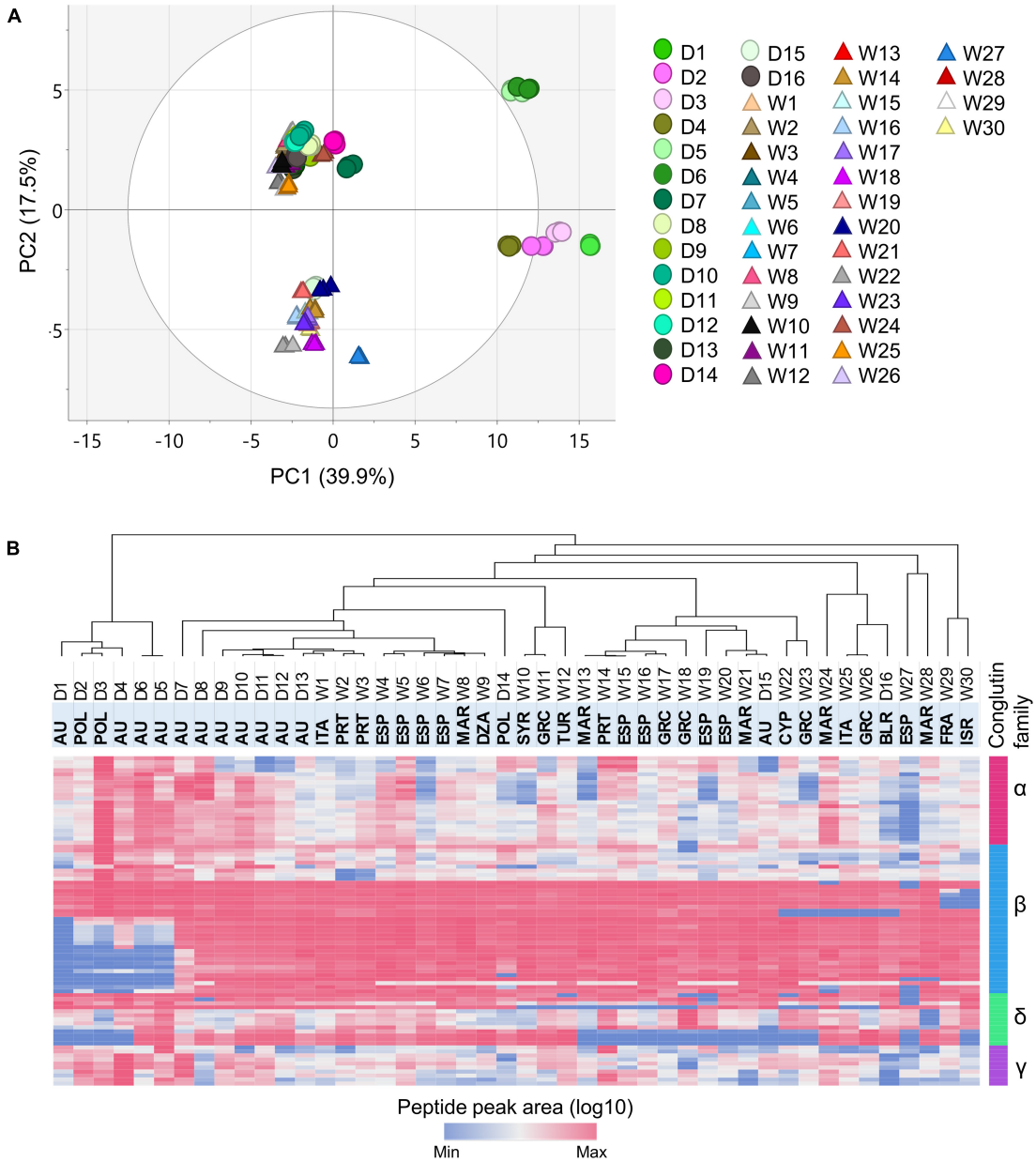

FIGURE 2 | Multivariate analysis of the peptide level abundance data. **(A)** The PCA scores plot, PC1 vs. PC2, showing the separation of 46 NLL genotypes based on the conglutin-derived peptide data (log10). Each color represents an NLL genotype where the wild (triangles) and domesticated (circles) varieties are indicated. **(B)** The heatmap displaying the monitored marker peptide peak areas (log10) across the analyzed lines. The dendrogram from the unsupervised HCA illustrating the similarity of the NLL varieties in terms of conglutin abundance. Each column corresponds to a genotype and every row represents a peptide. The highlighted row at the top illustrates the counties of origin for the lines under study. The country names are abbreviated as follows: DZA (Algeria), AU (Australia), BLR (Belarus), CYP (Cyprus), FRA (France), GRC (Greece), ISR (Israel), ITA (Italy), MAR (Morocco), POL (Poland), PRT (Portugal), ESP (Spain), Syria (SYR), and TUR (Turkey).

These proteins also have high homology with the α-conglutin sequences from distant lupin species and 11S globulins from other legumes (37). In fact, it has been highlighted that the α-conglutins are potentially associated with the immunologic cross-reactivity phenomenon with other legumes, particularly with the Ara h 3 peanut and Gly m 6 soybean allergens (38). The *in silico* investigation of the presence of known linear epitopes in the α-conglutin proteins identified Gly m 6 related epitopes, which were more frequently present in α1-conglutin (**Supplementary Figure 2A**). These epitopes were mainly found in the acidic subunit of these proteins which is a known immunoreactive fraction from lupin (13). The discrimination and the quantitation of the α 1-, α 2-, and α3-conglutins were achieved through monitoring the relative abundance of 11, 4, and 7 specific peptides, respectively. The α-subfamily specific peptides demonstrated similar patterns with

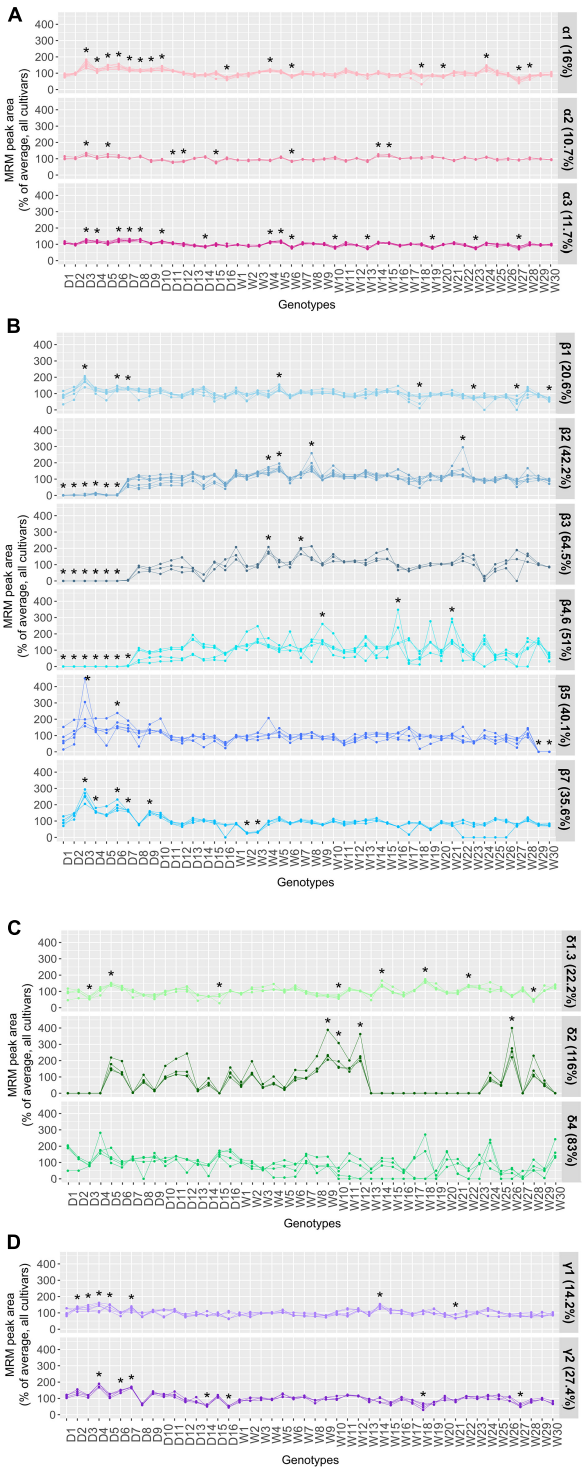

FIGURE 3 | Relative quantitation of the marker peptides monitored for **(A)** α-, **(B)** β-, **(C)** δ-, and **(D)** γ-conglutin families. The line graphs in each panel compare the relative abundance of the peptides measured for each conglutin subfamily. For ease of comparison the MRM peak area of each peptide was converted to percentage relative to the average peak area for all accessions. One-way ANOVA with Dunnett test was conducted for the multiple comparisons of the experimental groups and determining the significant differences (*$p < 0.05$).

small variations ranging from 11 to 16% across the NLLs examined (**Figure 3A**). The consistency of the α-conglutin protein levels across different NLL modern cultivars have been demonstrated previously (16); however, distinct α-conglutin RNA expression patterns were found between different lupin species (7). The Polish domesticated D3 cultivar with relatively high total protein content (22.8% above average; **Supplementary Table 2**) exhibited the highest α-conglutin levels and the Spanish W27 wild accession with comparatively lower total protein estimated value (22.4% lower than average; **Supplementary Table 2**) was found to have the lowest overall α-conglutin content (**Supplementary Figure 3A**).

Beta-Conglutin Protein Family

The vicilin-like β-conglutins, which belong to 7S globulin family, are the most abundant proteins in NLL seeds. These proteins are involved in multifunctional roles including seedling growth, plant development and antifungal defense response. They also display nutraceutical characteristics such as anti-inflammatory, hypoglycemic and antioxidant effects (9); however, anti-nutritional factors are also attributed to these proteins, which are characterized as the major allergens from lupin (in NLL named as Lup an 1; WHO/IUIS). Besides being involved in the primary lupin allergy the β-conglutins are also reported to be the target of immune system cross-reactivity from other legume allergens mainly peanut Ara h 1 and soybean Gly m 5 (38). The epitope mapping analysis revealed the presence of two soybean Gly m 5 epitopes, which were conserved across all the proteins of this family (**Supplementary Figure 2B**). These highly conserved sequence regions may have an involvement in the cross-reactivities observed for these legumes.

Seven protein subfamilies (β1 to β7) with relatively high amino acid sequence similarities (>75.6%) are included in the β-conglutin family, the quantitative evaluation of which was achieved through monitoring 3 to 8 quantitative peptides, across the 46 NLL accessions (**Supplementary Table 3**). Among the β-conglutin subfamilies the β4- and β6-conglutin proteins have the highest degree of sequence identity (97.8%). Specific peptides from β4 (GLTFPGSTEDVER and QLDTEVK) and β6 (QSAYER) proteins were identified, their relative abundance displayed similar patterns to the shared peptides (FGNFYEITPNR and ILLGNEDEQEDDEQR) from these two proteins (β4,6 group) across the 46 NLLs under study. This is suggestive of a common regulatory mechanism to produce these two highly similar proteins. A similar observation was also noted for the other β-conglutin subclasses; for instance, the proteins in the β2 group (including UniProt IDs: F5B8W0, A0A1J7FNI8, A0A1J7G5F6, A0A1J7GN79) have been quantitatively assessed using eight peptides, four of which are unique to F5B8W0 and the others are shared among these proteins; however, the trend of all peptides correlated across the analyzed NLL varieties. Altogether, larger biological differences (between 20.6 and 64.5%) were observed for the relative abundance of the β-conglutin protein subfamilies (**Figure 3B**); the variability was particularly notable for β2 to β7 conglutin subfamilies.

The distinct interspecific β-conglutin RNA expression patterns (7) and the presence of these proteins at different

levels across a number of Australian domesticated cultivars (16) have been demonstrated previously. Herein, across the lower β-conglutin containing D1–D6 cultivars, the peptides monitored for β2, β3 and β4,6 protein subfamilies were either absent or present at significantly lower levels (∼99%). Similar β-conglutin peptide profiles were noted for D7 cultivar, apart from the higher abundant β2 peptides measured from this cultivar. In parallel, a comparative proteomics study of white lupin varieties also demonstrated the disappearance of a set of β-conglutins in the domesticated cultivar analyzed (39). This signifies the breeding strategies used during the domestication of these lines have led to development of lupin varieties with reduced β-conglutin content, which can potentially serve as hypoallergenic candidates for commercial cultivation or be exploited in breeding strategies to develop improved hypoallergenic lupin varieties. However, clinical trials will be required to determine if this degree of alteration in the β-conglutin profiles can prevent the adverse allergic reactions caused by lupin and/or influence the allergy-eliciting required dose.

Interestingly, among the wild accessions, the W27 with relatively low total protein estimate (∼23% lower than average; **Supplementary Table 2**) and smaller seed size, displayed a substantially reduced β-conglutin content, wherein all the β-conglutin subfamilies were present but downregulated. The levels of this protein class were found the most elevated in a Moroccan W8 accession with relatively high estimated total protein content (∼12% higher than average; **Supplementary Table 2** and **Supplementary Figure 3B**). In some instances, the downregulation of the β-conglutin subfamilies were compensated for by an elevation in the other conglutin subfamilies. For example, in the low β-conglutin containing D3 cultivar, significantly higher levels of α 1-, α 2-, α 3-, β 1-, β 5-, β 7-, γ1-conglutin peptides were measured (**Figures 3A,B,D**), which contributed to the high total protein content measured for this cultivar (**Supplementary Table 2**).

Delta-Conglutin Protein Family

The four δ-conglutin subfamilies (δ1 to δ4) include 2S sulfur-rich albumin proteins, which belong to prolamin superfamily. This class of proteins in lupin are relatively small and are proposed to be mainly involved in storage function; however, they may also have a defense role in the seed due to the structural similarities with the plant cereal α-amylase/trypsin inhibitor family (40). The δ-conglutin fraction were also found to have low digestibility properties (41) and allergenicity potential (42, 43). Within this family the δ1 and δ3 sequences share a high sequence identity (over 98%), for which the discriminatory peptide (SSQESEESEELDQCCEQLNELNSQR, unique to δ1) and the shared peptides demonstrated similar trends and were employed for evaluation of these proteins over the study genotypes. Among the δ-conglutin subfamilies, substantial divergence was observed in the abundance of the δ2 and δ4 peptides where 83 and 116% overall variance were estimated for these proteins, respectively (**Figure 3C**). The δ2-conglutin peptides were found to be significantly increased in abundance in the wild accessions W26, W9, W26, and W10; on the contrary, these peptide markers were absent or present at low levels in 21 of the investigated genotypes (including 8 domesticated and 13 wild accessions). The δ4-conglutin is the most distinct protein within this family, the peptides monitored for which were comparably low in abundance and exhibited inconsistent trends. For ALQPVMEK and YCYSEAK peptides, this may be explained by genotype-specific amino acid substitution in these peptide regions. Overall, D5 and W18 are among the highest δ-conglutin containing accessions, whilst the lower levels were notable for D3 and W28 (**Supplementary Figure 3C**). The protein digestibility level study can determine if the genotypes with lower overall δ-conglutin levels have the potential to offer a higher nutritional quality for feed or food purposes.

Gamma-Conglutin Protein Family

This protein family has garnered special interest due to their key role in the lupin antihyperglycemic properties (44). Two γ-conglutin subfamilies are included in this protein class, which are basic 7S globulins and account for the least abundant fraction of conglutins in NLL seeds (37). Similar to other conglutin families, the γ-conglutins are also predominantly stored in the storage vacuoles; however, they are also found in the extracellular apoplectic regions and exhibit stability during germination, which suggests that these proteins may not fall in the classical category of the seed storage proteins (40). The aspartic-type endopeptidase activity GO term is assigned to γ-conglutins, which are also proposed to be involved in plant defense mechanisms (44). In terms of allergenicity, there is some level of discrepancy regarding the immunogenicity of these proteins ranging from weak to strong in different reports (38). Herein, the *in silico* epitope mapping evaluations revealed the presence of two overlapping epitopes from soybean 7S basic globulin 2 (UniProt ID: Q8RVH5) protein within the structure of the two γ-conglutins (**Supplementary Figure 2C**), which indicates the possible involvement of these proteins in the cross-allergic reactions with soybean.

The γ-conglutin sequences have relatively few tryptic cleavage sites and unlike the other conglutin families (α, β, and δ) were previously reported to be undetectable through the untargeted analysis of the Tris-HCl extracted lupin samples (45). Nevertheless, herein unique tryptic peptides corresponding to the two γ-conglutin subfamilies were determined and measured for the evaluation of these proteins. This indicates the resistance of γ-conglutins in native state to proteolytic degradation and highlights the ability of the denaturing extraction buffer used to assist in peptide liberation using trypsin digestion. Overall, a low variance was observed for γ-conglutins across the genotypes studied (14.2% for γ1 and 27.4% for γ2) (**Figure 3D**). Wherein, the relative abundances measured for the γ1-conglutin peptides were considerably higher compared to the γ2-conglutin peptide responses, a pattern which was also displayed in the RNA expression levels of the NLL γ-conglutins. The Portuguese wild accession (W14) with the highest protein estimate result was noted to have the highest overall γ-conglutin levels, whilst the Moroccan accession (W21) exhibited the lowest γ-conglutin content (**Supplementary Figure 3D**). The relative abundances of the γ1-conglutin peptides were significantly higher in D2-D5, and D7, whilst the γ2 specific peptides were found at significantly higher levels in the D4, D6, and D7 cultivars, this elevation may implicate the compensation for the suppressed β-conglutin

levels across these domesticated lines, for ensuring the overall protein quantity within the seed, a phenomenon which has been previously reported for other seeds such as ultra-low-gluten barley cultivars (46) and transgenic soybean seeds (47). These NLL cultivars with lower allergenic β-conglutin content and higher bioactive γ-conglutin levels may offer enhanced health benefits; however, the lupin seed proteome also includes several thousand moderate or low abundant proteins (19). For deeper understanding of the proteome composition and rebalancing mechanisms a comprehensive investigation of the proteome-wide alterations is essential.

CONCLUSION

Knowledge of the conglutin abundance diversity within NLL germplasm can be used to identify superior genotypes and facilitate the development of NLL varieties with optimal protein composition and traits. The targeted LC-MS/MS assay developed herein allowed the discrimination and quantitative evaluation of the conglutin proteins' subclasses across a diverse range of domesticated and wild NLL accessions. Distinct differences were observed across the analyzed lines based on their conglutin profiles, wherein the major variability was associated with the β- and δ-conglutin protein contents. Importantly, the absence/lower abundance of the β2- to β6-conglutin subfamilies were noted across several Australian and Polish domesticated cultivars. This led to the suppression of the overall allergenic β-conglutin levels in these NLLs, for which some degree of compensatory elevation of α- and γ-conglutin families was noted. The identified potential hypoallergenic NLL varieties can be used for commercial cultivation or be exploited in breeding strategies to enhance the quality of lupin gain a food ingredient. Further studies focusing on the digestibility values and clinical immunogenicity of these lines can unravel the association of the conglutin profiles with nutritional quality of lupin varieties.

DATA AVAILABILITY STATEMENT

The datasets presented in this study have been uploaded to the CSIRO Data Access Portal. Data access link: https://doi.org/10.25919/pfkr-s130.

AUTHOR CONTRIBUTIONS

MC conceived the design of the study, provided guidance to AT through the sample preparation, data acquisition and processing steps. AT prepared the samples, analyzed, visualized the data, and drafted the manuscript. AJ provided AT guidance to build the lupin specific database, sequence analysis, epitope mapping, and data visualization. JB provided AT guidance to design the targeted MRM assay, develop scripts for data curation, statistical analysis, and visualization. MN-W provided assistance with the sample preparation and data acquisition. TL provided guidance to AT for sample preparation and targeted assay development. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.842168/full#supplementary-material

Supplementary Data Sheet 1 | Supporting figures and tables.

Supplementary Data Sheet 2 | MRM assay transition list.

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REVIEW
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Soft Tribology and Its Relationship With the Sensory Perception in Dairy Products: A Review

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Nowadays, dairy products, especially fermented products such as yogurt, fromage frais, sour cream and custard, are among the most studied foods through tribological analysis due to their semi-solid appearance and close relationship with attributes like smoothness, creaminess and astringency. In tribology, dairy products are used to provide information about the friction coefficient (CoF) generated between tongue, palate, and teeth through the construction of a Stribeck curve. This provides important information about the relationship between friction, food composition, and sensory attributes and can be influenced by many factors, such as the type of surface, tribometer, and whether saliva interaction is contemplated. This work will review the most recent and relevant information on tribological studies, challenges, opportunity areas, saliva interactions with dairy proteins, and their relation to dairy product sensory.

Keywords: soft tribology, sensory analysis, dairy products, friction coefficient, yogurt

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INTRODUCTION

Oral processing, also known as mastication or chewing, is a complex mechanism involving many physical, chemical and biochemical changes with plenty of superficial interactions taking place (1, 2), such as teeth grinding, tongue-palate, tongue-teeth, teeth-food, and tongue-food (3). This is a complex process characterized by a shift from rheology-dominant to tribology-dominant activities being the first process of food consumption that yield energy and essential nutrients to our body (4) and includes all muscle activities, jaw and tonge movements contributing to prepare food for swallowing (1). Oral processing can be defined as the procedure of changing from solid food to a bolus ready to be swallowed, through reducing particle size of the food and mixing them with saliva where mechanical, enzymatic, and even microbiological takes place (4). This process is associated with the description of food quality that all of us made based on the food appearance, flavor, nutrition and texture, being the latter a factor where most of people have an anchored idea based on their past experiences. Texture as already said is related to rheological properties and tribological characteristicas of the food bolus in the different oral processing phases (4), being texture a multidimensional experience perceived during all stages of oral processing. Returning to the beginning to this paragraph, it is important to write that rheology is the study of the deformation of materials, whereas tribology (as described in the following sections) is the science of interacting surfaces in motion and studies lubrication and friction, applied first to study roughness of engineering materials (1).

Dairy products (yogurt, cream, cream cheese) are classified as semi-solid foods because most of them can change their rheological properties depending on the temperature, and one important characteristic is that they normally reside short time during oral processing, exhibiting fluid-dominant attributes, such as thickness: a sensory property highly related to rheological studies. Besides thickness, other attributes perceived commonly in dairy food are creaminess, fattiness, smoothness, stickiness, and astringency, for which classical rheology is not enough to describe them (5). For that reason, many researchers have put their attention on tribology. Recent results have demonstrated the potential of this discipline to predict traits in developing new food products due to the relationship between friction, food composition, and sensory attributes (6).

Owing to the high impact on consumer preferences, the food industry always analyzes sensory perception. The most used equipment to analyze rheological behaviors is the texturometer, the viscometer, and the rheometer. However, these tests are based on shear deformation or destruction of the food, giving a good result on the mechanical properties of the product, but without describing the geometric or surface properties of the sample, which are obtained by rubbing or squeezing the food against some surface. Due to these techniques being insufficient to describe very important characteristics such as creaminess or astringency, the use of tribology and its correlation with sensory analysis has begun to be evaluated (7, 8). Nowadays, there are already some studies of tribological analysis on numerous dairy foods such as yogurt, cream, cream cheese, custards, and others (9–17). The purpose of this review article is to collect and discuss the most relevant and novel information about the tribological analysis of dairy products and the relationship shared by the coefficient of friction (CoF) and the sensory attributes of smoothness, creaminess, and astringency. Finally, we discussed the latest topic in lubrication analysis: adsorption studies.

TRIBOLOGY

Tribology can be used to study the complex process of food oral processing, a mechanical function of the human body that undergoes chewing, transportation, and swallowing. The procedures can be divided into three main stages: the first oral phase that relies on the rheological characteristics of food such as brittleness, adhesiveness, and hardness. The second step involves surface and lubrication mechanisms like smoothness and creaminess. The third step is a transitional phase that relies upon tribology factors like thickness, creaminess, and consistency, which may be a critical part of sensory intensity and profile (18).

Tribology is defined as the study of lubrication, friction, and wear between two surfaces in relative motion and is commonly used to study mechanical engineering phenomena and materials, oils, and lubricants (6, 19). Several factors influence this parameter, like the surfaces' material, average load, sliding speed, contact area, and temperature. Typically, tribological results are presented as a Stribeck curve (**Figure 1**), which plots CoF against the sliding speed, and is divided into the following lubrication or friction regimes: (1) *the boundary regime*, where the lower and upper surfaces are in almost complete contact, only separated by a thin layer of lubricant, or in this case food, (2) *the mixed regime*, in which some parts of each surface are still in contact, and (3) *hydrodynamic or fluid regime*, in which the food sample or lubricant withstands the average force applied by surfaces, keeping them completely separated (19, 20). These three regimes represent different food samples between the tongue and palate (21).

Regarding food science, tribology provides information about the CoF resulting from the interactions between food and the surfaces on the oral cavity: tongue, palate, cheeks, and teeth (22). In order to mimic the surfaces within the oral cavity, at least one of the materials in contact with the food is not rigid or, in other words, is soft. These measurements provide insights into several sensory perceptions related to texture, even though obtaining quantitative empirical relationships between frictional interactions of foods with surfaces is challenging. In order to overcome these challenges, the selection of an appropriate tribological system, including instrument, surfaces, food system model, and analysis of the role of saliva, among other variables, is the most relevant element for the use of tribology as an indicator for texture perception (23). Soft tribology is intended primarily to evaluate the geometric properties of a food or beverage during the stages of its consumption. In other words, it aims to explain the relationship between friction properties and food processing in the mouth to describe parameters such as creaminess and smoothness, giving information about consumer preference (19, 23). Nowadays, mouthfeel attributes, such as creaminess, roughness, or astringency commonly found in dairy products, have been linked to friction on the tongue and palate; thus, friction screening tests are increasingly used in product development (22). Despite all the work already reported about oral processing and soft tribology, linking friction characteristics with sensory perception remains a challenge because CoF cannot be defined as a property of the material or food but rather a complex system-dependant related to many factors.

Among them are the surfaces type used for the test (soft, hydrophilic and elastic like the human tongue, or hard, synthetic and hydrophobic like polydimethylsiloxane -PDMS-, **Figure 2**), the food properties itself (solid, semi-solid, fluid), the measurement tribometer system or type (being the most commonly used the Mini-Traction Machine (MTM), the Optical Tribometer Configuration (OTC), the Anton-Paar rheometer equipped with a special attachment and the High-Frequency Reciprocating Rig (HFRR), **Figure 3**, and whether or not saliva interaction is considered in the tribological analysis (23). As mentioned before, the selection of all these factors are important for reliable results in lubrication analysis; however, in this review, we will not discuss them since multiple past works are related to this topic (1, 6, 7, 23–25).

PRINCIPLE, MECHANISM AND SALIVA INTERACTIONS WITH DAIRY PROTEINS

Saliva plays an important role in oral processing because helps to wet food, reduce friction in mastication process and influence in the sensory perception of foods (26). The saliva proteins

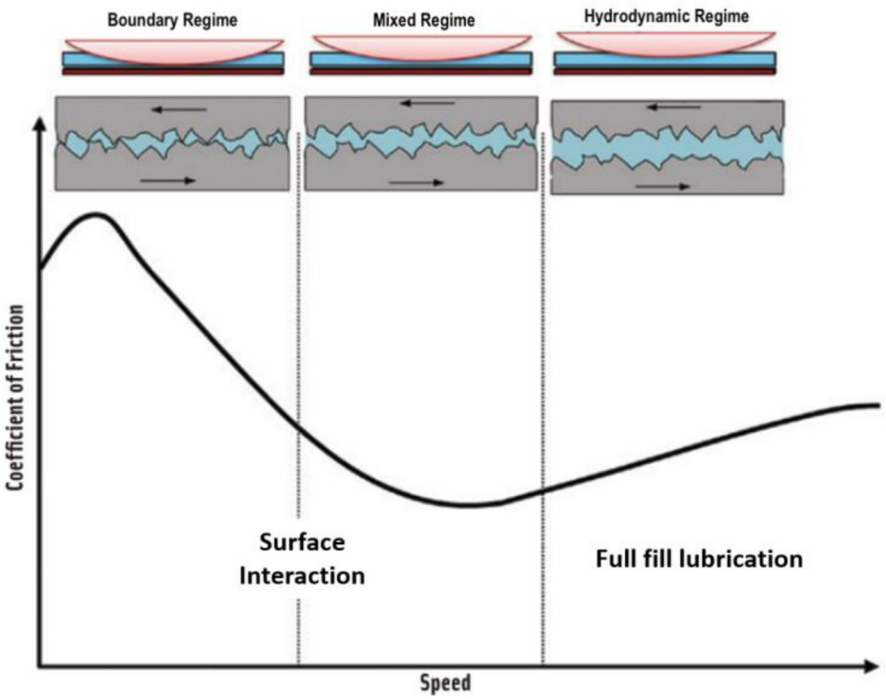

FIGURE 1 | Typical stribeck curve with the three different lubrication regimes. From: Mermelstein (22).

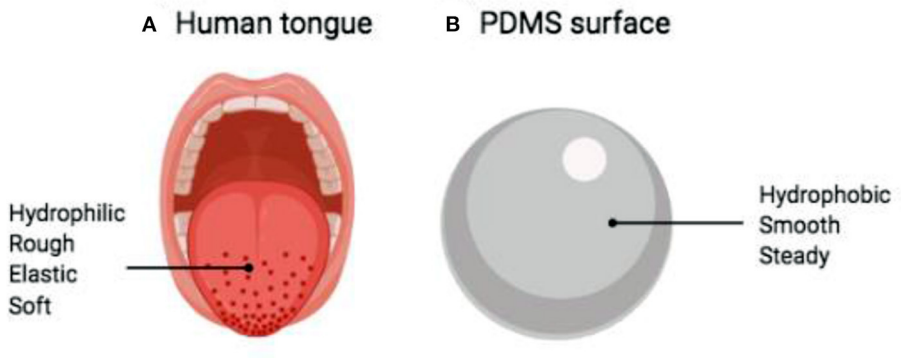

FIGURE 2 | Comparison between **(A)** human tongue and **(B)** untreated PDMS surface. PDMS: Polydimethylsiloxane. Figure edited from Rudge et al. (24).

are responsable of the oral lubrication for the food processing and digestion. The main proteins found in saliva are mucins, statherin, prolinerich glycoproteins, acidic protein-rich proteins, and lactoferrin. During oral processing saliva proteins mixes with food to form a bolus which increase the ease of swallowing and give the humans a sensory perception and textural attributes (27). Despite a number of studies addressing the interactions of saliva proteins with polysaccharides, studies related to the interaction with dairy products are still limited. Hence, the interactions between saliva proteins and dairy products are reviewed.

Dairy products contain caseinates and whey proteins like β-lactoglobulin (B-LG), bovine serum albumin, α-lactalbumin, lactoferrin, and immunoglobulins (18). According to Çelebioglu et al. (18), the electrostatic interaction between positively charged whey proteins and negatively charged saliva proteins at low pH, cause astringency for its precipitation around the oral cavity or binding to oral epithelial. Moreover, (28) showed that caseins attract the salivary proteins only *via* non-covalent interactions giving as result the perception of mouth drying. Based on this, the complexity of the dynamic saliva model and dairy proteins, in addition to the variety of parameters such as concentration, pH, heat, and surface properties that effect the friction, the tribology could be a valuable instrument to study the sensory perception.

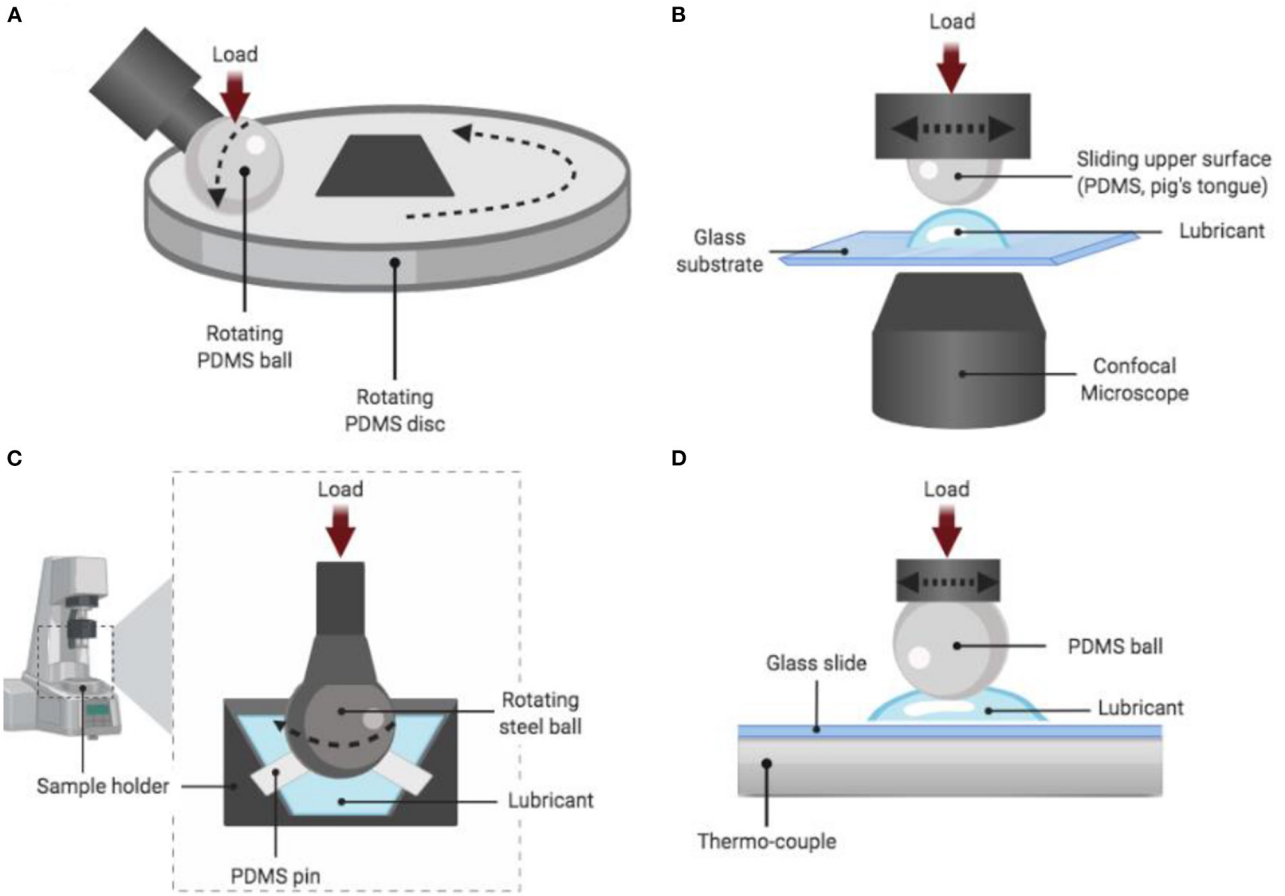

FIGURE 3 | Scheme of the most used equipment for tribological measurement. **(A)** Mini-traction machine in a ball on disk configuration (MTM); **(B)** Optical tribometer configuration (OTC); **(C)** Anton-Paar ball on three pins rheometer attachment and **(D)** High-Frequency Reciprocating Rig (HFRR) device. NOTE: Polydimethylsiloxane (PDMS) is an example of the surface, but other materials can be used. Figure edited from Shewan et al. (6) and Tsui et al. (17).

DAIRY PRODUCTS, SENSORY PERCEPTION, AND TRIBOLOGY

Dairy products are among the most studied or preferred foods for tribological analysis. Cream, yogurt and custard are semi-solid foods, defined as oil-in-water emulsions that often contain a dispersed solid phase as proteins (17). Usually, they also contain carbohydrates (lactose and polysaccharides), fat (low or high content) and other soluble components as minerals. These dairy foods require minimum effort for mastication because they promptly form a bolus ready to be swallowed (9). As a result of the rapid swallowing, these foods spend a few seconds in the mouth (29), and therefore they have limited oral interactions consisting just of tongue rolling and swallowing with little or no chewing. As the food contact period with the oral cavity surfaces is short, it is important to pay attention to the flow and the sensations that remain after swallowing, that is, the posterior sensations such as after-mouth feel or astringency. These characteristics will dominate in the tongue of the consumer, who will evaluate the texture of the semi-solid food mainly due to its fluidity and lubricating properties during retention in the mouth (29). In recent years, the relationship between lubrication properties and dairy sensory perception has gained a broad interest in food science and industry over other food types (30, 31). **Table 1** summarizes a compilation of the work carried out in recent years on the tribology of dairy products, summarizing the conditions and parameters evaluated, along with the additional studies carried out for obtaining complementary information.

Stribeck Curve in Dairy Products

As mentioned before, the traditional Stribeck Curve is divided into three regimes. However, for dairy products a particular situation occurs, since more zones could be observed, and this is due to the complexity of the matrix involving different hydrocolloids. Nguyen et al. (13) described 4 zones: Zone 1: this zone is characterized by having a very narrow space between the two surfaces. So the CoF in this zone is governed by the soluble substances and small particles dispersed in the liquid whey, such as whey protein and free fat globules, which migrated from the gel

TABLE 1 | Summary of tribological studies on dairy products.

Dairy product	Sample composition parameters	Tribometer and test	Tribometer conditions	Surface Material	Saliva interaction	Additional studies	References
Milk	• Skim milk (0.08% fat) • Milk (1% fat) • Whole milk • (3.5 % fat)	• Discovery hybrid rheometer-3 (TA Instrument, Newcastle, USA) • Double ball on plate	• Load: 1 N • Temperature: 25°C • Sample: 1 ml • Speed: 0.15–750 mm s^{-1}	• Upper: 2-polypropylene balls (15.88 mm diameter) • Lower: PDMS disk (40 mm diameter)	Yes	• Rheological • (Viscosity) • Sensory analysis (Astringency) • Microbial (Aerobic plate count and coliform) • Proximate analysis (Lactose, Protein, Fat, Solids)	(5)
Custard	• Starch (1, 2, 3% wt/wt) • κ-carrageenan (0, 0.15, 0.3% wt/wt) • Fat content (0, 3, 6% wt/wt)	Discovery Hybrid Rheometer with ring-on-plate tribo-rheometry (TA Instrument, USA)	• Load: 2 N • Temperature: 35°C • Speed: 0.01 to 6.5 rad s^{-1} for 1 min with 20 points per decade during 10 min.	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Static light scattering (Particle size) • Rheological (Viscosity and dynamic oscillation) • Sensory analysis (Thickness, Smoothness, Powderiness, Creaminess and Oiliness)	(9)
Milk Yogurt Cream cheese	• Whole milk (3.6 wt% fat) • Skim milk (0.1 wt% fat) • Full-fat yogurt (4.2 wt% fat) • Fat-free yogurt (0 wt% fat) • Full fat (21.5 wt% fat) • Low fat (2.5 wt% fat)	• Mini Traction Machine (MTM, PCS instruments, UK) • Ball on disk	• Load: 2 N • Contact pressure: ∼100 kPa • Speed: 1,000–1 mm s^{-1} sliding-to-rolling ratio of 50%	• Upper: PDMS ball (19 mm diameter) • Lower: PDMS flat plate	Yes	• Static light scattering (Particle size) • Sensory analysis (appearance, mouthfeel, after-feel and taste) • Rheological (Viscoelasticity and dynamic oscillation)	(10)
Milk	Milk (0.08, 2, and 5% fat)	• Discovery Hybrid Rheometer-3 (TA Instrument, Newcastle, USA) • Double ball on plate	• Load: 1 N • Temperature: 25°C • Sample: 1 ml • Speed: 0.15–750 mm s^{-1}	• Upper: 2-polypropylene balls (15.88 mm diameter) • Lower: PDMS disk (40 mm diameter)	No	• Rheological (Viscosity) • Confocal laser Scanning microscopy • Fat globule size • Protein separation (SDS-PAGE) • Proximate analysis (Milk solids, fat, and protein contents) • Sensory analysis (astringency)	(11)
Milk Cream cheese	Pasteurized milk (0.1, 1.3, 2, 3.8, 4.9% Fat) (3.9, 3.5, 4.1, 3.6, 3.6 % protein) (0.5, 5.5, 11.6% Fat) (13.9, 11.5, 9.4% Protein)	Discovery hybrid rheometer, using ring on plate tribo-rheometry (TA Instrument, USA)	• Load: 1 and 2 N • Temperature: 35°C • Speed: from 0.01 to 100 s^{-1} with 20 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Rheological (Viscosity) • Laser scattering (Particle Size).	(12)
Pot-set yogurt	• Commercial yogurt (0.1, 1.3, and 3.8% wt fat) • Yogurt (0.1% fat) with: • Gelatin (0.5–1.5%) • Xanthan gum (0.005–0.015%) • Carrageenan (0.01–0.08%) • Modified starch (0.5–1.5%)	Discovery hybrid rheometer with ring-on-plate tribo-rheometry (TA Instrument, USA)	• Load: 2 N • Temperature: 35°C • Sample: 2 g • Speed: from 0.01 to 100 s^{-1} with 20 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Texture analysis (firmness/hardness) • Syneresis analysis • Rheological (Viscosity and dynamic oscillation) • Microscopy (Distribution of fat and protein)	(13)

(Continued)

TABLE 1 | Continued

Dairy product	Sample composition parameters	Tribometer and test	Tribometer conditions	Surface Material	Saliva interaction	Additional studies	References
						• Sensory analysis (Thickness, Smoothness, Creaminess, Powderiness, Stickiness, Lumpiness, Oily coating, Residual coating)	
Butter	• Dairy cream (38% fat wt/wt) • Emulsifiers • Sodium Caseinate (0.5, 1.5 % wt/wt) • Tween 80 (0.5 % wt/wt)	Discovery Hybrid Rheometer and 3- ball on plate tribo-rheometry (TA Instrument)	• Load: 2 N • Temperature: 5–35 °C • Speed: 15,000 mm s^{-1}	• Upper: Not specified • Lower: 3M^{TM} transpore surgical tape	No	• Static light scattering (Particle size) • Confocal laser Scanning microscopy • Differential scanning calorimeter (Solid Fat content) • Cryo-SEM imaging • Proximate analysis (Fat, protein and moisture content) • Texture (Hardness) • Rheological (Viscosity) • Color	(15)
Stirred yogurt	• Fat content (0.1, 6, and 12/100 g) • Protein content (3.5, 4.5, and 6/100 g) • Casein to whey protein ratio • (80:20, 60:40, and 40:60)	• Rheometer (Physica MCR 301) with a tribology accessory attached • Ball on pyramid	• Load: 3 N • Temperature: 10°C • Sample: 1.5 g • Speed: 0.001–100 min^{-1}	• Upper: Stainless steel ball • Lower: elastic pad made of styrene-butadiene rubber	No	• Laser diffraction spectroscopy (Particle size) • Rheological (Viscosity and dynamic oscillation) • Sensory analysis • Appearance: Grainy and viscous • Texture: Grainy, viscous, fatty mouthfeel, slimy, creamy.	(16)
Pot-set yogurt	Fat Content (0, 4.2, 9.5% wt fat)	HFRR high-frequency reciprocating rig (PCS Instrument, UK)	• Load: 2 N (Pmax = 0.25 MPa) • Temperature: 23–25°C • Time: 60 s • Reciprocating sliding: 1 mm stroke 10 Hz • Sliding Speed: Mid stroke 20 mm/s	• Upper: PDMS ball (19.8 mm diameter) • Lower: Glass microscope slide	No	None	(17)
Pot-set yogurt	Fat content (0.1, 2.0, and 4.7%)	• Mini Traction Machine (MTM2, PCS Instruments Ltd., UK) • Ball on disk	• Load: 1 N • Temperature: 35°C • Sliding and rotational speed: 1–1,000 mm s^{-1} sliding-to-rolling ratio of 50%	• Upper: PDMS ball • Lower: PDMS disk	Yes	Rheological (Viscosity, viscoelasticity, and dynamic oscillation)	(20)
Custard	Fat content (0.9, 2.7, and 6.4%)						
Thickened cream	Fat content (13, 21 and 35%)						

(Continued)

TABLE 1 | Continued

Dairy product	Sample composition parameters	Tribometer and test	Tribometer conditions	Surface Material	Saliva interaction	Additional studies	References
B-LG solutions	• Protein content (0.5, 1, 2, 4, 7, and 10% wt) • pH (3.5 and 7.0)	• Mini Traction Machine (MTM, PCS Instruments Ltd., UK) • Ball on disk	• Load: 1 N • Temperature: 25 ± 2°C • Speed: 5 mm s^{-1}	• Upper: PDMS ball (18.6 mm diameter) • Lower: PDMS disk (22.5 mm diameter and 4 mm thickness)	Yes	Sensory analysis (Astringency)	(32)
Stirred yogurt	• Yogurt with added: • Extra skimmed milk powder (100 g of skimmed milk powder/500 mL) • Whey protein concentrate (50 g of skimmed milk powder + 21.38 g of whey protein/500 mL) • Extra skimmed milk powder + 2% starch • Whey protein concentrate + 2% starch	• Texture analyzer equipped with Exponent software version 3.2 (both from Stable Micro Systems, Godalming, UK) • Ball on disk	• Load: 0.27 N • Temperature: 25°C • Sliding speed: 0.1–10 mm s^{-1}	• Upper: Three stainless steel balls • Lower: 1 mm thick silicone elastomer	Yes	Sensory analysis (Free-choice term)	(33)
Fat-free yogurts	• Constant protein (5%) • Lactose (6%) • Casein to whey protein ratios (80:20, 70:30, 60:40, and 50:50)	• Rheometer MCR 301 Anton Paar Physica • with a tribology accessory (T-PTD200, BC12.7, Anton Paar Physica) • Ball on plate	• Load: 3 N • Temperature: 10°C • Speed: 0.001–1,000 min^{-1} • Sample: 1.5 g	• Upper: Stainless steel ball • Lower: Rubber pads	No	• Dynamic light scattering (Particle size) • Rheology (Viscosity) • Microscopy (Microstructure) • Sensory analysis (19 textural attributes)	(34)
Milk	Skim milk to full-fat milk (0.06, 0.15, 0.3, 0.5, 0.7, 1, 2, 3, 4, 6.5, 8.68% wt fat) (3.3% wt protein)	• Mini Traction Machine (MTM; PCS Instrument Ltd., London, UK) • Ball on disk	• Load: 5 N • Temperature: 20°C • Speed: from 500 to 5 mm s^{-1}	• Upper: Stain ball • Lower: 3 mm thick disk made of silicone, neoprene or Teflon	No	• Rheological (Viscosity) • Microscopy (coalescence of the fat on the surface) • Sensory analysis (categories smell/taste, mouth-feel, mouth/after-feel and after-taste/feel sensation)	(35)
Milk	• Skim milk (50% carbohydrates, 35% protein, 1.5% fat, and 4% moisture) (10% wt/wt) • Microparticulated whey protein (0.5, 3, 6, 9, 12, 15, 18, 20% wt/wt) • Commercial homogenized cream (0.5, 3, 6, 9, 12, 15, 18, 20% wt/wt) • Hydrocolloids (0.5% wt/wt)	• Rheometer (MCR 302, Anton Paar) with a tribology accessory attached • Ball- on-three-plates	• Load: 1 N • Temperature: • 25 and 37°C • Speed: 0.0447–940 mm s^{-1}	• Upper: PDMS ball (radius 6.35mm) • Lower: PDMS plates (size 3 × 6 × 16 mm) • Both treated with a high-frequency generator	No	• Microscopy (Morphological characterization) • Rheological (Viscosity)	(36)

(Continued)

TABLE 1 | Continued

Dairy product	Sample composition parameters	Tribometer and test	Tribometer conditions	Surface Material	Saliva interaction	Additional studies	References
Milk	• Skim milk (0.1 g/100 ml milk fat) + Phytosterols ester (0.8, 1.2, 1.6, and 2.0/100 g) • Commercial milk (0.1, 1.3, 2 g/100 ml fat) • Commercial milk (1 g/100 ml fat) + Phytosterols ester (0.32 g/100 ml)	Discovery Hybrid Rheometer, using ring on plate tribo-rheometry (TA Instrument, USA)	• Load: 2 N • Temperature: 35°C • Speed: from 0.1 to 100 s^{-1} with 20 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Color • Rheological (Viscosity) • Static light scattering (Particle size) • SPME-GC (Volatile compounds) • Sensory analysis (Creaminess, Graininess, Thickness/viscosity, Oily mouth coating, and Off-flavor)	(37)
Cream cheese	• Cream cheese • 5.5% milk fat • 3.2% (wt/wt) phytosterols (emulsion or esters) • 3% (wt/wt) β-glucan • Commercial cream cheese (13.7, 24.2% fat)	Discovery Hybrid Rheometer with ring-on-plate tribo-rheometry (TA Instrument, USA)	• Load: 2 N • Temperature: 35°C • Sample: 2 g • Speed: 30–0.01 rad s^{-1} with 20 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Moisture analysis • Static light scattering (Particle size) • Microscopy (Distribution of fat and protein) • Texture analysis (firmness, spreadability, adhesiveness) • Rheological (Viscosity)	(38)
Yogurt	• 4.5% (w/v) protein • Complex solutions • Fish Gelatin–Arabic gum/Xanthan gum/ κ-carrageenan • Ratios 10:0, 9:1, 7:3, 5:5, 3:7, 1:9, 0:10 (wt/ wt)	Discovery Hybrid Rheometer, using ring on plate tribo-rheometry (TA Instrument, USA)	• Load: 1 and 2 N • Temperature: 35°C • Speed: from 0.01 to 273 s^{-1} with 10 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Zeta potential • Static light scattering (Particle size) • Rheological (Viscosity) • Texture analysis (Firmness and adhesiveness) • Water holding capacity	(39)
Stirred yogurt	• Skim yogurt (0.1% fat) • Full fat yogurt (3.8% fat) • Inulin (7, 8 9%) • Pectin (0.2, 0.25, 0.3%) • GOS (9.1, 11.3, 13.6%) • β-glucan (0.1, 0.2,0.3%).	Discovery Hybrid Rheometer, using ring on plate tribo-rheometry (TA Instrument, USA)	• Load: 2 N • Temperature: 35°C • Speed: from 0.01 to 100 s^{-1} with 20 points per decade	• Upper: Stainless steel ring • Lower: 3M^{TM} transpore surgical tape	No	• Color analysis • Syneresis analysis • Texture analysis (Firmness and stickiness) • Static light scattering (Particle size) • Microscopy (Microstructure) • Rheological (Viscosity) • Sensory analysis (creaminess, astringency, thickness, smoothness, lumpiness, chalkiness, fatty feel, stickiness, oily coating, residual coating)	(40)

B-LG, Beta-lactoglobulin; GOS, Galactooligosaccharides; PDMS, Polydimethylsiloxane; SPME-GC: Solid-Phase Microextraction followed by Gas Chromatography.

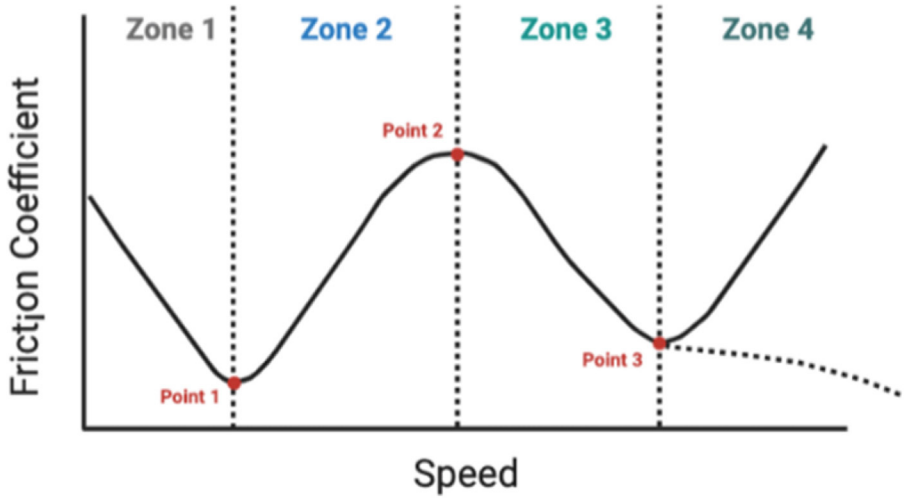

FIGURE 4 | Graph of the coefficient of friction divided into 4 zones possibly observed in dairy products. Figure edited from Nguyen et al. (13).

matrix. And when there are a lot of small particles, the friction is gradually reduced from dry contact to a minimum value that will be the guideline for zone 2. Zone 2: the rest of the fluid (in the form of a gel) begins to enter the contact zone, gradually increasing friction. A thin film of lubrication is created between the contact surfaces that increases friction until it reaches its maximum value (this will be a second transition point, which will mark the end of zone 2 and the beginning of zone 3). Unlike the traditional Stribeck curve, here the friction is no longer a constant value but increases linearly with speed. Zone 3: the friction begins to decrease due to the fact that the lubrication film grows even more and the thickness increases. In this area, viscosity plays an important role. Zone 3 can be compared with the mixed regime in the conventional Stribeck curve. Once again the CoF reaches a minimum point and marks the start of zone 4. Zone 4: from the minimum point of friction generated in zone 3, the friction curve changes slope. The friction can reach another minimum point at the end of the mixed regime (point T3) if the fluid retains its structure, and increases again with increasing speed. Now the surfaces are completely separated because the hydrodynamic film is fully developed, this zone is the same as the hydrodynamic regime in the traditional Stribeck curve. The CoF is somewhat determined by the internal friction (or fluid viscosity) and increases linearly with speed.

On the other case, if the speed is so high that it breaks down the fluid structure, the friction can be further reduced with speed. So not only the viscosity plays an important role in this zone, but also the gel strength of the sample.

What was described by Nguyen et al. (13) is similar to a cosine wave as can be seen in **Figure 4**. However, (30) in their work shows that there may be more forms of the curve in dairy products. They reported, in addition to the traditional Stribeck curve, other shapes such as: "S," "C," "U," and "W." These were obtained depending on the starter culture, the composition of the milk base (stabilizers such as starch and gellan gum) and the production process. They also mention that the physical and chemical properties such as adhesion, wettability, viscoelasticity, plasticity, hardness, and roughness of the compound played a key role in each zone/regime.

Sensory Perception

Taste and texture are critical factors in designing and developing food to reach the final consumer's desired characteristics. The consumer evaluates these descriptions during the oral processing of different foods (7, 41). **Table 2** shows the definition of the essential dairy product attributes associated with their textural description.

One of the most important components of sensory perception is the amount and type of fat present in dairy products. Fat can be retained as globules during oral processing, released as free fat, or mixed with other food ingredients depending on the type of food. This is a crucial factor in the attributes of smoothness, creaminess and astringency, among others (43).

Nevertheless, non-fat ingredients have recently become a valuable alternative for enhancing food texture by replacing fats. For instance, ice cream can use carbohydrate-based molecules to decrease ice crystal formation, improve texture and mouthfeel, and reduce fat content (44). In the same way, proteins are fundamental to dairy products texture, hardness, and organoleptic properties. In addition, recent studies have shown insights about controlled protein aggregation, which could relate to a similar texture, creamy appearance, and consistency of full-fat products, such as whey proteins that provide fat-like functions in terms of flavor, mouthfeel, and texture in light and low-fat formulations (45, 46).

Astringency

Astringency in dairy products is associated with fat content and the percentage and type of protein (11, 32, 43). Examples of astringent compounds are tannins, polyphenols, positively charged proteins, and polysaccharides (5, 6). The astringency attribute is believed to occur when there is an aggregation

TABLE 2 | Definitions of sensory attributes in dairy products.

Attributes	Definition	References
Thickness	• The degree to which the sample looks and feels thick • Mechanical property perceived when compressing the product between the tongue and the palate • Resistance to flow in the mouth before saliva modifies the sample	(13, 32, 42)
Smoothness	• The degree to which the sample feels smooth in the mouth, with an absence of lumps or granules. Assessed by swirling the sample in the mouth • Perceived smoothness of the sample squeezed between palate and tongue	(13, 32)
Creaminess	• The perceived silky smoothness of the sample. Assessed by moving the tongue parallel to the palate in circles • Silky smooth sensation in the mouth • Combined perception of fat, smoothness, and viscosity	(13, 32, 42)
Cohesiveness/Stickiness/Adhesiveness	• Degree to which the sample sticks around the mouth and coats the mouth surfaces (roof, cheeks, teeth). Assessed by pressing the sample between palate and tongue • Mechanical attribute relating the degree to which a substance can be deformed before it breaks • Degree to which the sample sticks to the teeth and palate	(13, 42)
Lumpiness/Grainy	• The degree to which large, soft lumps are perceived in the mouth. Assessed by swirling the sample in the mouth • Amount of soft lumps or graininess present in the sample	(13, 42)
Mouth coating/Residual coating	• The mouthfeel of the product once swallowed, the perception of a thin layer covering the palate • Intensity of residues left in the mouth after swallowing	(13, 32, 42)
Astringency	• Drying-out and puckering sensation that follows the consumption of particular food or drinks	(6)

and precipitation of salivary proteins, causing saliva to lose its lubricating capacity (11). Therefore, tribology appears as a valuable tool to link this molecular phenomenon with sensory perception because astringency is described as reducing the lubrication capacity provided by saliva in the oral cavity (6).

Vardhanabhuti et al. (32) were the first to study the effect of β-Lactoglobulin (B-LG) found in whey on saliva and its relationship with astringency. In their work, they evaluated this protein at different pHs (3.5 and 7.0), depicting an increase in the CoF almost instantly when B-LG at pH 3.5 was added. The authors established that this increase could be due to a depletion of the lubricating salivary protein film and/or the complexes formed between salivary proteins and B-LG. However, more research is required to elucidate the precise mechanism. On the other hand, B-LG's CoF at pH 7 did not have relevant saliva changes, suggesting that it would have low astringency. Finally, in the same work, they compared the B-LG concentrations at pH 3.5 from 0.5 to 10.0% w/w. The results showed inconsistencies between the concentrations and CoF. The coefficient of the highest protein concentration was below the values observed for the lowest protein concentrations. It was concluded that there was no simple relationship between sensory results and tribology, suggesting that the complex and dynamic system in the oral environment plays a key role in the perception of astringency. An important aspect is that (32) did not consider the particle sizes of the sample, which is not surprising since they carried out the first tribological studies in dairy.

Additionally, (33) found a relationship between the type of protein and astringency. This study tested yogurt samples with two protein types: whey protein concentrates and skimmed milk powder. The results showed less acceptability by the consumer panel with descriptions such as graininess or astringency and a significant reduction in the CoF for samples containing whey protein. Since the authors suggest that this coefficient's reduction is due to particle size and hypothesized that this reduction could be due to the inability of the whey particle (due to its large size) to fit between the asperities of the sliding surfaces when producing a lubrication effect. These observations complement the (32) report, where B-LG in whey protein also reduced the CoF (**Table 1**).

Regarding milkfat, this component reduces the sensation of astringency. Li et al. (11) compared the astringent effect of ultra-pasteurization (UP) and high-temperature short-time pasteurization (HTST) in milk with <0.2, 2.0, and 5.0% fat. According to the sensory analysis with a trained panel of consumers, it was shown that UP increased the astringency of the milk compared to HTST pasteurization. However, the tribological results were inconsistent, so the relationship between friction and the astringent sensation was not fully determined. Nevertheless, the authors reported lower CoFs values associated with the high-fat samples (**Table 1**).

Furthermore, (47) evaluated the sensory perception in cottage cheese with 0, 1, 3, 8, and 11% fat content, reporting that astringency and bitterness perceived by an experienced panel decreased as the fat increased. In this study, tribological analyses were not performed. However, (14) performed a tribological analysis in cream cheese with different fat concentrations (0.5, 5.5, 11.6% w/w) (**Table 1**), determining an inversely proportional relationship between fat percentage and CoF. The authors stated that low-fat cream cheeses were associated with higher CoF, firmer texture and reduced spreadability. They attributed the higher CoF values to a thin layer of fat between the surfaces, discussed later. There are still many analyses for other astringent substances to describe the relationship between the precipitation of proteins from saliva and the astringency of those substances (11). It cannot be assured that the CoF will increase for all of them because other aspects are involved, such as the particle size as previously commented and layers formed on the surfaces, as

will be discussed in Section Dairy Products, Sensory Perception, and Tribology.

Smoothness and Creaminess

Smoothness is associated with a movement in both surfaces (upper and lower) inside the mouth (48), whereas creaminess focuses on the movement of the tongue parallel to the palate in circles (34). In dairy products, fat is the most crucial factor related to these attributes, being then important to understand its relationship with CoF. One of the first works to associate CoF with fat and the sensory attributes of creaminess was done by Chojnicka-Paszun et al. (35), who evaluated the sensory perception of homogenized and pasteurized milk with fat content between 0.06 and 8.7%, correlating CoF with viscosity values. An MTM tribometer with a ball was used for the disk test, in which three types of 3 mm thick disks surfaces were compared: silicone, neoprene, or Teflon (**Table 1**). The authors reported correlation coefficients between creaminess and CoF of −0.92 and −0.94 for neoprene and silicone, respectively, when the milk's fat content was between 0.06 and 4.0%. This effect was attributed to forming a thin layer because of the coalescence of fat globules on the surfaces. This layer could be observed at low speed, above the fat content of 1 and 2% in silicone and neoprene, respectively. The high correlation coefficient for silicone rubber may indicate that it is the best material to represent the oral environment for homogenized and pasteurized milk tested under the described conditions. Fused fat droplets were separated at high speeds, causing an increase in CoF (35).

Selway and Stokes (20) studied the potential of soft tribology to differentiate commercial dairy products with similar rheological properties using an MTM with PDMS. They worked with a different fat percentage in custard (0.9, 2.7 and 6.4%), pot-set yogurt (0.1, 2.0 and 4.7%), and thickened cream (13, 21 and 35%). In their analysis, the function of pre-absorbed saliva was contemplated (**Table 1**). Results indicated that there were no significant differences in the bulk rheological profile in almost all the products, except high-fat custards. Due to their weak gel structure, the products exhibited a non-Newtonian flow behavior and some solid-like properties. However, the differences found in the CoF were interesting. In general, all low-fat products exhibited higher CoF than medium and high-fat counterparts. In yogurt, as in thickened cream, the medium and high-fat content samples had very similar CoFs. This could be attributed to fat being an excellent lubricant and reduced adhesion. This work by Selway and Stokes (20) demonstrated that tribology is a valuable tool for dairy characterization and food differentiation because, despite the similar bulk rheological profiles of the products, they exhibited unique tribological properties. Laguna et al. (10) evaluated the relationship between the rheological and tribological properties with the sensorial characteristics of commercial milk, yogurt and cream cheese (full fat and free fat versions) using an MTM tribometer with a PDMS flat plate and a 19 mm diameter ball. Two fat concentrations for each product were used: milk (3.6 and 0.1%), yogurt (4.2 and 0 %), and cream cheese (21.5 and 2.5%) (**Table 1**). In the sensorial results, untrained panelists were able to distinguish between the full fat and fat-free/low samples of the three dairy products, whereas the product's flow behavior (in the presence and absence of saliva) was similar, only with cream cheese depicting a moderately higher G' in full-fat samples. In the case of the tribological results, at low entrainment velocities 1–100 mm s^{-1}, CoFs, as in sensorial results, depicted differences in full-fat yogurt and cream cheese compared to fat-free/low-fat alternatives. The only exception was the traction coefficients for milk.

Sonne et al. (16), besides analyzing different fat contents (0.1, 6, 12/100 g) in stirred yogurt, also evaluated the effect of protein content (3.5, 4.5, 6.0/100 g) with different ratios of casein/whey proteins (80:20, 60:40, 40:60) on lubricating properties and correlated the results to properties as creaminess. The authors used a stainless-steel ball as a palate and a styrene-butadiene rubber elastic pad simulating the human tongue. Although different tribological methods and conditions were used, their conclusion supports (35) idea regarding fat content and coalescence in which increasing fat was related to a decrease in CoF and the formation of an interfacial film between surfaces. Higher friction discrimination was observed at speeds below 10 mm s^{-1}. Moreover, CoF decreased with increasing protein levels and when reducing whey proportion, suggesting that the content and type of protein also affected important attributes such as creaminess and smoothness. The authors concluded that the in-mouth creaminess of yogurt is a multi-sensory experience and related to the combination of rheological, tribological and particle size characteristics. They were able to deduce three important features to produce a fat-reduced yogurt with comparable in-mouth creaminess to a full-fat yogurt: (1) small particle size ($d_{90,3} \leq 50\,\mu m$); (2) high viscosity (shear stress $\geq 80 s^{-1}$, at 100 s^{-1}) and (3) low friction (friction coefficient ≤ 0.3, at 1 mm s^{-1}).

Adding alternative structural compounds such as hydrocolloids (13), polymeric thickening agents (49), microparticulate whey protein (36), phytosterols (37, 38), and microbubbles (50) could improve the creaminess and smoothness without using extra fat, achieving healthier product options. Recently, tribological research has focused on the effects of some hydrocolloids, such as xanthan gum, gelatin (13), fish gelatin (39), carrageenan (9, 13), and starch (9, 13, 33) (**Table 1**). Nguyen et al. (13) researched and compared yogurts with three ratios of fat (0.1, 1.3, and 3.8%) with skim yogurt (0.1% fat) added with gelatin (0.5, 1.0, 1.5%), modified starch (0.5, 1.0, 1.5%), xanthan gum (0.005, 0.010, 0.015%), and carrageenan (0.01, 0.04, 0.08 %). This study established one methodology for tribological studies in dairy products, using a Discovery Hybrid Rheometer, with a ring-on-plate on a rough plastic surface of 3M Transpore Surgical Tape as the lower surface. Samples with xanthan gum, starch, and carrageenan exhibited a higher CoF than the control (skim yogurt), so they were less creamy. These samples were described by consumers as lumpy and chalky, while counterparts produced with gelatin gave the greatest resemblance to a full-fat yogurt. The sample containing the hydrocolloid showed similar thickness, smoothness, and creaminess and had a comparatively lower CoF than full-fat yogurt.

In addition to the hydrocolloids mentioned above, (40) also investigated the effects on the CoF and sensory perception when adding functional ingredients such as inulin (7, 8, and 9%),

pectin (0.2, 0.25, and 0.3%), galactooligosaccharides (GOS, 9.1, 11.3, and 13.6%), and β-glucan (BG, 0.1, 0.2, and 0.3%) as a replacement for fat in skim stirred yogurt (0.1% fat, **Table 1**). The results were compared with full-fat stirred yogurt without any addition of ingredients. The overall conclusions indicated that none of the ingredients significantly improved the sensory attributes of skim yogurt, which makes sense since the CoFs did not have significant changes compared to the CoF of the full-fat yogurt. In fact, in samples supplemented with pectin and BG, the smoothness and creaminess were reduced. They caused an undesirable mouthfeel of lumps and coatings due to the presence of large gel particles, probably due to the poor solubilization or inadequate incorporation of the ingredients during yogurt manufacturing. However, the authors did not discuss this possibility, and they determined that GOS and inulin were the best ingredients in this group, as they showed a lower CoF and did not affect the texture, gel particle size, rheological and sensory characteristics compared to pectin and BG. Apparently, single fat replacers cannot completely substitute or fulfill all the fat functionality. Therefore, the combination of two or more fat substitutes was the subject of an investigation by Ningtyas et al. (38) since it represents a promising option to compensate for the lack of texture and sensory attributes. They analyzed the effect on the textural, microstructural, and lubrication properties of five combinations of phytosterols (PS, emulsified, and esterified form) and β-glucan (BG) reduced-fat cream cheese. The final composition of the samples was 5.5% milk fat, 3.2% (w/w) PS, and 3% (w/w) BG (**Table 1**). The results were compared with low-fat cream cheese (LFCC) with any fat replacer and with commercial cream cheeses: high-fat (24.2% fat, P1) and reduced-fat (13.7% fat, P2).

General conclusions showed that BG combined or alone increased the viscosity similarly to a high-fat cheese. Moreover, it showed relatively high moisture content, firmness, and adhesiveness due to its ability to bind water. The addition of PS improved the spreadability of cream cheeses, improved lubrication, and reduced CoF values. However, it did not provide any significant effect on viscosity compared to P2 and LFCC. Recently, a similar study was carried out on milk by Goh et al. (37) (**Table 1**). In this work, the addition of PS ester (0.8, 1.2, 1.6, and 2.0 /100 g) into skim milk (0.1 g/100 mL milk fat) was evaluated and compared to commercial milk (0.1, 1.3, 2 g fat/100 mL, and 1.3 g fat/100 mL with 0.3 g PS/100 mL). This study concluded that the viscosity and lubrication resulting from milk enriched with PS are comparable with commercial products at the same total fat content. As the PS ester concentration increased, CoF decreased, while the size of the fat globules and the viscosity increased.

RELATIONSHIP OF ADSORPTION STUDIES WITH TRIBOLOGY AND DAIRY PRODUCTS

Tribology has certainly attracted the attention of food research. Although many works related to the sensory aspects of foods have already been reported, there is still much to investigate in other areas to guide the decision-making process when foods are being formulated or reformulated. As described earlier, fat substitution aimed to maintain the functionality and sensory properties in dairy products is among the main interests worldwide. Kew et al. (51) discussed the most viable options to replace fat in food products, including protein concentrates from animal and plant and microparticulated forms of proteins, concluding that whey protein is the best and most commonly used for substitution. Following this statement, researchers are currently investigating replacing whey protein in dairy products with vegetable protein since it could have great potential in the industry, but its understanding, application and characterization are still limited. Omrani Khiabanian et al. (52) studied the replacement of whey protein with pea protein in feta cheese to understand the effect on the chemistry, rheology, texture, and microstructure. They varied the proportions of both proteins to reach 12% of the total composition and concluded that by increasing pea protein above 3%, feta cheese began to have undesirable sensory properties, especially flavor, and texture.

Additionally, by gradually increasing the vegetable protein concentration, the hardness, cohesiveness, springiness, gumminess, and chewiness of the cheese were reduced. Although this research did not include tribological analyses, it was the beginning to understand the effect of this vegetable protein on dairy. Recently, (53) hypothesized that rheology, lubrication, and adsorptive properties of pea protein could cause these unpleasant effects on food texture. Adsorption is described as an augmentation in the concentration of a substance, known as adsorbate, at the interface of a liquid or gas layer, because of the operation of surface forces (54). Once adsorbed, the adsorbate resides in a surface known as adsorbent, being adsorption classified according to two main mechanisms: physisorption and chemisorption (42). The first one occurs when binding interactions are <40 kJ/mol, and adsorbate-adsorbent interactions mainly are associated with van der Waals forces, whereas chemisorption is the adsorption mechanism with binding interactions higher than 40 kJ/mol and intramolecular association governed by covalent binding (42, 54). The adsorption studies in soft tribology allow a better understanding of the boundary lubrication regime, where CoF is dependent on non-hydrodynamic characteristics.

There are still few studies of friction in dairy foods; nevertheless, the techniques for investigating adsorption have attracted attention as a complementary tool for tribological analysis. Among the experimental techniques reported to study the boundary regime are those focused on solid surfaces in contact with the lubricant or food, used to study the structural, physical and chemical aspects from macroscopic to atomic-scale (54). The instruments used are optical, scanning tunneling, atomic force microscopes, and stylus profilometer. To measure the amount of mass and thickness of adsorbate are Quartz Crystal Microbalance (QCM) and the ellipsometer (54). The most widely reported study on the adsorption phenomena in food is the QCM with Dissipation Monitoring (QCM-D), which measures adsorption in real-time.

Moreover, QCM-D can record the frequency and energy dissipation changes as a function of time to monitor adsorption (53, 55, 56), providing information about the adsorption kinetics,

mass and viscoelasticity of the adsorbate. Zembyla et al. (53) used PDMS-coated sensors on this equipment to mimic the human oral surfaces besides the gold-coated alternatives. Zembyla et al. (53) compared the rheological, lubrication, and adsorbent properties of whey and pea protein dilutions with and without heat treatment in the presence or absence of salivary mucin on the surface of the PDMS sensors. The QCM-D results showed that pea protein adsorbed twice as much on PDMS surfaces and forms a slightly more viscous film than whey protein. Pea protein can be better adsorbed in salivary mucins than whey protein, forming films with similar viscoelastic properties. The high adsorption capacity of pea protein resulted in better lubrication for concentrations lower than 10 mg/ml, which was not observed for whey protein. However, when increasing the concentration to 100 mg/mL, the pea protein tended to form aggregates which negatively affected the lubrication and increased CoF, while whey protein improved lubrication. This demonstrates that whey protein needs higher concentrations to saturate the contact surfaces than pea protein. In conclusion, replacing whey protein with high pea protein concentrations could negatively affect the sensorial characteristics of foods developed with this ingredient.

Omrani Khiabanian et al. (52) reported that feta cheeses with low concentrations of pea protein were texturally more compact and hard, which could mean an increase in the CoF. The most recent study on the lubrication and adsorption of alternative proteins for fat substitutes was carried out by Kew et al. (57), evaluating dilutions of five types of proteins (1) Whey protein isolate (WPI), (2) Pea protein concentrate (PPC), (3) Potato protein isolate (PoPI), (4) Insect protein concentrate (IPC, *Alphitobius diaperinus*) and (5) Lupine protein isolate (LPI). The QCM-D results demonstrated that the final film viscoelasticity was higher for IPC followed by PPC, LPI, WPI, and PoPI, respectively, revealing that PoPI and WPI formed the most rigid layers, with low CoF, whereas the most viscous films as PPC led to high friction coefficient. At concentrations of 5%, all proteins showed effective lubrication, but when increasing the concentration to 10%, CoF of LPI, PoPI, and IPC increased, while WPI showed the lowest boundary friction coefficient and the best lubrication performance. Perhaps it is advisable to use alternative proteins in low concentrations for food development. However, to verify this information, it is necessary to study the direct effect of these proteins in the food system, including the adsorption studies, which can be useful better to understand lubrication and friction in the boundary regime. With the mixed regime, this section is where CoF tends to correlate with a range of sensory properties such as smoothness, slipperiness, and pastiness (57). The use of specific methodologies and instruments to study adsorption is an opportunity area not fully explored yet.

CONCLUSIONS

In recent years, several investigations have been carried out dealing with tribology and its relationship with the sensory perception of foods, especially smoothness, creaminess, and astringency. There is a constant relationship in dairy products, where the higher the fat in the food, the greater the smoothness and creaminess, thus reducing the coefficient of friction generated during the tribological analysis and, in turn, reducing the sensation of astringency in the oral cavity. However, it has also been observed that dairy proteins can produce a sensory perception of astringency when these interact with salivary proteins because both have a different charge that can leads to an increase in friction and/or the loss of saliva lubrication if these are at low pH. It is important to mention that this document has not added other sensory attributes such as ropiness, chalkiness, powderiness and others due to the lack of research that studies them explicitly. Due to their components (fat, protein, carbohydrates, stabilizers), dairy products represent an interesting matrix for tribological studies, in which multiple factors come into play since they can cause high variability in the results. In previous review articles on lubrication and food, great emphasis was placed on determining exactly the best system to use for the type of food wanted to study. For example, the type of tribometer and surface material to obtain more reliable results and if using saliva provides more realistic scenarios. Today, we discussed adsorbent properties as the newest topic that complements tribology. So far, there are not still many studies on adsorption and tribology for dairy products. However, it is expected that just as the particle size is essential for friction analysis, so will adsorption. To this day, tribological results have offered significant advances in sensory analysis not only for dairy products but for the food industry in general.

Furthermore, it proves to be a promising predictive tool that supports and complements conventional systems (texture and rheology) in new product development. However, despite the evident advantages of tribology, its use in industrial and commercial applications has some important challenges because it cannot be applied in solid foods, and the analytical equipment is expensive. Besides, instruments, measurements, sample preparation, and personnel training are important parameters for obtaining reliable results that yield case-specific data. Therefore, the use of tribometers with convectional systems as rheometers and trained sensorial panels is needed.

Currently, few food companies have integrated tribology as one of their tools for food analysis. However, in a short time, more institutions are likely to become aware of this technique and its several applications for decision making in the development of new and healthy food products contributing to research and a better understanding of the use of soft tribology in the food industry.

AUTHOR CONTRIBUTIONS

BC-P collected data, drafted the manuscript, and adjusted information same that AS-R, who besides reviewed and edited the manuscript. DM and SS-S reviewed and edited the manuscript. RE-C collected data and drafted the manuscript. Finally, CC-H conceptualized the article, drafted, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Hydrothermal-induced changes in the gel properties of Mung bean proteins and their effect on the cooking quality of developed compound noodles

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Mung bean proteins (MBPs) are highly nutritious food ingredients, but their lack of gluten limits their use in staple foods such as noodles. In this study, MBPs were modified by hydrothermal treatment, and their gel properties and the major structural changes were analyzed at different heating temperatures (25, 65, 75, 85, 95, and 105°C), moisture contents (0, 15, 20, 25, 30, and 35%), and hydrothermal treatment times (0, 15, 30, 45, 60, and 75 min). Thereafter, the modified MBPs (MMBPs) were added to wheat noodles at substitution levels of 3, 6, and 9% to evaluate their effect on the quality of the noodles. The results showed that the hydrothermal treatment significantly improved the gel properties and water absorption capacity of the MBPs and slightly increased their disulfide bond content. When MBPs with a 25% moisture content were heated at 85°C for 60 min, their gel properties notably improved, and their structural changes were maximal. The structural changes revealed that the MBP molecule formed a macromolecular polymer because a significant protein band appeared at about 66.2 kDa. Secondary structure and microstructure analyses revealed that the MBP structure was significantly damaged and that the β-sheet structure increased because of changes in the degree of aggregation between the protein molecules. Compared to the untreated MBPs, the MMBPs significantly improved the cooking quality and texture properties of the noodles, and the addition amount reached more than 6%, whereas that of the untreated MBPs was less than 3%. At this time, the cooking loss and the broken rate of the untreated MBPs group were about 2 times higher than that of the 6% MMBP-treated group. An analysis of changes in the water distribution, rheological properties, and microstructure revealed that intermolecular cross-linking occurred between the MMBPs and wheat dough, which improved the quality of the MMBP-treated noodles. The findings demonstrated that the MMBPs obtained by hydrothermal treatment had a positive effect on

the wheat dough properties and noodle quality. These results provide a technical foundation for incorporating novel protein supplements into staple foods.

KEYWORDS

hydrothermal treatment, Mung bean protein, modified Mung bean protein, gel properties, cooking quality

Introduction

Noodles are a traditional and popular staple food in China, and they are mainly made of wheat and other grains. Wheat flour has a protein content of 12–15%, but lysine is the first limiting amino acid in its protein composition, making it unable to completely satisfy the requirement of essential amino acids (1). People are becoming increasingly aware of the importance of consuming healthy food as the prevalence of non-communicable nutritional diseases (such as hypertension, hyperlipidemia, and coronary heart disease) increases, and consumer demand for healthy food is also growing rapidly (2–5). Since the middle of the twentieth century, researchers have used food fortification with essential nutrients to improve nutritional deficiencies (6). Tazrart et al. indicated that adding legumes to grains could compensate for lysine deficiency in grains and methionine deficiency in beans, resulting in amino acid complementarity (7). These characteristics and market demand considerably enhance the value of legume proteins as potential ingredients.

Mung bean proteins (MBPs) are important high-quality plant-derived proteins with a high essential amino acid content (43.5% of the total amino acid content), and their nutritional score exceeds the recommended value of Food and Agriculture Organization (FAO)/World Health Organization (WHO) (8). Lysine, tyrosine, phenylalanine, and leucine are particularly abundant in MBPs (9). MBPs have been reported to have many beneficial physiological effects, such as improving glycolipid metabolism, preventing and controlling non-alcoholic fatty liver disease, and regulating antioxidant enzyme activity (10–12). However, MBPs are mostly used for producing protein beverages, emulsion stabilizers, etc., because they lack gluten, which limits their application in staple foods such as noodles. Numerous studies have verified that modification treatments such as high static pressure modification, extrusion and steam explosion, ultrasonic acid digestion, and hydrothermal treatment broaden the application areas of MBPs, soybean, and wheat bran (9, 13–16). In particular, hydrothermal modification treatment, which is the most common processing treatment in the food industry, has been proven to be very effective in modifying proteins. Yang et al. demonstrated that thermal modification treatment (95°C for 30 min) improved the gel properties of soybean proteins (17). Yan et al. discovered that quinoa protein gels produced in a water bath at 80–95°C had high chewiness, elasticity, cohesion, hardness, and adhesion (16). Some studies have also confirmed that the noodles made by mixing edible legume protein (peas, broad beans, soybeans, etc.) with wheat flour not only endows the noodles with higher protein content, but also significantly improves their taste, palatability and cooking characteristics (18–20). Gel properties are important for maintaining the quality of noodles, and improving the gel properties of MBPs will be an effective strategy to broaden its application in noodles. The objective of this study was to improve the gel properties of MBPs using a hydrothermal modification method based on gel property indicators such as gel strength, water absorption capacity, and disulfide bond content. Thereafter, the modified MBPs (MMBPs) obtained via hydrothermal treatment were added to noodles, and the effect of the gelled MBPs on the noodle quality was analyzed.

Materials and methods

Materials

Wheat flour (protein content 15%, w/w) and MBPs (protein content 92%, w/w) were purchased from a local market in Daqing, China. Additionally, β-Mercaptoethanol (β-ME) and sodium dodecyl sulfate (SDS) were obtained from Solarbio Co. (Beijing, China).

Hydrothermal treatment of the Mung bean proteins

The MBPs were hydrothermally treated using the method described by Yan (16). MBP powder was laid flat on a tray and evenly sprayed with certain amounts of water (0, 15, 20, 25, 30, and 35%). Subsequently, they were sealed with cling film, placed in a blast drying oven (Yiheng Instruments Co., Shanghai, China), and heated at 25, 65, 75, 85, 95, and 105°C for 0, 15, 30, 45, 60, and 75 min, respectively. The hydrothermally treated MBPs were cooled to room temperature.

Disulfide bond contents

The disulfide bond content in the control and hydrothermally treated MBP sample was measured using the total sulfhydryl group content and free sulfhydryl group content, as outlined by Ellman (21). In this experiment, 75 mg of the sample was dissolved into 10 mL of Tris-glycine buffer (8 mol/L), and then 1.0 mL of the protein solution was pipetted into 4.0 mL of Tris-glycine buffer and 0.05 mL Ellman's reagent. The solution was rapidly mixed before being left at room temperature for 5 min. The free sulfhydryl content was measured at 412 nm. The total sulfhydryl content in the MBP samples was determined using the following method: 1 mL of the protein solution was pipetted into 0.05 mL of β-ME and 4.0 mL of Tris-glycine buffer, and the solution was mixed and kept at room temperature for 1 h. Next, 10 mL of 12% trichloroacetic acid was added and allowed to react for another 1 h. Thereafter, the protein solution was centrifuged at 4,000 r/min for 10 min. Subsequently, 1.0 mg of the protein precipitate was dissolved in 4.0 mL of Tris-glycine buffer and 0.04 mL of Ellman's reagent. After rapid mixing, the solution was kept at room temperature for 5 min and measured at 412 nm.

Gel strength

Texture profile analysis (TPA; TA. XT plus texture analyzer, Shanghai, China) was used to measure the gel strength according to a method described by Yang et al. (17). Each protein sample was diluted in deionized water to 20%, placed in a water bath, and heated at 80°C for 30 min before being cooled to room temperature and stored overnight at 4°C. The protein gel samples were analyzed by TPA, and the determination parameters were as follows: P/0.5 probe model, 1.0 mm/s probe running speed, 0.5 mm/s puncture running speed, 10 mm/s return speed, 5 mm puncture distance, and 1 g trigger type.

Water absorption

The protein sample (1.0 g) was mixed with 20 g of deionized water and centrifuged at 4,500 r/min for 10 min after standing for 80 min at room temperature. The supernatant was weighed and used to calculate the adsorbed water content as a percentage of the protein content.

Electrophoresis

The method of Zhang et al. (22) was used to perform SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Here, 1 mL of the protein sample (0.5 mg/mL) was mixed with 1 mL of buffer (containing 0.25 mol/L of Tris–HCl, 0.5% bromophenol blue, 10% SDS, 50% glycerin, and 5% β-ME) and heated in boiling water for 5 min to denature the protein. The stacking and separating gels consisted of 5 and 12% acrylamide, respectively. Aliquots of 40 μL of protein per lane were loaded onto the stacking gels, and electrophoresis (DYY-8C, Beijing, China) was performed (laminated gel voltage: 80 mV; separation gel voltage: 120 mV). When the protein band reached the bottom of the electrophoresis machine, the gel was stained with Coomassie brilliant blue for 30 min and subsequently destained in a mixed solution with 5 w/v% methanol and 7.5 w/v% acetic acid until the band edge became colorless.

Fourier-transform infrared analysis

Fourier-transform infrared (FTIR) spectroscopy was used to identify changes in the protein structure of the control and hydrothermally treated MBPs according to the method proposed by Zhang et al. (22). The FTIR spectra of the samples were recorded by scanning the MBP solution at a wavelength range of 400–4,000 cm^{-1} using a MAGNA-IR560 FTIR instrument (Thermo Nicolet Co., Wis. American).

Modified Mung bean proteins-treated noodle preparation

The hydrothermally treated MMBPs were mixed with 3, 6, and 9% wheat flour (human daily protein intake of 68 g). Thereafter, 100 g of the mixed flour and 44% water were mixed in a kneading machine (ODA-5HM1, Beijing, China) for 10 min at medium speed to make a dough, which was then allowed to rest for 25 min in sealed containers at room temperature. The roll gap was adjusted to 2 mm, and the sheeted dough was doubled over and passed through the same gap five times. Thereafter, the roll gap was reduced to 1 mm, and the dough was cut into 2 mm wide noodles.

Cooking quality

Cooking loss was determined using the method of Zhao et al. (23). Noodles (20 strips) were cooked in 100 mL of boiling tap water for 9 min and subsequently drained before drying the cooking water in an air oven at 105°C. The residue was weighed, and the results were expressed as a proportion of the uncooked dried noodles.

The broken rate of cooked noodles was determined using the same method described by Zhao et al. (23). Noodles (20 strips) were cooked in 100 mL of boiling tap water for 9 min. The cooked noodles were then removed, and the number of unbroken noodles was counted immediately.

Noodles (20 strips) were cooked in 100 mL of boiling tap water for 9 min, then removed and weighed. Water absorption

capacity was expressed as the ratio of the weight of the cooked noodles to the weight of the uncooked noodles.

Noodle texture analysis

The method of Zhao et al. (23) was used to perform a TPA on the cooked noodles. Noodles were cooked until done, placed in cold water for 2 min, and then drained for 30 s. The texture properties of the noodles were analyzed using a TA. XTplus texture analyzer (Ruibin Co., Shanghai, China). The TPA parameters for the cooked noodles were set as follows: P50 probe model, 2.0 mm/s pretest speed, 1.0 mm/s posttest speed, 1.0 mm/s test speed, 70% strain, 5 g trigger type, and a 1 s interval between two compressions.

Low-field nuclear magnetic resonance

Transverse relaxation time (T2) of the dough samples was measured using a low-field nuclear magnetic resonance (LF-NMR) system (NMI20-015V-I, Suzhou, China). The test parameters were as follows: the number of sampling points (TD) was 139994, regulated analog gain 1 (RG1) was 10.0, the regulated first data (RFD) was 0.002, the regulated digital gain (DRG) was 3.0; the preamplifier regulate gain (PRG) was 1, and the number of sampling (NS) was 4. The T2 relaxation map was obtained after inverting the exponential attenuation curve. There were three states of water in the dough: bound water (T21), weakly bound water (T22), and free water (T23).

Rheological properties

The samples were measured with a DHR-1 hybrid rheometer (TA Instruments, Inc., New Castle, Delaware, American) in the oscillation mode using the method described by Chen et al. (24). The samples were loaded within a 1 mm gap between two parallel plates (upper plate diameter = 30 mm). A thin layer of glycerin was applied around the sample to prevent dehydration. The samples were equilibrated at 25°C for 2 min and then heated from 25 to 100°C at a controlled heating rate (0.1°C/min). The dynamic rheological properties were determined by recording the storage modulus (G′) or elastic component (G″).

Microscopic observation

The sample microstructures were observed with a scanning electron microscope (SEM) (SU3400, Tokyo, Japan) using the method of Yuan et al. (25). The samples were covered with a thin gold layer and immediately observed using an SEM at 1,000× magnification.

Statistical analysis

All the data points in the experiment were acquired in triplicate. The statistical differences ($p < 0.05$) between the average values were analyzed using the Statistics 9.0 software and Tukey's HSD (Honestly Significant Difference) comparison test.

Results

Determination of Mung bean proteins and hydrothermally treated Mung bean proteins gel properties

Figure 1 depicts the effect of hydrothermal treatment on the gel strength of the MBPs at different heating temperatures (25, 65, 75, 85, 95, and 105°C), moisture contents (0, 15, 20, 25, 30, and 35%), and treatment durations (0, 15, 30, 45, 60, and 75 min). When the MBPs were heated at or above 75°C, their gel strength increased significantly and reached 97 g/cm^2 at 85°C, which was higher than that of other treatment groups. Interestingly, when they were heated at 95 and 105°C, their gel strength decreased gradually to 90 and 74 g/cm^2 (**Figure 1A**).

The disulfide bond contents had a positive correlation with the gel strength, and the gel network structure was mainly connected by disulfide bonds. **Figure 1B** depicts the variation in the disulfide bond content in the MBPs under different treatment conditions. The effect of heating time on the disulfide bond content varied slightly, except for the group that was treated for 75 min. However, the effect of moisture content and temperature on the disulfide bonds was particularly significant, indicating that the intermolecular interactions of the MBPs increased at an appropriate temperature and moisture content and that the number of protein molecules that favored the formation of a gel network structure increased, promoting the formation of disulfide bonds between the MBP molecules and increasing the gel strength. When the MBPs were hydrothermally treated at 85°C for 60 min with a moisture content of 25%, their disulfide bond contents and gel strength reached about 10 μmol/g and 183 g/cm^2, respectively, which were significantly higher than those of the untreated group.

Figure 1C shows the water absorption capacity of the MBPs under different treatment conditions. There was no significant difference in the water absorption capacity of the MBPs when the temperature was lower than 85°C ($p > 0.05$); however, the water absorption capacity significantly decreased ($p < 0.05$) when the temperature was above 85°C. This may be because the denaturation temperature of the MBPs was 85°C, and the peptide chain of the MBPs unfolded as the temperature increased, resulting in a decrease in the hydration of the MBP hydrogen and ionic bonds. The water absorption capacity of the

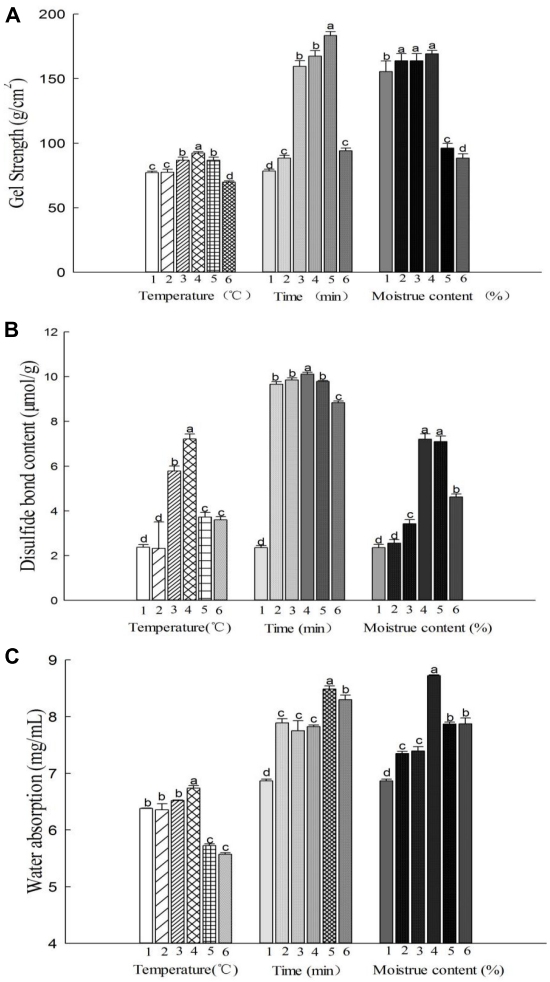

FIGURE 1
Gel properties of the MBPs under different hydrothermal treatment conditions. Numbers 1–6 for the temperature represent the MBP groups treated with different hydrothermal temperatures (25, 65, 75, 85, 95, and 105°C, respectively). Numbers 1–6 for the time represent the MBP groups treated with different hydrothermal times (0, 15, 30, 45, 60, and 75 min, respectively). Numbers 1–6 corresponding to the moisture content represent the MBP groups treated with different moisture contents (0, 15, 20, 25, 30, and 35%, respectively). The letters a–d represent significant differences between the groups. **(A)** Gel strength. **(B)** Disulfide bond content. **(C)** Water absorption.

MBPs increased as the hydrothermal treatment time increased. The MBPs had the highest water absorption capacity when heated at 85°C for 60 min, but the water absorption capacity decreased when the treatment time was 75 min. The moisture content had a significant effect on the water absorption capacity of the MBPs, and this effect was notably strong when the moisture content was 25% but significantly weak when the moisture content exceeded 25%, which is consistent with the gel strength and disulfide bond content results. The above results indicate that the gel properties of the MBPs increased when treated with a 25% moisture content at 85°C for 60 min. The hydrothermal treatment caused the MBP structure to unfold and exposed the sulfhydryl groups originally buried in the center of the protein, which easily formed disulfide bonds between the protein molecules and enhanced intermolecular forces, forming a dense gel network structure and improving its water absorption capacity.

Determination of changes in the Mung bean proteins and hydrothermally treated Mung bean proteins structures

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis

SDS–PAGE was performed to investigate the effects of the hydrothermal treatment methods on the molecular weight and patterns of the MBPs (Figures 2A–C). The major protein band of the MBPs was observed at 45, 35, 30, 25, and 16 kDa. However, the protein bands of the hydrothermally treated MBPs were observed at nearly 66.2 kDa, indicating that the treated MBP produced new macromolecules. The disulfide bonds were the main force affecting gel properties; meanwhile, there were also hydrophobic bonds, hydrogen bonds, and ionic bonds. Yang et al. found that there were four kinds of forces in heat-treated soybean protein gel, which were disulfide bond, hydrophobic force, hydrogen bond, and classical force in order of magnitude (17). And some studies showed the existence of Tyr-Tyr and carbonyl-NH_2 in proteins (26, 27). This research confirmed the presence of disulfide bonds in MBPs gels, but the difference was not significant. Moreover, FTIR results showed the existence of hydrogen bonds in MBPs. In particular, the 66.2, 45, 30, and 25 kDa protein bands of the hydrothermally treated MBPs thickened as the heating time increased at 85°C ($p < 0.05$), indicating that high temperatures cause protein degradation and internal structural damage of MBPs. However, all the protein bands of the MBPs narrowed at temperatures above 85°C and almost disappeared at 100°C. There was no significant change under different moisture conditions.

Fourier-transform infrared spectroscopy

Figures 2D–F show changes in the MBP secondary structure observed by FTIR spectroscopy under different treatment conditions. An amide I band at 1,600–1,700 cm^{-1} was observed in all the samples. Spectral shifts at 1,600–1,700 cm^{-1} occurred in the hydrothermal temperature-treated and time-treated groups. The MBP samples treated at a temperature above 85°C for over 30 min exhibited an increase in peak intensity and a significant redshift in the amide I bands (Figures 2D,E). At the same time, the secondary structure contents (α-helix, β-sheet, and random coils) of the MBPs changed significantly ($p < 0.05$)

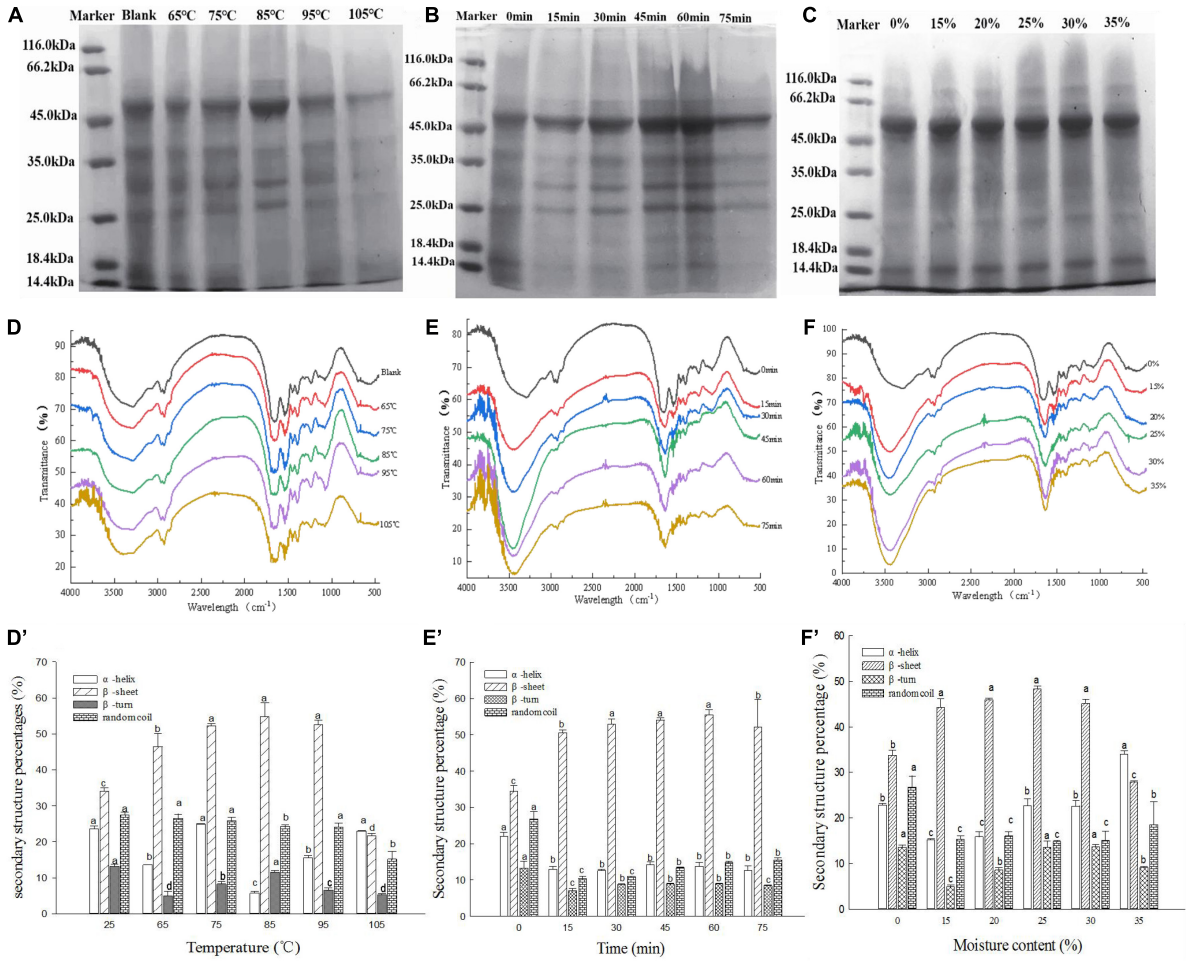

FIGURE 2
Changes in the structure of the hydrothermally treated MBPs at different controlled temperatures (25, 65, 75, 85, 95, and 105°C), processing times (0, 15, 30, 45, 60, and 75 min) and moisture contents (0, 15, 20, 25, 30, and 35%). **(A–C)** SDS–PAGE of the MBPs under the different treatment conditions, respectively. **(D–F)** FTIR spectra of the MBPs under the different treatment conditions, respectively. **(D'–F')** Secondary structure percentages of the MBPs under different treatment conditions based on FTIR analysis. The letters a–d indicate significant differences between the groups.

(Figures 2D'–F'). These results indicate that the increase in temperature and duration caused the secondary structure of the MBPs to change or the peptide chains of the MBPs to rearrange. Compared to those of the temperature-treated and time-treated MBPs, the spectral shifts in the amide I band of the MBPs with different moisture contents did not change significantly ($p > 0.05$). However, spectral blue shifts and increasing peak intensities were observed at 3,250–3,500 cm^{-1} (associated with O–H stretching vibration) as the moisture content increased (particularly above 25%), and the result was similar to that of the heat-treated and time-treated groups. The hydrothermally treated MBPs were believed to have undergone changes in their original spatial structure, which disrupted intramolecular hydrogen bonds and increased intermolecular forces, thereby increasing the β-sheet content and facilitating the formation of a gel network structure.

Noodle quality evaluation

Effect of modified Mung bean proteins addition on the cooking properties of noodles

Figures 3A,A' depict the microstructures of the hydrothermally treated and untreated MBPs. The native MBPs had prominent depressed spherical particles, whereas the MMBPs had relatively large spherical expansion structures. As shown in the SEM images, the MMBP particle surfaces had larger holes, and there were noticeable cross-linked aggregates between the particles, which were different from those of the native MBPs, indicating that the ordered structure of the protein was destroyed by the hydrothermal treatment, thereby exposing hydrophobic groups and allowing protein molecules to interact with water molecules and other groups.

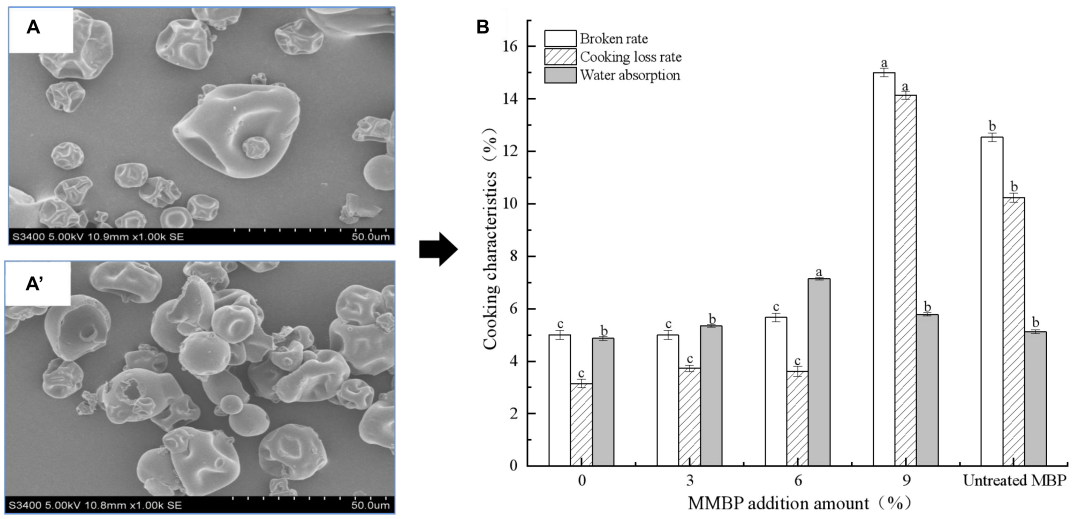

FIGURE 3
Cooking properties of noodles treated with different MMBP amounts (0, 3, 6, and 9%). **(A)** and **(A')** Microstructure of the hydrothermally treated and untreated MBPs, respectively. **(B)** Cooking properties of the noodles. The letters a–c indicate significant differences between the groups.

Figure 3B shows the effects of MMBP addition on the cooking loss rate, broken rate, and water absorption capacity of the noodles. During cooking, the cooking loss and broken rates increased as the substitution level increased, but no statistically significant differences were found between the 3 and 6% substitution levels. The broken and cooking loss rates of the noodles with 3% untreated MBPs were found to be similar to those of the 9% MMBP-treated noodle group, indicating that the MMBPs increased both the protein content and quality of the noodles. During cooking, the MMBP-treated noodles had a significantly higher water absorption capacity than the control group; however, the 9% MMBP-treated noodle group had a significantly lower water absorption than the 6% MMBP-treated noodle group, but it had a higher water absorption capacity than the control group, which may be due to strong water—starch—gluten interactions forming a highly dense structure and decreasing the water absorption capacity. Overall, the 6% MMBP-treated noodles had the best cooking characteristics.

Effect of modified Mung bean proteins addition on the texture properties of noodles

Among the TPA parameters of the noodle samples, gumminess, chewiness, and cohesiveness had a significant positive correlation with hardness (Table 1). Hardness depends on the degree of formation of a gluten network structure in noodles, and it plays an important role in maintaining the quality of noodles. The hardness and springiness of the cooked noodles increased linearly as the MMBP amount increased. When the MMBP amount increased to 9%, the hardness of the noodles increased, but the springiness decreased sharply due to the decrease in water absorption capacity of the high-protein noodles (Figure 3B).

Effect of modified Mung bean proteins addition on the water distribution of wheat dough

Moisture plays an important role in noodle processing, and the macro quality characteristics of noodles (such as texture) are mostly determined by the water absorption capacity and internal moisture state of the dough. The MMBP-treated wheat dough had a slightly shorter relaxation time (T21, T22, and T23) than the wheat dough group (Figure 4A), indicating that the water molecules and macromolecules such as protein and starch were closely integrated. The relaxation time of the different MMBP-treated doughs shifted to the left as the added MMBP amount increased, indicating that the high-protein dough had a denser and stronger ability to bind water than the low-protein dough. This also indicated that the compact gluten network inhibited the swelling of the noodles, thereby affecting the dry matter water absorption capacity of the noodles, which is consistent with the findings on the edible properties of the noodles (Figure 3B).

Effect of modified Mung bean proteins addition on the rheological properties of wheat dough

Figures 4B,C depict the rheological properties of MMBP-treated wheat dough. Using MMBPs to partially replace wheat resulted in a noticeable increase in G′ and G″ values. Additionally, G′ was higher than G″ for all the groups, indicating that the dough was more elastic than sticky. The 6% MMBP-treated group had the highest G′ and G″ values among the MMBP-treated groups, indicating that the MMBP-treated dough had an increased resistance to deformation and that the

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TABLE 1 Texture properties of the noodles treated with different MMBP amounts.

Addition (%)	Hardness (g)	Springiness(%)	Cohesiveness (g˙s)	Gumminess (g)	Chewiness (g˙s)
0	11629.13 ± 150^{d}	0.64 ± 0.05^{c}	0.61 ± 0.01^{d}	7845.27 ± 143^{d}	6640.68 ± 125^{a}
3	13778.32 ± 154^{c}	0.67 ± 0.02^{b}	0.63 ± 0.03^{c}	9717.01 ± 103^{c}	6567.32 ± 132^{a}
6	15553.59 ± 134^{b}	0.74 ± 0.04^{a}	0.65 ± 0.02^{b}	10507.60 ± 201^{b}	6369.66 ± 132^{b}
9	16661.92 ± 121^{a}	0.68 ± 0.02^{b}	0.69 ± 0.02^{a}	12442.68 ± 209^{a}	6062.90 ± 197^{c}

$^{a-d}$ Indicates significant differences between the groups.

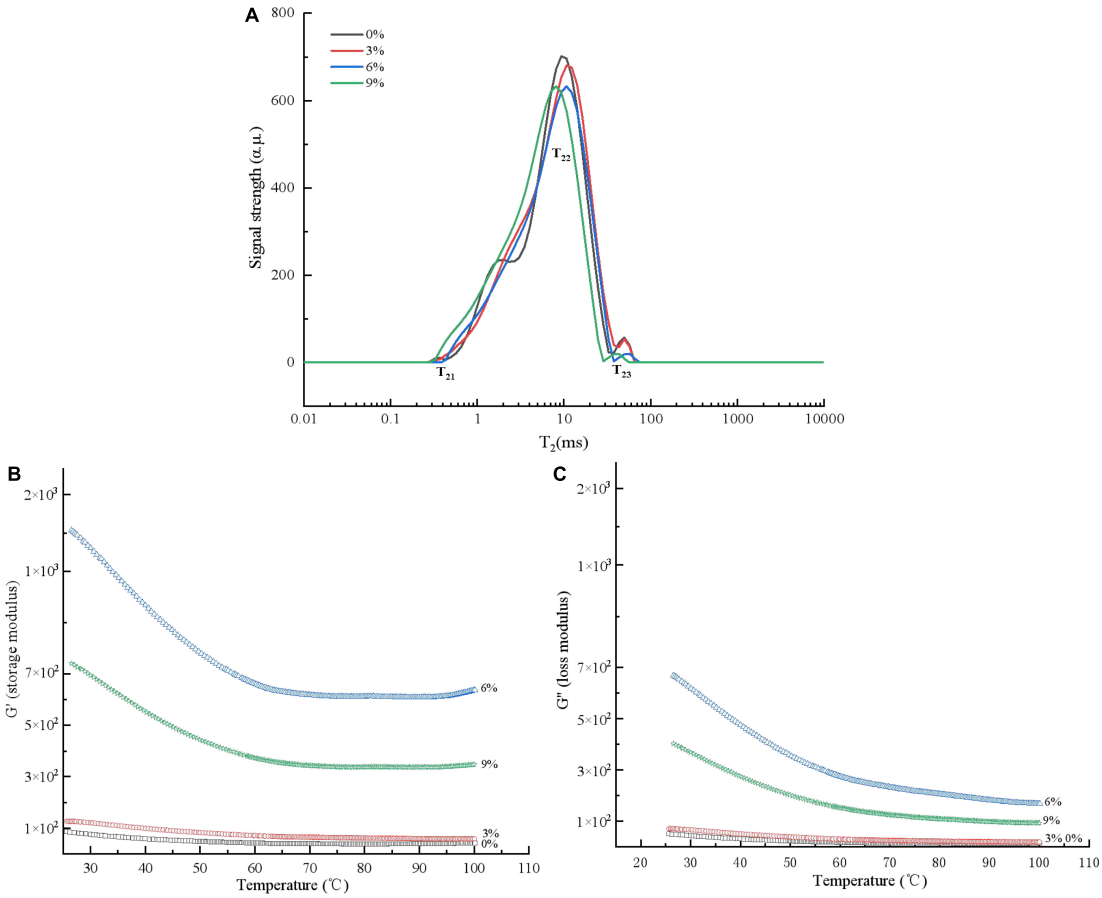

FIGURE 4
Water distribution and rheological properties of the dough treated with different MMBP amounts (0, 3, 6, and 9%). **(A)** Water distribution, **(B)** storage modulus, and **(C)** loss modulus.

MMBPs cross-linked with the wheat proteins to form a network structure. However, compared to those of the 6% MMBP-treated group, the G′ and G″ values of the 9% MMBP-treated group significantly decreased, reaching 53 and 50%, respectively. The rheological results at different temperatures also revealed that the MMBPs increased the G′ and G″ values of the dough, which is consistent with the texture property results.

Effect of modified Mung bean proteins addition on the wheat dough microstructure

Figure 5 shows the microstructure of the dough network treated with MMBPs. Starch granules were wrapped or embedded in the dough gluten protein, forming a relatively dense and uniform network structure, but some cracks were observed in the network structure (Figure 5A). The MMBPs were able to repair the dough cracks. The 3% MMBP-treated dough group had a denser network structure and smaller cracks than the untreated dough group (Figure 5B). The 6% MMBP-treated dough group had a relatively dense gluten network structure, and their cracks disappeared completely (Figure 5C). The 9% MMBP-treated dough group had the most compact network structure, with starch particles tightly wrapped (Figure 5D). These results confirm that MMBPs improve the ability of doughs to form stabilized cross-linked

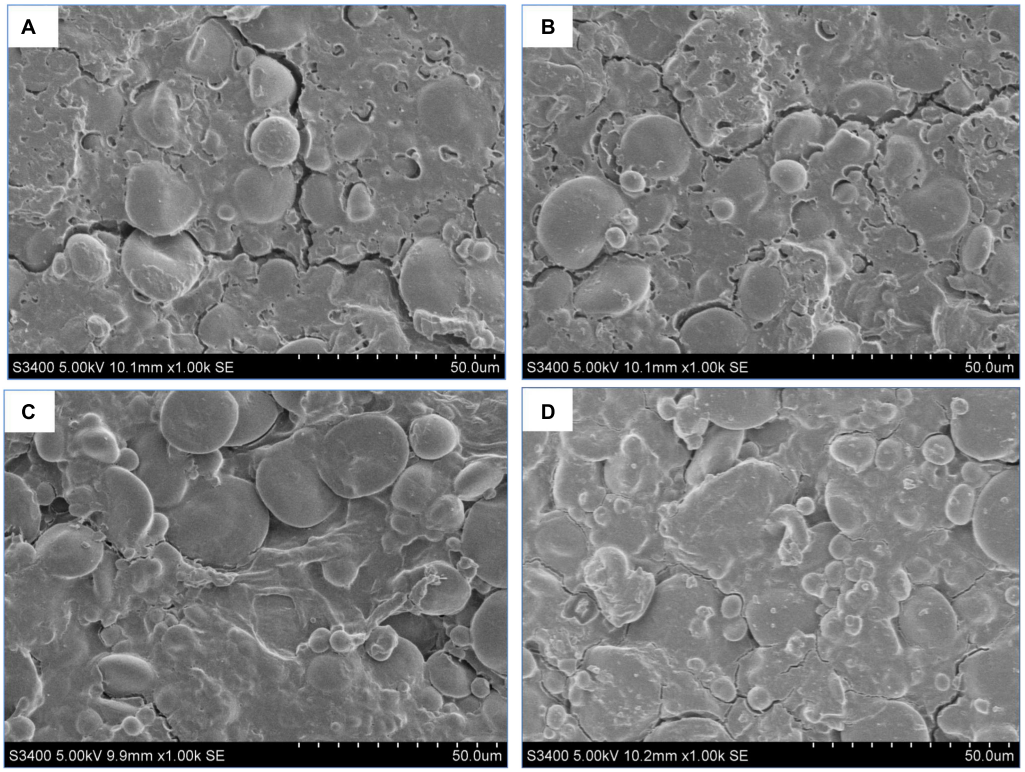

FIGURE 5
Optical microscopic images of the microstructures of the dough treated with different MMBP amounts (0, 3, 6, and 9%). Original magnification: 1000x. **(A)** Wheat dough, **(B)** 3% MMBP-treated dough, **(C)** 6% MMBP-treated dough, and **(D)** 9% MMBP-treated dough.

structures. However, the 9% MMBP-treated dough group had lower textural properties than the other groups, which may be because MMBP altered the force of gluten proteins (**Figure 5D**).

Discussion

MBPs are excellent plant-derived proteins that can provide nutritional supplements to a grain-based diet. However, their use in staple foods is limited because they lack gluten. Current research has found that improved protein gelation can broaden the application of MBPs in food products such as sausages, tofu, and noodles. In this study, hydrothermal treatment (temperature, time, and moisture content) was found to significantly affect the gel properties of MBPs because it disrupts the MBP structure, thereby exposing hydrophobic groups and intensifying protein interactions (28, 29). Chunkao et al. demonstrated that 85°C is the denaturation temperature of MBPs and that denatured MBPs expose more hydrophobic regions and sulfhydryl groups, resulting in high gel strength (30). The formation to stabilization stages of the MBP gels were discovered to occur at 85°C for 0–60 min. However, prolonged high-temperature treatment of MBPs causes dissociation of protein–peptide chains, which decreases gel strength. Yang et al. confirmed that soybean protein subjected to prolonged high temperature treatment leads to complete denaturation and also hydrolysis of protein to produce peptide chains or free amino acids, and these small molecular units cause a reduction in gel strength (17). MBPs with more than 25% moisture contents disrupted the intermolecular and protein—water binding capacities, affecting gel formation and maintenance. The increase in protein moisture contents caused a decrease in protein denaturation temperature due to the critical influence of hydration and water permeation in the protein molecular conformation. Wang et al. demonstrated that the water molecules of proteins with high moisture content partially penetrate into the surface of the protein structure, causing the protein to swell, thereby changing the flexibility of the protein-peptide chains and making it easier to form peptide hydrogen bonds (18). This result is consistent with the water absorption capacity results of the MBPs. In this study, the gel strength had a positive correlation with the disulfide bond content because the denatured MBPs were randomly aggregated to form disulfide bonds through intermolecular hydrophobic interactions, resulting in the cross-linking, folding, and rearrangement of the spatial protein structure (31). The above results suggest that the covalent polymerization of MBP molecules enhances their gelation

properties. The SDS–PAGE and FTIR results confirmed this hypothesis. The SDS–PAGE analysis revealed that the hydrothermally treated MBPs produced a new protein band near 66.2 kDa, which is similar to previous findings (9). However, prolonged high temperatures thinned the protein bands, indicating that the MBP peptide chains degraded, resulting in decreased gel strength. The FTIR analysis results revealed that the treated MBPs had a significantly higher β-sheet content than the untreated MBPs (**Figure 2**). Protein aggregation had a positive correlation with the β-sheet content, and high β-sheet contents facilitated the formation of gel networks (32). The microstructural changes confirm that the treated MBP structure was altered, which improved its functional properties.

Recent studies have demonstrated that adding proteins to noodles is an effective strategy for improving their nutritional value. However, it can also cause a decrease in noodle quality. In this study, the broken rate of the 3% untreated MBP noodles reached about 13.21% (5.02% for wheat noodles). The MMBP-treated noodles did not have different cooking characteristics from wheat noodles. When 9% of MMBPs was added, there was a significant difference because adequate amounts of MMBPs could cross-link with wheat dough and enhance the gluten network structure, favoring the noodle steaming characteristics. However, excess MMBPs were further cross-linked with the wheat dough, resulting in increased hardness and decreased water absorption capacity, which is consistent with the texture property results (**Table 1**). The changes in moisture distribution, rheological properties, and microstructure of the MMBP-treated dough indicated the cross-linking reaction between the MMBPs and wheat dough. The relaxation time of the MMBP-treated dough shifted to the left, indicating that the water in the dough changed from weakly bound water to strongly bound water under the forces of hydrogen bonding, hydrophobic interaction, and van der Waals forces with the MMBPs and wheat proteins (33). The 9% MMBP-treated dough had the strongest bound water, indicating that the changes in the forces between the MMBPs, wheat proteins, and water molecules affected the gluten network structure of the dough, decreasing the cooking characteristics of the noodles (34–36). Strong cross-linking between molecules in mixed dough increases the molecular mass of polymers in the dough, thereby increasing the G′ and G″ values, which is consistent with the findings of this study. The microstructure results confirmed the strong cross-linking reaction between the MMBPs and wheat dough, which resulted in a dense gluten network structure in the MMBP-treated dough (**Figure 5**).

Conclusion

In conclusion, these results indicate that MMBPs, as a type of foreign protein, promoted an exchange reaction between sulfhydryl and disulfide bonds in the dough, increased the probability of protein–peptide chain cross-linking, folding, and rearrangement, and enhanced the ability of the protein to wrap starch particles, resulting in a more compact dough and a strengthened internal network structure.

Data availability statement

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

Author contributions

JD designed the study, interpreted the results, and drafted the manuscript. YT performed the analysis. HC collected the test data. DZ and CW modified the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The current research status and strategies employed to modify food-derived bioactive peptides

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The ability of bioactive peptides to exert biological functions has mainly contributed to their exploitation. The exploitation and utilization of these peptides have grown tremendously over the past two decades. Food-derived peptides from sources such as plant, animal, and marine proteins and their byproducts constitute a more significant portion of the naturally-occurring peptides that have been documented. Due to their high specificity and biocompatibility, these peptides serve as a suitable alternative to pharmacological drugs for treating non-communicable diseases (such as cardiovascular diseases, obesity, and cancer). They are helpful as food preservatives, ingredients in functional foods, and dietary supplements in the food sector. Despite their unique features, the application of these peptides in the clinical and food sector is to some extent hindered by their inherent drawbacks such as toxicity, bitterness, instability, and susceptibility to enzymatic degradation in the gastrointestinal tract. Several strategies have been employed to eliminate or reduce the disadvantages of peptides, thus enhancing the peptide bioactivity and broadening the opportunities for their applications. This review article focuses on the current research status of various bioactive peptides and the strategies that have been implemented to overcome their disadvantages. It will also highlight future perspectives regarding the possible improvements to be made for the development of bioactive peptides with practical uses and their commercialization.

KEYWORDS

food-derived bioactive peptides, inherent drawbacks, bioactivity, modification, functional foods, therapeutic drugs

Introduction

Among the bioactive substances, bioactive peptides, including those of food origin, exert the ability to influence human health (1) positively and have caught the spotlight as potential bioregulators and their utilization in food, cosmetics, and clinical field, has been massive since their discovery. Food-derived peptides are produced *in vivo* or *in vitro* from

plant, insect, animal, or marine proteins. Thousands of bioactive peptides with different functionalities have been isolated from food proteins.

Examples are listed in Table 1. According to PlantPepDB, there are 3,848 plant-based peptides, among which 2,821 have been studied at the protein level (1). Plants such as oats, rice, sorghum, barley, and wheat (11, 12), legumes such as beans, peas, and lentils (13–15), mushrooms (16), nuts (17), vegetables such as broccoli (18), and fruits (19) are primary plant sources of bioactive peptides. Over 253 peptides have been identified from various marine sources (20), including fish (several species, e.g., tilapia, carp mackerel), crustaceans, and algae (21, 22). Marine peptides can also be derived from fish byproducts such as skin, viscera, scales, and heads (23, 24), usually discarded into the environment, causing pollution. The utilization of marine peptides is gaining more popularity, for instance, in the cosmetics industry, due to their anti-aging and anti-inflammatory properties (25, 26). Bioactive peptides from dairy sources have been widely exploited and isolated from milk, meat, cheese, and eggs, among other animal products (27, 28) and also from byproducts. Due to their experimentally proven health claims, functional foods and supplements containing these peptides (based on the bioactivity of interest) are commercially available. An example of such

TABLE 1 Sources of bioactive peptides from food-derived proteins.

Source	Protein	Production process	Identification tool	Peptide/sequence	Potential activity	Reference
Dairy	Sheep whey	Enzymatic hydrolysis: trypsin, papain, alcalase	*In silico* docking using Autodock Vina software	RLYLHENK (RL8) MQEHFTCCR (MQ9)	Dipeptidyl peptidase-IV inhibitor (DPP-IV)	(2)
	Goat whey, casein	Alcalase-assisted fermentation by Lactiplantibacillus plantarum L60 and Lacticaseibacillus rhamnosus LR22	liquid chromatography–tandem mass spectrometry (LC–MS/MS)	FFDDK, NMAHIPR, SCQDQPTTLAR	Angiotensin-1-converting enzyme inhibitior (ACE) and antioxidant	(3)
	Camel and bovine casein	Simulated gastrointestinal digestion	LCMS QTOF	FLWPEYGAL, ACGP, HLPGRG, GPAHCLL	Antidiabetic	(4)
Plant	Kiwicha	*In vitro* gastrointestinal digestion	(LC–MS/MS)	FLISCLL, SVFDEELS and DFIILE	ACE & DPP-IV inhibition, antioxidant	(5)
	Yam (*D. cayennensis*)	*In vitro* gastrointestinal digestion	nanoLC-ESI-MS/MS, MALDI-TOF-MS, *in silico* analysis done using PEAKS Studio 8.5 software, BIOPEP,	DDCAY, LLTW, LAPLPL, QLVHESQDQKR, LRPEW among others.	Antimicrobial, antioxidant effect, ACE inhibition and DNA protection.	(6)
	Adzuki Bean	Simulated digestion	Liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)	KQSESHFVDAQPEQQQR	Anti-inflammatory	(7)
Marine	Rainbow trout	Alcalase-hydrolysis, simulated digestion		NI	ACE inhibitor, antioxidant	(8)
	Nile tilapia	Trypsin digestion	Molecular docking	GPEGPAGAR & GETGPAGPAGAAGPAGPR	ACE- inhibitor	(9)
	Marine snail	Enzymatic hydrolysis	nano-LC-ESI-MS/MS	YSQLENEFDR YIAEDAER	ACE- inhibitor, antioxidant, antidiabetic	(10)

a product is a dietary supplement, "Bioactive Milk Peptides" containing casein decapeptide intended for stress relief and help with sleep.

Bioactive peptides are diverse in functionality, conformity, length, and size. The amino acid composition and sequence determine the peptides' physiological function. These peptides can be classified as antimicrobial (antifungal, antibacterial) (29, 30), anti-inflammatory (31, 32), anticancer (33), antihypertensive (34, 35), immunomodulatory (36), mineral-binding (37), opioids (38), and antidiabetic (39–41), based on the experimentally determined functionality. It has been reported that some peptides are multifunctional; thus, they can exhibit more than one biofunction (5, 42).

Based on the size, the majority of these bioactive peptides are of small size, typically consisting of 2–20 amino acid residues and a molecular weight of <6000Da (43). For instance, the antioxidant peptides are generally small in size (<1000Da), consisting of 5–16 amino acids per chain, with hydrophobic amino acids making a more significant proportion of their composition, contributing to higher antioxidant activity (44–46). Antihypertensive peptides vary significantly in length and are classified as tiny, small, medium, and large peptides based on the number of amino acid residues (47). However, other peptides, such as the antimicrobial peptides, may consist of up to 30- 100 amino acid residues (48, 49); an example of such is PR-39, a proline-rich antibacterial peptide derived from the pig intestine consisting of 39 amino acids (50). Several review articles on food-derived bioactive peptides are available, providing in-depth information on their production, purification, biological functions, and mechanism of action (51–55).

Peptides are inactive within their parental protein, thus requiring hydrolysis of the protein for their release. Peptide isolation from the native protein is widely conducted using conventional approaches such as enzymatic hydrolysis and microbial fermentation, with reports that they are safe (56). Several reviews have documented the production process of bioactive peptides, their purification, and analysis (51, 57). Enzymatic hydrolysis, as the name suggests this approach uses proteases (such as papain, bromelain, pepsin, trypsin, and chymotrypsin) to hydrolyze the parental protein, discharging the peptides of interest (58). Microbial fermentation involves using microorganisms capable of producing enzymes such as bacterial cultures of *Bacillus subtilis and Lactobacillus plantarum* to induce protein cleavage (59–61). Technology advancement has made the isolation of peptides from their parent protein possible through *in vitro* simulated gastrointestinal digestion, which involves using models that mimic the human digestion system to isolate peptides (62–64). Recombinant DNA technology uses microbial cells (E.coli is the most preferred since it is easy to culture and has been well characterized) for peptide yield by adding the peptide into the food matrix (65, 66).

Compared to the existing bioactive peptides that have been documented and proved to have potential benefits, it is noteworthy to mention that the translation of peptides into commercial products is still lagging. Only a few peptide-based products for human use are available on the market. The significant challenges hindering their translation are their inherent drawbacks (including toxicity, bitterness, instability, and susceptibility to enzymatic degradation in the gastrointestinal tract), regulatory obstacles, and higher production costs (67, 68). Numerous strategies have been applied to produce modified peptides, such as improved activity, reduced toxicity, and increased stability, thus subduing the drawbacks. Such techniques include modification of the peptide backbone: either by (1) the substitution of the amino acid residues, (2) insertion of new fragments, or (3) synthesis of peptidomimetics with similar bioactivity of a particular peptide of interest; microencapsulation, use of delivery systems for the release of peptides to the target site, assembly of peptides into supramolecular structures. This review highlights the research status of food-derived peptides and discusses how different techniques have been applied to overcome the drawbacks native to these peptides. The food-derived peptides reported in this review are those mainly utilized in the food and medical fields, with the ability to exert antimicrobial, antidiabetic, and antioxidant bioactivities, among others. In this article, the term modified peptides are referred to as the ones that have been altered to demonstrate improved properties: reduced toxicity, increased bioactivity, sensory quality, and stability.

Research status of bioactive peptides

Since their discovery, bioactive peptides have attracted many researchers and have garnered widespread acceptance. They have found several applications in the food processing, cosmetics, and pharmaceutical areas, as shown in the illustration below (Figure 1), due to their exceptional ability to exert physiological function(s).

They offer several benefits, such as food preservatives, dietary supplements, and functional foods in the food sector (69, 70), and some are commercially available in the market. For instance, in 2020, BASF launched PeptAlde4.0, a rice-derived peptide anti-inflammatory product, and intends to launch two more products: PeptiStrong and PeptiYouth, derived from fava beans and peas, respectively, later this year (https://agfundernews.com/nuritas-raises-45-million-to-scale-its-plant-based-peptides-discovery-platform) [Accessed on April 20, 2022], Creatine PepForm® Peptides, a whey protein derived food supplement for enhancing muscle mass. Other studies have reported that peptides from food proteins such as soybean, milk, wheat germ, peanut, and sesame can be promising carriers for zinc supplements (71), replacing the salt inorganic

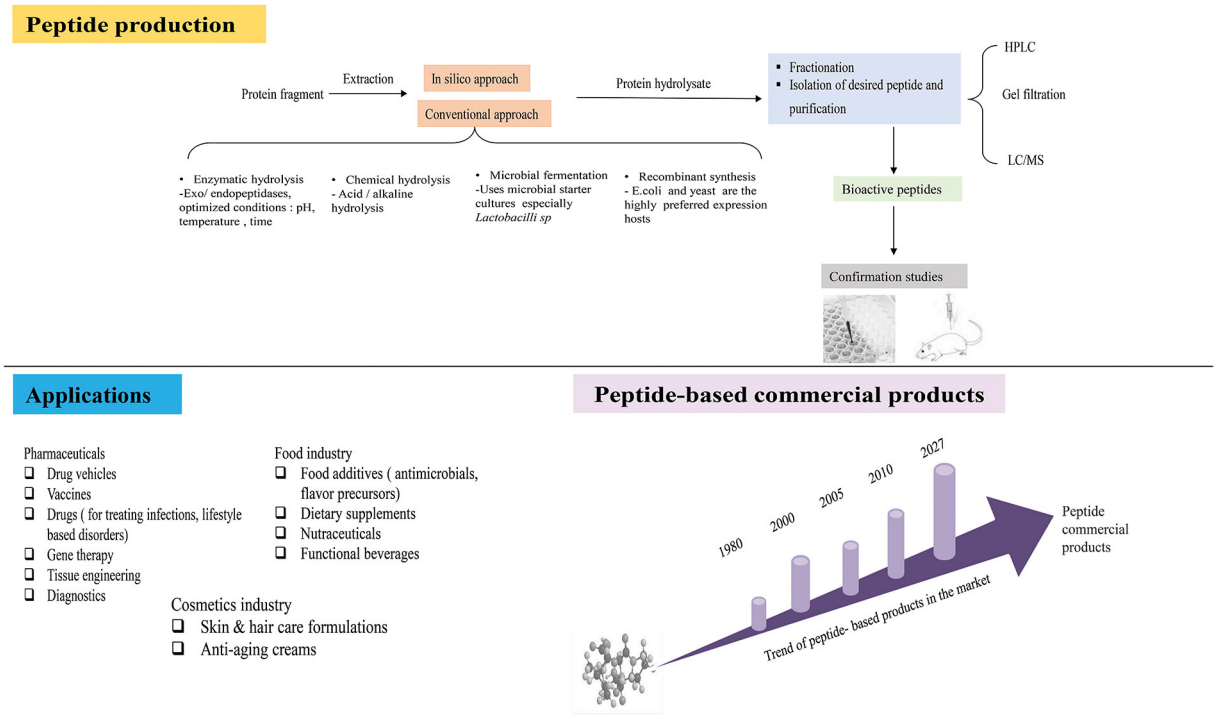

FIGURE 1
Illustration showing the experimental flow of peptide production, application of peptides and trend of commercial peptide products.

derived supplements, in particular, zinc sulfate, which causes inflammation of the gastrointestinal tract (72). Nanomaterials fabricated from food-derived peptides have been applied in food emulsions (73, 74), act as building blocks for hydrogels (75), and systems for delivery and improvement of the physiochemical properties of functional compounds (76–78).

In the therapeutic sector, these peptides are used as leads for drug design and alternatives to conventional drugs in treating several non-communicable/lifestyle disorders, such as obesity and diabetes, and cardiovascular and infectious diseases (79). The key characteristics that foster their translation into drugs are their high specificity, low toxicity, and ability to effectively interact with biological targets (challenging to treat with small molecules) (80) (see Figure 2). Most synthetic drugs in the market have been documented to have detrimental effects on human health. For instance, phentermine, liraglutide, bupropion/and naltrexone intended for anti-obesity have side effects; thus, their use is restricted (81). These effects leave room for the design and adaptation of peptide-based drugs because they are considered less harmful. After acting on target molecules, peptides usually disappear rapidly by proteolytic degradation, their byproducts amino acids with little toxicity (82).

The growing knowledge about the functions of peptides, increasing public awareness, and acceptance of health-promoting bioactive substances and the prevalence of diseases have boosted the global market for bioactive peptides, with North America, Europe, and the Asia Pacific being the central market (https://www.verifiedmarketresearch.com) [Accessed April 20, 2022]. Approved peptide drugs to treat several diseases are now common in the market. Peptide-based drugs account for a remarkable proportion of the pharmaceutical market income. According to the Verified Market Research (VMR) report, the revenue was valued at $48.62 billion by 2017 (https://www.verifiedmarketresearch.com) [Accessed April 20, 2022] and predicted to earn vast profits of 388 billion by 2024 (83).

Drawbacks of food-derived bioactive peptides

Bioactive peptides as health-enhancing components are incorporated as food ingredients, dietary supplements, and nutraceuticals (84), and lead compounds for the design of therapeutic drugs intended to promote human health by treating and reducing the risk of diseases (85, 86). Unfortunately, few peptides have been successfully translated into drugs, functional foods, nutraceuticals, and food preservatives due to factors including bitter taste, high susceptibility to degradation, and poor water solubility that hinder their intensive application and commercialization (87). Table 2 contains examples of peptides with relevant disadvantages.

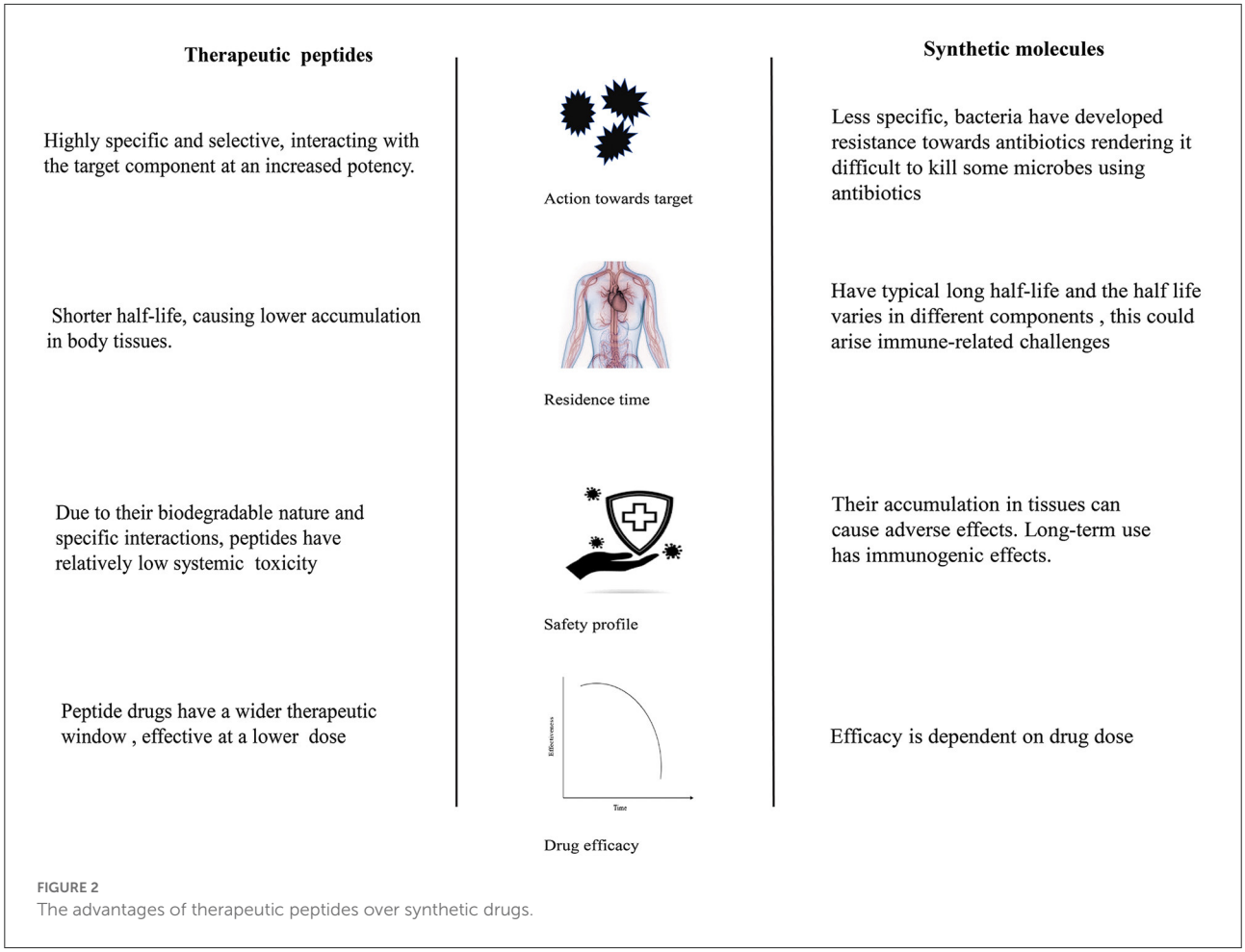

FIGURE 2
The advantages of therapeutic peptides over synthetic drugs.

TABLE 2 Examples of bioactive peptides with *in vivo* drawbacks.

Peptide	Drawback(s)	Reference
F2,5,12W	Poor *in vivo* stability. Susceptible to enzymatic degradation and rapid clearance after being treated with serum for 5 h	(88)
F2,5,12W	Cytotoxic toward mammalian cells	(89)
BMAP-28	Cytotoxic activity against the human cells human red blood cells (hRBCs) and 3T3 cells	(90)
Piscidin-1 (fish-derived AMPs)	Extreme cytotoxic hemolysis of red blood cells	(91)

Relatively lower activity

Generally, peptides have lower activity *in vivo*. Lower activity could be partly due to their inability to effectively penetrate the target cell and exhibit their action in the cellular environment. Cell permeability is an essential factor in designing therapeutic agents if the molecule is intended to target a component within the cell (92). Peptides are membrane-impermeable; thus, they cannot cross the cellular membrane easily. Impermeability limits their efficacy to the extracellular or transmembrane space. Their high molecular weight and polarity (due to multiple hydrogen bonding donors/acceptors in the peptide backbone) account for their impermeability (93, 94). For instance, some studies have demonstrated that a vast majority of cyclic peptides, both natural and synthetic, are cell impermeable, and their impermeability is intrinsic to the peptide structure (92).

Poor *in vivo* stability

Peptides are naturally unstable and highly susceptible to protein degradation. Instability toward proteases in the biological systems severely hamper the translation of different bioactive peptides such as antimicrobial peptides (AMPs), antidiabetic peptides, and other therapeutic drugs. Their smaller size favors their administration *via* the oral route, the accustomed delivery route for small molecules due to patient

appliance, and the lower production costs of oral drugs (95). Most peptides, especially those containing basic amino acids such as lysine and arginine, are highly prone to protease degradation in the digestive tract, tissues, and plasma (96). Upon oral administration, they can be readily hydrolyzed in acidic or enzymatic conditions causing cleavage of their amide bonds. Such cleavage causes only a tiny portion of the peptide to reach its target, resulting in low bioavailability (97–99).

Many peptides function as hormones, neurotransmitters, enzyme substrates or inhibitors, growth promoters (100), or other regulatory molecules that selectively bind to their target receptors when necessary. They can be removed rapidly when their request is expired (101), accounting for their rapid renal clearance (since the kidney usually filters out molecules below 60kDa) and a shorter half-life lasting for a few minutes, thus losing their activity (102). Also, upon administration into the human body, peptides may be susceptible to inactivation due to the concentration of salts and serum binding (103, 104), limiting their thorough clinical transformation into novel drugs.

Safety concerns and customer preference

Since they are isolated from food proteins, most food-derived peptides are considered safe and insignificantly toxic, but this concept is still a mystery, and available evidence of their safety is scarce. Some peptides may induce toxicity and allergenicity to a certain degree (105). Bioactive peptides with toxic nature can be produced at various stages: during protein extraction, pre-treatment, hydrolysis, or upon intake (106). There are also chances for immunological uncertainties due to the complex interactions of the peptides with the host environment (107). Intestinal wall disruption, lymphocyte toxicity, production of free radicals, cytotoxicity, and immunopathic tissue damage are the major problems linked with using peptides in the biological system (108).

To meet consumers acceptance, taste is critical in developing functional foods, including peptide-based foods (68, 109). Some peptides have a bitter taste upon oral consumption (110). The bitterness property of peptides could be produced during enzymatic hydrolysis of the protein hydrolysates (111), especially those containing amino acid residues with hydrophobic side chains (109). The bitter taste reduces the peptides' sensory quality, limiting the market opportunities despite their potential health benefits.

Alternative delivery approaches such as subcutaneous and intramuscular injections are used to administer peptide-based drugs to avoid biological barriers such as proteases. This route still has limitations, including a shorter *in vivo* half-life of the drugs, thus demanding multiple injections a day, causing discomfort and poor patient adherence (112).

Strategies employed to modify bioactive peptides

Peptide-based products legally proved and commercially available are still few despite their potential benefits. As discussed in the previous section, several challenges limit their thorough commercialization. Several strategies have been employed to overcome the obstacles. Some techniques have focused on modifying the peptide's amino acid composition (such as substituting L- with D- amino acids), terminal regions, or entrapping peptides into delivery systems resulting in novel peptide analogs with higher activity, stability, bioavailability, and reduced toxicity. Numerous bioactive peptides of different natural protein sources, including food, have been modified in this manner (113).

Peptide backbone modification

Several bioactive peptides have been modified *via* selectively adjusting the peptide backbone. Some synthetic analogs of natural peptides have also been designed and produced in this manner. For example, the novel antimicrobial peptide L10 (WFRKQLKW) developed from the amino acid substitution of the N-terminal domain of bovine lactoferrin (114). Modifying the primary amino acid sequence can improve the overall activity, stability, and selectivity and minimize the toxicity of bioactive peptides. Since only a tiny portion of crucial amino acids are responsible for its function, some modification strategies such as the substitution of the other residues with potential amino acids (for example, D- and unnatural amino acids), the extension of the peptide chain, and the introduction of essential fragments can improve the activity without hindering its primary function (115–117).

Amino acid substitution

Amino acid substitution is one of the common strategies employed to improve the activity of bioactive peptides. One of the significant advantages of this strategy is that it does not cause any remarkable change in the peptide's secondary structure. Thus, the peptide's functionality is intact. Substitution of amino acids at specific positions within the peptide sequence may increase the resistance of the peptide toward proteolytic degradation (118).

For instance, numerous antimicrobial peptides have been modified and designed *via* this strategy, as depicted in Table 3. The functionality of antimicrobial peptides is highly dependent on their cationic nature, which allows electrostatic binding to the anionic components of the target cell membrane, hydrophobicity which prolongs the association of the peptide with the membrane, and amphipathicity, which enables bilayer

TABLE 3 Peptide modification *via* amino acid substitution.

Target peptide	Substitution/Insertion	Advantage(s)	Reference
Sushi 1 peptide from horseshoe crab hemocyte	Arginine	Broader spectrum of antibacterial activity against both and gram-negative bacteria, including the methicillin-resistant *Staphylococcus aureus*,	(119)
Amyl-1-18,	Aspartic acid with arginine	Enhanced antifungal activity against *Candida albicans*	(120)
buCATHL4B	Tryptophan with non-natural amino acid Azulenyl-Alanine	Enhanced proteolytic stability and cytocompatibility with human cells.	(121)
HPA3N-T3	Arganine and Tryptophan with lysine and leucine, respectively	Significant decrease in hemolytic activity than the native peptide	(122)
HPA3NT3-A2	l-Lysine residues with d-Lysine residues	Enhanced stability and antimicrobial activity against E. coli, S. aureus in serum,	(123)
RRWWRWWRR	Tryptophan with histidine	Increased antimicrobial activity, lower cytotoxic and hemolytic activity	(124)
WRWRW	N-terminal arginine residue with a metallocene moiety	Improved antibacterial activity	(125)
F2,5,12W	Phenylalanine → Tryptophan	Enhanced antimicrobial activity against bacteria *Bacillus anthracis* and *Yersinia pestis,* increased LPS neutralizing activity, and decreased salt sensitivity.	(88)
F2,5,12W	Insertion of cysteine	Increased plasma stability	(126)
AMP Jelleine-1	Arginine and tryptophan	Higher antimicrobial activity toward the multidrug-resistant *P. aureginosa*	(127)
CAMP	Incorporation of non-natural amino acid residues	Increased hydrophobicity and enzymatic stability	(128)
Pep05.	Substitution of L-Arg & L-Lys residues with D- and unnatural amino acids (D-Lys, D-Arg)	Significant protease resistance and acute toxicity *in vitro*	(129)
Piscidin-1	Threonine residues with lysine	Reduced cytotoxicity, higher antibacterial activity than native peptide	(130)
CPF-C1	Introduction of Lys, tryptophan, and D-amino acids	Enhanced antimicrobial activity against multidrug-resistant strains	(131)
Chicken cathelicidin-2	D-amino acid substitution	Improved serum stability	(132)

penetration and disruption causing cell death (133, 134). The substitution with amino acid residues such as arginine (Arg), lysine (Lys), and tryptophan has been shown to positively influence the factors mentioned above, thus improving the activity of AMPs (134–138). In contrast, the replacement of arginine and tryptophan in the peptide chain has enhanced peptides' antimicrobial activity (see Table 3). L-amino acids, unlike D-amino acids, are easily susceptible to enzymatic degradation by proteases. Due to its spatial configuration, which is not recognized by proteases or immune receptors, Peptides with D- amino acids are characterized by increased resistance toward proteases degradation (139, 140). Previous studies demonstrated that D-substitution was a convenient technique to heighten the *in vivo* activity of antimicrobial peptides (141, 142). Substitution with bulky aromatic amino acids produces modified peptides with higher functionality and increased stability in physiological conditions, for example, salt (143). The replacement of tryptophan residues with bulky aromatic amino acids in the antimicrobial peptide FKCRRWQWRMKKLGA derived from Lactoferricin bovine (LFB) enhanced the peptide's antibacterial activity (144).

Chemical modification

Chemical modification of peptides has significantly improved their enzymatic stability and intestinal permeability. Conjugation of therapeutic peptides with potent macromolecules and metals seems ideal for delivering

TABLE 4 Chemical modification of food-derived bioactive peptide (s).

Peptide	Chemical modifier	Outcome	Reference
PMAP-37 (F34-R)	Cholesterol fragments binded to the N-terminal	Enhanced antibacterial and anti-biofilm activities, improved stability, wound healing activity *in vivo*	(148)
Clavanin A VFQFLGKIIHHVGNFVHGFSHVF-NH2),	Zn^{2+}	A remarkable increase in antimicrobial activity,	(149)
AWKR6	XTEN generating a potent XTENylated -AWKR6 conjugate	Prolonged plasma half-life by nearly 5-fold, higher GLP-1R-binding	(150)
Exenatide	mPEG	Improved hypoglycemic activity,	(151)
Bac7 (1–35)	PEG through a cleavable ester bond or through a non-hydrolyzable amide bond	Reduced renal clearance	(152)

peptides and protein-based peptides and improving the pharmacokinetic properties of peptides, including their *in vivo* stability and overall activity (145). Conjugation with macromolecules may bestow peptides with increased resistance to protease hydrolysis since macromolecular chains can shield the enzymatic sites on peptides (126). Peptide-conjugates can be formed *via* attachment of the macromolecules to peptides either through cleavable or non-cleavable linkers, resulting in releasable and stable conjugates. Releasable conjugates as the term suggests, the drug is separated and released from the carrier in its native form. In comparison to stable conjugates, releasable conjugates are more effective. Loss of drug potency is a major drawbacks associated with the use of stable conjugates. The bulky PEG moiety tends to reduce drug activity, and higher concentration of the conjugate is needed so as to maintain the drug activity (146). Conjugation with albumin-binding molecules is commonly used to increase peptides' half-life and *in vivo* stability (147). Examples of such molecules include polyethylene glycol polymers (PEG), [COSAN]- and XTEN. Table 4 shows examples of chemically modified peptides.

PEGylation

PEGylation, a process that involves the addition of a polyethylene glycol chain to a biomolecule, has been the first and most frequently applied approach to enhance peptide and proteins' pharmacokinetic (PK) properties drugs for over 25 years (153). PEG polymer is FDA-approved, non-toxic, non-immunogenic, and highly water-soluble. The polyethylene glycol (PEG) moiety is typically attached to peptide and protein drugs shielding the surface and increasing its molecular size, lowering its susceptibility toward proteolytic degradation and the clearance rate *via* renal ultrafiltration. Generally, PEGylation improves the *in vivo* efficacies of these peptides and peptide-based drugs by conveying its physio-chemical characteristics to the peptides without interfering with their biological function(s) (154).

A massive trend in the development of PEGylated drugs has been observed since the launch of ADAGEN (pegademase bovine), the first approved PEGylated protein manufactured by Enzon Pharmaceuticals in 1990 (155). PEGylated protein and peptide therapeutics are available on the market, and many more are still under development and clinical trials. Over the predicted period of 2021-2026, the market for PEG protein therapeutics is expected to rise at a compound annual growth rate (CAGR) of 9.3%. The rise in the number of chronic diseases (such as kidney diseases, cancer, and rheumatoid arthritis), awareness of detrimental effects of chemo and radiotherapy treatments (for instance, in cancer treatment), and the demand for drugs with suitable pharmacological activity attribute to the rapid increase of PEG protein therapeutics (https://www.mordorintelligence.com/industry-reports/pegylated-proteins-market) [Accessed April 22, 2022].

Several reviews have discussed PEG-peptide conjugates, their advantages over native peptides, and their application in drug delivery (99, 156, 157). The majority of the PEGylated drugs on the market have shown the benefits of improved pharmacological activity such as *in vivo* stability (e.g., Cimzia, Neulasta, PegIntron, Adynovate, Oncaspar), circulation time (e.g., Eligard, Renagel), delivery to target (e.g., Eligard), extended-release and reduced toxicity (e.g., AmBisome, Albecet). However, despite the successful application of PEG in the pharmaceutical field, PEG has been known to raise some safety concerns. Zhang and colleagues have discussed the drawbacks of PEG in-depth, highlighting that it is both immunogenic and antigenic, non-biodegradable, and growing concerns about the effects of its remains (158).

XTENylation

The conjugation of therapeutic peptides with XTEN has been reported to exhibit an extended half-life and reduced clearance from circulation compared to the native non-conjugated peptides. XTEN is a hydrophilic and biodegradable non-structural protein-polymer designed to mimic the biophysical properties of PEG (159). XTEN offers a biodegradable alternative to PEG. It is stable in serum conditions, but unlike PEG, it can be easily degraded by proteolytic enzymes after subsequent internalization into cells, reducing the risk of kidney vacuolation resulting from continuous treatments. Also, its biodegradable nature reduces its accumulation in tissues, preventing toxicity under normal circumstances (160). So far, no evidence has been documented on the adverse effects of XTEN.

Modification of the terminal regions

Functionalization of the terminal regions of the peptide chain may help shield the peptidefrom proteolysis degradation, thus increasing its stability *in vivo* (161). For instance, the modifications of the extremes through acetylation of the N-terminus and transformation of the C-terminus into a primary amide can increase peptides' overall stability and activity, given that these two regions are not involved in binding interactions (94).

Synthetic peptidomimetics

Peptidomimetics are tiny, protein-like chains that mimic traditional peptides and retain the ability to interact with biological targets and produce a similar natural effect. They offer advantages that make them excellent candidates over the physiologically active peptides in the pharmacology field. These functional mimics circumvent the pharmacokinetic hurdles of native peptides (162, 163), such as their prolonged stability in biological matrices (164). Peptidomimetics have gained much attention, and to date, numerous mimics in the market developed, some being analogs of food-derived bioactive peptides that function as mediators and are helpful in therapy. Cyclizing the linear peptide and coupling unnatural amino acids are common preparations for peptidomimetics. Significant advancement has been attained in the past concerning the development of peptidomimetics, for instance, those that mimic the bactericidal activity and mode of action of AMPs (165).

Cyclization of peptides

Studies have confirmed that cyclization increases the resistance of peptides toward enzymatic degradation since the amide bond of the peptide is hidden inside the helix, thus increasing resistance toward proteases (94, 166). Cyclic peptides, as the name suggests, these peptides take up a cyclic ring structure and are formed by linking together the two side chains of the same peptide *via* a stable bond (such as amide, disulfide, ether) to maintain and stabilize the helical structure of the chain (167). For instance, stapling is a cyclization technique that provides the peptide with an external brace that limits its flexibility and improves its affinity and selectivity to the target. This closed conformation hides the amide bond inside the helix, increasing resistance toward proteases and allowing an easier permeation into the target cellular membrane (94, 166). Among the nine overall methods for synthesizing cyclic peptides, head-to-tail fashion is the most straightforward and frequently used cyclization technique in which the linear peptides are stapled *via* the C- and N- terminals (168). The application of cyclic peptides (both naturally occurring and synthetic) as novel therapeutic paradigms in the modern pharmaceutical field has proliferated. For instance, from 2006 to 2015, nine cyclic peptide drugs have been approved and are currently available in the market (https://www.biochempeg.com/article/121.html) [Accessed April 22, 2022] with a wide range of biological functions such as enzyme inhibition, antimicrobial, anticancer, and antidiabetic. Cyclic peptides are characterized by increased cell permeability, protease stability, and pharmacological activity (higher retention time in blood, oral absorption) compared to their linear counterparts (169). The absence of amino and carboxyl ends accounts for the resistance of the cyclic peptides toward exogenous proteases. The increased cell permeability of these cyclic peptides results from exposure of the hydrophobic region to the surface while the hydrophilic regions are concealed inside the structure. Despite being suitable drug-like substances, it has been reported that cyclic peptides are not generally cell-permeable compared to their liner equals and have poor oral bioavailability (170, 171).

Extended-release technology

Excellent drug delivery systems shield the therapeutic molecule from premature degradation upon administration, enhance drug activity and reduce the occurrence of toxicity (172). Such delivery systems are the controlled release systems, which have been studied as an effective means for drug delivery compared to conventional delivery systems (capsules, tablets, ointments, granules, syrup, medicated gums). In traditional delivery, drugs are rapidly eliminated from the body; thus, a frequent dosage is required to maintain its therapeutic index, unlike in a controlled release system, whereby drugs are delivered at a specific target site and at a controlled rate while offering the intended therapeutic effect. The decrease in total dosage, such that a drug is administered weekly, monthly, or quarterly, is a significant advantage associated with the controlled release of drugs (173). Examples of delivery systems

TABLE 5 Examples of controlled delivery systems for bioactive peptides.

Delivery system	Example material	General features	Clinical application(s)	Drawback(s)	Reference
Hydrogels	Chitosan-based, alginate-based, hyaluronic based	Three-dimensional polymer, its high affinity for water absorption gives it a resemblance to living tissues, higher compatibility to biological systems than other synthetic polymers, biodegradability	Localized drug delivery: can deliver the drug through the hostile environment of the stomach and at specific sites within the gastrointestinal tract (GI) such as the colon. Also valuable for diagnostics and tissue engineering as scaffolds.	Conventional hydrogels are associated with toxicity, challenging to sterilize, limited curative use	(174, 175)
Liposomes	siRNA	Possess the ability to capture both hydrophilic & lipophilic molecules,	Reduces systemic toxicity of peptides, efficient delivery of the peptide to its target site.	Poor stability and circulation time in the blood, rapid clearance	(176)
Nanoparticles	Acrylic-based polymers, polyanions (e.g., Eudragits), polycations (e.g., chitosan)	Stable in the GI tract	Encapsulate drugs hence protecting them from low pH conditions and enzymatic degradation		(177)
Microencapsulation (Microsphere, microcapsules, microparticles)	Hyaluronate, calcium alginate (CA)-carboxymethyl cellulose, PGLA	Good compatibility	Improves stability, target delivery of drugs	Water insolubility, anaphylactic reactions, poor mechanical strength	(178)

such as microcarriers, hydrogels, liposomes, and nanoparticles have been used for the controlled release of peptides into the human body, as shown in Table 5.

Peptide self-assembly

Self-assembled peptides, both natural and synthetic, have become a popular hotspot in pharmaceutical and food processing, among other industries, due to their advantageous features such as resistance to proteolytic degradation, biocompatibility, and biodegradability (179–181). Apart from the self-assembled peptides which occur naturally in a food protein matrix (180), single peptides can be modified depending on the amino acid sequence *via* non-covalent interactions such as hydrogen bonding, aromatic stacking, and Van der Waals forces to form supramolecular nanostructures such as nanofibers, nanoparticles, hydrogels, and nanovesicles with specific properties (182, 183). This can be initiated through the chemical addition of a moiety, for instance, using protected amino groups or lipids to provide the driving force necessary to foster self-assembly (184). The activity (185), cell selectivity, and stability (186) of several antimicrobial peptides have been enhanced through self-assembly. An in-depth review of the self-assembled peptides, their types, their characteristics, and their applications has been made by several scholars (181, 187–189). Despite its remarkable features, the self-assembly of peptides is associated with some limitations, for instance, difficulty in the purification of the nanostructures, their lower stability under physiological conditions, and safety-related concerns (75). The peptide hydrogels can instigate biofilm formation, thus causing an imbalance of microorganisms in humans (190).

Other techniques

Among other factors, instability, bitter taste, and hygroscopicity limit the direct application of peptides in the development of functional foods (191). Studies have reported

that encapsulation of peptides can overcome these challenges, thus improving peptides' sensory property, bioactivity, and stability (87). The stability of antioxidant peptides from flaxseed protein was improved *via* spray-drying encapsulation of the peptide along with the use of surfactants (192). Bitter peptides were less bitter and had improved gastrointestinal stability following their encapsulation in water-in-oil high internal phase emulsions (193). The entrapment of egg white-derived peptides into chitosan- tripolyphosphate nanoparticles improved its bioavailability (194).

The chelation of bioactive peptides with metals also enhances the activity affecting the peptide's structure, charge, and mode of action. Binding divalent metals such as copper and zinc have regulated the antimicrobial activity of the peptide. Piscidin 1& 3 peptides from fish mast cells had improved antibacterial activity, membrane insertion, and increased cytotoxicity against cancer cells when chelated with copper Cu^{2+} (195, 196). The antibacterial activity was improved upon the zinc Zn2+ to ClavaninA, demonstrating the ability to cleave the bacterial chromosomal DNA (197).

Future perspective and conclusion

Generally, the advancement and development of technology have facilitated the research and utilization of bioactive peptides. The availability of peptide-based products: therapeutic drugs, functional foods, and additives in the modern market is a vivid outcome of the tremendous progress made. Despite their limitations, bioactive peptides have great potential. As discussed in the previous section, peptides have been modified using several methods to eliminate their disadvantages. The modification of bioactive peptides has been successful to a satisfactory level, in enhancing the bioactivity and physiochemical properties of peptides. However, more studies need to be conducted since the outlined strategies have limitations also.

Addressing the peptides' intrinsic drawbacks alone does not foster their translation into commercial products. Other hindrances, including manufacturing costs and regulatory challenges, are yet to be overcome since they impede the commercialization of peptides. Establishing universal criteria for approval of peptide-based products will expand the international market for these products. Time is yet another significant obstacle facing manufacturers since it takes a relatively long time to approve a peptide drug legally. The 10–12-year lag between a peptide drug candidate passing into clinical trials and potential approval (https://www.polypeptide.com/wp-content/uploads/2019/10/1401702726538c49464a6f5.pdf) [Accessed April 22, 2022] is somewhat challenging. Even after peptide drugs are approved for treatment, they can still be withdrawn/recalled from the market due to drug failure. Such circumstances become a burden on the manufacturers due to the costs incurred in producing the drugs. An example of this was the withdrawal from the market of Omontys (Peginasatide), a peptide drug intended to treat symptomatic anemia and related chronic kidney disease, due to reports of hypersensitive reaction associated with the drug (https://www.takeda.com/en-us/newsroom/news-releases/2014/affymax-and-takeda-announce-termination-of-omontys-peginesatide–product-collaboration-and-license-agreement/) [Accessed April 22, 2022].

Also, the information on the peptides' mode of action *in vivo*, their specific safety level, and their interaction(s) with the human systemis scarce; this calls for more investigation to be carried out. Addressing these challenges and discovering new bioactive peptides brightens the future for bioactive peptides, which could result in extensive utilization and huge market demand.

Author contributions

JM: investigated, conceptualized, and wrote the original manuscript. WC: conceptualized, reviewed, edited, and responsible for the supervision of the entire manuscript. ZC, KH, WL, RD, and PZ: proofread and edited. HZ and JG: reviewed the final draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparative study of the most commonly used methods for total protein determination in milk of different species and their ultrafiltration products

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Milk ultrafiltration is a widely used membrane filtration process that allows the recuperation of whey proteins in a concentrate high in total solids, which can later be transformed in multiple healthy dairy products with great prospects for the food industry. Protein content is a decisive factor for the technological performance of milk concentrates and currently, the ISO standard method for its determination is Kjeldahl, which is time-consuming and requires specific instrumentation. For this reason, the use of rapid methods to quantify protein would greatly facilitate the monitoring of the milk ultrafiltration process. In this study, the bicinchoninic acid assay (BCA), the detergent compatible Bradford assay and the Dumas method were compared to Kjeldahl protein determination to select a quick and accurate methodology suitable for milk of different species and its ultrafiltration products (retentates and permeates). The protein content obtained from Bradford assay and Dumas method in origin milk and retentate samples was consistent with Kjeldahl values. In contrast, BCA protein levels were significantly different when compared to Kjeldahl and no method was proved to be suitable for protein determination in permeate samples. The use of sodium dodecyl sulfate was also examined to improve protein measurements without success. In comparison with the official method, Bradford assay quantitatively provided the best results, and it would be recommended for a quick, economic and easy determination of total protein content in milk and retentate samples.

KEYWORDS

ultrafiltered milk, whey protein, Kjeldahl, Dumas, BCA, Bradford, SDS-PAGE

Introduction

Milk is defined as the normal mammal secretion obtained by milking one or more times, without any type of addition or extraction of substances (1). It is a complex food containing a good balance between major nutrients (proteins, fat, and carbohydrates) and also rich in minerals and vitamins. Milk furnishes a broad range of nutritionally relevant compounds such as caseins, whey proteins, bioactive fatty acids, polar lipids and other minor constituents, which have functional properties both physiologically and technologically (2). Depending on the animal species, milk shows differences on its composition, from milk fat globule size to protein concentration. Each composition factor contributes to milk technological performance and the final characteristics of dairy products (3).

Whey proteins have aroused great interest not only for their physiological properties, but specially for being a by-product of the cheese industry produced in massive quantities (4). There are multiple approaches for the valorization and recovery of whey components, being membrane processes the most widely used in the dairy industry (5). The ultrafiltration (UF) systems concentrates caseins, whey proteins, total solids and colloidal salts in proportion to the amount of permeate removed (6, 7). Then, ultrafiltered milk can be processed and transformed into a wide variety of high-protein dairy products. The ISO standard method for quantifying the protein content of milk and milk products is Kjeldahl digestion, which consists of the determination of total nitrogen by oxidation of the sample with sulfuric acid and subsequent titration of ammonium sulphate with NaOH (8). However, this method is complex, time-consuming, and requires specific instrumentation and contaminant agents. Moreover, Kjeldahl typically uses large sample volumes and it is a destructive procedure. The Dumas method also determines the total nitrogen of the sample by combustion, but this methodology is faster and simpler than Kjeldahl, not requiring toxic chemicals. Dumas and Kjeldahl determinations may give rise to different results depending on the non-protein nitrogen content of the analyzed sample, since these methods are not capable of distinguishing non-protein nitrogen from protein nitrogen (9). Therefore, both methods are susceptible to interferences by organic and inorganic compounds containing nitrogen (9, 10).

In recent years, the alternative of using colorimetric assays for protein determination has spread widely because they are fast, easy to use and require a small amount of sample. There are two main colorimetric methods that differ in their basis: the detergent compatible Bradford assay that relies on the binding of the dye Coomassie Blue G250 to protein (11) and the bicinchoninic acid (BCA) assay, based on the Biuret reaction and cupper ion reduction in alkaline conditions (12). Although BCA is extensively used in food analysis to determine total protein content, non-accurate results have been observed in complex matrices (13, 14). In this line, it has been reported that reducing sugars (15) and phospholipids (16) can interfere with the Biuret reaction. Regarding dairy samples, it has also been observed that thermal treatments may affect the BCA protein measurements, which has been related to reducing substances originated during the heating processes of milk (17). These reducing substances would also be present in milk UF products due to the heat-treatment that occurs in both milk pasteurization and dairy products manufacture. On the other hand, the composition of the food matrix could also affect protein determination in the Bradford assay. Gazzola et al. (18) observed that the protein content in wine samples was underestimated due to the presence of ethanol and polyphenols. Other substances commonly used in electrophoresis, such as sodium dodecyl sulfate (SDS) or Triton-X100, have also been reported to interfere with the Bradford assay (19). In contrast, several modifications to the Bradford assay have been attempted to achieve correct protein determinations for specific samples like collagen, gels or plant proteins (20, 21).

The analytical methodology used to determine the protein content in milk and final dairy products must be precise and accurate, since total protein concentration can modify the technological performance of milk (22). In the present research, a comparative study between 4 different total protein determination methods (i.e., Kjeldahl, Dumas, BCA and Bradford) was carried out in milk from cows, goats and sheep and their UF products. Samples covered a wide range of protein concentrations and results were compared to the official method in order to select an accurate, quick and quantitative method that could be used for protein monitoring in the dairy industry. In addition, to our knowledge, this is the first comparative study that evaluates the addition of SDS as a mean to troubleshoot inaccuracy in protein determination by rapid colorimetric methods in dairy matrices. Milk, retentates and permeates were also analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) to control the UF process and determine which proteins were retained or lost within the permeate.

Materials and methods

Materials

Whole pasteurized milk from cow, goat and sheep were purchased in local supermarkets (Madrid, ES). Pierce^{TM} BCA Protein Assay Kit and Pierce^{TM} Detergent Compatible Bradford Assay Kit were purchased from ThermoFisher Scientific (Rockford, IL). SDS and BluSafe dye were acquired from Sigma-Aldrich (St. Louis, MO) and Nzytech (Lisbon, PT), respectively. Criterion^{TM} XT precast gels with 12% Bis-Tris, sample buffer, Precision Prestained Protein Dual Xtra Standard (250 kDa to 2kDa) and Bis-Tris SDS running buffer were obtained from Bio-Rad Laboratories (Hercules, CA).

Skimming of milk and ultrafiltration

Milk skimming was carried out by centrifugation at 9000 rpm for 30 min at 25°C. Whole and skimmed milks were ultrafiltered using a Centramate^{TM} 500 S Tangencial Flow Filtration System. The UF was performed in duplicate using a minimum protein binding polyethersulphone membrane with a pore size of 30 kDa and a mean transmembrane pressure (TMP) of 0.25 bar. To obtain similar concentration factors, the UF process was always carried out with 1 L of milk and it was stopped when 570 mL of permeate were measured in a graduated cylinder. Original milks and their UF products (retentates and permeates) were collected in falcon tubes and kept at −20°C until analysis. Before protein determination, samples were thawed in a thermoblock to 40°C and then well mixed using a vortex.

TABLE 1 Advantages and disadvantages of the most commonly used methods for total protein determination.

Method	LOQ[a] (μg/mL)	Basis	Advantages	Disadvantages
Kjeldahl	500	Total nitrogen determination by sulfuric acid digestion and ammonium titration	· Robust technique suitable for different sample matrices · Official ISO method for protein quantification in milk and dairy products	· Chemicals needed · Specific instrumentation required · Long analysis time · Indirect measurement of total protein that requires a matrix-dependent correction factor
Dumas	100	Total nitrogen determination by high temperature combustion and inorganic nitrogen detection	· No chemicals needed · High correlation to Kjeldahl determinations · Fast analysis · High level of automation	· Specific instrumentation required · Indirect measurement of total protein that requires a matrix-dependent correction factor
Bradford	100	Protein determination by Coomassie dye-binding and absorbance measurement	· Simple, easy, fast and cost-effective analysis · No specific equipment is required · Small amount of sample · Direct measurement of protein content ·Not affected by non-protein nitrogen	· Protein determination is influenced by the presence of common protein surfactants · Quantification sensitive to amino acid composition
BCA	20	Protein determination by biuret reaction and absorbance measurement	· Simple, easy, fast and cost-effective analysis · No specific equipment is required · Small amount of sample · Direct measurement of protein content · High sensitivity · Wide detection range	· Protein determination is influenced by reducing agents and chelators · Colorimetric reaction does not have an end-point

[a]LOQ, Limit of quantification.

Electrophoresis

SDS-PAGE assay was performed as described in Villas-Boas et al. (23). Briefly, samples were diluted with PBS to achieve a protein concentration of 4 mg/mL. Then, sample dilutions were well-mixed with sample buffer to a 1:4 ratio (sample:buffer). After heating samples at 95°C for 5 min, samples were analyzed on a precast Criterion XT 12% Bis-Tris gel, through a separation carried out at 120 V. Bands were finally stained directly with BlueSafe dye for 1 h.

Colorimetric assays for protein determination

The BCA assay was performed in 96-well microplates following the user guide. Briefly, 25 μl of each sample and 200 μl of BCA working reagent were added to each well. The microplate was shaken for 15 seconds and then incubated for 30 min at 37°C using a BioTek® Cytation 5 Cell Imaging Multi-Mode Reader. Absorbance was measured at 562 nm and protein concentration in samples was determined by interpolation on the bovine serum albumin (BSA) standard curve.

The Bradford assay was performed in 96-well microplates following the user guide. Briefly, 10 μl of each sample and 300 μl of Coomassie reagent were added to each well. After 10 min incubation at room temperature, absorbance was measured at 595 nm using the same BioTek® Cytation 5 Cell Imaging Multi-Mode Reader and protein concentration in samples was determined by interpolation on the BSA standard curve.

Both colorimetric assays were performed in triplicate on three different days ($n = 9$). Sample dilutions and standard curves were made with MilliQ water. When SDS was used, both samples and standard curves were diluted with 2% SDS in MilliQ water since both Bradford and BCA kits are detergent resistant. Table 1 summarizes the main advantages and disadvantages of using colorimetric methods for protein determination.

Dumas and Kjeldahl methods for protein determination

The Dumas method was performed in a LECO Corporation TruMac (St. Joseph, MI) in duplicate. One mL of sample

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TABLE 2 Total protein content (g/100 mL) of cow, goat and sheep milk, before and after ultrafiltration (UF), determined by Kjeldahl.

			Average ± SD[a]	PCF[b]
COW	Skimmed	Starting milk	3.26 ± 0.03	2.38
		Retentate	7.75 ± 0.66	
	Whole	Starting milk	3.36 ± 0.02	2.36
		Retentate	7.92 ± 0.03	
GOAT	Skimmed	Starting milk	3.04 ± 0.03	2.44
		Retentate	7.41 ± 0.03	
	Whole	Starting milk	3.56 ± 0.01	2.25
		Retentate	8.01 ± 0.16	
SHEEP	Skimmed	Starting milk	5.27 ± 0.02	1.84
		Retentate	9.68 ± 0.06	
	Whole	Starting milk	5.28 ± 0.08	2.10
		Retentate	11.09 ± 0.03	

[a] Each value is obtained from two UF performed on different days and duplicate Kjeldahl analysis.
[b] PCF, Protein Concentration Factor.

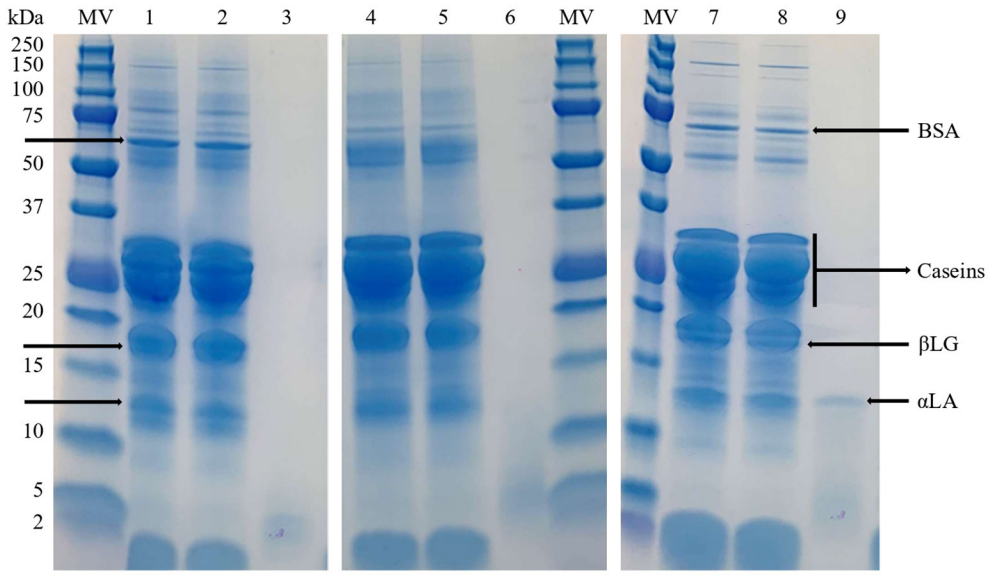

FIGURE 1
SDS-PAGE analysis of cow milk (1), cow milk retentate (2), cow milk permeate (3), goat milk (4), goat milk retentate (5), goat milk permeate (6), sheep milk (7), sheep milk retentate (8) and sheep milk permeate (9). MW, Molecular weight standard; αLA, α-lactalbumin; βLG, β-lactoglobulin; BSA, bovine serum albumin.

was combusted at 1100°C under oxygen atmosphere and the gas was drawn by a He flux through filters and a cooler to remove water and particles. Then, a 10 mL aliquot was passed through a catalytic filter where NO_x are reduced to inorganic nitrogen and CO_2 is removed by adsorption. Finally, the inorganic nitrogen reached the heat conducting cell and the electronic signal (measured as area) was compared to pure helium flux.

Kjeldahl method was carried out in duplicate, following the Standard ISO (8). Briefly, samples were homogenized with sulfuric acid and digested at 420°C in a digestion system that neutralizes fumes. After digestion, ammonium was distilled and titrated, providing the total nitrogen content of the sample. Total protein was calculated using the correction factor of milk (6.38). Table 1 summarizes the main advantages and disadvantages of using Dumas and Kjeldahl methods for protein determination.

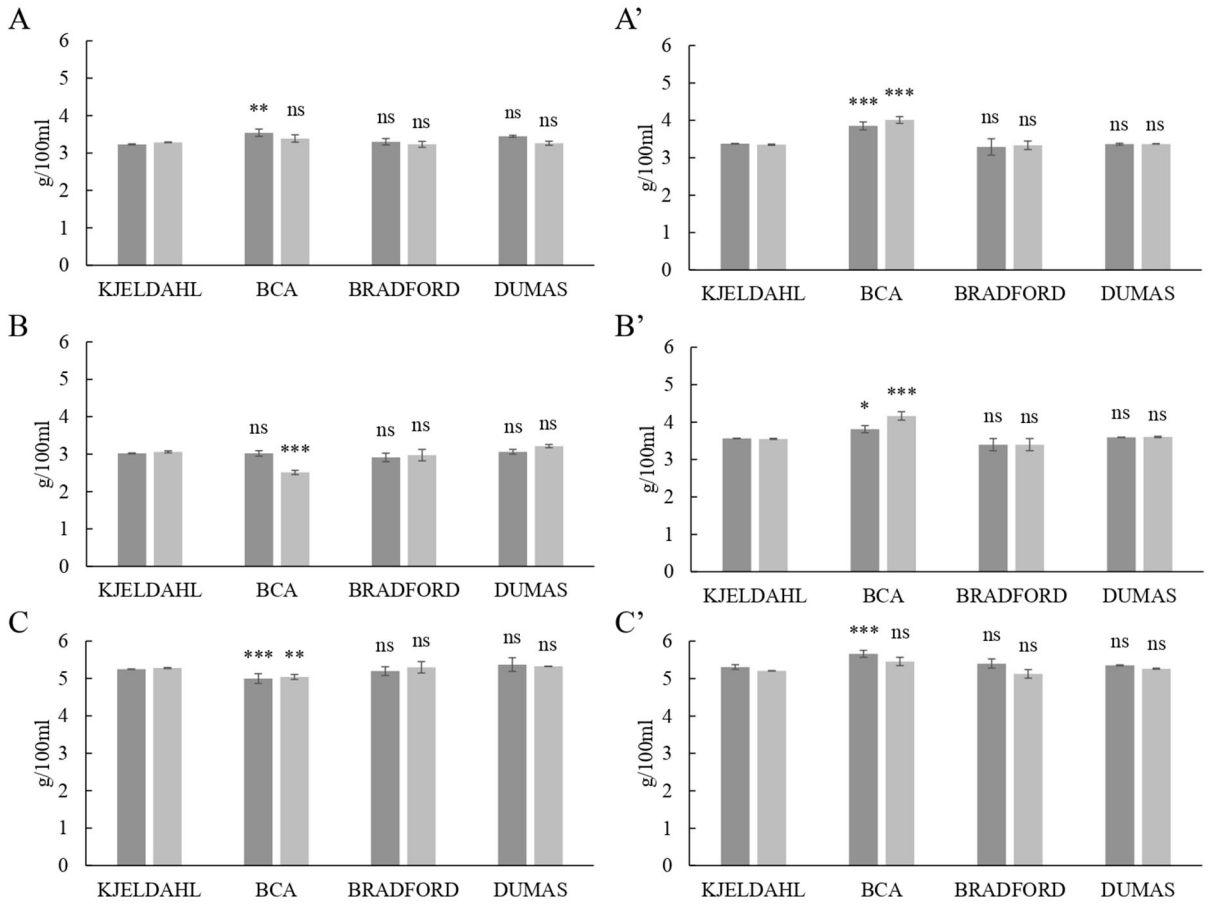

FIGURE 2
Comparison of protein levels in milk (g / 100 mL) obtained by Kjeldahl official reference method with the contents determined by the bicinchoninic acid assay (BCA), the detergent compatible Bradford assay and the Dumas method. **(A)** Cow's skimmed milk, **(A')** cow's whole milk, **(B)** goat's skimmed milk, **(B')** goat's whole milk, **(C)** sheep's skimmed milk, and **(C')** sheep's whole milk. Different shades of gray indicate different ultrafiltration processes. *P*-value: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; ns = $P > 0.05$.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (San Diego, CA). A two-way Anova test was used to compare the protein content obtained by Kjeldahl with the other methods used. Multiple pairwise comparisons were carried out when SDS was used for troubleshooting tests (Section Use of SDS for troubleshooting). Differences were considered as statistically significant at $P < 0.05$.

Results and discussion

Ultrafiltration process and protein recovery

Table 2 shows the total protein content of milks and their corresponding retentates as determined by the official Kjeldahl method. During the UF process of milk, a theoretical volumetric concentration factor (VCF) is usually calculated, which indicates the expected concentration of total solids and other molecules larger than the pore size of the UF membrane. To evaluate the UF performance, an experimental protein concentration factor (PCF) was also calculated based on protein concentration (Table 2). Small milk components (e.g., lactose) are able to pass through the membrane pore and do not have a concentration value near the theoretical VCF. The pore size used in the present research (30 KDa) was selected to retain whey proteins in the retentate fraction. Thus, a correct performance of the UF process would be proven if calculated PCF is similar to the VCF value. All PCF (Table 2) were close to the theoretical VCF value (2.33). On the other hand, the protein content of whole and skimmed milks did not differ substantially. Comparing between species, sheep's milk and sheep's milk retentate showed the highest protein contents (Table 2).

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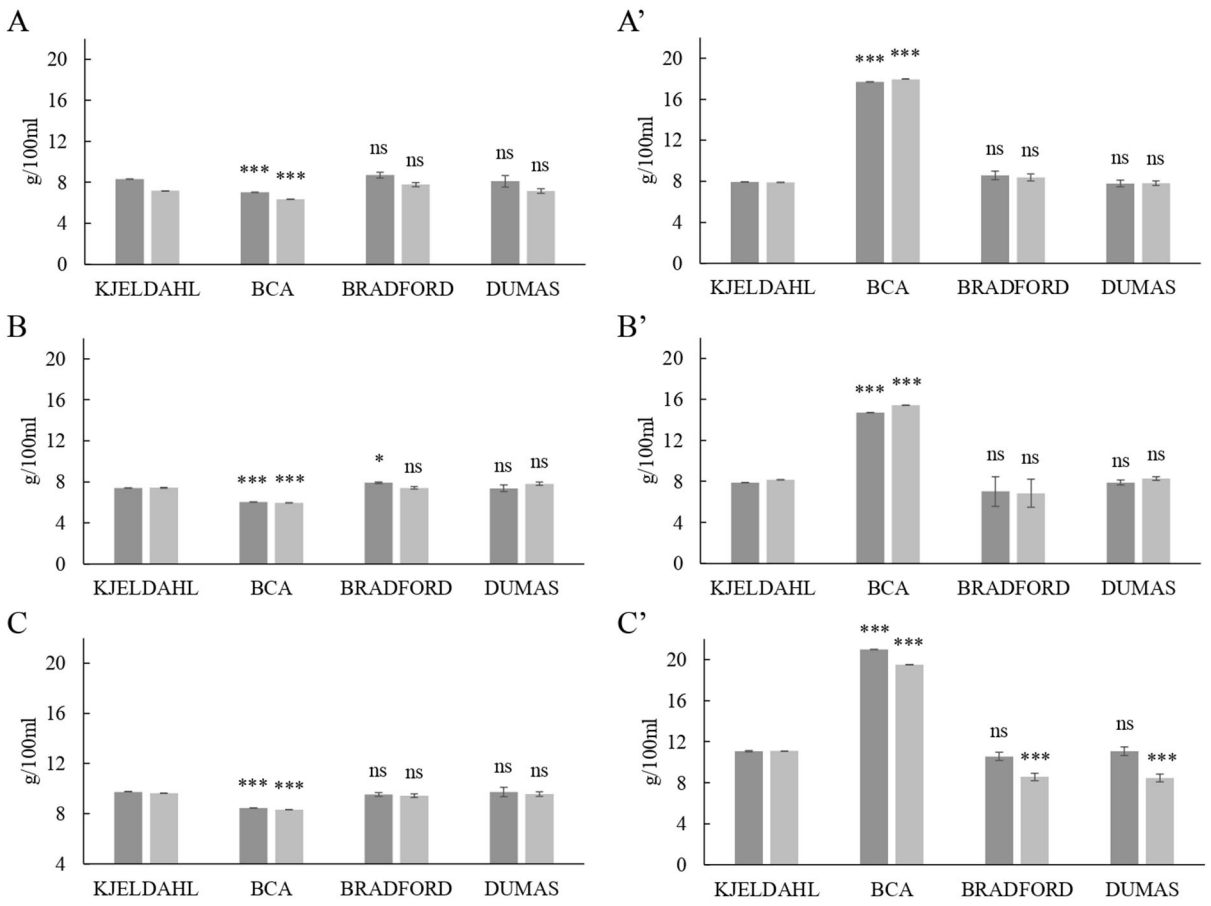

FIGURE 3
Comparison of protein levels in milk retentate (g / 100 mL) obtained by Kjeldahl official reference method with the contents determined by the bicinchoninic acid assay (BCA), the detergent compatible Bradford assay and the Dumas method. **(A)** Cow's skimmed retentate, **(A')** cow's whole retentate, **(B)** goat's skimmed retentate, **(B')** goat's whole retentate, **(C)** sheep's skimmed retentate, and **(C')** sheep's whole retentate. Different shades of gray indicate different ultrafiltration processes. *P*–value: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$; ns = $P > 0.05$.

The SDS-PAGE assay confirmed that whey proteins were successfully retained in milk retentates by UF with a 30 kDa membrane (Figure 1). The protein profiles of the retentates were identical to their respective original milks for all ruminant species. It is important to note that these UF retentates, which will be transformed into dairy products by coagulation, preserve high-value whey proteins such as bovine serum albumin, β-lactoglobulin and α-lactalbumin. Regarding milk permeates, their protein profiles differed greatly from cheese whey derived from traditional milk coagulation (24). Electrophoresis of sheep's milk permeates, from both whole and skimmed milks, revealed a band between 10–15 kDa that corresponds to α-lactalbumin. It would indicate that α-lactalbumin is able to pass through the membrane pores, but it was not detected in cow's or goat's permeates due to the higher relative concentration of α-lactalbumin in the original sheep's milk samples. To completely retain whey proteins, the membrane pore size must be at least smaller than the size of α-lactalbumin, considering the normal fluctuation of protein size. The bands below 5 kDa (Figure 1) would be related to peptides from protein degradation naturally occurring in milk (25, 26).

Comparison of total protein determination methods in milk

Total protein contents in skimmed and whole milks from the three ruminant species determined by Dumas and colorimetric assays were compared with the Kjeldahl method (Figure 2). Even though duplicates of UF were performed with the same type of milk, each liter was processed at different days so original milk samples from UF1 were not mixed with the ones from UF2 and they were analyzed separately. The Bradford and Dumas determinations were not significantly different from the Kjeldahl value, in agreement with previous research. For

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TABLE 3 Comparison of total protein content in permeate samples (g/100 mL) determined by Kjeldahl official reference method with the bicinchoninic acid assay (BCA), the detergent compatible Bradford assay and the Dumas method.

		Kjeldahl	BCA	Bradford	Dumas
COW	Skimmed	0.14 ± 0.02	0.15 ± 0.02	<LOD	0.21 ± 0.01***
	Whole	0.16 ± 0.04	0.15 ± 0.02	<LOD	0.21 ± 0.16
GOAT	Skimmed	0.23 ± 0.01	0.16 ± 0.02***	<LOD	0.27 ± 0.01***
	Whole	0.18 ± 0.02	0.16 ± 0.02	<LOD	0.25 ± 0.05**
SHEEP	Skimmed	0.25 ± 0.07	0.22 ± 0.02	<LOD	0.28 ± 0.07
	Whole	0.27 ± 0.02	0.24 ± 0.02*	<LOD	0.37 ± 0.02***

P–value: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$ (Indicates significant differences with Kjeldahl reference value).
< LOD, Values lower than the limit of detection.

instance, Wiles et al. (27) reported that there is no evidence for a generic difference between Dumas and Kjeldal methods for multiple dairy products. In addition, Kamizake et al. (28) established that the Bradford assay could be used for the direct determination of total protein content in reconstituted whole and skimmed milks. Our results would also indicate that the Bradford colorimetric method successfully determined the total protein content in pasteurized milk samples with different levels of fat and total solids. In contrast, BCA protein determinations were significantly different from Kjeldahl ($P < 0.05$) in every pasteurized whole milk sample, regardless of the species (Figure 2).

BCA assays are widely used for the determination of total protein content in food and biological samples. However, there is some controversy regarding the reliability of BCA results. Keller and Neville (29) observed that the BCA assay was the most consistent and reliable method for determination of total protein in milk compared to Kjeldahl and, more recently, Giuffrida et al. (30) reported that the total protein content determined by BCA was not significantly different from the Kjeldahl value in human milks. In contrast, Lonnerdal et al. (31) showed that the BCA assay consistently overestimated Kjeldahl protein values by 30%. Bergqvist et al. (32) determined that lactose would contribute to an overestimation of up to 15% of the total protein content when using the BCA assay in human milk. The results presented in Figure 2 indicate that the BCA methodology is not reliable for the quantitative determination of total protein in milk samples.

Comparison of total protein determination methods in milk UF products

The comparison of the total protein contents in milk retentates after UF is displayed in Figure 3. Similar to the results obtained in the original milk samples, Bradford and Dumas quantifications did not substantially differ from those of Kjeldahl, although a slight decrease was observed in sheep's whole retentate. However, when comparing BCA assay to Kjeldahl, the protein contents for skimmed and whole milk retentates were significantly different. The BCA underestimated protein content in skimmed milk retentates from all species, but it was overestimated on average by 2-fold in whole milk retentates samples (Figure 3). For instance, cow's whole retentate determined by BCA showed a total protein content of 17.81 ± 0.40 g/100 mL, which was significantly different to Kjeldahl determination 7.92 ± 0.03 g/100 mL. These results confirm that the dairy matrix affects BCA determination and that milk fat would interfere in total protein quantification. It is important to note that milk fat is dispersed in the form of triglyceride globules covered by a lipid trilayer membrane rich in phospholipids (33). Those phospholipids would interfere with the BCA Biuret reaction (16) and thus the assay would overestimate protein content. This would explain the high protein contents obtained in the retentates from whole milk, which are underestimated in skimmed milk retentates (Figure 3). These differences in protein determination, when compared to Kjeldahl, indicate that the BCA assay is not suitable for concentrated samples such as milk retentates.

For milk permeates, the protein values were very different depending on the method used. The protein contents obtained by the Kjeldahl reference method were in the range of 0.138–0.273 g per 100 mL of permeate (Table 3). Despite this, the Bradford assay failed to determine protein levels as the values obtained were below the detection limit (100 µg/mL, LOD). Dumas analysis was not suitable to determine the protein content in milk permeates, since the results obtained were overestimated and significantly different from Kjeldahl (Table 3). BCA protein concentrations were more similar to Kjeldahl than the other methods tested, which would be related to the absence of fat in milk permeates.

Kjeldahl reference method does not directly determine protein and it uses a matrix-dependent correction factor to quantify protein. For this reason, and since there is no specific factor for milk permeates, the protein content determined by Kjeldahl could be overestimated. It has been shown that the Kjeldahl method is not capable of detecting melamine

TABLE 4 Total protein content (g/100 mL) determined by colorimetric assays, with and without sodium dodecyl sulfate (SDS), and Kjeldahl in milk samples and ultrafiltration retentates from cow, goat and sheep milks.

		Kjeldahl		BCA		BCA + 2% SDS		Bradford		Bradford + 2% SDS	
		Milk	Retentate	Milk	Retentate	Milk	Retentate	Milk	Retentate	Milk	Retentate
COW	Skimmed	3.26 ± 0.03^{a}	7.75 ± 0.66^{A}	2.90 ± 0.10^{b}	6.69 ± 0.42^{B}	2.63 ± 0.11^{c}	5.50 ± 0.39^{C}	3.26 ± 0.09^{a}	8.09 ± 0.53^{A}	4.16 ± 0.12^{d}	9.77 ± 0.42^{D}
	Whole	3.36 ± 0.02^{a}	7.92 ± 0.03^{A}	3.10 ± 0.08^{b}	17.81 ± 0.40^{B}	2.74 ± 0.14^{c}	6.32 ± 0.37^{C}	3.23 ± 0.20^{ab}	8.38 ± 0.29^{A}	4.64 ± 0.23^{d}	10.94 ± 0.14^{D}
GOAT	Skimmed	3.04 ± 0.03^{a}	7.41 ± 0.03^{A}	2.71 ± 0.10^{b}	6.00 ± 0.18^{B}	2.17 ± 0.09^{c}	6.13 ± 0.68^{B}	2.95 ± 0.14^{a}	7.65 ± 0.41^{A}	3.86 ± 0.07^{d}	9.38 ± 0.46^{D}
	Whole	3.56 ± 0.01^{a}	8.01 ± 0.16^{A}	3.29 ± 0.17^{b}	15.05 ± 1.41^{B}	2.81 ± 0.10^{c}	4.80 ± 0.16^{C}	3.38 ± 0.15^{ab}	6.95 ± 0.23^{A}	4.32 ± 0.18^{d}	10.25 ± 1.24^{D}
SHEEP	Skimmed	5.26 ± 0.02^{a}	9.68 ± 0.06^{A}	4.73 ± 0.11^{b}	8.38 ± 0.17^{B}	3.54 ± 0.15^{c}	6.46 ± 0.35^{C}	5.26 ± 0.11^{a}	9.49 ± 0.25^{A}	6.18 ± 0.13^{d}	11.51 ± 0.10^{D}
	Whole	5.28 ± 0.08^{a}	11.09 ± 0.03^{A}	4.73 ± 0.07^{b}	20.25 ± 0.86^{B}	3.83 ± 0.08^{c}	6.46 ± 0.35^{C}	5.22 ± 0.17^{a}	9.44 ± 1.18^{D}	6.71 ± 0.39^{d}	12.08 ± 0.60^{A}

^{a,b,c,d} Means within a row with different lowercase superscripts indicate significant differences between milk samples.
^{A,B,C,D} Means within a row with different uppercase superscripts indicate significant differences between retentate samples.

adulteration in milk (10), thus amino acids and other non-protein nitrogen naturally present in milk permeates could be incorrectly measured as protein nitrogen when applying the correction factor. It is well known that the Bradford assay would not interfere with non-protein nitrogen (17, 34). The inability to determine the protein levels in milk permeates by Bradford (i.e., values < LOD, Table 3), together with the absence of bands in the electrophoresis gel (Figure 1), indicates that protein contents in permeates were at trace levels and none of the methods tested provided reliable results for this UF product.

Use of SDS for troubleshooting

The determination of total protein content in food samples using the BCA assay is a widespread methodology in food science and technology research (35–37). The inaccurate results obtained for milk and UF products were attempted to be solved with SDS, as previously described by Morton and Evans (38). These authors overcame BCA assay overestimations by the addition of 2% SDS and we followed a similar approach in both colorimetric assays (Table 4). Results were not satisfactory as the addition of 2 % SDS not only did not improve BCA protein quantification, but it also worsened Bradford determination when compared to Kjeldahl reference method (Table 4).

BCA protein determinations in milks, with or without the addition of SDS, were underestimated when compared to Kjeldahl in milk samples, with an average reduction of 11% and 33% in standard BCA and BCA+2% SDS, respectively. In whole retentate samples, the addition of SDS appeared to troubleshoot fat interference but it was not able to provide accurate results, as BCA+2% SDS underestimated protein content by 24% on average when compared to Kjeldahl values (Table 4). Skimmed retentate samples showed a similar behavior to the original milks, with an average underestimation of 15% for standard BCA and 26% for BCA+2% SDS. In contrast, the addition of 2% SDS to the Bradford assay produced a mean overestimation of 25% for milk and retentate samples.

Conclusions

After comparative testing of the most frequently used methods to determine the total protein content, when used for milk and their retentates after UF, the Bradford assay would be the most suitable since it allows the obtention of fast and accurate results compared to the Kjeldahl reference method. Moreover, the protocol is simple to perform, requiring neither expensive equipment not experienced analysts for data acquisition. The Dumas method also provided accurate results, but it is a destructive procedure that requires a larger sample size and specific instrumentation. Regarding the BCA assay, it was

significantly affected by the composition of the matrix and its use for protein quantification in dairy samples is not advised. Further research is encouraged to develop a quick routine method capable of determining trace level protein content in milk UF permeates.

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 author.

Author contributions

JF and PG-C contributed to the conception, design of the study, oversaw the research and the statistical analysis, reviewed, and edited the manuscript. DH wrote the first draft of the manuscript, carried out the laboratory work, and performed the data analysis. All authors have read and approved the final submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Improvements in extraction yield by solid phase lipid extraction from liquid infant formula and human milk, and the fatty acid distribution in milk TAG analyzed by joint JOCS/AOCS official method Ch 3a-19

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The Röse-Gottlieb method is one of the most widely used methods for extracting lipids from milk samples. However, we found that lipid recovery from liquid infant formula and human breast milk was lower than expected. Better lipid recovery from these liquid matrices was obtained by solid phase extraction using silica gel; ~10% more could be recovered from liquid infant formula and ruminant milk, and 25% more from human breast milk. However, the method is not recommended for lipid extraction from dried whole milk powders.

KEYWORDS

Röse-Gottlieb extraction, human breast milk, solid phase extraction (SPE), ruminant milk fat, TAG, positional distribution of fatty acids

Introduction

The nutritional value of infant formulas is becoming increasingly important as the equalization of social and household roles of mothers and fathers progresses. The decrease in the number of babies per couple might also lead parents to prefer infant formula of better quality and safety. Manufacturers of infant formula are constantly striving to match the nutritional value of human milk, including lipids. Human milk lipid is unique in that the structure of the triacylglycerols is largely symmetrical, with oleic acid in the *sn*-1 and -3 positions, and palmitic acid in the *sn*-2 position (1, 2) (OPO, 18:1-16:0-18:1). We recently compared the lipid composition and structure of several infant formulas in the Japanese market to human milk after extracting lipids from the infant formula samples by the Röse-Gottlieb method (3). The Röse-Gottlieb method (4, 5) is one of the most common methods for extracting neutral

lipids from dried milk products. In our hands, 92–96% of the lipids could be extracted from most infant formula samples. However, <80% of the lipid could be extracted from a liquid formula (3). Improvement in yield was not obtained by increasing the time and temperature of sample heating with ammonia water prior to solvent extraction, by increasing the volume of extraction solvent per volume infant formula, or by using chloroform or dichloromethane as an extraction solvent. The chloroform/methanol extraction of milk samples increased gravimetrical yield than Röse-Gottlieb method. However, the increased matter was found not to be ether soluble, and was indicated to be carbohydrates mainly by IR spectrum. We postulated chloroform/methanol extracted some glycolipids from milk. The target of this study is neutral lipids, TAG, in milk. Thus, chloroform/MeOH was not desirable as the extraction solvent this time. This study presents that solid phase extraction using silica gel resulted in an improvement in neutral lipid recovery from both liquid infant formula and ruminant milk of ∼10% over the Röse-Gottlieb method. Solid phase extraction was even more effective in extracting lipids from human milk, increasing the lipid recovery more than 25%.

Materials and methods

Materials

Liquid infant formula, goat and cow milk were obtained in the local market. Dried cow milk powder was from Yotsuba Milk Product Co. (Sapporo, Japan). Dried goat milk powder was purchased from Shinko Kikaku Co. (Yokohama, Japan, produced in the Netherlands) and dried camel milk powder was from Aadvik Foods and Products Private Co. (Nokha, India). Human breast milk samples, each from a single donor, were purchased from Lee Biosolutions, Inc. (Maryland Heights, MO, USA). Their lipid contents were determined using a Miris Human Milk Analyzer (Uppsala, Sweden). Milk samples were divided into 2 groups and mixed to prepare ∼25 ml each of high- and low-fat human milk. Immobilized *Candida antarctica* lipase B preparation, lipase CL "Amano" IM, was provided by Amano Enzyme (Gifu, Japan). Inert-Sep-SI silica gel columns (0.69 g) were from GL Science (Tokyo, Japan). Ethanol was dried over molecular sieves 3A. Silica gel 60, ethanol, methanol, hexane, diethyl ether, potassium hydroxide, and other reagents were purchased from FUJIFILM Wako Pure Chemical Corp. (Tokyo, Japan). Reagents were of analytical grade.

Extraction of lipids by Röse-Gottlieb method

Lipids in milk and liquid infant formula were extracted by the Röse-Gottlieb method as described in AOAC Official Method 932.02 (4, 5). In brief, 10 ml milk was mixed with 1.5 ml of 28% ammonia water, and the mixture was incubated at 65 ± 5°C for 15 min with occasional mixing. To the solution, 10 ml of ethanol and several drops of 1% phenolphthalein/ethanolic solution were added, and this mixture was vigorously shaken with 25 ml of diethyl ether. Petroleum ether (25 ml) was added, and shaking was repeated. The ether layer was recovered after phase separation. An additional 4 ml of ethanol was added to the water layer, which was then extracted with 15 ml diethyl ether followed by extracting with 15 ml petroleum ether. This extraction step was repeated once more. All the ether layers were combined and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure until the weight of the extracts reached to give the constant values. Extractions were conducted twice, and mean values are presented.

Extraction of lipids by silica gel

Silica gel 60 and liquid infant formula or milk (10 ml) were added to a 300 ml flask and mixed. The suspension was evaporated using a rotary evaporator at 120 hPa and 50°C initially, gradually decreasing pressure to 15 hPa and increasing water-bath temperature to 80°C. Evaporation was occasionally stopped, and lumps of milk-adsorbed silica gel was crushed with a spoon into smaller pieces to assist in removing water. Drying continued until the silica gel/milk texture was powdery again. The silica gel/milk powder was loaded into a glass chromatography column with a diameter of 30 mm, and lipid was eluted with 100∼200 ml of diethyl ether: petroleum ether (1:1, v/v). For human breast milk, 15 g silica gel was mixed with 5 ml milk, dried, and eluted with 100 ml solvent. The solvent was removed from the eluents under reduced pressure to obtain the recovered lipids.

Lipid content in goat milk

The goat milk was freeze-dried (FDU-1110, EYELA, Tokyo, Japan), and crushed to obtain a dried goat milk powder. A fixed amount of the internal standard, undecanoic acid methyl ester, was added to the dried milk powder and methyl esters were prepared by 0.5 M potassium hydroxide methanolic solution. Lipid amounts were calculated based on the peak areas of the internal standard and of other fatty acid methyl esters.

Fatty acid distribution analysis by joint JOCS/AOCS official method Ch 3a-19

Fatty acid distribution of lipids were analyzed according to Joint JOCS/AOCS official method Ch 3a-19 (6). Extracted lipids (0.25 g), dehydrated ethanol (2.5 g), and Lipase CL "Amano" IM (0.10 g) were added to a glass vial and shaken at 30°C for 3 h. The reaction mixtures were filtered through absorbent cotton to

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remove immobilized lipase, transferred to a new glass tube or evaporation flask, and stored at −20°C until further use.

Ethanol in the 2 ml reaction mixtures was removed with a rotary evaporator. The resulting oil (0.1 ml) was immediately loaded onto an Inert-Sep-SI SPE column, pre-equilibrated with 10 ml hexane:diethyl ether, 8:2 (v/v). The column was eluted with 10 ml of the same solvent mixture to obtain fraction 1, consisting mainly of fatty acid ethyl esters (FAEEs); then rinsed with 20 ml of the same solvent to flush out diacylglycerols (DAGs). Finally, the column was eluted with 10 ml diethyl ether to obtain fraction 2, which consisted mainly of *sn*-2 MAG. This fraction was brought to dryness under reduced pressure and methylated using 0.5 M potassium hydroxide methanolic solution.

The methylated samples were analyzed by GC (Agilent Technologies, Santa Clara, USA) equipped with a DB-23 column (0.25 mm, 0.25 μm, 30.0 m, Agilent Technologies). The column temperature was controlled at 60°C for 3 min, raised to 180°C at the rate of 8.0°C/min, and raised to 240°C at the rate of 3.0°C/min. Injector and detector (FID) temperatures were set at 245 and 250°C, respectively. All analyses were carried out twice and the mean values are presented.

The FID responses of the GC were corrected to the molar ratios by the theoretical FID response factors according to Joint JOCS/AOCS official method Ch 3a-19 (6, 7). Briefly, the area percent of each FA alkyl ester was divided by the respective active carbon numbers (carbon number of each FA alkyl esters−1) and by the atomic weight of carbon (12.01) to obtain the molar ratios of FA.

The molar ratio of each fatty acid (MX) is calculated as follows:

$$Mx = A/\left[ACN \times AW_C\right]$$

where

Mx is the molar ratio of each fatty acid methyl ester;

A is the area percentage of fatty acid methyl ester;

ACN is the Active Carbon Number (Carbon number of each fatty acid methyl ester minus 1);

AW_C is the atomic weight of carbon (12.01).

The percentage by mole of each fatty acid (Y) is calculated as follows:

$$Y = M_x/M_T \times 100$$

where

Y is the molar percentage of each fatty acid;

M_X is the molar ratio of each fatty acid methyl ester

M_T is the sum of M_X.

Results

Solid phase extraction of lipid from ruminant milk and liquid infant formula

Initial experiments establishing the conditions required for lipid extraction were carried out with liquid goat milk. Goat milk (10 ml; lipid content, 2.8%) was added to 5∼15 g silica gel, mixed, and water was removed under reduced pressure using a rotary evaporator. Lipids were eluted from the dried silica gel by a mixture of diethyl ether and petroleum ether (1:1, v/v). The recovered lipid was weighed after the removal of the solvent. The recovery was calculated by comparing the amounts extracted with the theoretical amounts. The recovery of lipids from goat milk by solid phase extraction reached *ca*. 0.26 g/10 ml milk, which was an increase in the yield of ∼10% compared to the Röse-Gottlieb method (*ca*. 0.23 g/10 ml milk, Table 1).

TABLE 1 Effect of the amount of silica gel on lipid recovery.

	Lipid content[a] (%)	Silica gel (g/ml milk)	Lipid yield (g/10 ml milk)
Goat milk	2.9[b]	RG[c]	0.225 ± 0.013
		0.5	0.253 ± 0.018
		1	0.266 ± 0.018
		1.5	0.254 ± 0.019
Cow milk	4.3	RG[c]	0.381 ± 0.005
		0.5	0.359 ± 0.001
		1	0.419 ± 0.015
		1.5	0.423 ± 0.021
		2	0.401 ± 0.034
Liquid	3.8	RG[c]	0.277 ± 0.008
Infant formula		1	0.287 ± 0.023
		1.5	0.295 ± 0.014
		2	0.312 ± 0.009
Cream	47	2	4.41 ± 0.424
(Cow milk)		3	4.63 ± 0.311
		4	4.37 ± 0.417

[a]The lipid content displayed on the product label. [b]The lipid content was determined as described in Materials and Method Section Lipid content in goat milk. [c]Röse-Gottlieb extraction method.

The recovery from cow milk (lipid content, 4.3%) was also improved to 0.42 g/10 ml milk by using 1∼2 g silica gel/ml milk from 0.38 g/10 ml milk by the Röse-Gottlieb method (Table 1), with the improved yield of *ca.* 10% (Supplementary Figure 1). Then, the lipid in the liquid infant formula (lipid content, 3.8%) was extracted by SPE. The recovery reached 0.31 g/10 ml milk when 2 g silica gel was used per 1 ml milk, whereas it was 0.28 g/10 ml milk by the Röse-Gottlieb method (Table 1) The extracted lipid was predominantly TAG when analyzed by TLC and TLC-FID (data not shown). The fatty acid compositions of the liquid infant formula extracts obtained by both methods were similar (Supplementary Table 1). When solid phase extraction was applied to a cream of cow milk with lipid content of 47%, 4.4∼4.6 g/10 ml milk was achieved with 2∼4 g silica gel/ml cream (Table 1). It was unfortunate that the appropriate amount of silica gel to a volume of milk could not exactly be fixed due to the experimental error. However, we estimate that silica gel in a range of 1∼2 g/ml milk would be good.

Solid phase extraction of lipid from reconstituted dried ruminant milk

Commercially dried goat milk powder (1 g, lipid content 15%) was dissolved in 8.5 ml 40°C water, 10 g silica gel was added, and drying and elution with 200 ml diethyl ether:petroleum ether (1:1, v/v) was carried out. However, no lipid was obtained. From 1 g of commercially dried whole cow milk powder (lipid content, 30%), only 0.21 ± 0.002 g lipid was recovered where 0.3 g was expected. From commercially dried camel milk (lipid content 33%) adsorbed on 30 g silica gel, only 0.22 ± 0.02 g oil was obtained where 0.33 g was expected. Thus, the lipid recoveries were 0∼66% from the dried ruminant milk powders. Thus, optimization of the solid phase extraction will be required for dried milk powders.

TABLE 2 Röse-Gottlieb and solid phase extraction of lipids from human breast milk.

Extraction method	Recovery (%)	
	High fat milk	Low fat milk
Solid phase	85.1 ± 5.7	89.0 ± 7.9
Röse-Gottlieb	60.3 ± 2.7	61.5 ± 6.4

The lipid contents of the high fat milk was 4.54 g/100 ml, and that of the low fat milk was 1.89 g/100 ml.

Solid phase extraction of lipid from human breast milk

When the Röse-Gottlieb extraction method was applied to human breast milk, ∼60% lipid recovery was obtained, as indicated by the Miris human milk analyzer (Table 2). When solid phase extraction using 3 g silica gel/ml was applied to human milk samples, the recovery from high fat milk samples (lipid content, 4.5%), improved from 62% obtained by the Röse-Gottlieb method to 89% by SPE (Table 2). From low fat milk sample (lipid content, 1.89%), solid phase extraction achieved 85% recovery. Thus, lipid recovery from human breast milk was increased more than 25% using solid phase extraction.

The fatty acid compositions of lipids extracted by the two methods from both high- and low-fat human breast milk were similar (Table 3). The major FAs were 16:0, 18:1, and 18:2.

TABLE 3 FA composition of lipid (TAG) extracted by SPE or Röse-Gottlieb method from human milk.

Fatty acid	Composition (mol%), high fat milk		Composition (mol%), low fat milk	
	SPE	Röse-Gottlieb	SPE	Röse-Gottlieb
6:0	0.82 ± 0.32	0.95 ± 0.18	4.10 ± 0.64	5.94 ± 0.48
8:0	0.93 ± 0.01	1.21 ± 0.08	1.06 ± 0.00	0.44 ± 0.01
10:0	1.08 ± 0.02	1.19 ± 0.05	1.18 ± 0.01	1.49 ± 0.05
10:1	0.73 ± 0.01	0.90 ± 0.05	0.78 ± 0.02	0.34 ± 0.02
12:0	5.78 ± 0.06	5.96 ± 0.03	5.45 ± 0.06	5.91 ± 0.10
12:1t	0.49 ± 0.08	0.56 ± 0.09	0.56 ± 0.00	0.26 ± 0.01
12:1	0.49 ± 0.02	0.60 ± 0.01	0.56 ± 0.01	0.13 ± 0.19
14:0	6.68 ± 0.03	6.70 ± 0.02	7.23 ± 1.62	6.38 ± 0.05
15:0	0.27 ± 0.00	0.26 ± 0.01	0.13 ± 0.18	0.31 ± 0.01
16:0	26.55 ± 0.01	26.19 ± 0.10	25.77 ± 0.18	24.80 ± 0.07
16:1	2.23 ± 0.02	2.26 ± 0.09	1.88 ± 0.29	2.01 ± 0.07
17:0	0.23 ± 0.01	0.24 ± 0.00	0.30 ± 0.04	0.30 ± 0.00
17:1	0.11 ± 0.02	0.11 ± 0.00	0.13 ± 0.02	0.12 ± 0.01
18:0	6.72 ± 0.05	6.64 ± 0.00	6.49 ± 0.07	6.37 ± 0.03
18:1	31.14 ± 0.35	30.99 ± 0.04	29.40 ± 0.23	29.70 ± 0.06
18:2 n-6	13.02 ± 0.05	12.89 ± 0.02	13.03 ± 0.09	13.32 ± 0.06
18:3 n-3	0.82 ± 0.04	0.77 ± 0.02	0.82 ± 0.02	0.89 ± 0.00
CLA	0.34 ± 0.04	0.28 ± 0.02	0.16 ± 0.22	0.20 ± 0.02
20:0	0.28 ± 0.02	0.28 ± 0.03	0.14 ± 0.19	0.17 ± 0.01
20:1	0.41 ± 0.22	0.26 ± 0.03	0.14 ± 0.20	0.29 ± 0.01
others	0.88 ± 0.29	0.76 ± 0.02	0.71 ± 0.44	0.62 ± 0.15
Sum	100	100	100	100

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TABLE 4 FA composition at *sn*-2 and FA distribution in high fat milk analyzed by Joint JOCS/AOCS Official Method Ch 3a-19.

Fatty acid	SPE extraction		Röse-Gottlieb extraction	
	Composition (mol%)	Distribution	Composition (mol%)	Distribution
	sn-2[a]	1,3[b] : 2[c]	*sn*-2[a]	1,3[b] : 2[c]
6:0	2.48 ± 0.11	−1 : 101	2.87 ± 2.23	−1 : 101
8:0	0.25 ± 0.33	91 : 9	–	100 : 0
10:0	0.61 ± 0.01	81 : 19	0.56 ± 0.06	84 : 16
10:1	–	100 : 0	–	100 : 0
12:0	7.03 ± 0.08	59 : 41	6.92 ± 0.20	61 : 39
12:1t	0.24 ± 0.05	83 : 17	0.41 ± 0.12	76 : 24
12:1	0.14 ± 0.18	91 : 9	0.16 ± 0.22	91 : 9
14:0	11.94 ± 0.17	40 : 60	12.19 ± 0.48	39 : 61
15:0	0.57 ± 0.02	31 : 69	0.57 ± 0.02	28 : 72
16:0	55.65 ± 0.72	30 : 70	55.97 ± 0.73	29 : 71
16:1	2.00 ± 0.04	70 : 30	2.06 ± 0.05	70 : 30
17:0	0.22 ± 0.00	67 : 33	0.24 ± 0.04	67 : 33
17:1	0.12 ± 0.00	63 : 37	0.13 ± 0.02	61 : 39
18:0	1.26 ± 0.01	94 : 6	1.07 ± 0.19	95 : 5
18:1	8.59 ± 0.13	91 : 9	8.31 ± 0.69	91 : 9
18:2 n-6	6.37 ± 0.05	84 : 16	6.14 ± 0.17	84 : 16
18:3 n-3	0.44 ± 0.01	82 : 18	0.59 ± 0.25	75 : 25
CLA	0.14 ± 0.01	86 : 14	0.14 ± 0.00	83 : 17
20:0	0.15 ± 0.01	82 : 18	0.15 ± 0.00	82 : 18
20:1	0.33 ± 0.17	73 : 27	0.18 ± 0.00	77 : 23
others	1.45 ± 0.48	45 : 55	1.34 ± 0.68	41 : 59
Sum	100	67 : 33	100	67 : 33

[a]The value is expressed as β. α = [TAG in mol% (Table 3)] x 3 - β.
[b]100 x α / ($\alpha + \beta$).
[c]100 x β / ($\alpha + \beta$).

Fatty acid distribution analysis of human breast milk by joint JOCS/AOCS official method Ch 3a-19

As sufficient amounts of lipids were extracted from high-fat human breast milk, its fatty acid distribution was analyzed by Joint JOCS/AOCS Official Method Ch 3a-19 (Table 4). The FA compositions of human milk lipids at the *sn*-2 position extracted by both methods were similar. The molar distribution of each FA at the *sn*-1(3) and *sn*-2 positions is also shown in Table 3. Of the total 16:0 in TAG, 70% was located at *sn*-2, and 30% was at *sn*-1(3). As for 18:1, 90% was located at *sn*-1(3) and 10% at *sn*-2. These observations agreed well with previous reports (1, 2). Here, it was confirmed that SPE increased the lipid recovery compared to the conventional Röse-Gottlieb method without affecting total FA composition of the extracts (Table 3) and the FA distribution as determined by Joint JOCS/AOCS Official Method Ch 3a-19 (Table 4).

Discussion

Liquid infant formula is a drink-ready product for babies, freeing parents and caretakers from preparing hot water, measuring and dissolving the proper amount of powdered infant formula, and cooling the prepared formula to drinkable temperature, while the baby is crying for milk. The product is especially beneficial under the circumstances of natural disaster, where the supply of drinking water and electricity are limited. Liquid infant formula is highly homogenized to prevent phase separation during storage and re-warming and provide long shelf-life. This stable homogeneous state might reduce lipid recovery when extracted by the conventional Röse-Gottlieb method (3). We postulated that silica gel may help disrupt the emulsion of the formula and improve the recovery of the lipid. We postulated this based on the observation that during liquid-liquid extraction in the Röse-Gottlieb method, especially at the second and third extraction step, emulsions often form in the organic phase even in the presence of ethanol.

In the Röse-Gottlieb method, milk is treated with ammonium water for 15 min at 65°C prior to the liquid-liquid extraction step to disrupt the stable assembly of milk protein and lipid. This step was not needed in the SPE method, as the recovery was not changed with or without treatment. However, the step of dehydrating silica gel after the adsorption of milk could not be omitted. We postulate that the presence of water might inhibit the adsorption of milk lipid to silica gel, and/or interfere with contact between elution solvent and milk-absorbed silica gel.

The increase in the recovery by the SPE method compared to the conventional Röse-Gottlieb method was *ca.* 10% from liquid infant formula and ruminant milk and was 25% from human breast milk. In cases where recovery by the Röse-Gottlieb method is lower than expected, the SPE method would be worth trying.

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 author.

Author contributions

YW conceived and designed the study, carried out the research, and wrote the first draft of the manuscript. MH, AF, and LK carried out the research. HT and AM provided valuable resources in support of this research. All authors contributed to the article and approved the final draft of the manuscript.

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Conflict of interest

Authors MH and HT are employed by Ezaki Glico Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.970837/full#supplementary-material

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Uridine affects amino acid metabolism in sow-piglets model and increases viability of pTr2 cells

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Background: As an important nucleoside precursor in salvage synthesis pathway of uridine monophosphate, uridine (UR) is the most abundant nucleotide in sow milk. This study aimed to investigate the effects of maternal UR supplementation during second trimester of gestation on reproductive performance and amino acid metabolism of Sows.

Results: Results showed that compared to CON group, the average number of stillborn piglets per litter was significantly reduced ($P < 0.05$) with higher average piglet weight at birth in UR group ($P = 0.083$). Besides, dietary UR supplementation significantly increased TP in sow serum, BUN content in cord serum, and TP and ALB in newborn piglet serum ($P < 0.05$); but decreased AST level in sow serum and BUN level in piglet serum ($P < 0.05$). Importantly, free amino acids profile in sow serum newborn piglet serum and colostrum was changed by maternal UR supplementation during day 60 of pregnancy, as well as the expression of amino acids transporter ($P < 0.05$). In addition, from 100 to 2,000 μM UR can increased the viability of pTr2 cells. The UR exhibited higher distribution of G1/M phase of cell cycle at 400 μM compared with 0 μM, and reduced S-phases of cell cycle compared with 0 and 100μM ($P < 0.05$).

Conclusion: Supplementation of uridine during day 60 of pregnancy can improve reproductive performance, regulate amino acid metabolism of sows and their offspring, and increase the viability of pTr2 cells.

KEYWORDS

uridine, reproductive performance, placental transport, amino acid, sows

Introduction

The gastrointestinal tract and visceral organ and skeletal muscle protein synthesis of piglet are influenced by sow milk (1). Interestingly, in milk from various mammals, soluble nucleotides contribute to as much as 20% of its non-protein fraction (2). Exogenous nucleotides would increase nucleic acid and protein synthesis, promoting cell growth and proliferation under pathological conditions (3).

Attractively, the most abundant nucleotide is uridine monophosphate (UMP), which accounts for 86–98% of total 5′ nucleotides and has the highest content in sow' colostrum (4). Therefore, UMP may be the most needful nucleotide not only for piglets but also for sows due to excessive secretion. UMP can be synthesized *in vivo*, but the synthesis of endogenous nucleotides from amino acids requires a great deal of energy (5). UR is an important nucleoside precursor in salvage synthesis pathway of UMP and most normal tissues rely on it for the recovery synthesis of pyrimidine nucleotides (6). Tissues could not synthesize uridine, therefore plasma uridine is used to maintain basic cellular functions in tissues (7). It was shown in our previous study that dietary UR bettered to improve the intestinal barrier and nucleotide transport in weaned piglets more than UMP (8).

A restricted feeding program is currently adopted by the swine industry to prevent excess weight gain during gestation, which can ease farrowing difficulties in gilts and sows, as well as appetite reduction of sows during lactation (9). However, sows need sufficient nutrients to support optimal fetal growth during gestation. Thus, accurate nutrition supply is very important for sows. In addition, the placenta regulates and supplies nutrient composition from mother to fetus and controls the source of hormonal signals that affect maternal and fetal metabolism (10). Consequently, the capacity of the placenta to transport nutrients is crucial to fetal growth. The specific transporter proteins in the plasma membrane of the syncytiotrophoblast are vital to the capacity of the placenta to transport nutrients (1). Our previous study showed that cytokine secretion and intestinal mucosal barrier function in suckling piglets could be regulated by maternal dietary supplementation with UR, leading to reduced incidence of diarrhea (11).

Therefore, the objectives of the present study were to explore the effects of maternal UR supplementation on reproductive performance of sows, serum biochemical parameters, serum amino acids profile of sow and piglet, placental amino acids transporter, and the growth of porcine trophectoderm cell.

Materials and methods

Ethics statement

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (2013020).

Animals and experimental treatments

60 multiparous crossbred sows (Landrace × Yorkshire) were selected according to parity (parity 4–6), expected delivery and body shape, and divided into two groups which fed a basal diet (CON) or a basal diet with UR supplementation (120 g/t) from 60th day of pregnancy to delivery. All sows were fed twice daily at 06:30 and 15:00 h and had free access to water during the experiment period. Each sow was fed 2.5–3.0 kg/d from days 60 to 107 of pregnancy and approximately 1.8 kg/d on day 5 before delivery. Farrowing was not induced and was constantly supervised. Sows were identified by ear tags and the reproductive performance of sow was recorded at delivery. Newborn piglets were individually weighed, and IUGR piglet weigh approximately 618–869 g, defined as weighing approximately 65% of the birth weight of the largest littermate in each litter (12).

Sample collection

After delivery, eight sows were randomly selected to collect blood sample in each group by the way of ear border vein in 5 ml vacuum tubes with one of them containing heparin lithium. The blood samples of eight neonatal piglets were collected (0800 h) before eating colostrum from jugular vein (13), and centrifuged for 15 min at 3,000 × g,

4°C, to obtain serum and plasma, and then stored at −20°C until analysis.

One allantochorion tissue sample (n = 8 sows/group) in the similar place of placenta (great vessels were avoided) of piglets with BW about 1.5 kg were obtained immediately after farrowing. Part of placenta was stored in liquid nitrogen for RT-qPCR analyses (14).

A total of 16 sows (Yorkshire × Landrace, n = 8 sows/group) were randomly selected to collect colostrum (30–40 mL/sow) from the 4–6th mammary glands of each sow within 12 h postpartum by massaging breasts (15). Colostrum was aliquoted and frozen at −70°C for 5 min, then stored at −20°C until it was assayed.

Biochemical analyses

Automated Biochemistry Analyzer (Synchron CX Pro, Beckman Coulter, Fullerton, CA, USA) was used for analyze the concentrations of serum triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL), and low-density lipoprotein (LDL) according to the commercial kits and manufacturer's instructions which was purchased from Beijing Chemlin Biotech Co., Ltd. (Beijing, China).

Cell culture and treatment

The porcine trophectoderm cell line-2 (pTr2), which has been previously characterized and used for functional studies of porcine trophectoderm cell line, was originally isolated from the elongated porcine blastocysts, and collected on day 12 of pregnancy in this study (16). The cells (passages 30–35) were grown in 75 cm 2 flasks containing medium with 15 ml DMEM/F-12 with 5% FBS, 1% P/S, and 0.05% insulin, which was changed every 2 days. At confluence, the cells were collected using 0.125% trypsin solution (1.25 ml of 2.5% trypsin in 23.75 ml of 0.02% EDTA). After counting the number of cells, they were diluted to 4 × 104 cells/ml in DMEM/F-12 containing 5% FBS, 1% P/S, and 0.05% insulin.

Cell counting kit-8

The pTr2 cells were subcultured in 96-well plates (30% confluence) in growth medium until the monolayer confluence reached 40% and then switched to custom medium free of serum and insulin. After starvation for 24 h, cells were added uridine (0, 50, 100, 200, 400, 600, 1,000, 1,500, and 2,000 μM) for 48 h to each well (n = 6 wells/treatment). Cells were treated with 10% CCK-8 (Dojindo; Kumamoto, Japan), diluted in normal culture medium at 37°C until visual color conversion occurred. OD (absorbance) values for each well were converted to relative cell number using standard curves.

Cell cycle assay

The pTr2 cells were subcultured in 6-well plates (30% confluence) in medium until the monolayer confluence reached 40% and then switched to customized medium free of serum and insulin. After starvation for 24 h, cells were added uridine (0, 50, 100, 200, and 400 μM) for 48 h to each well (n = 3 wells/treatment). The cultured pTr2 cells were trypsinized and fixed in 75% cold alcohol overnight. The fixed cells were stained by propidium iodide (PI), and analyzed by flow cytometer (FACSCallbur, Becton-Dickinson). ModiFit software was used to analyze the proportion of cells in G0/G1, S, or G2/M phase.

Ribonucleic acid extraction, reverse transcription and real-time PCR analysis

Total ribonucleic acid (RNA) was extracted from liver tissue using Trizol reagent (Beyotime Biotechnology, Shanghai, China). The total RNA was reverse transcribed into cDNA using reverse transcription kit from Takara Biomedical Technology, Japan. All primers were designed using Primer-BLAST on the National Center for Biotechnology Information (NCBI) website (**Table 1**). Using SYBR Green I Dye (Thermo fisher scientific, New York USA) according to the manufacturer's instructions, Quantitative real-time RT-PCR (qRT-PCR) was performed using the lightcycler 480II real-time PCR system (Roche, Basel, Switzerland). The PCR cycling conditions were as follows: 95°C for 5 min, 98°C for 2min, followed by 40 cycles of 5 s at 98°C, 5 s at 60°C, 10 s at 95°C, and a final step of 1 s at 65°C. Gene expression analysis was performed by relative PCR amplification analysis ($2^{-\Delta\Delta Ct}$).

Statistical analysis

Data are expressed as the mean ± standard deviation of at least three independent experiments. The statistical significance between the two groups was assessed by the student's t-test. In the cell experiment, Statistical significance was assessed by the one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparisons. Differences were considered statistically significant at $P < 0.05$. The IBM SPSS Statistics for Windows software,

TABLE 1 Primer used for real-time PCR.

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
GLUT4	GGCCATCGTCATTGGCATTC	GTCAGGCGCTTCAGACTCTT
LAT1	TTTGTTATGCGGAACTGG	AAAGGTGATGGCAATGAC
LAT2	TCTCCATCCCACTGGTCACA	CGCTTCACATGGATCATGGC
SNAT1	AAGAACCTGGGCTATCTCGG	TGTTGCGTTAGGACTCGTTG
SNAT2	TACTTGGTTCTGCTGGTGTCC	GTTGTGGGCTGTGTAAAGGTG
ENT3	GGTTGGACTACGCCAGGTACT	GAGGGACTCGATGTTGGTGG
SANT4	GCTGTGGCAATCCTGTCACT	CCATCCAAATGCTTTTTCACCCA
VEGFA	GCCTTGCTGCTCTACCTCCA	TGGCGATGTTGAACTCCTCAGT
VEGFR2	GAGTGGCTCTGAGGAACGAG	ACACAACTCCATGCTGGTCA
EAAT1	GATGGGACCGCCCTCTAT	CGTGGCTGTGATGCTGATG
EAAT2	GGCTGCTGGACAGGATGA	TAAATGGACTGGGTCTTGGT
EAAT3	ATAGAAGTTGAAGACTGGGAAAT	GTGTTGCTGAACTGGAGGAG
PAT1	TGTGGACTTCTTCCTGATTGTC	CATTGTTGTGGCAGTTATTGGT
CAT-1	TGCCCATACTTCCCGTCC	GGTCCAGGTTACCGTCAG
PEPT1	CATCGCCATACCCTTCTG	TTCCCATCCATCGTGACATT
GAPDH	GTCTGGAGAAACCTGCCAAA	CCCTGTTGCTGTAGCCAAAT

Version 22.0 (IBM Corp., Armonk, NY, USA), was used for statistical analyses.

Results

Effect of uridine on reproductive performance of sows

The reproduction performance of the sows was summarized in Table 2. The average number of born, live born, mummification, healthy, and IUGR piglets per litter was not affected by maternal UR supplement. Interestingly, the average number of stillborn piglets per litter was significantly reduced in UR group compared with CON group ($P < 0.05$). In addition, the average piglet weight at birth was higher in UR group than CON group ($P = 0.083$).

Effects of uridine on serum biochemical parameters in sow serum, cord serum and piglet serum

The serum biochemical parameters in sow serum, cord serum and piglet serum were summarized in Table 3. Dietary UR supplementation significantly increased serum TP in sow serum ($P < 0.05$), but decreased AST level ($P < 0.05$). The BUN content in cord serum was significantly higher by maternal UR supplementation ($P < 0.05$). Meanwhile, the serum TP and ALB was significant increased, but the BUN level was decreased in piglet serum by UR administration during pregnancy ($P < 0.05$).

Effects of uridine on free amino acids profile in sow and piglet serum and colostrum

Sows that were fed the UR-supplemented diet had lower serum contents of Ser and greater serum contents of Cys ($P < 0.05$, Table 4). Meanwhile, in piglets serum, the level of urea was significant decreased, but the Ile and Met level was increased by maternal UR administration during pregnancy ($P < 0.05$, Table 5). However, dietary UR supplementation significantly decreased Asp, Ser, Val, Leu, βAla, and His content in colostrum of sows ($P < 0.05$, Table 6).

TABLE 2 Effect of UR on reproductive performance of sows.

Items	CON	UR	SEM	*P*-value
Total born piglets per litter, *n*	12.93	12.56	0.305	0.558
Live born piglets per litter, *n*	11.30	11.89	0.306	0.348
Stillborn piglets per litter, *n*	1.41[a]	0.50[b]	0.149	0.002
Mummification piglets per litter, *n*	0.26	0.22	0.101	0.860
Healthy piglets per litter, *n*	10.63	11.39	0.312	0.238
IUGR piglets per litter, *n*	0.78	0.56	0.152	0.480
Average weight of piglets at birth, kg	1.54	1.68	0.039	0.083

The a,b means columns with different letters are significantly different ($P < 0.05$), and columns with the same letters or no superscript are considered when $P > 0.05$. $n = 8$ replicates. The same below.

TABLE 3 Effect of UR on serum biochemical parameters in sow serum, cord serum and piglet serum.

Items	Sow serum				Cord serum				Piglet serum			
	CON group	UR group	SEM	*P*-value	CON group	UR group	SEM	*P*-value	CON group	UR group	SEM	*P*-value
TP (g/L)	71.91^{b}	78.75^{a}	1.70	0.039	22.65	23.86	1.63	0.725	24.00^{b}	27.34^{a}	0.87	0.050
ALB (g/L)	47.08	47.33	0.98	0.901	5.67	5.49	0.46	0.852	5.63^{b}	6.87^{a}	0.26	0.012
ALT (U/L)	34.67	35.4	1.58	0.824	6.51	6.18	0.65	0.807	15.51	18.67	1.24	0.216
AST (U/L)	45.89^{a}	34.38^{b}	2.81	0.036	23.14	23.57	2.17	0.926	47.29	45.38	2.63	0.731
ALP (U/L)	72.78	56.67	4.57	0.077	1826.63	1993.89	249.53	0.750	1794.38	1727.75	87.3	0.717
BUN mmol/L	4.29	3.81	0.24	0.335	5.79^{b}	7.77^{a}	0.39	0.007	4.24^{a}	3.50^{b}	0.16	0.018
GLU (mmol/L)	6.26	6.00	0.20	0.520	0.50	0.55	0.11	0.831	3.66	4.29	0.20	0.119

Effects of uridine on amino acids transporter in the placenta of sows

The expression of amino acids transporters in the placenta of sows was shown in Figure 1. Supplementing the maternal diet with additional UR significantly increased the mRNA expression of Glut4 and EAAT3 transporters in sow placentas ($P < 0.05$). In addition, a significant enhanced in VEGFR2 was also detected in sow placentas ($P < 0.05$).

Effects of uridine on cell viability in the pTr2 cells

Cell viability was assessed by CCK8 assay. Figure 2 showed that UR at the concentration of 100–2,000 μM increased cell viability of pTr2 cells ($P < 0.05$). Importantly, cell viability is the highest at 200, 400, 600, and 1,000 μM of UR.

TABLE 4 Effects of UR on free amino acids profile in sow serum.

Items (μg/ml)	CON	UR	*P*-value
Urea	4832.23 ± 239.87	4372.51 ± 438.48	0.375
Tau	242.87 ± 17.15	270.51 ± 27.99	0.427
Asp	34.68 ± 1.35	30.84 ± 3.79	0.364
Thr	296.56 ± 29.83	395.01 ± 46.45	0.094
Ser	210.06 ± 10.19^{a}	180.21 ± 8.48^{b}	0.042
Glu	603.63 ± 53.12	527.31 ± 47.78	0.301
Gly	999.69 ± 23.14	929.07 ± 78.74	0.411
Ala	1031.02 ± 60.45	957.57 ± 99	0.536
Val	416.13 ± 27.97	429.3 ± 19.63	0.705
Cys	51.04 ± 5.25^{b}	98.94 ± 16.74^{a}	0.022
Met	63.34 ± 4.56	73.52 ± 8.79	0.325
Ile	237.28 ± 16.55	227.51 ± 12.28	0.642
Leu	284.19 ± 23.78	276.9 ± 15.48	0.796
Tyr	357.08 ± 22.87	321.41 ± 19.63	0.254
Phe	263.07 ± 17.72	240.89 ± 10.57	0.298
Lys	373.84 ± 39.13	329.3 ± 36.21	0.416
His	178.28 ± 12.34	187.43 ± 12.63	0.611
Arg	411.95 ± 44.23	349.99 ± 27.55	0.267
Pro	570.23 ± 44.84	576.92 ± 66.45	0.935

Effects of uridine on cell cycle assay in the pTr2 cells

Since cell cycle progression is also an important regulator of cell proliferation, we next explore whether UR regulated cell cycle (Figure 3). The results suggested that UR at 400 μM cells exhibited higher distribution of G1/M phase of cell cycle compared with 0 μM, and reduced S-phases of cell cycle compared with 0 and 100μM ($P < 0.05$, Table 7).

TABLE 5 Effects of UR on free amino acids profile in piglet serum.

Items (μg/ml)	CON	UR	*P*-value
Urea	4907.43 ± 252.62^{a}	3730.29 ± 163.77^{b}	0.002
Tau	302.82 ± 51.67	268.42 ± 60.46	0.670
Asp	109.43 ± 15.37	107.52 ± 18.58	0.937
Thr	243.41 ± 32.66	262.34 ± 31.48	0.684
Ser	388.07 ± 30.53	385.57 ± 41.19	0.961
Glu	610.31 ± 108.5	797.73 ± 158.09	0.345
Gly	1223.72 ± 115.42	1005.21 ± 69.4	0.137
Ala	1313.72 ± 97.54	1225.06 ± 159.45	0.634
Cit	128.79 ± 10.23	110.75 ± 5.8	0.159
Val	443.74 ± 30.61	464.34 ± 41.25	0.694
Cys	112.19 ± 5.77	107.34 ± 10.15	0.684
Met	29.5 ± 2.79^{b}	44.47 ± 6.4^{a}	0.041
Ile	53 ± 4.22^{b}	85.77 ± 12.87^{a}	0.040
Leu	133.17 ± 8.49	148.38 ± 23.54	0.558
Tyr	263.86 ± 35.41	279.45 ± 33.93	0.756
Phe	154.03 ± 30.47	138.12 ± 14.75	0.658
Lys	630.95 ± 64.99	574.7 ± 92.69	0.627
His	107.43 ± 10.26	98.84 ± 10.7	0.571
Arg	106.31 ± 14.28	110.34 ± 14.68	0.847
Pro	344.97 ± 11.99	325.88 ± 33.58	0.601

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TABLE 6 Effects of UR on free amino acids profile in colostrum.

Items (μg/ml)	CON	UR	*P*-value
Tau	3043.18 ± 507.8	2363.96 ± 166.43	0.155
Urea	3707.17 ± 250.42	3367.26 ± 397.54	0.482
Asp	12.76 ± 1.3	9.49 ± 0.81	0.051
Thr	28.71 ± 4.15	25.02 ± 2.94	0.479
Ser	24.86 ± 2.38 [a]	17.71 ± 2.14 [b]	0.040
Glu	84.7 ± 8.03	75.32 ± 6.93	0.389
Gly	12.15 ± 1.08	11.51 ± 1.85	0.769
Ala	30.03 ± 3.18	26.77 ± 2.28	0.418
Val	27.96 ± 4.59 [a]	14.08 ± 1.94 [b]	0.018
Cys	14.17 ± 2.79	10.28 ± 1.63	0.236
Ile	11 ± 2.56	7.2 ± 0.77	0.158
Leu	19.33 ± 2.82 [a]	11.88 ± 1.82 [b]	0.038
Tyr	18.21 ± 5.64	6.91 ± 0.97	0.137
Phe	18.28 ± 1.59	46.59 ± 19.94	0.194
βAla	80.85 ± 13.59[a]	40.02 ± 8.14[b]	0.025
Orn	4.85 ± 1.07	4.82 ± 0.76	0.985
Lys	21.85 ± 1.64	17.6 ± 2.45	0.182
His	9.01 ± 1.25[a]	4.85 ± 0.4	0.011
Arg	28.95 ± 2.7	26.17 ± 2.66	0.477
Pro	40.65 ± 5.01	37.91 ± 3.53	0.694

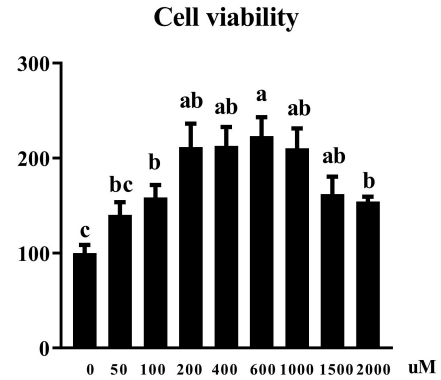

FIGURE 2
Effects of UR on cell viability in pTr2 cells. The a,b means columns with different letters are significantly different ($P < 0.05$), and columns with the same letters or no superscript are considered when $P > 0.05$. $n = 6$ replicates.

Discussion

Maternal nutrient supply may act as an inducer of fetal development and can permanently affects anatomy, physiology, and metabolism of fetus (17). Meanwhile, dietary nucleotides are non-protein nitrogenous compounds that modulate protein synthesis and is important for growth, repair, and differentiation of body tissue, including liver and small intestine (18). Dietary nucleotides supplement increased ADFI of weaned pigs and ADG, ADFI, and G: F of nursery pigs (19, 20). Consistently, our previous studies also showed UR supplement promoted nucleotide transport in weaned piglets and improved the growth performance in early weaned piglets (8, 21). Attractively, IUGR pig had improved nutrients utilization, intestinal function and immunity as a result of dietary nucleotides supplementation (22). Pregnancy is a critical period for fetal growth and development, however, there no research about dietary nucleotides supplement in sows that restricted feeding during gestation and excessive secreted milk during gestation. The results from the present study showed that UR supplement in sow diet during pregnant period decreased the number of stillborn piglets per litter and enhanced piglet weight at birth by 9.09%.

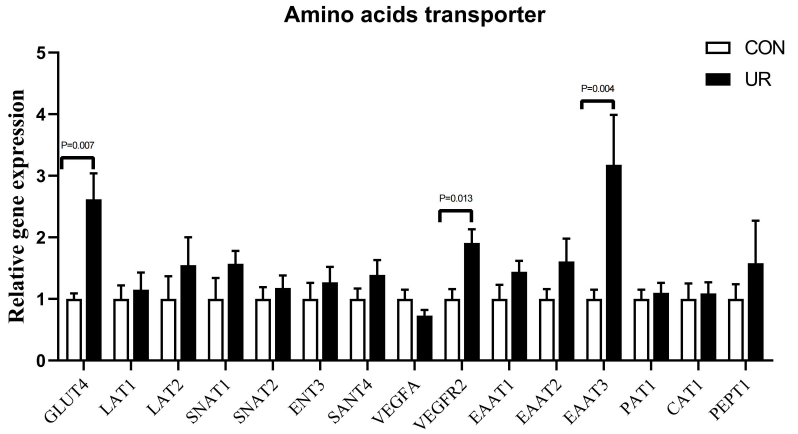

FIGURE 1
Effects of UR on amino acids transporter in the placenta of sows. The data was presented as mean ± SEM, significantly different was considered when $P < 0.05$, $n = 6$ replicates.

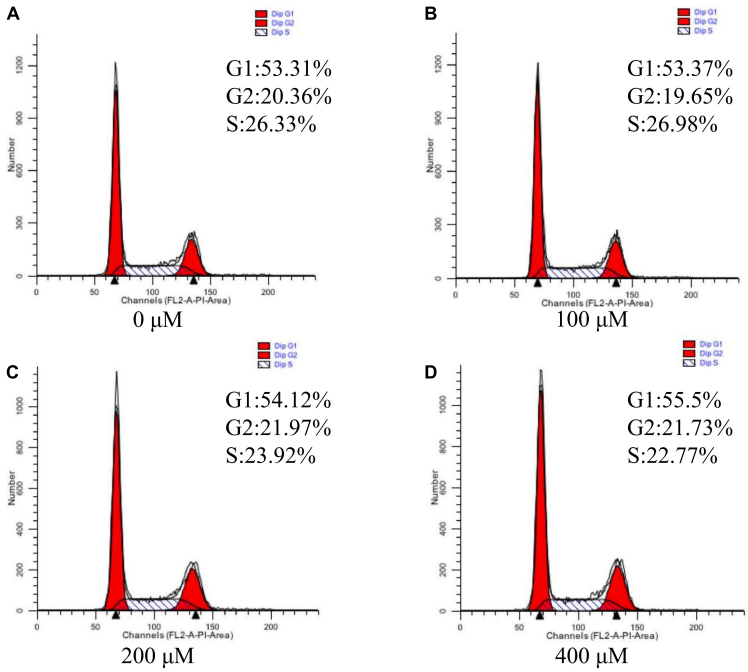

FIGURE 3
Effects of UR on cell cycle in pTr2 cells. Representative flow-cytometry diagrams of cells treated with 0 μM **(A)**, 100 μM **(B)**, 200 μM **(C)**, and 400 μM **(D)** UR for a 48-h 4-days period after starvation for 24 h.

TABLE 7 Effects of UR on the distribution of cell cycle in pTr2 cells.

Items (%)	0 μM	100 μM	200 μM	400 μM	*P*-value
G1	53.8 ± 0.28^{ab}	53.4 ± 0.26^{b}	55.02 ± 1.05^{ab}	55.72 ± 0.2^{a}	0.066
G2	20.46 ± 0.36	20.92 ± 0.64	21.1 ± 0.62	21.91 ± 0.42	0.326
S	25.75 ± 0.57^{a}	25.67 ± 0.71^{a}	23.89 ± 1.39^{ab}	22.37 ± 0.61^{b}	0.077

The a,b means columns with different letters are significantly different ($P < 0.05$), and columns with the same letters or no superscript are considered when $P > 0.05$. $n = 3$ replicates.

Maximizing placental protein synthesis and placental growth (including vascular growth) is related to maternal amino acid metabolism (23). Nucleotides modulate protein synthesis in the liver, and improve nutrients utilization (18, 22). TP indicates the body's nutritional status, protein absorption, and metabolism, while BUN indicates protein degradation (24). In the present study, the results showed that UR supplementation during the gestation period increased serum TP content of both sows and piglets and deceased serum BUN content, which may suggest UR improved the protein metabolism. Similarly, nucleotide-supplemented augmented the process of protein synthesis in liver injury rats (3). Piglets receiving diet with nucleotides additives experienced an accelerated carbon turnover during the post-weaning period (25). In addition, one-carbon metabolism is an essential material for the synthesis of purines, dTMP, and methionine (26). Addition of yeast RNA contributed to significantly reversing the effects of folate/methyl deficiency (27). In present study, UR supplement significantly affected the profile of serine and cysteine in sow serum, as well as the profile of methionine in piglet serum. It demonstrated that UR can improve methionine by affecting one-carbon metabolism both in sows and piglets mediated by placenta transmission. Moreover, nucleotides can alleviate the decrease of leucine concentration in the liver caused by alcoholic liver injury (28). However, our results showed no effect on the leucine content which may due to the different animal model. Moreover, maternal amino acid metabolism plays an important role in maximizing placental protein synthesis and placental growth (including vascular growth) (23). The insulin independent 1 (GLUT1) and insulin-dependent 4 (GLUT4) glucose transporters could regulate the entry of glucose into the adipocyte for *de novo* fatty acid and triglyceride synthesis, vital for the lipogenesis (29). It indicates the changes of glucose metabolism in sow's placenta. In this experiment, EATT3 gene expression also changed, which is the excitatory amino acid transporters family, but the content of glutamate in sow and piglet serum and colostrum did not change. It may due to the glutamate transporter EAAT3 regulates extracellular glutamate levels,

but its contribution to glutamate uptake is much lower than that of the astrocytic transporters EAAT1 and EAAT2 (30).

The size, morphology, and nutrient transfer capacity of placenta plays a direct decision role to the prenatal growth trajectory of the fetus, which further influence the birth weight (31), because of placenta acting as the key organ for the exchange of oxygen, nutrients, and wastes between the maternal-fetal circulations (32). Vasculogenesis is *de novo* formation of blood vessels from mesoderm precursor cells, and angiogenesis is creation of new vessels from a preexisting blood supply, which is very important for the maternal-fetal exchange (31). In the Yorkshire pig, fetal growth from mid to late gestation is associated with an increase in area of endometrial attachment (placental exchange area) (10). In present study, uridine can increase the relative mRNA expression of VEGFR gene in placenta, so as to increase the birth weight of piglets. In pigs, the elongation of placenta is the main factor determining the size of placenta. Around the 13th day of pregnancy, the pig toe nail began to attach to the uterine epithelium, more stable adhesion was carried out on the 15th–16th day, and formal implantation was carried out on the 18th–30th day (33). The blastocyst is composed of two distinct cell layers, with the trophectoderm accounting for two thirds of the cells (16, 34). The *in vitro* experiments also showed that uridine at an appropriate concentration can improve cell viability and cell cycle of pTr2 cells, which suggested the vital role of uridine in pTr2 cell proliferation.

Conclusion

The supplement of uridine at day 60 of pregnancy reduced the average number of stillborn piglets per litter with higher average piglet weight at birth, and UR may affect the growth of placental tissue by increasing the viability of pTr2 cells, and the amino acid metabolism both of sows and piglets.

Data availability statement

The datasets used during the study are available in the figshare repository, accession numbers doi: 10.6084/m9.figshare.21324357; doi: 10.6084/m9.figshare.21324366; and doi: 10.6084/m9.figshare.21324384.

Ethics statement

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (2013020).

Author contributions

XW and Y-LY: conceptualization. YL: data curation and writing—original draft. H-LW, LG, and TZ: formal analysis. H-LW and YL: project administration. JL: resources. H-LW, LG, TZ, and XW: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of mixture design approach on nutritional characteristics and sensory evaluation of steamed bread added rice flour

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This study was designed to evaluate the effects of different rice nutrient compounds on steamed bread's nutritional characteristics and sensory evaluation. The mixture design approach was used to research the interactions between different rice flours and wheat flours on the sensory evaluation of steamed bread. The arginine content of different rice flour (long-grained rice, polished round-grained rice, and black rice) was higher at 44.19, 21.74, and 34.78% than that of the common wheat, respectively. When the added amount of mixed rice flours exceeds 15%, the steamed bread gradually reduces its elasticity, and sensory score, and has a smaller specific volume. Rice is a widely consumed grain product, which provides energy and nutrients for more than half of humanity, especially in Asia. Different rice varieties have received increased attention from researchers for their high bioactive substances and other health benefits. The results of the current study provide a theoretical basis for the nutritional steamed bread and noodle industries to use different rice flour as an ingredient for enhancing or to improving the nutritional value of flour products.

KEYWORDS

mixture design approach, steamed bread, flour, rice flour, nutritional characteristics

Introduction

A significant number of researches have recently been carried out to develop gluten-free products by removal of gluten from wheat flour products, such as noodles, biscuits, cake, and steamed bread, which introduces some significant technological challenges (1–3). These challenges are indispensable for achieving desired farinograph properties, gas-holding ability, and texture characteristics of dough (4–11). Alternative compositions such as starch and protein from different flour sources have been recently used to acquire previously mentioned properties to satisfy its technological functions.

However, the use of different flour sources is limited because manufacturers are still accustomed to using refined, enriched other flour sources (5, 12–14).

Rice is a widely consumed grain, which provides energy and nutrients for more than half of humans, especially with regard to Asians (15). Recently, different rice varieties have received increased attention from researchers for their high bioactive substances, resistant starch, and other health benefits (16). Rice flour is utilized in a wide variety of food products, so it can affect the final product quality (17, 18). Different rice flours have also become a popular raw material in many health food products because of consumers' health concerns (19). It is deemed to be a more nutritious and healthier product than non-organic flours due to the wide use of heavy metal contamination, pesticide residue, herbicides, and chemical fertilizers (20). Variations in the properties of different rice flours have significant effects on the overall quality of the food products (21). Oil absorption capacities of different rice flours are associated with rancidity that naturally occurs in food products, which have a major predictor of shelf-life (12, 22, 23). The high-viscosity of different rice flours could be properly employed in high-viscosity products as a thickening agent (24).

Recently, more attention has been focused on color-grained rice varieties, which contain polyphenols in the aleurone and the pericarp, respectively, whereas common rice varieties contain small amounts of polyphenols (25–28). Polyphenols, such as phenolic acids, anthocyanins, and proanthocyanidins, have been reported as the primary antioxidants in different rice varieties (29–32). Generally, white rice contains mainly phenolic acids, and the presence of procyanidins characterizes red rice; however, black rice is characterized by the presence of anthocyanins (33–35).

However, little is known about the effect of different rice flours on steamed bread's nutritional characteristics and performance (36–39). Therefore, it is necessary to illustrate how different color-grained rice flours contribute to steamed bread's nutritional characteristics and performance (40). So, three different rice varieties (long-grained rice, polished Round-grained rice, and black rice) and the common wheat variety were milled into different flours with the mixture design approach to clarify the effect of proteins, fat, microelement, and total dietary fiber (TDF) on the quality of steamed bread. Thus, this research aimed to investigate the effect of different rice flours on the nutritional characteristics and sensory evaluation of steamed bread.

Materials and methods

Materials

The common wheat (Wenmai 2504, Kato, Prosky) and three different rice varieties were obtained from the 2021 harvest in Henan, Hubei, and Heilongjiang provinces. Long-grained rice (LGR) variety (*Oryza sativa* L. subsp. *indica* Kato) was provided by Jingshan Hongda grain and oil industry Co., Ltd. (Jingmen, Hubei, China). The polished round-grained rice (PRGR) variety (*Oryza sativa* L. subsp. *japonica* Kato) was provided by Yuanyang Kangjian Co., Ltd. (Xinxiang, Henan, China). Black rice (BR) variety (*Oryza sativa* L. cv. Khao Niaw Dam Peuak Dam) was provided by Wuchang Changxinxing Food Co., Ltd. (Heilongjiang, China), and Wenmai 2504 (W 2504) as the common wheat was purchased at farmers' markets (Jiaozuo, Henan, China).

Sample preparation

The common wheat was manually cleaned and stored in a 4°C freezer. The incomplete wheat and other impurities were removed. The moisture content of wheat before milling was controlled at 15 ± 0.5% for 16 h. LFS-30 (Buhler, Wuxi, China) was used in milling of the common wheat, and assembling at 3B flour system. Whole wheat kernels were first used to remove the bran layer using a grain polisher (TYT200, Tianyang Machinery Co., Ltd., Shandong, China) before preparing the flour. The different rice flours were collected by the universal grinder. The mixed flour was prepared with LGR (A), polished round-grained rice (B), and black rice (C). According to the orthogonal test, the ratio of A: B: C was 1: 1: 1, 1: 2: 2, 1: 3: 3, 2: 1: 2, 2: 2: 3, 2: 3: 1, 3: 1: 3, 3: 2: 1, and 3: 3: 2, respectively. The dough was prepared using the method of Chen (41). After preparation, the dough was put in a 4°C refrigerator to cool. For each dough, samples were prepared in duplicate.

Chemical analyses of the different rice flours

The lipid content of the different rice flours and wheat flour was determined using the method of Moreau with some modifications (42). Moisture was measured according to the National Standard of the People's Republic of China GB 5009.3-2016. The protein content of the different flours was determined by the Kjeldahl method. In a 100 ml round bottom flask, 0.5 g of various rice and common wheat samples were mixed with 4 ml of concentrated sulfuric acid, and the mixture was heated to 440°C with a conventional convection-conduction heating system until boiling. However, the heating time should not exceed 3–5 min.

Analysis of amino acids

Amino acids analysis of the rice and common wheat samples was accomplished according to the method of Du (43). The

TABLE 1 Physicochemical index of different rice and common wheat.

Cultivars	Moisture content (%)	Protein (%)	TDF (%)	Fat (%)
W 2504	12.7 ± 0.207	10.9 ± 0.014*	1.20 ± 0.707*	1.5 ± 0.236
LGR	14.2 ± 0.216*	7.93 ± 0.007	0.72 ± 0.028	0.9 ± 0.038
PRGR	13.7 ± 0.124	7.70 ± 0.014	0.62 ± 0.071	0.6 ± 0.017
BR	11.6 ± 0.141	7.50 ± 0.134	0.81 ± 0.071	1.9 ± 0.070*

*Means within each wheat category followed by the same letter are not significantly different at $p < 0.05$, the water content is dry base content.

rice and common wheat samples (100 mg) were hydrolyzed with 10 ml 5 M NaOH at 110°C for 20 h. The hydrolyzate was transferred and dissolved with deionized water in a 50 ml volumetric flask. The mixture was then filtered through a 0.45 μm of nylon syringe filter (Filtrex Technology, Singapore). The analysis of amino acids from different rice and common wheat samples was determined with an automatic amino acid analyzer (Biochrom 30+, Cambridge, UK). Amino acids were derivatized with ninhydrin reagent for postcolumn (0–50 ml/h) and determined at wavelengths of 570 nm (for the quantitation of α-amino acids) and 440 nm (for the imino acids). Amino acids of different samples and standard solution were analyzed under the same conditions, and all the above measurements were performed in triplicate.

Determination of total dietary fiber and microelement

The content of TDFs was determined according to the method of Prosky (44). Microelement determinations of the rice and common wheat samples were performed using an Agilent 240FS atomic absorption spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with flame atomization [a mixture of acetylene (2.9 L/min) and air (13.5 L/min)] (45). The emission mode of K is set to 766.5 nm.

Preparation of rice dough and steamed bread

Different ratios of rice flours into wheat flours were made to steamed bread according to the sponge dough method of He with some modification (46). The recipe of steamed bread is as follows: The common wheat flour is the base flour, and the different rice flours are added by 5, 10, 15, 20, 25, and 30% with the total amount being 100 g. Dry yeast, 1 g (Anqi, Anqi Yeast Co., Ltd., Hubei, China) was added into water (30°C). A total of 100 g samples were mixed with yeast/water which Farinograph water absorption rate up to 85% in a dough maker (B10, Henglian Food Machinery Co., Ltd., Guangdong, China). After mixing at medium speed for 10 min with the appropriate amount of warm water (38°C), and using a rolling pin to press the sheet 20 times, the dough was divided into three parts, and shaped by hand into a round, long, and straight dough with a smooth surface. Then, the dough pieces were proofed for 2 h at 30°C with 80% RH, and remixed flour and dough by hand until the dough was sticky. After that, the proofed steamed bread was put in a steamer (ASD, Zhejiang, China), when the water boiled, and steamed for 20 min.

Sensory evaluation of steamed bread making

The numbers of panelists were 18 (10 men and 8 women). Panelists included 18 adults between the ages of 27 and

TABLE 2 Amino acid composition of different rice and common wheat (g/100 g).

AA	W 2504	LGR	PRGR	BR
Asp	0.5	0.67	0.68	0.65
Thr	0.31	0.29	0.28	0.28
Arg	0.43	0.62	0.68	0.56
Pro	1.32	0.33	0.32	0.3
Tyr	0.3	0.24	0.29	0.22
Ala	0.37	0.44	0.42	0.42
Val	0.45	0.46	0.46	0.44
His	0.21	0.2	0.2	0.19
Met	0.15	0.13	0.13	0.1
Ile	0.38	0.32	0.3	0.28
Cys	0.16	0.11	0.12	0.11
Phe	0.48	0.45	0.43	0.38
Ser	0.58	0.44	0.44	0.39
Glu	4.26	1.5	1.48	1.26
Gly	0.44	0.32	0.33	0.33
Lys	0.23	0.28	0.27	0.31
Leu	0.8	0.62	0.62	0.55
TAA (g/100g)	11.37	7.42	7.45	6.77
EAA (g/100g)	3.42	2.9	2.9	2.67
EAAI	91.64	78.86	78.27	73.38

*Tryptophan content is less, no determination results.
**TAA, total amino acid content; EAA, essential amino-acid content; EAAI, essential amino acid index.

65. They were selected, screened, and recruited according to international standards (47). A total of 15 of them were university faculty, and 13 of them had received theoretical and practical training in sensory analysis and experience in tasting different flour products such as bread, steamed bread, or noodles. The other five had not participated in the panel discussion before. Assessors were not paid for participating in the panel.

Statistical analysis

Data were collected for analysis of variance to determine differences between different rice flours and to investigate the effect of mixture design approach on farinograph and extensometer performance. Significance analysis was performed using Statistica 7.0 software to study the relationship between rheological properties and mixture design approach. Data were reported as the mean (standard deviation SD) of triplicates.

Results and discussion

Physicochemical properties of different-grained rice flours

The chemical composition of the different rice flours and wheat flour was shown in **Table 1**. The protein content of different rice flours was 27.24–43.04% lower than that of the common wheat. Protein is the main food component of humans and other animals. However, there are still challenges in formulating gluten-free products with rice because rice flour does not function as wheat gluten, which is known as an essential structural protein (48). The fat and total TDF content were almost lower than the common wheat. The fat of wheat is mainly concentrated in the germ; however, the fat of rice flour is mainly concentrated in the bran (49). TDF was mainly concentrated in the pericarp and seed coat, while the content of endosperm was very few. The content of fat was abundant in black rice flour because the rice bran was reserved in the process.

Distribution of amino acid, total amino acid, and essential amino acid content

The amino acid contents for different rice flours and wheat flour were shown in **Table 2**. The nutritional characteristics of different rice flours mainly refer to the balance of protein content and amino acid composition, and the essential amino acid content is the key to determining the nutritional quality of different rice flours (50). TAA and EAA contents of different rice flours were lower than those of common wheat flour, and TAA content was 34.12–40.46% lower than that of common wheat flour. The essential amino acid content of different rice flours was 15.2–21.93% lower than that of common wheat. The results showed that the quantity and quality of protein and amino acids of common wheat flour were greater than those of different rice flours. Due to the amino acid composition characteristics of different rice flours, people can directly eat them to meet nutritional needs, and can also be used as breeding materials (51). The arginine content of different rice flours was higher than that of common wheat: Long-grain rice flour increased by 44.19%, polished round-grained rice by 21.74%, and black rice flour by 34.78%.

Determination of the microelement in different rice flours

The microelement contents for different rice flours and wheat flour were shown in **Table 3**, and the content of zinc in black rice flour was 132.32% higher than that of W 2504. Zinc has a role in helping intellectual growth and development (52). The content of sodium in black rice flour was 160.81% higher than that of W 2504. Sodium is an essential trace element for the human body, an important part of water balance, and a complex component of pancreatic juice, bile, sweat, and tears. Therefore, sodium can promote physical development and increase the body's ability to resist disease. At the same time, sodium can also regulate tissue respiration and prevents fatigue (53). However, the content of other microelements in different rice flours was lower than that of W 2504. Therefore, the mixture design approach can improve their nutritional value.

TABLE 3 Microelement content of different rice and common wheat.

Cultivars	Zn^{++}	Fe^{++}	Mg^{++}	Na^{+}	Ca^{++}
W 2504	16.4 ± 0.071	35.2 ± 1.13*	320 ± 2.828*	27.3 ± 1.414	270 ± 1.414*
LGR	14.7 ± 0.141	16.2 ± 0.283	280 ± 2.828	17.1 ± 0.141	120 ± 4.243
PRGR	11.2 ± 0.028	16.4 ± 0.057	158 ± 2.828	19.3 ± 0.141	191 ± 1.414
BR	38.1 ± 0.283*	16.2 ± 0.156	147 ± 1.414	71.2 ± 1.414*	120 ± 2.828

*Means within each rice category followed by the same letter are not significantly different at $p < 0.05$.

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TABLE 4 Nutritional characteristics of different rice flour with different proportions.

	Combination	Protein (%)	TDF (%)	EAAI	Microelement (mg/kg)
1	($A_1B_1C_1$)	7.71 ± 0.028	0.70 ± 0.014*	76.84	181.09 ± 1.541
2	($A_1B_2C_2$)	7.67 ± 0.028	0.70 ± 0.014*	76.43	187.17 ± 3.068*
3	($A_1B_3C_3$)	7.65 ± 0.042	0.70 ± 0.014*	76.26	189.77 ± 2.503*
4	($A_2B_1C_2$)	7.71 ± 0.014	0.72 ± 0.014*	76.55	173.59 ± 2.248
5	($A_2B_2C_3$)	7.68 ± 0.014	0.72 ± 0.014*	76.34	180.08 ± 1.301
6	($A_2B_3C_1$)	7.74 ± 0.049*	0.67 ± 0.028	77.65	188.51 ± 2.135*
7	($A_3B_1C_3$)	7.71 ± 0.028	0.73 ± 0.028*	76.43	170.38 ± 2.291
8	($A_3B_2C_1$)	7.78 ± 0.042*	0.69 ± 0.014*	77.75	177.21 ± 1.117
9	($A_3B_3C_2$)	7.74 ± 0.028*	0.69 ± 0.028*	77.27	181.98 ± 2.80

*Means within each rice category followed by the same letter are not significantly different at $p < 0.05$.

Effect of mixture design approach on nutritional characteristics

Flour products are a major part of the Chinese daily diet, with wheat as the main source, but are mainly made from the seeds of common wheat. However, different rice flours contain most of the valuable protein, micronutrients, and phytochemicals found in grains, and if included in flour or used as food ingredients, they would go to a long way toward improving the nutritional quality of human food (54). Nutritional characteristics of mixed flours from different rice flours and wheat flour were shown in Table 4. The results showed that the highest protein content was no. 8 ($A_2B_3C_1$), 6 ($A_2B_3C_1$), and 9 ($A_3B_3C_2$). However, the highest value of EAAI was no. 8 ($A_3B_2C_1$) and 6 ($A_2B_3C_1$). The microelement content highest is the no. 3 ($A_1B_3C_3$) and 6 ($A_2B_3C_1$). Therefore, when paired with these factors, we choose no. 6 ($A_2B_3C_1$) as a nutritional content more rich.

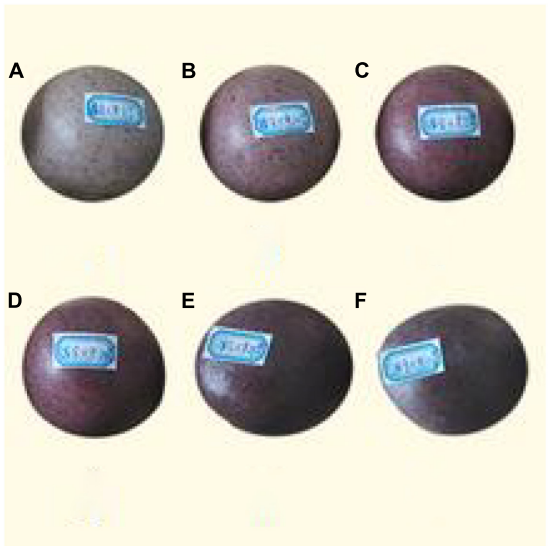

FIGURE 1
Images of steamed bread made by different rice flours [the added amount of panels **(A–F)**: 5, 10, 15, 20, 25, and 30%].

Effect of mixed flours on sensory analysis of steamed bread

As shown in Figures 1,2, with the increase in the amount of different rice flours, the specific volume of steamed bread is getting smaller and smaller. Because different rice flours do not contain gluten, the steamed flours cannot form a gluten network structure. The results have shown that the different rice flours have some effects on weakened gluten network structure. The amount of different rice flours exceeded the ultimate value, so small size, low specific volume, bouncy toughness and bite, and poor shape would appear in the steamed bread. When the added amount of different rice flours exceeds 15%, the steamed bread gradually reduces elasticity and has a smaller specific volume.

As shown in Figure 2A, the specific volume of steamed bread is becoming smaller and smaller with the increase of colored rice composite powder. This is because rice does not contain gluten, which cannot form a gluten network structure. The research shows that the medium gluten flour is more suitable for making steamed bread, while the coarse grain flour has a certain role in weakening the gluten strength. Excessive addition will lead to low gluten strength, making steamed bread small in volume, low in specific volume, poor in elasticity and bite strength, poor in shape, and large and uneven in pulp structure (1, 3). So the appearance, structure, elasticity, and toughness are slightly reduced. The evaluation value of color and smell has been rising due to the special color and aroma of rice. As shown in Figure 2B, with the increase of colored rice composite powder, the total sensory evaluation score of steamed bread increased first and then decreased. When the added amount exceeds 15%, the elasticity of steamed

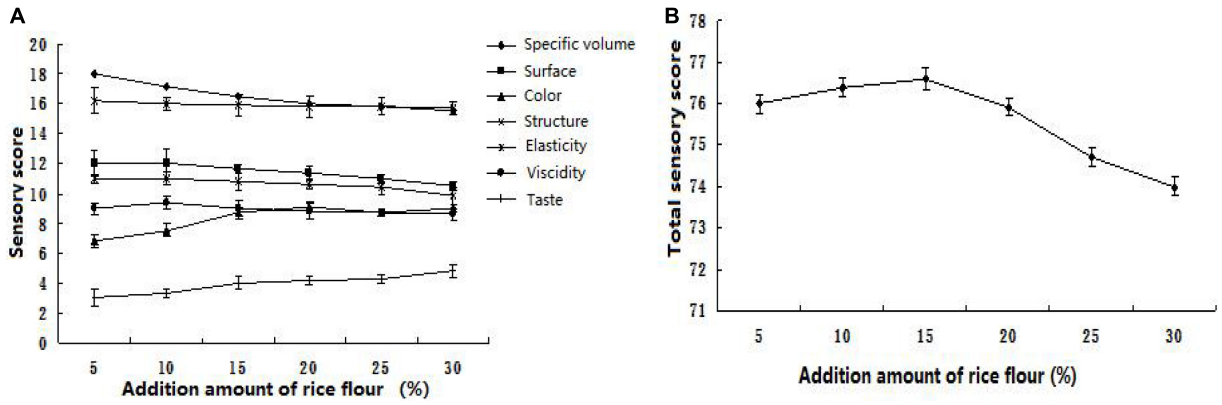

FIGURE 2
Sensory analysis of different rice flour with different proportions (**A:** Sensory score; **B:** Total sensory score).

bread gradually decreases, the taste becomes worse, and the specific volume becomes smaller. When the addition amount was 15%, the total score of colored rice steamed bread was the highest. As mentioned in the color section above, the reduced whiteness of the crust and crumbs can easily be attributed to the color in the rice flour. Color change of cereals may appeal to consumers (40).

Conclusion

The effects of additions of different rice flours with different proportions on the nutritional characteristics and quality of steamed bread were investigated. Based on the analysis of protein, TDF, fat, amino acid composition, microelement, physicochemical index, nutritional characteristics, and quality of steamed bread, this study demonstrated that additions of different rice flours with different proportions primarily affected physicochemical index, nutritional characteristics, and quality of steamed bread. When the addition amount was 15%, the total score of colored rice steamed bread was the highest. The results of the current study offer opportunities for the steamed bread and noodle industry to use different rice flours as an ingredient for enhancing the nutrient-value amount of dough. The acceptability of dough with different proportions of different rice flours needs further study. The different rice flours have the potential to be utilized as nutrient-value foods.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

ST: data curation (equal), formal analysis (equal), investigation (equal), writing—original draft and review (equal), and editing (equal). YW: conceptualization (equal) and writing—review and editing (equal). ZC: formal analysis (equal), methodology (equal), writing—original draft (supporting), funding acquisition (equal), and writing—review and editing (equal). All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Separation and purification of antioxidant peptides from purple speckled kidney bean by macroporous adsorption resin and analysis of amino acid composition

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The protein hydrolysate of purple speckled kidney bean (PSKB) was used as the raw material in this study, and the antioxidant peptide of the PSKB protein hydrolysate was purified using macroporous resin. The XAD-7HP macroporous resin was selected as the best purification material, and the static adsorption-desorption of the purified PSKB antioxidant peptide was optimized. The optimum static adsorption and desorption conditions were as follows: the adsorption capacity reached 11.93 ± 0.11 mg/ml at pH 7 for 24 h, and the desorption capacity was 5.24 ± 0.04 mg/ml with 60% ethanol for 30 min. Under this condition, the amount of antioxidant peptide obtained by adsorption-desorption was the highest. The optimum process conditions were as follows: the appropriate flow rate was 1 ml/min, and the optimal injection volume was 40 ml. The adsorption amount at this time can reach 12.19 ± 0.15 mg/ml. The components with an elution time of 10–30 min were separated using the reversed-phase high-performance liquid chromatography (RP-HPLC) technique to obtain three main components, namely, RP_1, RP_2, and RP_3. The DPPH free radical scavenging ability reached 56.26 ± 0.56, 66.42 ± 0.56, and 78.57 ± 0.56%, respectively, which were 36.65, 46.34 ± 0.56, and 54.39 ± 0.56% higher than those before purification. The amino acid sequences of the three components were identified as Phe-Leu-Val-Asp-Arg-Ile, Phe-Leu-Val-Ala-Pro-Asp-Asp, and Lys-Asp-Arg-Val-Ile-Ser-Glu-Leu.

KEYWORDS

purple speckled kidney bean protein, antioxidant peptide, separation, purification, macroporous resin

Introduction

Purple speckled kidney bean (PSKB), commonly known as *Phaseolus vulgaris* L., is one of the most globally important edible legume crops in humans, and it is rich in components of high amounts of proteins (20–25%), complex carbohydrates (50–60%), and many essential vitamins and minerals (copper, calcium, iron, magnesium,

manganese, and zinc) (1). PSKB protein peptide is an oligopeptide, which is an intermediate product between proteins and amino acids. In special food applications, protein hydrolysates have been used as high value-added ingredients, with high protein digestibility and amino acid selection. This type of food is indispensable for people with chronic conditions such as Crohn's disease, who have difficulty absorbing the protein in its natural form. Studies found that small-molecule peptides have good biological activity and strong antioxidant properties (2).

To improve the value of PSKB, protein hydrolysates were separated from the protein. Using PSKB albumin as raw material, Hongxiu Fan et al. found that, after enzymatic hydrolysis, the antioxidant activity, foaming properties, and PSKB albumin stability were significantly improved, and ultraviolet absorption was enhanced (3). Using PSKB peptide as a test material, Mehnaza Manzoor et al. found that the protein peptide can reduce the oxidative damage of $HepG_2$ cells caused by H_2O_2 to a certain extent, can also regulate the cell's redox system, remove intracellular reactive oxygen species, and improve intracellular antioxidant enzyme activity (4). Macroporous adsorption resin was developed in the 1960s and was used as a new type of non-ionic porous polymer adsorbent. Its separation characteristics are adsorption and the molecular sieve principle (5). At present, macroporous resins are widely used in the separation and purification of active ingredients such as total flavonoids, polyphenols, anthocyanins, polysaccharides, alkaloids, and saponins (6–11). Tingting Bu et al. (12) used macroporous adsorption resin to adsorb the small molecular peptide in whey protein hydrolysate and found that the percentage of peptide below 600 Da was 93.60%, which was 42.81% higher than that before adsorption. The adsorption effect was significant. Macroporous adsorption resin has potential application prospects in the field of protein-peptide adsorption. Using different types of macroporous resins, Cerrone Cabanos et al. (13) compared the adsorption effects of alfalfa leaf protein peptides, screened the best macroporous adsorption resin as DA201-C, and studied its adsorption-desorption characteristics for alfalfa leaf protein peptides. Mingzhu Zhuang et al. (14) explored the adsorption and desorption performances of 4 medium-sized macroporous adsorption resins on soybean peptides. The results showed that different resins had significant differences in the purification of raw materials and the enrichment of peptides. The non-polar resins with large specific surface area and small pore size had good adsorption and desorption properties.

At present, there are many studies on the physicochemical properties and single-enzyme hydrolysis technology of PSKB protein. However, the amino acid sequence of PSKB is less studied. At the same time, the amino acid sequence also limits the production, processing, and application of the antioxidant peptides from PSKB. So, in this study, the PSKB antioxidant peptides were prepared by simultaneous hydrolysis of complex protease, the static adsorption and desorption conditions were optimized, and the structure was identified, which laid a certain research foundation for the deep processing of PSKB protein and for the development and utilization of PSKB peptides.

Materials and methods

Materials

The purple speckled kidney bean, which was produced in Heilongjiang Province in China, was provided by the National Cereal Engineering Technology Research Center in Daqing, Heilongjiang Province.

Pepsin (3,000 U/g) and trypsin (74,000 U/g) were purchased from Germany's Saiguo Biotechnology Co., Ltd. The HPD-400, HPD-400A, and XAD-7HP resins were purchased from Cangzhou Baoen Adsorption Material Technology Co., Ltd. Potassium sodium tartrate was purchased from Liaoning Quanrui Reagent Co., Ltd. Anhydrous ethanol was purchased from Shandong Deyan Chemical Co., Ltd. DPPH, ferrous sulfate, and ferrous chloride were of analytical grade and were purchased from Aladdin Reagent Co., Ltd. The PHS.2C precision pH meter was purchased from Mettler Toledo, USA. RP-HPLC was purchased from Agilent, USA.

Extraction of PSKB protein and preparation of hydrolysate

PSKB protein extraction is based on the experimental methods of Li Yuqiong (15, 16) and so on. The two enzymes with the best hydrolysis effects, neutral protease and alkaline protease, were screened through preliminary experiments. The double-enzyme simultaneous composite hydrolysis experiment was performed. The single-factor combined orthogonal experiment was used to optimize the dual-enzyme simultaneous hydrolysis process. The optimal hydrolysis conditions were as follows: The temperature was 55°C, the pH was 10, the alkaline protease:neutral protease ratio was 1:2, and 5 ml of the mixed enzyme was added. Under this condition, PSKB had a proteolysis degree of 42.2% and had good antioxidant activity.

TABLE 1 Physical structure parameters of different macroporous resins.

Type of resin	Specific surface area/(m^2.g)	Types of polarity	Average pore size/A
XAD-7HP	500–550	Polarity	75–80
HPD-400	500–550	Polarity	85–90
HPD-400A	480–500	Weak polarity	450–500

Macroporous resin pretreatment

Three resins, HPD-400, HPD-400A, and XAD-7HP, were selected. The adsorption and desorption properties of the three resins were investigated, and the types of macroporous resins suitable for the separation and purification of the antioxidant peptide from PSKB were selected (see Table 1 for details).

Static adsorption of PSKB proteolysate by macroporous resin

Determination of the best resin

A total of 15 g of pretreated resin was added to a 250-ml stoppered Erlenmeyer flask, and 100 ml of PSKB antioxidant peptide solution was added after enzymatic hydrolysis to completely soak the resin. The flask was sealed and shaken at 180 r/min in a constant temperature shaker for 24 h to completely contact the resin with the crude peptide solution. After it was fully adsorbed, 1 ml of the supernatant was absorbed, mixed with 4 ml of the biuret reagent, and measured at 540 nm after 30 min. The remaining peptide concentration in the adsorption solution was used to calculate the resin adsorption (17).

$$\text{Adsorption capacity (mg/g)} = \frac{C - C_0}{m} \times V$$

$$\text{Adsorption rate (\%)} = \frac{C - C_0}{C} \times 100$$

In the formula, C and C_0 are the protein concentrations (mg/ml) before and after adsorption, respectively; V is the volume (ml) of the PSKB antioxidant peptide solution.

Static adsorption curve

A total of 10 g of screened resin was added to a 250-ml Erlenmeyer flask, 100 ml of PSKB protein hydrolysate was added after concentration treatment, and the bottle mouth was sealed and shaken in a constant temperature shaker (30°C, 180 r/min). The solution was collected every 1 h, the peptide content in the solution was determined after adsorption, the adsorption rate and adsorption amount of PSKB peptide were calculated according to the formula, and a static adsorption curve was drawn.

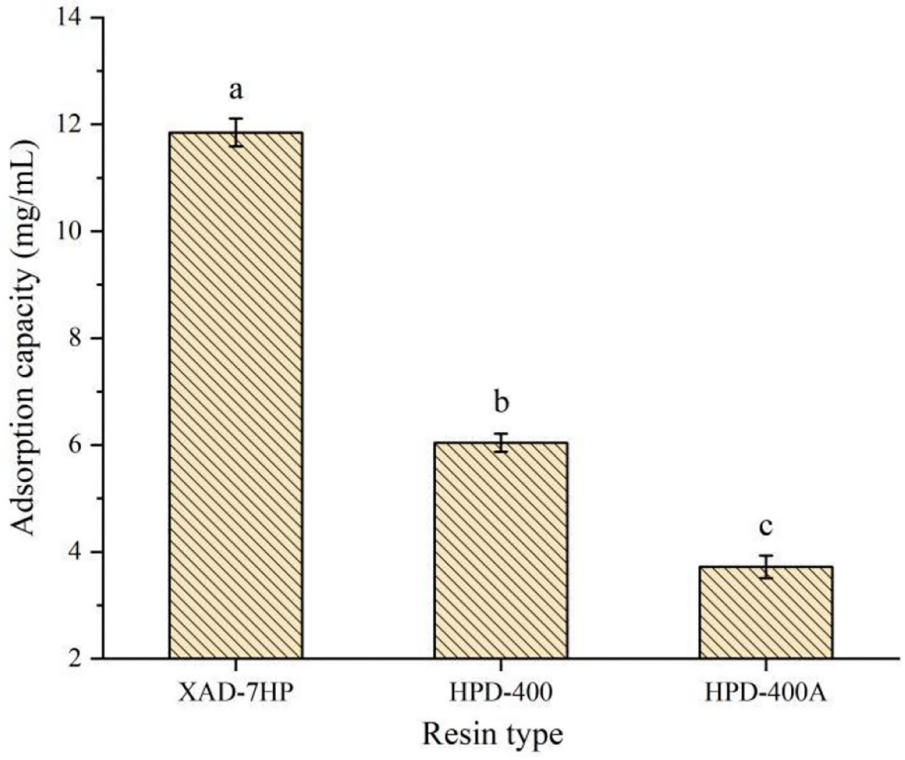

FIGURE 1
Adsorption capacity of different types of resins to protein peptides of PSKB. There is a significant difference between different small letters ($P < 0.05$).

Static desorption and selection of eluent

Static adsorption was performed as described in the "Determination of the best resin" section, shaken in a constant temperature shaker for 12 h, the filter was removed when it was fully adsorbed, and then the adsorbed macroporous resin was placed in a 250-ml conical flask again. After the adsorption of the PSKB antioxidant peptides, the resin was eluted. The eluents were deionized water (0%) and 20, 40, 60, and 80% ethanol solutions of 20 ml each, which were shaken under the same conditions. After 10, 20, 30, 40, and 50 min of desorption, the volume and the protein content of the desorption solution were measured, and a static desorption curve was drawn.

$$\text{Desorption (mg/mL)} = \text{Desorbed protein concentration} \times \text{Desorption volume}$$

Static adsorption capacity test of PSKB hydrolysate with different Ph values

A total of 10 g of the treated macroporous adsorption resin was weighed, placed in a 250-ml triangle bottle, and 50 ml of PSKB protein hydrolysate with different pH values was added (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) so that the resin was completely soaked and placed in a constant temperature shaker (180 r/min) at 30°C. After adsorption for certain periods of time, it was filtered, and the volume of the hydrolysate and the peptide content before and after the adsorption were used to calculate the amount of adsorption.

Dynamic adsorption and desorption of PSKB antioxidant peptides by macroporous adsorption resin

We weighed 100 g of the treated XAD-7HP resin into a 1.0 × 100 cm chromatography column, and the PSKB antioxidant peptide solution at pH 7.0 was 1.0, 1.5, and 2.0 ml at 30°C. The column was loaded at a minimum flow rate, the mass concentration of the effluent was checked, and the adsorption curve of the baseline was considered as the transmission point to obtain the optimal loading volume and loading quality. A 60% ethanol solution was used as the eluent (flow rate of 2 ml/min). The eluent was collected at a certain dose every 10 min. The absorbance of the collected solution was measured with a UV detector at a wavelength of 540 nm. The eluted

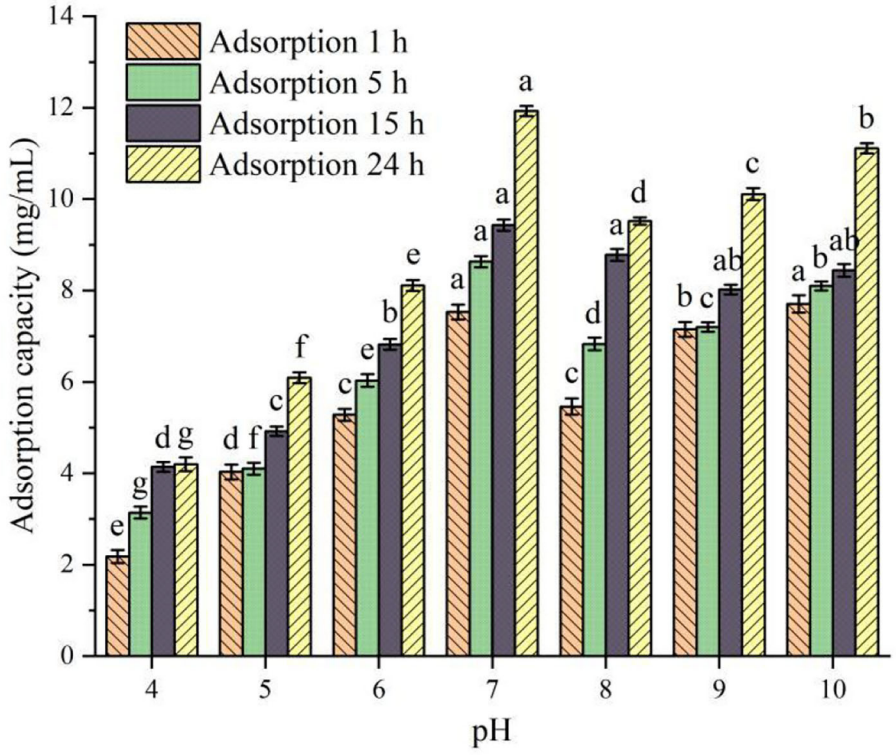

FIGURE 2
Adsorption capacity of PSKB protein hydrolysate at different pH values. There is a significant difference between different small letters ($P < 0.05$).

peaks were collected and dried for later use. With reference to the method of Pablo R. Salgado et al. (18), the antioxidative capacity of each component peptide solution was tested at a concentration of 1%. In a stoppered test tube, 0.5 ml of PSKB protein hydrolysate and 3.5 ml 1×10^{-4} mol/L DPPH absolute ethanol solution were collected, mixed well, and let stand at room temperature for 30 min. Absolute ethanol was used as a blank control. Using VC as a positive control, the absorbance value was measured at a wavelength of 517 nm, and the DPPH clearance ability was calculated according to the following formula.

$$DPPH \text{ clearance rate } (\%) = \left(\frac{A_1}{A_2 - A_3}\right) \times 100\%,$$

where A_1 is the absorbance value of the DPPH solution after adding the PSKB protein hydrolysate; A_2 is the absorbance value of the PSKB protein hydrolysate solution; A_3 is the absorbance value of DPPH solution without adding the PSKB protein hydrolysate.

RP-HPLC for the detection of antioxidant peptides in PSKB

The hydrolysate and the purified antioxidant peptide of PSKB protein were, respectively, treated by reversed-phase high-performance liquid chromatography, and the components and antioxidant capacity of each component were analyzed. The experimental conditions were as follows: Agilent C18; mobile phase: acetonitrile:water:trifluoroacetic acid: 20:80:0.02%); flowrate: 1.0 ml/min; column temperature: 25°C; ultraviolet detection; and detection wavelength: 540 nm.

Amino acid sequence determination of antioxidant peptides from PSKB

The antioxidant peptide of PSKB was isolated from macroporous resin for the identification of amino acid sequence, and the method was adapted from Jiaqi Fang et al. (19, 20) and modified appropriately. Chromatographic conditions were as follows: mobile phase A (mass spectrometric ultrapure water containing 0.1% formic acid), mobile phase B (mass

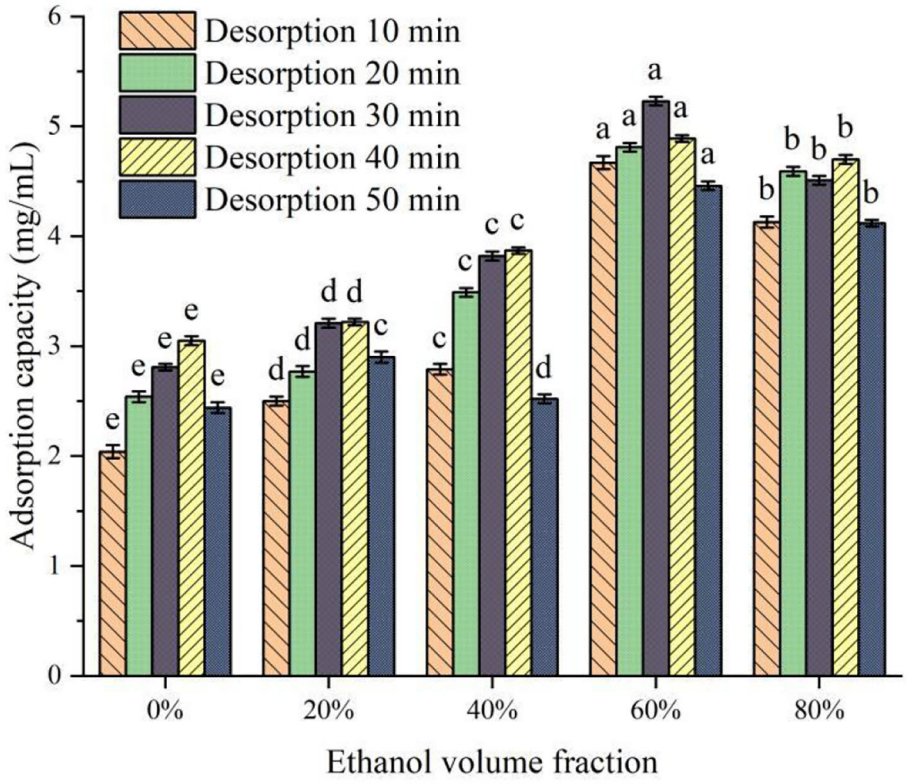

FIGURE 3
Effect of ethanol solution with different volume fractions on desorption. There is a significant difference between different small letters ($P < 0.05$).

spectrometric acetonitrile containing 0.1% formic acid), a flow rate of 200 μl/min (2 μl/min after shunt), and a gradient of 120 min (5% B 15 min, 5–32% B 45 min, 90% B 35 min, 5% B 5 min, 5% B equilibrium 20 min). The mass spectrometry of electrospray operating voltage was 3.5 kV; the ion migration tube temperature was 250°C; fragments of the spectrum diagram to make the tandem mass spectrometry (MS), according to the same energy cracking, was set to collision-induced dissociation (collisionally induced dissociation, CID); collision energy was 35%; the ionization method was electrospray ionization (ESI); the scanning range, in terms of mass-to-charge ratio (m/z), was 400–1,800. The secondary mass spectrum retrieval software uses Proteome Discovery 1.2, and the retrieval algorithm is a sequence.

Statistical analysis

All experiments were done in triplicates and were expressed as means ± SD. Data were analyzed using a one-way analysis of variance (ANOVA) using SPSS statistics 20.0 (SPSS Inc, USA). The significant differences were determined using Duncan's multiple range tests at a *P*-value of < 0.05.

Results and analysis

Screening of macroporous adsorption resin

Due to the different specific surface areas, surface electrical properties, and hydrophobic interaction with adsorbed substances, different resins have a significant impact on the adsorption capacity of antioxidant peptides in PSKB (21, 22). The structural parameters of the three macroporous adsorption resins selected in the test are shown in Table 1. Table 1 shows that XAD-7PH and HPD-400 are polar resins with small pore diameters; HPD-400A is a weak polar resin with a large pore size. The adsorption capacity of antioxidant peptides in PSKB by different types of resins is shown in Figure 1. It can be seen from Figure 1 that XAD-7PH polar resin with the smallest pore size has the strongest adsorption capacity, which is 11.85 ± 0.26 mg/ml. With the increase in pore size, the adsorption capacity of macroporous adsorption resin gradually decreased, in which

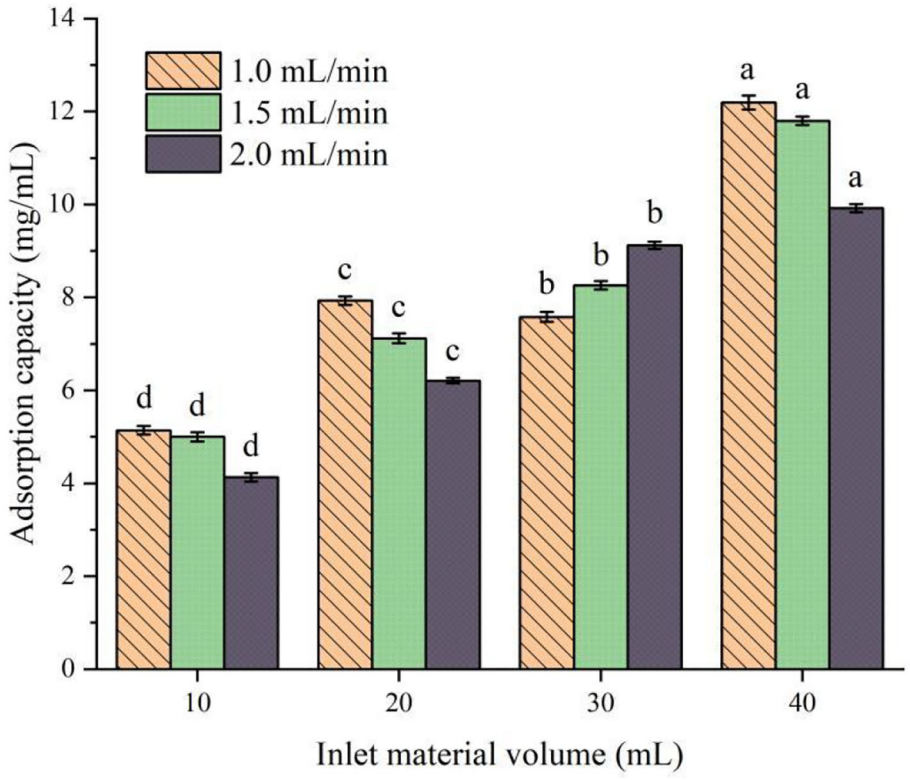

FIGURE 4
Effects of different flow rates and feed amounts on the adsorption of protein peptides in PSKB. There is a significant difference between different small letters ($P < 0.05$).

HPD-400 resin was 6.04 ± 0.17 mg/ml and HPD-400A resin was 3.72 ± 0.21 mg/ml. The adsorption capacities of the three resins were significantly different ($P < 0.05$). XAD-7PH polar resin with the smallest pore size has better adsorption capacity for antioxidant peptides, which mainly depends on dipole ions and hydrogen bonds in the molecule (23). Therefore, we used XAD-7HP resin to separate antioxidant peptides from PSKB.

Study on static adsorption of protease hydrolysate from PSKB by macroporous resin

Static adsorption curves of PSKB protein hydrolysates at different pH values

The relationship between the static adsorption capacity of XAD-7HP macroporous adsorption resin and pH value is shown in Figure 2. It can be seen from Figure 2 that, under the same adsorption time, the adsorption capacity of XAD-7HP resin under different pH conditions for PSKB protein hydrolysates is significantly different ($P < 0.05$). Among them, with the increase in pH, the adsorption capacity of XAD-7HP resin on PSKB protein hydrolysates shows an upward trend, reaching the maximum at pH = 7, which is 7.53–11.93 mg/ml. With the continuous increase in pH, the adsorption capacity of XAD-7HP resin on PSKB protein hydrolysates slightly decreases as the adsorption capacity of the resin is related to van der Waals force and hydrogen bond, and its network structure and specific surface area have certain screening performance (24). Under the same pH condition, the adsorption capacity of XAD-7HP resin to PSKB protein hydrolysate was gradually enhanced with the extension of adsorption time ($P < 0.05$). At pH = 7 and an adsorption time of 24 h, the adsorption capacity was the strongest, reaching 11.93 ± 0.11 mg/ml.

Static desorption experiment and selection of desorption agent

The molecular polarity of ethanol solution with different volume fractions is different, and the binding ability with protein peptides and the adsorption ability of covalent bonds are different (25). The change in desorption capacity of XAD-7HP macroporous adsorption resin with different ethanol volume fractions is shown in Figure 3. It can be seen from Figure 3 that the desorption ability of desorbers with different ethanol volume fractions on PSKB protein hydrolysate is significantly different ($P < 0.05$). With the increase in ethanol volume fraction, the desorption capacity of the desorber to PSKB protein hydrolysate showed an overall upward trend, reaching the highest when the ethanol volume fraction was 60%, which was 4.46–5.23 mg/ml. The volume fraction of ethanol continued to increase, and the desorption capacity slightly decreased. When the volume fraction of ethanol was 80%, it was 4.12–4.70 mg/ml. This is because the volume fraction of ethanol is too high, which makes it difficult for proteins and peptides to dissolve. With the prolongation of desorption time, the desorption amount of protein hydrolysate by the desorber increased first and then decreased. When the desorption time reached 30 min,

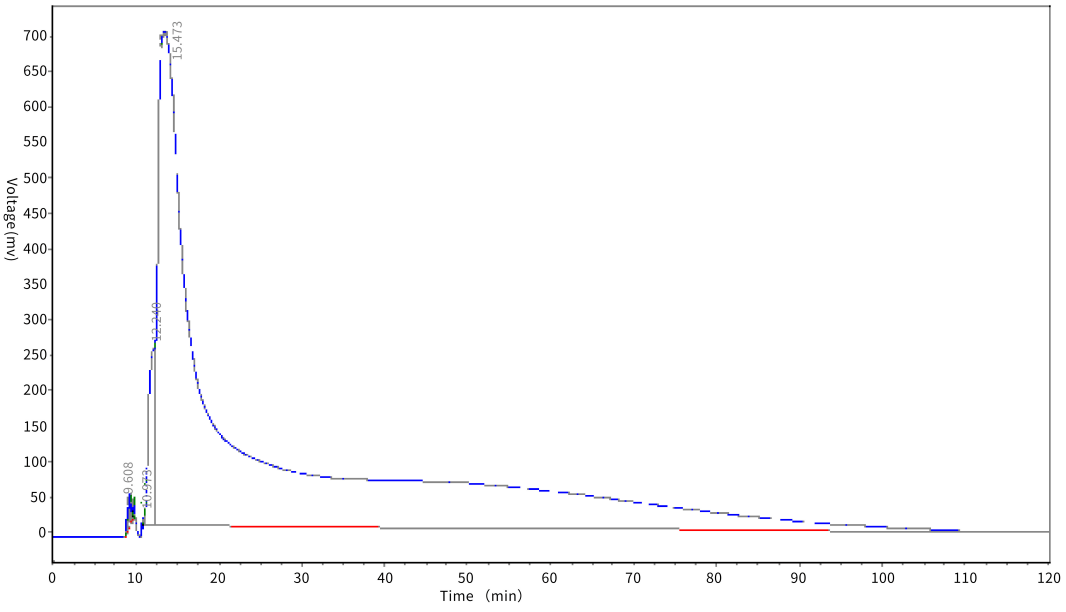

FIGURE 5
Antioxidation peptide desorption test of PSKB.

the desorption amount reached the maximum. This is mainly because, with the desorption time extension, the probability of contact between the desorption solution and the protein peptide of PSKB adsorbed on the resin is increased, more easily elution PSKB polypeptide from the resin. However, with the increase in desorption time, the resin reached saturation resolution, and

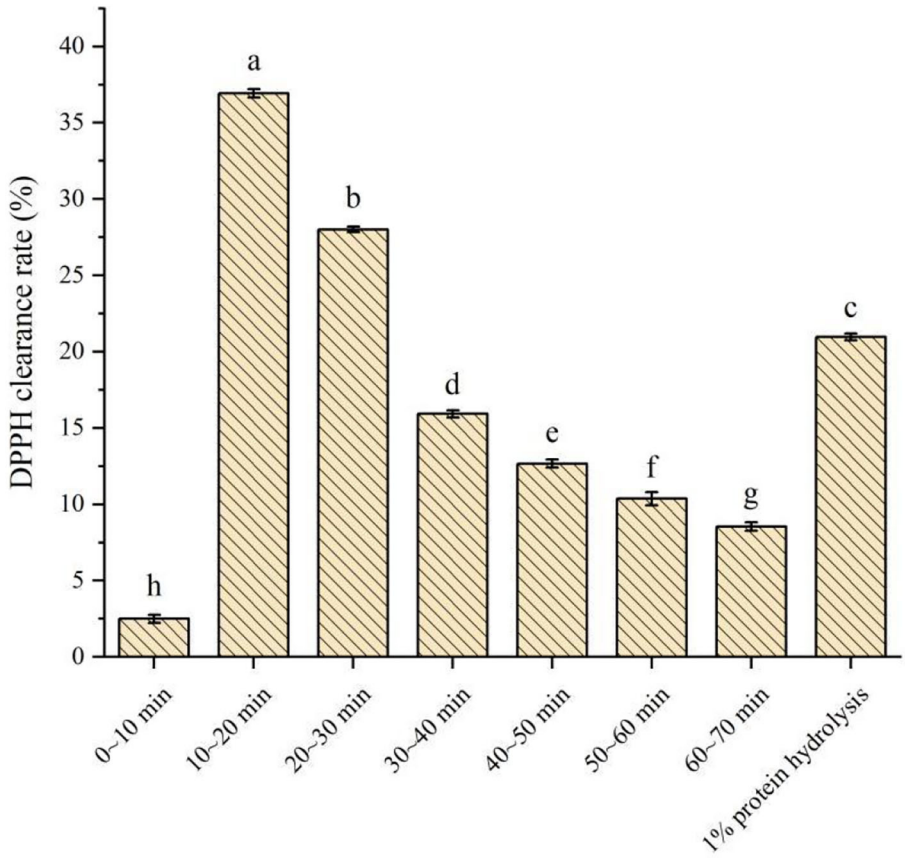

FIGURE 6
Determination of antioxidant capacity of purified components of PSKB protein peptide. There is a significant difference between different small letters ($P < 0.05$).

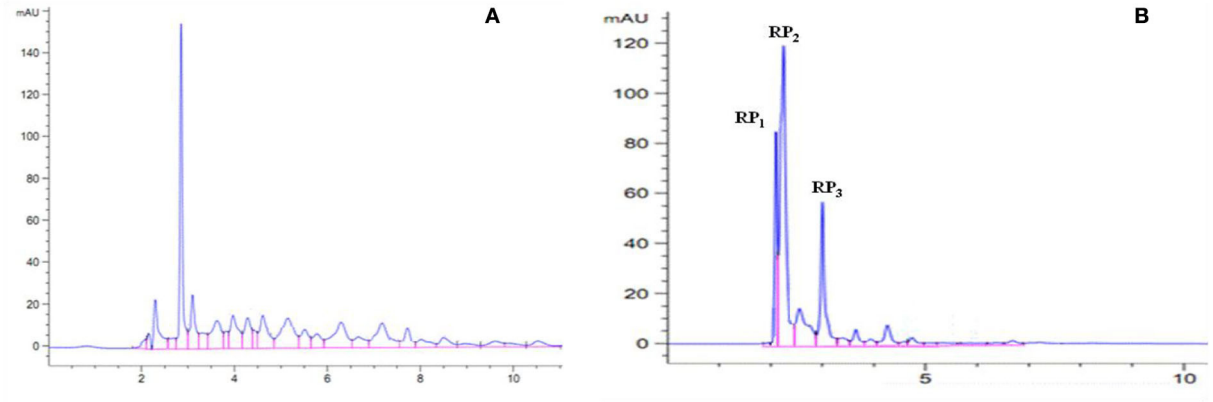

FIGURE 7
Composition of PSKB protein hydrolysate. **(A)** The composition of the unpurified PSKB antioxidant peptide. **(B)** The composition of PSKB antioxidant peptides purified by XAD-7HP resin.

its resolution did not increase with time. Therefore, the volume fraction of ethanol in the desorption solution was 60%, and the desorption for 30 min was a suitable analytical condition, at which time the desorption volume reached 5.23 ± 0.04 mg/ml.

Dynamic adsorption and desorption of macroporous resin

Selection of the best feeding speed and quantity

The effect of different flow rates and feeding amounts on the adsorption of protein peptides in speckled kidney beans is shown in Figure 4. It can be seen from Figure 4 that, when the control flow rate is the same and the feed amount is different, there is a significant difference among the components ($P < 0.05$). With the increase in feed amount, the adsorption capacity of XAD-7HP resin to PSKB protein peptide increases with the increase in PSKB and reaches the maximum when the feed amount is 40 ml, which is 9.92–12.19 mg/ml. Under the same feeding amount, the increase in the flow rate decreases the adsorption capacity of PSKB protein peptide, and the adsorption capacity decreases significantly when the flow rate is greater than 1.5 ml/min ($P < 0.05$). This is mainly because the feed flow rate directly determines the diffusion degree and binding law of the adsorbed substance to the resin. Although the sample can fully contact the resin when the flow rate is too low, it will prolong the industrial treatment time and increase production cost, and if the flow rate is too high, the adsorbed substance will be washed down before it combines with the resin in time, resulting in lower adsorption (26, 27). Therefore, the loading flow rate of 1 ml/min was selected as the appropriate flow rate, and the feeding volume of 40 ml was the optimal injection volume. At this point, the adsorption volume could reach 12.19 ± 0.15 mg/ml.

Dynamic desorption test of macroporous resin

The desorption effect of XAD-7HP macroporous adsorption resin on antioxidant peptides in PSKB is shown in Figure 5. It can be seen from Figure 5 that the protein peptide of PSKB began to be eluted at about 10 min, and then, the voltage value of the desorption curve rose rapidly and then dropped sharply, which may be due to the high content of PSKB protein peptide on the resin before desorption. At 25 min, the desorption curve began to show a flat state and the elution rate slowed down. This is mainly because the remaining polypeptide is mainly composed of relatively high molecular weight components. The components with relatively high molecular weight are less soluble in 60% ethanol solution and are not easily desorbed. Therefore, the desorption curve is relatively stable in the latter stage (28). With the extension of elution time, the content of protein peptide in the resin decreases, and the PSKB protein peptide adsorbed is desorbed at approximately 60 min, which indicates that XAD-7HP macroporous adsorption resin can adsorb PSKB antioxidant peptides, and the desorption is easier.

DPPH clearance rates of PSKB protein peptides with different desorption fluids

The DPPH clearance rates of different desorption fluids of PSKB protein peptides are shown in Figure 6. It can be seen from Figure 6 that the DPPH clearance rate of PSKB antioxidant peptide samples at different time periods was significantly different ($P < 0.05$). Among them, the antioxidant activity was very low at 0–10 min, and the DPPH clearance rate was only 2.5 ± 0.26%, indicating that the PSKB peptide with strong antioxidant activity was not removed at the first 10 min, which was consistent with the dynamic desorption results above. From 10 min, the collected liquid has certain activity, and the activity is concentrated in 10–30 min. Among them, the DPPH clearance rate of 10–20 min desorption solution was 36.93 ± 0.27% and that of 20–30 min desorption solution was 28.02 ± 0.18%. With the prolongation of the desorption time, the DPPH clearance rate of the desorbed solution gradually decreased, and the lowest was 8.54 ± 0.28% at 60–70 min. The DPPH clearance of 1% unpurified PSKB protein hydrolysate was 20.97 ± 0.22%, which was significantly lower than the antioxidant activity of PSKB peptides collected in 10–30 min ($P < 0.05$). This indicated that the antioxidant peptides of PSKB after purification by XAD-7HP resin were concentrated in the components with an elution time of 10–30 min when the antioxidant capacity of PSKB polypeptide was the highest.

Characterization of antioxidant peptide in PSKB

RP-HPLC analysis

After the enzymatic hydrolysis of proteins, peptides with different molecular weights were produced. The length of the peptide chain was closely related to the biological activity of peptides (29, 30). The wheat embryo polypeptide with a

TABLE 2 Antioxidant peptide scavenging DPPH clearance rate before and after separation.

Sample	DPPH Clearance rate/%
Before the separation	35.64 ± 0.56^{d}
After the separation	64.32 ± 0.56^{bc}
RP_1	56.26 ± 0.56^{b}
RP_2	66.42 ± 0.56^{c}
RP_3	78.57 ± 0.56^{a}

There is a significant difference between different small letters ($P < 0.05$).

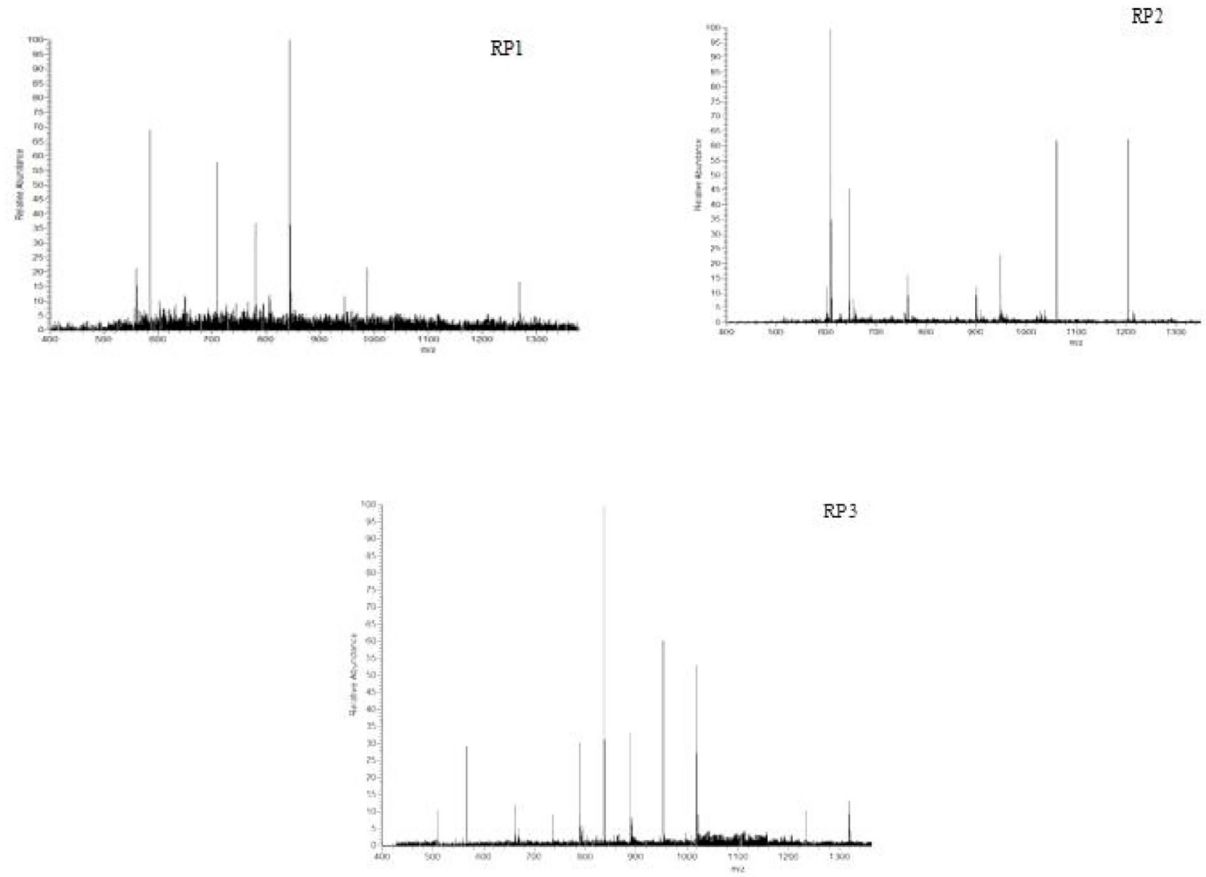

FIGURE 8
First-order structure mass spectrum of PSKB antioxidant peptides.

molecular weight of 180–2,000 Da has stronger antioxidant activity than the macromolecular polypeptide (31). Therefore, the PSKB protein hydrolysate was further analyzed using RP-HPLC in this study. The composition of PSKB protein hydrolysate is shown in Figure 7. Figure 7A shows the composition of the unpurified PSKB antioxidant peptides. It can be seen from Figure 7A that the components of the purified PSKB antioxidant peptide are relatively complex, and the peaks are dispersed in 2–12 min; Figure 7B shows the composition of PSKB antioxidant peptides purified by XAD-7HP resin. The peaks are concentrated in 2–7 min, with few miscellaneous peaks. The three most important peaks are RP_1, RP_2, and RP_3.

The purified PSKB antioxidant peptide was prepared into 1% protein solution, and the antioxidant activity of each component of the sample before and after purification was characterized, as shown in Table 2. It can be seen from Table 2 that there was a significant difference in the DPPH clearance rate of PSKB protein peptide before and after purification ($P < 0.05$). Among them, the DPPH clearance rate of PSKB protein peptide before purification was 35.64 ± 0.56%, and the DPPH clearance rate of PSKB protein peptide after purification was 64.32 ± 0.56%. After purification, there was also a significant difference in DPPH clearance among the components ($P < 0.05$). Among them, RP_3 had the highest antioxidant activity, and the DPPH clearance capacity was 78.57 ± 0.56%.

Amino acid sequencing of antioxidant peptides in PSKB

Mass spectrometry is a method for the separation and determination of samples according to the mass-to-charge ratio of ions (m/z), which can be qualitative and quantitative analysis (32–34), and has a high accuracy of qualitative analysis and quantitative analysis of the characteristics of the reliability. The mass spectrogram of antioxidant peptides RP_1, RP_2, and RP_3 in PSKB is shown in Figure 8. The

composition of antioxidant peptides was complex and consisted of a variety of peptides. Among them, the RP_1 showed that it consisted of 6 amino acid residues, the amino acid sequence was Phe-Leu-Val-Asp-Arg-Ile (confirm whether these amino acids are PSKB protein amino acids, the same as below) (Figure 8 RP_1), and the relative mass was 885.1; RP_2 was made up of 7 amino acid residues, the amino acid sequence was Phe-Leu-Val-Ala-Pro-Asp-Asp (Figure 8 RP_2), and the relative mass is 961.06; RP_3 was composed of 8 amino acid residues, the amino acid sequence is Lys-Asp- Arg-Val-Ile-Ser-Glu-Leu (Figure 8 RP_3), and the relative mass was 1,043.13.

Conclusion

In this study, PSKB protein hydrolysate was purified by macroporous adsorption resin, and suitable adsorption resin and adsorption conditions were selected. The results showed that XAD-7HP resin had a strong adsorption capacity, and the adsorption capacity was the strongest at pH 7, adsorption time was 24 h, the sample flow rate was 1 ml/min, and the feed rate was 40 ml. Desorption in 60% ethanol solution for 30 min was the best. The elution time of the enriched antioxidant peptide was concentrated at 10–30 min. Further purification by RP-HPLC yielded three antioxidants with high components, of which RP_3 had the strongest antioxidant activity, and the DPPH scavenging capacity reached $78.57 \pm 0.56\%$. The amino acid sequence was Lys-Asp-Arg-Val-Ile-Ser-Glu-Leu. This study provides a theoretical basis for the deep processing of PSKB and the development of antioxidant peptide drugs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization: NZ and FZ. Writing—original draft preparation: DL. Writing—review and editing: YY. Data curation: X-yX and NW. Formal analysis: Z-qM. Methodology: X-yX. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Analytical and functional approaches to assess the immunogenicity of gluten proteins

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Gluten proteins are the causative agents of celiac disease (CD), a lifelong and worldwide spread food intolerance, characterized by an autoimmune enteropathy. Gluten is a complex mixture of high homologous water-insoluble proteins, characterized by a high content of glutamine and proline amino acids that confers a marked resistance to degradation by gastrointestinal proteases. As a consequence of that, large peptides are released in the gut lumen with the potential to activate inflammatory T cells, in CD predisposed individuals. To date, several strategies aimed to detoxify gluten proteins or to develop immunomodulatory drugs to recover immune tolerance to gluten are under investigation. This review overviews the state of art of both analytical and functional methods currently used to assess the immunogenicity potential of gluten proteins from different cereal sources, including native raw seed flours and complex food products, as well as drug-treated samples. The analytical design to assess the content and profile of gluten immunogenic peptides, described herein, is based on the oral-gastrointestinal digestion (INFOGEST model) followed by extensive characterization of residual gluten peptides by proteomic and immunochemical analyses. These approaches include liquid chromatography–high-resolution mass spectrometry (LC-MS/MS) and R5/G12 competitive ELISA. Functional studies to assess the immune stimulatory capabilities of digested gluten peptides are based on gut mucosa T cells or peripheral blood cells obtained from CD volunteers after a short oral gluten challenge.

KEYWORDS

gluten immunogenic peptides (GIP), INFOGEST digestion method, gastrointestinal proteases, T-cell assays, short gluten challenge, celiac disease

1. Introduction

1.1. Epidemiology, diagnosis, and therapy

Celiac disease (CD) is a common immune-mediated enteropathy caused in genetically susceptible individuals by the consumption of gluten proteins, contained in wheat, barley, and rye cereals (1). The genetic predisposition is given by specific alleles of the HLA class II DQ genes that encode for the DQ2.5 (DQA1*05 and DQB1*02) or DQ8 (DQA1*03 and DQB1*0302) molecules (2). CD can arise at any age, with a global prevalence ranging from 0.5 to 2%, with an average of 1% in wheat consuming countries (1).

Clinically, CD may manifest with gastrointestinal symptoms, such as abdominal pains, diarrhea, anemia, and malabsorption syndromes (typical CD forms), or may present with extra-intestinal symptoms, such as skin lesions, ataxia, infertility, and frequent aphthous ulcerations (atypical CD forms). However, it may manifest symptomless, despite an evident intestinal mucosa inflammation (silent CD forms) (3). Diagnostic criteria include serological tests, such as the detection of anti-tissue transglutaminase antibodies (tTG-IgA) and anti-endomysium (EMA-IgA). Anti-deamidated gliadin peptides (DGP-IgG) are also included but have a specificity lower than tTG-IgA (average value: 97.9 and 87.81% for tTG and DPG, respectively) (4). Notwithstanding the high sensitivity and specificity of these serological tests, histological evaluation of duodenal mucosa after an esophagogastroduodenoscopy (EGD) is still considered the gold standard to make a diagnosis of CD, in particular in adults (5). Instead, in childhood, less invasive criteria are recommended according to recent guidelines of the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN). A diagnosis of CD can be performed in the presence of high titers of tTG-IgA (at least 10-fold the cutoff), and the presence of clear CD-associated symptoms, thus no more requiring the EGD (6).

Furthermore, approximately 10% of children with positive tTG-IgA show duodenal mucosa with normal villous architecture and low inflammation. These patients are considered to have a "potential" CD. Notably, most of the subjects included in this group are at high risk for developing a typical CD later in life (7, 8).

To date, no drug is available for celiac disease patients, and the only safe and efficient treatment is the gluten-free diet (GFD) which is to be strictly followed for life. Gluten removal from the diet results in symptom recovery and small intestinal lesion resolution in the great majority of dietary compliant patients (9, 10).

1.2. Genomic and chemistry of gluten proteins

Wheat is one of the world's major cultivated and consumed food crops along with rice and maize.[1] It is an essential staple food because of its important nutritional characteristics, technological properties, and long shelf life (11). Bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) are the most important varieties currently grown worldwide. Bread wheat accounts for 95% of global wheat production and is used for the manufacturing of bakery foods (i.e., bread, cakes, and cookies). Durum wheat is primarily used for the production of pasta. The modern cultivated wheat has passed a long evolution that took place for more than 10,000 years starting with polyploidization events between *Triticum urartu* (AA genome) and an *Aegilops speltoides*-related species (BB genome) and resulting in *Triticum turgidum ssp. dicoccoides,* and between *T. turgidum ssp. durum* (AABB genome) and *Aegilops tauschii* (DD genome), forming the modern hexaploid bread wheat (AABBDD genome) (12). Historically, the large diffusion of hexaploid and tetraploid wheat cultivars was due to their adaptability and high yield potential, as well as to the capacity of gluten proteins to confer viscoelastic properties that allow the dough to be processed into food products (13, 14).

Gluten proteins account for about 85–90% of wheat protein fraction, while the remainder is constituted by the water-soluble albumin and globulin proteins. According to Osborne's classification, gluten proteins are divided into alcohol-soluble monomeric gliadin and alcohol-insoluble polymeric glutenin. The gliadins are further classified into α-, γ-, and ω-gliadin fractions with molecular mass ranging from 28 to 55 kDa. Gliadins are important in determining the extensibility characteristics of dough. During the mixing of flour with water, gliadins take part in the development of the gluten network through the formation of intermolecular hydrogen bonds and hydrophobic bonds between non-polar amino acid side chains, which also interact with the flour lipids (15–17).

Glutenins consist of disulfide-linked proteins with molecular mass ranging from approximately 60,000 to more than 10 million. Following the reduction of inter-chain and intra-chain disulfide bonds, glutenins are divided into high molecular weight (70–90 kDa) glutenin subunits (HMW-GS) and low molecular weight (20–45 kDa) glutenin subunits (LMW-GS), which represent the 40 and 60% of glutenin composition, respectively (14). Structurally, HMW-GS consists of three domains characterized by a non-repetitive N-terminal domain and a C-terminal domain, which contain cysteine residues, that produce inter-chain and intra-chain disulfide bonds, and a repetitive central domain that promotes

1 http://faostat.fao.org/

intermolecular hydrogen bonding (18). The number of cysteine residues of HMW-GS affects the rheological properties of the dough, since disulfide bonds determine gluten extensibility and elasticity through the formation of larger glutenin aggregates (19).

1.3. Proteolytic resistance of gluten proteins and role in CD immunopathogenesis

Gluten proteins are poorly affected by proteases occurring in the gastric and intestinal tract (GI), including the enzymes of the small intestinal brush-border membrane. The marked resistance to proteolysis is due to the high percentage of proline and glutamine residues and to the lack of specific GI proteases with cleavage-site activity for intra-chain post-proline residues (20). As a consequence, large gluten oligopeptides reach the gut lumen, triggering adverse immune responses in patients with CD (21). To date, several gluten peptides resistant to digestive enzymes and harboring immune toxic sequences have been identified (22, 23). Two peptides derived from α- and γ-gliadin are particularly relevant for the activation of intestinal T cells: the 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) from α-2-gliadins and the 26-mer (FLQPQQPFPQQPQQPYPQ QPQQPFPQ) from γ-gliadins. These peptides contain shorter sequences that, after TG2-mediated deamination, bind HLA-DQ2 and HLA-DQ8 molecules and stimulate T cells in patients with CD (11, 23–25).

Among protease-resistant sequences, peptide 31–55 from α-gliadins (LGQQQPFPPQQPYPQPQPFPSQQPY) has been found to contribute to the onset and the development of CD (26, 27). Its shorter peptide, 31–43 (LGQQQPFPPQQPY) elicits an innate immune response in professional antigen presenting cells (monocytes, macrophages, and dendritic cells) and expression of stress signals on intestinal epithelial cells (28–30).

Several studies have demonstrated that intestinal CD4 + T cells have a key role in inflammatory responses in CD (25, 31–33), and that all gluten protein families contain peptides able to stimulate T-cell response (23). The activation of these T cells, resident mainly in the lamina propria compartment, triggers an inflammatory cascade mediated by interferon-γ (INF-γ) and interleukin-21 (IL-21). These gluten-specific CD4 + T cells can be isolated from CD intestinal mucosa and *in vitro* expanded, thus representing an important source for bioassays useful to

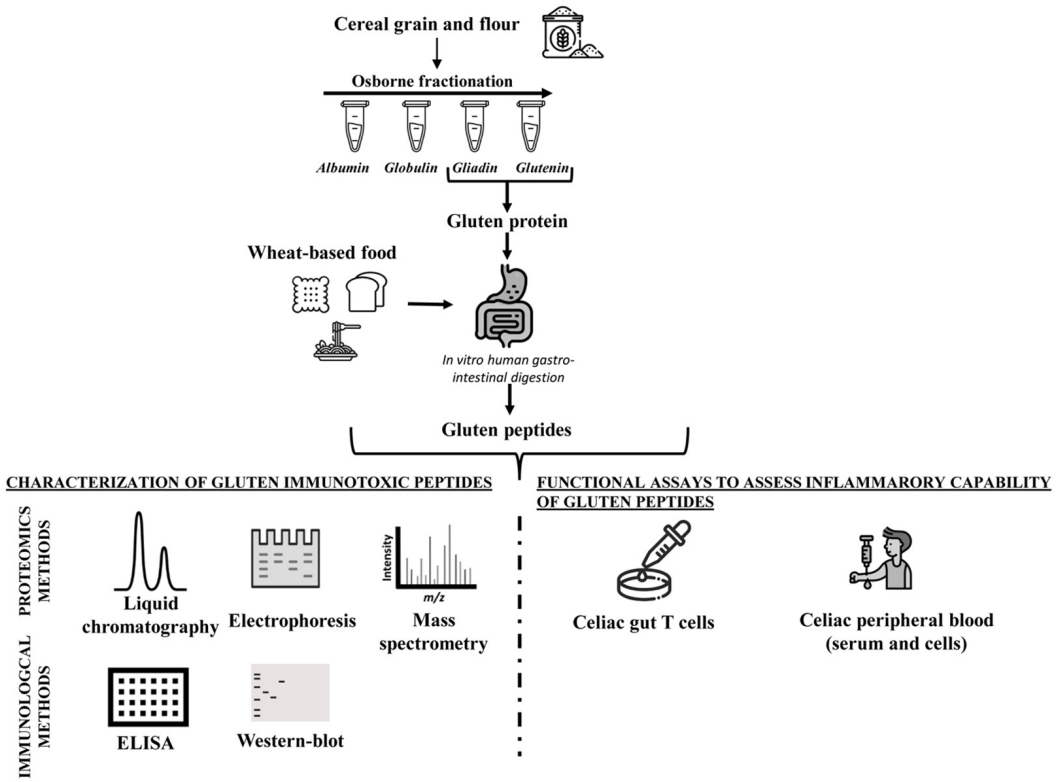

FIGURE 1
Workflow of analytical and functional approaches to assess the amount and immunogenicity of gluten proteins in raw seed flour and complex food matrices.

assess pathogenesis and to validate novel therapies for CD management, as reported below in more detail.

2. Analytical assessment of gluten peptides immunotoxicity

2.1. *In vitro* enzymatic digestion of gluten proteins

Understanding the outcome of the gluten proteins in the human digestive system (bioavailability) is an area of interest being these dietary proteins are the causative factor of CD.

Food digestion may be assessed by both *in vivo* (human or animal) and *in vitro* methods, although the latter procedures are preferred in research related to food and nutrition because of their speed, cost, and reproducibility compared to *in vivo* studies (34). Models of *in vitro* digestion have been proposed since the 1990s to study the metabolism of food components along with the gastrointestinal tract. Regarding the CD studies, early *in vitro* digestion experiments were limited to pepsin and trypsin proteolysis for mimicking the gastric and pancreatic stages, respectively (25). Shan et al. (35) used a more accurate *in vitro* digestion consisting of a pool of duodenal enzymes (trypsin, chymotrypsin, elastase, and carboxypeptidase) along with brush-border membrane enzymes to mimic the duodenal and intestinal digestion, respectively. With this strategy, the authors identify the well-known 33-mer from α-gliadin and 26-mer from γ-gliadin. A separate study employed the *in vitro* methods to demonstrate the digestive protease resistance of a 25-mer from γ-gliadin (26). Similarly, Gianfrani et al. assessed the immunogenicity of diploid wheat *Triticum monococcum* after *in vitro* digestion (22). The authors found that monococcum gluten proteins are more susceptible to the digestion of GI proteases compared to those from hexaploidy wheat leading to an overall lower immunotoxicity on T cells (22).

The *in vitro* digestive models have been improved extensively over the years. Noteworthy, an *in vitro* model has been successfully designed to reproduce the physiological process as close as possible to the *in vivo* one (36, 37). The INFOGEST digestion procedure consists of three consecutive phases: oral, gastric, and intestinal (small intestine), which include all enzymes, bile salts, digestive fluids (saliva, gastric, and intestinal juices), and incubation times of physiological condition. Following a pilot publication in 2016, the INFOGEST model is the most used *in vitro* digestion method throughout the research community aimed to simulate the behavior of foods within the human gastrointestinal tract (38). The INFOGEST method was also applied to the study of the immunogenic potential of peptides from wheat products, tree nuts, and peanuts, which are all resistant to gastrointestinal digestion (39–42).

2.2. Detection of gluten peptides after the *in vitro* gastroduodenal digestion

Because of the physicochemical properties of gluten proteins, there are, to date, few approaches validated to detect gluten toxic peptides present in different food matrices. In general, the main analytical methods employed to study the gluten protein are electrophoresis (43–46), reversed-phase

TABLE 1 Strengths and limitations of common analytical methods for the detection of gluten proteins/peptides.

Methods	Strengths	Limitations	References
ELISA	• Commercially available. • User friendly. • Rapid analysis. • Quantitative analysis of intact gluten.	• Cross-reactivity of antibodies. • Lack of unique kit procedures (different buffers, concentration of antibodies, extraction protocols, etc.). • Lack of certified reference materials. • Over- or under-estimation of the gluten content.	(55–61)
Western-blot	• Identification of more reactive proteins or peptides. • Support the ELISA test.	• Less sensitive compared to ELISA assay. • Problems relating to protein quantification.	(62, 63)
RP-HPLC	• Protein polymorphism of gluten. • Evaluation of gliadin/glutenin ratio.	• Need to couple to mass spectrometry for protein/peptide identification.	(47–50)
Electrophoresis	• Detect small variations in protein size. • Qualitative characterization of proteins/peptides.	• Difficult to separate gluten proteins having similar molecular weights. • Measure of molecular weights are not accurate. • Need to proteomic based analysis for protein sequencing.	(43–46)
Mass spectrometry	• Highly sensitive. • Accurate detection of proteins/peptides. • Quantitative analysis.	• Require experienced staff. • Expensive equipment. • Need certified reference materials for accurate quantitation. • Incomplete available databases.	(51–54)

(RP)-HPLC, size-exclusion HPLC (SE-HPLC) (47–50), mass spectrometry (MS) (51–54), enzyme-linked immunosorbent assay (ELISA) (55–61), and Western blot analysis (62, 63). The most used assays to detect gluten in food are based on immunological tests (such as ELISA kits) or proteomic approaches involving MS. However, none of them is considered universally adequate for the complete detection of gluten in food matrices. All technologies present advantages and disadvantages depending on specific tasks (**Figure 1** and **Table 1**).

2.2.1. Immunological method: ELISA assay

Immunological methods for gluten protein detection are the most widespread methodologies available so far. ELISAs are the most frequently used by food manufacturers or control authorities to assess the gluten content in food products. Several ELISA kits are available in the market; however, for some of them, the composition of extraction buffers, the nature of the antibodies, and the calibration material are not declared. Furthermore, some kits are specific for detecting wheat gluten, while others detect also rye and barley prolamins.

The majority of commercially available kits are based on the R5 monoclonal antibody, which is only validated by Codex Alimentarius legislation, while other tests are based on different antibodies, such as the G12 and α-20 monoclonal antibodies. Most of them detect only gliadin proteins, excluding the glutenin fraction. Usually, the gluten content is calculated by multiplying the prolamin content, which represents 50% of total wheat protein by a factor of 2. However, several issues have been reported for ELISA measurements (**Table 1**) that can result in an overestimation or underestimation of gluten amount since the ratio of prolamin/glutenin depends on the wheat variety and the type of food processing (55–61). Due to this overestimation or underestimation of the gluten content by ELISA, alternative analytical methods are urgently needed (64).

An important issue is the quantitative accuracy of processed foods. Bruins Slot et al. evaluated the accuracy and reproducibility of 14 ELISA kits for gluten detection in several food matrices with different degrees of complexity (55). The authors showed that there is no single ELISA method that can accurately detect and quantify gluten in all the different matrices. In a recent study, the gluten content resulted was overestimated, up to six times, in breakfast food products containing rye, or underestimated, up to seven times, in barley-containing food, thus representing a serious health risk for people with CD (59). Technological treatment may affect the limit of detection of gluten by ELISA. For instance, the exposure of wheat to microwave technology was claimed as an efficient method to remove the antigenic properties of gluten. This evidence was based on R5 ELISA, which detected the reduction of immunogenicity to over 99% (65) after microwave treatment. However, a separate study showed that the undetectably of gluten by the R5 antibody was actually due to a failure to extract the gluten proteins from the microwave-treated flour. In fact, gluten was found to be insoluble in the extraction buffers because of the microwave heat treatment. Conversely, after enzymatic hydrolysis, gluten peptides were easily extracted and analyzed by G12 ELISA and mass spectrometry analysis, confirming that microwave treatment does not abolish the immunogenicity of gluten proteins (66).

2.2.2. Proteomic methods: MS-based technologies

Mass spectrometry (MS)-based proteomic offers an alternative method to detect the gluten content and/or to confirm ELISA measurements. This method represents a complementary way that can provide specific information, such as peptide identification and relative peptide abundance. Furthermore, the analysis of intact proteins by MS has been used to develop low-resolution fingerprints for grain source identification for the implementation of gluten-free labeling regulations (67). Several approaches for the quantitation of immunogenic gluten peptides by targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) were published in recent years (68). A bottom-up proteomic approach, which is based on the enzymatic digestion of proteins and identification of the resulting peptides by LC-MS/MS, has been applied to gluten analysis and allowed to select specific peptides to be used as markers for the detection of gluten in complex food matrices. Sealey-Voyksner et al. used the LC-MS/MS method to select six CD-immunogenic wheat gluten peptides in native and processed food samples (51). Similarly, Fiedler et al. identified two wheat marker peptides from α-gliadins, to be used to detect wheat contamination in oats (67). Nine CD-immunogenic peptides from α-gliadins were quantitated by van den Broeck et al. using LC-MS/MS (52). Schalk et al. (64) were the first to establish a link between concentrations of 16 wheat marker peptides and gluten contents using a targeted-quantitative LC-MS/MS method. Gluten contents expressed as the sum of all determined protein types were comparable to those analyzed by RP-HPLC and R5 ELISA in wheat starches with high gluten contents.

More recently, Li et al. identified and quantified gluten peptides from wheat, rye, barley, and oats in different food products (53). Several peptide fragments, including immunotoxic peptides, were found in breakfast cereals, breakfast bars, and powdered drinks, such as the peptide RPLFQLVQGQGIIQPQQPAQLEVIR from γ-gliadin. This sequence comprises a known T-cell epitope, VQGQGIIQPQQPAQL. Another stimulatory peptide, QQPG QGQQPEQGQQPGQGQQGYYPTSPQQPGQGK, derived from the high molecular weight glutenin subunit, was found in both breakfast cereals and bars. Again, the SDQPQQSFPQQPQQK peptide that contained the immunotoxic sequence belonging to γ1-secalin was identified in gliadin/avenin-like seed protein in the breakfast cereals.

A comparison to ELISA showed similar results for the wheat-containing products, but discrepancies were noted for the barley-containing products. The authors highlighted the high relevance of mass spectrometry as a reliable tool for the detection of gluten proteins in food and, particularly, for extensive heat-treated foods (53). In conclusion, these studies have proven that proteomics is a useful strategy both for the detection of CD-toxic peptides and the quantification of gluten content in processed food products (53).

2.2.3. The INFOGEST protocol for digestion of gluten proteins in complex food matrices

To date, most of the studies on gluten proteins were conducted without taking into account the role of extensive gastrointestinal digestion, consequently obtaining results with little physiological relevance (21, 22, 26, 35, 69). The gastrointestinal digestion employing the INFOGEST model has recently been applied to study the bioavailability of immunotoxic gluten peptides in complex food matrices (70–73). Mamone et al. (74), investigated the metabolic fate of pasta proteins upon *in vitro* simulated gastrointestinal digestion including an additional step with porcine intestinal BBM hydrolases that mimics peptide degradation at the level of the jejunum. The authors demonstrated that bread and pasta gluten proteins were completely hydrolyzed during *in vitro* digestion. Ogilvie et al. (70) used proteomics and an immunochemical approach to assess the immunogenicity of gluten residues in wheat-based food during simulated *in vitro* digestion. The authors demonstrated that gluten toxic peptides were rapidly released from the food matrix during the intestinal phase.

Recently, Boukid et al. successfully combined the INFOGEST method and target proteomic analysis to detect and quantify gluten peptides relevant to CD pathogenesis (54). The authors demonstrated that 227 peptides were released after gastrointestinal digestion, and nine peptides harbored CD-immunogenic sequences. Interestingly, Olgivie et al. explored the kinetic of immunogenic peptides released from the sourdough bread upon INFOGEST digestion, using a quantitative proteomics analysis and ELISA. The authors demonstrated that although sourdough fermentation affected the degree of gluten hydrolysis, no difference in the immunogenicity profile was shown in the digested product, compared with yeast-fermented bread (73).

3. Glutenases: Gluten-degrading enzymes as a new therapeutic frontier in CD

Over the last few years, several strategies have been proposed to detoxify or decrease the immunogenicity of wheat-based food (75). An interesting approach is based on the addition of proteases that are able to hydrolyze immunotoxic sequences. For instance, the use of prolyl endopeptidase (PEP) from *Aspergillus niger* during the brewing process represents a useful method to produce gluten-free barley-based beers (76).

Food-grade protease-based strategy seems efficient in detoxifying moderate quantities of dietary gluten proteins. These glutenases have to degrade gluten immunogenic peptides prevalently in the stomach, thus avoiding any stimulation of the duodenal immune system. Endopeptidases, produced by various plants, bacteria, or fungi, are currently under investigation for their capability to degrade the proline/glutamine-rich gluten peptides in the gastric and upper intestinal tracts (77–82). Shan et al. have shown the capability of bacteria prolyl endopeptidases from *Flavobacterium meningosepticum* (FM-PEP), *Sphingomonas capsulate* (SC-PEP or ALV002), and *Myxococcus xanthus* (MX-PEP) in degrading successfully immunogenic gluten amino acid sequences (78). In detail, the gluten-degrading properties of FM-PEP, SC-PEP, and MX-PEP (stable and active at pH 6–7) were evaluated using two substrates, the 33-mer and smaller sequence, PQPQLPYPQPQLP. The results of this study showed that all PEPs preferentially cleave P-Q bonds that are usually found in immunogenic gluten peptides and that are resistant to gastrointestinal proteases. Edens et al. identified glutenase from

TABLE 2 *In vitro* T-cell-based assay to evaluate immunogenicity of gluten proteins and novel strategies to treat celiac disease (CD).

Purpose of the research	Outcomes by *in vitro* functional T-cell assays	References
Elucidation of immunopathogenic mechanisms of celiac disease	Generation and expansion of gliadin-specific T-cells and clones from celiac small intestinal mucosa.	(88–90)
	Characterization of tissue-resident memory T-cells in celiac disease.	(85, 86)
	Identification of immunogenic gluten peptides.	(23, 25, 35, 84)
Efficacy evaluation of novel enzymatic strategies to treat celiac disease in pre-clinical studies	Gluten peptide transamidation (mTG-transamidation) of wheat flour inhibits the response to gliadin.	(87, 96)
	Gluten proteolysis at gastric condition by the endoproteases «glutenases": (1) EP-B2/PEP (ALV003/Latiglutenase in clinical trial) (2) Kuma062 (3) E40.	(77, 81, 82, 91, 92)
Searching of less immunogenic wheat species for CD prevention	Assessment of the lower immunostimulatory activity of *Triticum monococcum*.	(22, 97, 98)

the fungus *A. niger* (AN-PEP or ASP) that efficiently hydrolyzed gluten and abolish T-cell stimulatory properties (79). Unlike SC-PEP, FM-PEP, and MX-PEP, which have an optimum pH activity between 7.0 and 8.0, AN-PEP is active at lower pH values between pH 4.0 and 5.0 explaining its functionality in the stomach and allowing early-digestion of gluten proteins.

Again, a mixture of aspergillopepsin from *A. niger* and DPP-IV from *Aspergillus oryzae* was used to hydrolyze small amounts of gluten *in vitro* (80). Other studies showed the proteolytic abilities of a glutamine-specific cysteine endoprotease derived from seeds of germinating barley (EP-B2). Different from PEP, EP-B2 has cleavage-site specificity for post-glutamine residues. Gass et al. proposed a new strategy based on the combination of EP-B2 and PEP enzymes, both with gastric activity and complementary substrate specificity (81). Both *in vitro* and *in vivo* functional experiments demonstrated that these enzymes synergically hydrolyzed gluten proteins, reducing their immunogenicity (81).

Recently, a novel microbial endopeptidase expressed in recombinant actinomycete *S. lividans* TK24, named the endoprotease-40 (E40), has been demonstrated to be a successful enzyme in detoxifying gluten proteins during the transit in the stomach, suggesting its use for oral enzymatic therapy (OET) in CD. The authors, using both proteomic and immunochemical approaches (SDS-PAGE, RP-HPLC, LC-MS/MS, and ELISA), showed that E40 efficiently hydrolyzed the most immunogenic 33-mer, as well as the whole gliadin proteins. Furthermore, the analysis of T lymphocytes from duodenal biopsies of patients with CD indicated a markedly reduced production of IFN-γ when exposed to gluten digested with E40, as described below (77, 82).

4. Functional approaches to assess the immunostimulatory properties of gluten peptides: Celiac T-cell-based bioassays

As mentioned previously, the peculiar amino acid composition of wheat gluten proteins, and homologous proteins of rye and barley, strongly hampers the degradation by gastrointestinal proteases, with a release of large peptides with immunogenic potential for T cells in the gut lumen of subjects with CD (24, 35, 83, 84). These partially digested gluten peptides are deamidated by the tTG enzyme highly expressed in the intestinal mucosa of patients with CD. This reaction increases the binding affinity of gluten peptides to the CD-associated HLA-DQ2/DQ8 molecules. The peptide-HLA complex is recognized by CD4 + T cells triggering an inflammatory reaction that leads to a profound remodeling of the intestinal mucosa tissue (24, 35, 83, 84). Of note, the gluten-reactive CD4 + T cells are exclusively found in the gut mucosa of patients with CD and persist in the celiac intestinal mucosa for decades as memory cells (85, 86).

For their high disease specificity, these memory T cells reactive to gluten peptides have been largely used in several studies aimed to elucidate the CD immunopathogenic mechanisms and to validate gluten detoxifying and immunomodulatory strategies. Through painstaking work, gluten-specific T cells can be isolated from gut mucosa biopsies of patients with CD and *in vitro* expanded as polyclonal CD3 + cell lines or cell clones, as reported in detail elsewhere (87–90). In brief, intestinal cells are isolated from jejunal explants by enzymatic digestion with collagenase and *in vitro* stimulated with autologous feeder cells and an enzymatic digest of gluten/gliadin proteins. The T-cell cultures are further expanded by repeated stimulations with allogenic feeders, the mitogen phytohemagglutinin, and growth factors IL-2 and IL-15. Usually, the T-cell line antigen specificity is assessed by the detection of IFN-γ, the dominant cytokine in CD pathogenesis, by enzyme-linked immunosorbent assay (ELISA or ELISPOT) and T-cell proliferation assays, as reported in the many studies mentioned earlier. Examples of studies using intestinal T-cell assays in CD are presented in **Table 2**.

4.1. Identification of gluten immunogenic peptides

For many years, studies aiming to identify the complete repertoire of gluten epitopes were supported by the use of intestinal T-cell lines and T-cell clones generated from the jejunal mucosa of patients with CD (25, 35). These studies demonstrated a great heterogeneity of immunogenic gluten peptides. T-cell epitopes were identified in all wheat gluten proteins (α-, γ-, ω-gliadins, high molecular weight-HMW, and low molecular weight-LMW glutenins) and in homologous proteins from hordeins and secalins (35). An update of the known HLA-DQ-restricted gluten T-cell epitopes, responsible for the induction and/or maintenance of intestinal mucosa inflammation in patients with CD, has been reported (23). The definition of the complete repertoire of gluten immunogenic peptides is a pivotal step for the development of alternative strategies to the gluten-free diet (GFD) for the treatment of CD, as it provides a useful tool to assess whether these novel strategies under investigation succeed in reducing the stimulatory capability of gluten epitopes known as the most dominant in patients with CD.

4.2. Analysis of glutenase capability to detoxify gluten peptides

Before moving on to *in vivo* clinical studies and subsequent clinical trials, the efficacy of potential detoxification strategies of gluten proteins from different foodstuff sources has been often

assessed using different gluten-reactive T cell lines (G-TCLs) characterized by a large repertoire of reactivity toward gluten peptides (25). Among the several alternative strategies under investigation for CD treatment, great attention is currently paid to enzymatic approaches intended to fastly digest gluten proteins that work at the same low pH occurring in the stomach. As extensively described earlier, many glutenases, produced by bacteria, fungi, and plants, as barley, or engineered recombinant proteins, have been investigated as possible drug therapy for CD alternative to GFD, or as oral supplements to support GFD and protect celiac diseases from unintentional gluten exposures (77, 81, 91, 92). Glutenases, thanks to their capability in cleaving the proline-rich and glutamine-rich gluten sequences, represent a promising drug for oral therapy in CD (93). Pioneering studies from Khosla et al. assayed a combination of two proline-specific endopeptidase (EP-B2/PEP) that operates under gastric conditions is able to detoxify gluten within 10 min of simulated duodenal conditions, as proved by chromatographic analysis and *in vitro* celiac T cells. In particular, they observed that polyclonal G-TCLs, reactive to a panel of known gluten epitopes, did not proliferate in response to all tested concentrations of digested and enzyme-treated gluten (81, 91). Following these encouraging *in vitro* findings, clinical studies have been performed to assess the efficacy of EP-B2 combined with an endopeptidase from *Sphingomonas capsulata* (SC-PEP) known as ALV003/Latiglutenase (94, 95).

Wolf et al. reported *in vitro* celiac T-cell studies to assess the glutenasic activity of a computationally designed kumamolisin endopeptidase known as Kuma030 or TAK062 (92). This recombinant protein recognizes tripeptide sequences in the immunogenic regions of gliadin or homologous proteins in barley and rye. A decreased immunostimulatory potential of gliadin proteins treated with Kuma030 was measured in G-TCLs generated from five patients with CD, as shown by IFN-γ and cell proliferation readouts (92).

Intestinal T-cell lines were also used in a functional assay to verify the reduced immunostimulatory capacity of gluten proteins digested with the E40, a promising protease of microbial origin (77) (**Figure 2**). A recent study from the same research group showed that E40 was efficient in hydrolyzing gluten epitopes in complex food matrices, such as pasta, bread, and wheat beer samples (82).

4.3. Validation of gluten detoxifying strategies aimed to obtain gluten-free wheat flour

An enzymatic strategy to reduce gluten load consists of wheat flour pre-treatment with microbial transglutaminase (mTG) and acyl-acceptor molecules (L-lysine, glycine ethyl ester, and hydroxylamine). This approach consists of inducing the transamidation of specific glutamine residues, thus blocking their deamidation by tTG (87, 96). As the deamidation is a crucial step for the immunotoxicity of gluten proteins, it has been demonstrated that this biochemical strategy strongly reduces the capability of transamidated gluten to stimulate G-TCLs obtained from 12 patients with CD and highly reactive to untreated gliadin (87). The transamidation reaction specifically masks the gluten epitopes rendering them not able to activate pro-inflammatory T cells. Of note, this approach does not alter the integrity of the gluten protein structure, as well as its viscoelasticity and technological properties.

4.4. Searching for safer wheat species for CD prevention

Although the minimal level of gluten consumption that is safe for patients with celiac disease has not yet been established, a number of studies demonstrated that the magnitude of inflammatory T-cell response to gluten strictly depends on the gluten peptide concentration that is loaded on HLA-DQ2/8 restriction molecules. As a consequence, great attention was paid to the research of cereal species with a low content of immunotoxic sequences, with the aim of limiting dietary exposure to gluten in the general population with genetic CD risk (11). As extensively described earlier, another crucial factor determining the immunostimulatory potentiality of gluten proteins is the digestibility by gastrointestinal proteases, strictly dependent on primary protein structure. Recent studies reported that diploid wheat species, such as *T. monococcum (T. monococcum)*, contain gluten proteins more susceptible to the digestion of gastro-pancreatic and brush-border membrane (BBM) enzymes if compared to those from hexaploid common wheat *T. aestivum* (97). For this peculiarity, the diploid wheat species have been tested *in vitro* to ascertain their low immunostimulatory activity on TCLs of patients with CD (22, 97). As convincingly demonstrated by functional T-cell assays, the extensive *in vitro* gastrointestinal digestion of monococcum gluten proteins drastically reduced the capability to elicit IFN-γ production and cell proliferation compared to hexaploidy wheat gluten (**Figure 3**) (97, 98). These *in vitro* studies were further corroborated by *in vivo* short oral challenge as reported below. All these data obtained from functional T-cell assays, combined with proteomic analysis, make diploid wheat crop species among the most suitable candidates for the prevention of CD in people at high risk of developing the disease due to genetic and familiar predisposition.

5. Short-term (3 days) oral gluten challenge

The entirety of limitations connected to a lifelong gluten-free diet (GFD), and the low percentage of patients

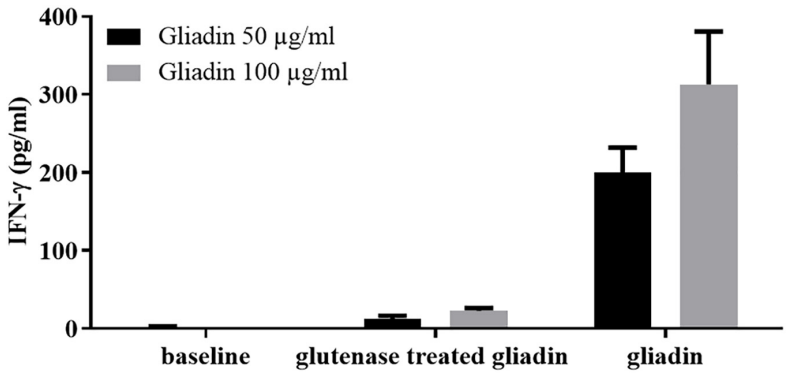

FIGURE 2
The digestion with the endoproteases "glutenases" strongly reduced the immunostimulatory capacity of gliadin on celiac intestinal T cells. INF-γ responses of intestinal T-cell line obtained from a celiac patient were evaluated after incubation of gliadin proteins both native or treated with glutenase E40 (77, 82). Gliadin was purified from hexaploid wheat flour and incubated with E40 at pH 4, 37°C for 120 min (enzyme:substrate ratio 1:20). Gliadin enzymatic digest was next deamidated by tTG treatment. IFN-γ production was measured by ELISA in cell supernatants collected after 48 hours of incubation. Native and E40-treated gliadin were assayed on T cells at 50 and 100 µg/ml.

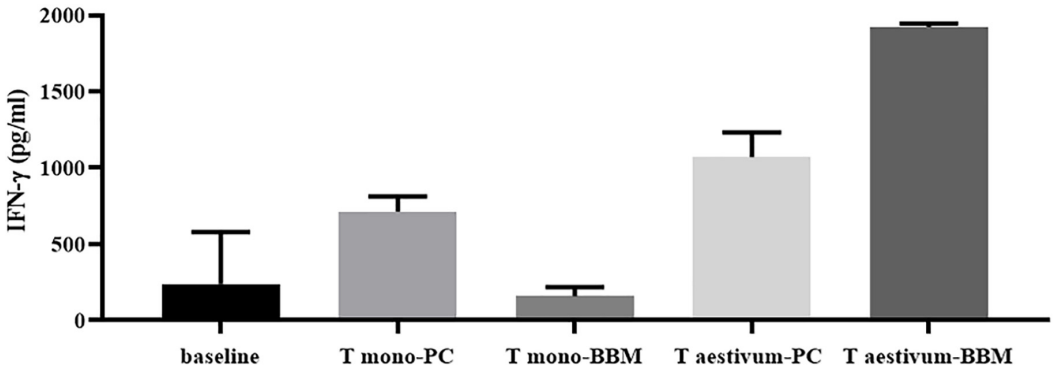

FIGURE 3
Marked reduction of specific T-cell response to gliadin from diploid wheat (*monococcum*) after the extensive *in vitro* gastro-intestinal digestion including brush-border membrane enzymes (BBM). IFN-γ responses of intestinal T-cell line obtained from a celiac patient were evaluated after stimulation with PC- (pepsin-chymotrypsin) or BBM- (pepsin-chymotrypsin-elastase and brush-border membrane enzymes) gliadin digests from *Triticum aestivum* and *Triticum monococcum* wheats (22). Gliadin samples were deamidated by tTG treatment and assayed at 50 µg/ml. IFN-γ production was evaluated by ELISA in cell supernatants after 48 hours of incubation.

with CD compliant with dietary therapy, mainly during adolescence, prompted the scientific community to search for an alternative treatment for CD (99, 100). Simultaneously with the development of therapeutic strategies, it became necessary to have functional tools to rapidly test their efficacy before moving forward to clinical studies. For decades, the evaluation of gut mucosa damage, in terms of villous eight and crypt depth (Vh/Cd ratio) and intraepithelial lymphocytes (IEL) infiltration, after a long (from 14 to 90 days) gluten consumption has been the gold-standard approach to assessing the validity of novel therapies (101–105). However, this procedure required an endoscopy and is invasive for most patients with CD; furthermore, the long-term gluten exposure, needed to induce intestinal damage, makes this approach too demanding to evaluate the effects of novel therapies.

In addition, the frequent risk of histological evaluation pitfalls and other possible causes, not gluten-dependent inducing mucosa villi atrophy, cannot be excluded. In the early 2,000 s, it developed a minimally invasive strategy to detect gluten-specific T cells in the peripheral blood of GFD-compliant patients with CD who underwent 3 days of clinically controlled gluten consumption, around 6–16 gr gluten/die supplied as bread slides, sandwiches, or cookies (106–109). Basically, gluten primed CD4 + T lymphocytes, usually undetectable (or low detectable) in the peripheral blood of treated patients with CD, are transiently mobilized in the blood and became detectable after 6 days of gluten consumption revealed by sensitive assays, mainly enzyme-linked immunospot (ELISPOT) assay detecting IFN-γ-producing T cells (106–109), and by tetramers technology at flow cytometry or by tetramer assays (110). Over

the past few years, the brief oral gluten challenge gave relevant support in the characterization of gluten epitopes active both in adult and pediatric patients with CD (33, 83), as well as allowed the elucidation of cell phenotype and pattern of cytokines produced by gluten-reactive T cells (90, 107, 108, 111, 112).

5.1. Short-term gluten challenge, as a pre-clinical evaluation assay of novel gluten detoxification and therapeutic strategies

A number of studies showed that the short-term gluten challenge is a sensitive tool for the pre-clinical assessment of new strategies to detoxify gluten proteins or therapeutic drugs. Tye Din et al. used this non-invasive short-term procedure to validate the efficacy of the combined cysteine endoprotease and prolyl endopeptidase (ALV003/Latiglutenase) (113). An *in vivo* study was conducted on 20 patients with CD on GFD, randomized to consume foods made with gluten (16 g/day) pre-treated with ALV003, or gluten pre-treated with a placebo. The T-cell reactivity against immunodominant α-gliadin epitope 33-mer or whole gliadin, as revealed by IFN-γ ELISPOT, was markedly decreased in volunteers receiving gluten pre-treated with ALV003/Latiglutenase compared to those receiving placebo-treated gluten, thus suggesting the huge potentiality of glutenase degradation to reduce the gluten immunotoxicity (113).

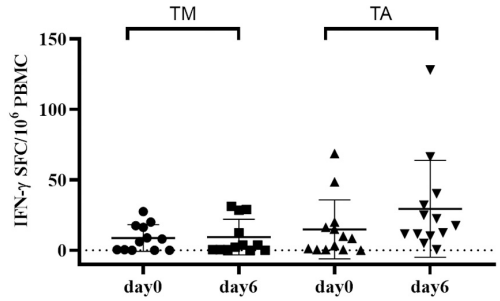

FIGURE 4
Short gluten oral challenge with the diploid *Triticum monococcum* wheat results in a lower immune response compared to soft *Triticum aestivum* wheat. Gliadin-specific T-cell responses were elicited by the short oral challenge with the diploid wheat *Triticum monococcum* (TM, left panel) and with the hexaploid wheat *Triticum aestivum* (TA, right panel). Celiac subjects (n = 11) consumed for 3 days sandwiches made with TM (cv Norberto-ID331) or with TA (cv Sagittario) corresponding to 12 g of gluten (119). PBMCs obtained soon before (day 0) and (day 6) after the gluten consumption were in vitro stimulated with deamidated, pepsin-chymotrypsin digests of gliadin from either TM wheat (left) or TA wheat (right). Digested samples were deamidated by tTG treatment and assayed at 50 μg/ml. IFN-γ secreting cells were revealed by EliSpot assay. Data are reported as IFN-γ spot-forming cells (SFC) detected in 10^6 PBMCs.

The relevance of the brief gluten challenge to monitor the efficacy of gluten detoxification strategies was further addressed by an Italian study aimed to assess whether the pre-digestion of wheat flour with selected lactobacilli and fungal proteases (hydrolyzed wheat gluten) might decrease, or even abolish, the T-cell immunogenicity in patients with CD (114, 115). Twenty subjects with celiac disease including pediatric subjects, following a GFD regimen for at least 3 years, were randomized to consume bread slices produced with hydrolyzed wheat flour (corresponding to 10 g of gluten/day). The hydrolyzed wheat gluten did not mobilize gluten-reactive T lymphocytes from the intestinal mucosa to the peripheral blood compared with that elicited after a short-term oral challenge with untreated wheat flour, with statistically significant differences in the number of circulating gluten-reactive T cells between the two experimental groups. Notably, the brief challenge of the study was a further confirmation of a previous long-term (60 days) challenge addressed to demonstrate whether the enzymatic pre-digestion with lactobacilli and fungal proteases abolish gluten

TABLE 3 Short oral gluten challenge to evaluate immunogenicity of gluten proteins and novel strategies to treat celiac disease (CD).

Purpose of the research	Outcomes by short gluten oral challenge	References
Elucidation of immunopathogenic mechanisms of celiac disease	Detection of gluten-specific T cells in peripheral blood by ELISPOT and tetramers technology.	(106–108, 110–112)
	Identification of immunogenic gluten peptides.	(33, 107)
Efficacy evaluation of novel enzymatic strategies to treat celiac disease in pre-clinical studies	Foods made with gluten pre-treated with glutenase ALV003/Latiglutenase were less immunotoxic for CD patients.	(112, 113)
	Enzymatic pre-digestion with lactobacilli and fungal proteases (hydrolyzed wheat gluten) abolishes gluten proteins toxicity in CD patients.	(114, 115)
Searching of less immunogenic wheat species for CD prevention	Reduced immunogenicity in CD patients of diploid *Triticum monococcum* compared to hexaploid *Triticum aestivum* soft wheat.	(119)

protein toxicity (114). No alteration was reported as histological mucosal change or in antibody seroconversion, thus indicating no relapse after pre-hydrolyzed wheat flour long consumption.

Another successful application of a brief gluten oral challenge was provided by a study aimed to assess the immune stimulatory properties of gluten derived from a diploid wheat species, that is, *T. monococcum*. After the promising *in vitro* T-cell findings (98, 116–118) and proteomic and mass spectrometry analysis demonstrating the pronounced digestibility of diploid gluten proteins (22) compared to the more evolved wheat genome, subsequently a brief oral gluten challenge study was performed (119). Volunteers with CD were enrolled to consume bread sandwiches made with diploid or hexaploid wheat flour (approximately 12 g of gluten per day for each study branch). The results confirmed as gluten from *T. monococcum* retains a reduced capability to recruit *in vivo* gluten-specific T-cell response compared to *T. aestivum* (soft) wheat. A significantly decreased number of IFN-γ secreting cells reactive to diploid gliadins were elicited in the peripheral blood of patients with CD eating sandwiches made with *T. monococcum* flour. Furthermore, the consumption of monococcum-based bread for a few days elicited a lower number of T cells reacting to five immune-dominant epitopes from α-, *ω*-, and γ-gliadins compared to those elicited by soft wheat. Overall, these findings confirmed the reduced *in vivo* immunogenicity in patients with CD of diploid *T. monococcum* compared to hexaploidy soft wheat (**Figure 4**). Additional studies in a larger celiac cohort, or in CD first-degree relatives, are required to address the applicability of this grain, with low immunogenic gluten, in the prevention of celiac disease in the general population (119).

Based on that, the short gluten challenge proved to be a reproducible assay to monitor the immune response to gluten, with great potential for its application in clinical practice. The design of clinical trials aimed to evaluate novel therapeutic drugs, or alternative cereals, could benefit greatly from this non-invasive short-term procedure. Examples of studies using short oral gluten challenges in CD are presented in **Table 3**.

6. Conclusion

Although nutritionally poor for humans, gluten proteins are largely used in the food industry, as they confer unique viscoelasticity properties to the dough and the palatability of gluten-based food products. Due to the widespread of gluten proteins in foodstuffs, compliance with gluten exclusion dietary therapy might be difficult for many patients with CD. To overcome this, several strategies are currently under investigation that are aimed at detoxifying gluten immunogenic peptides or finding specific therapeutic drugs that aim to recover immune tolerance to gluten.

This review overviewed the analytical and functional methods currently used to detect gluten immunogenic peptides in foodstuffs made with naturally gluten-free cereals, or gluten-containing cereals treated with various detoxifying strategies. The amount and amino acidic sequence of gluten immunotoxic peptides have been characterized in several studies by proteomic and immunochemical analysis, such as liquid chromatography–high-resolution mass spectrometry (LC-MS/MS) and R5/G12 competitive ELISA after the INFOGEST digestive *in vitro* model that mimics the human physiologic gastrointestinal digestion. The capability of digested gluten peptides to stimulate an inflammatory reaction has been dissected both *in vitro,* on intestinal human T cells, and *in vivo*, on peripheral blood of volunteers with CD after a brief oral gluten challenge. The large and successful application of such analytical and functional approaches in pre-clinical studies, aimed to validate biochemical, enzymatic, and immunotherapeutic strategies currently under investigation to treat celiac disease, has been also discussed.

Author contributions

GM and CG conceptualized the review contents. GM, LD, SV, SP, and CG revised the literature and wrote the manuscript. All authors approved the final manuscript.

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Conflict of interest

CG served as Scientific Advisor at Nemysis Limited.

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Publisher's note

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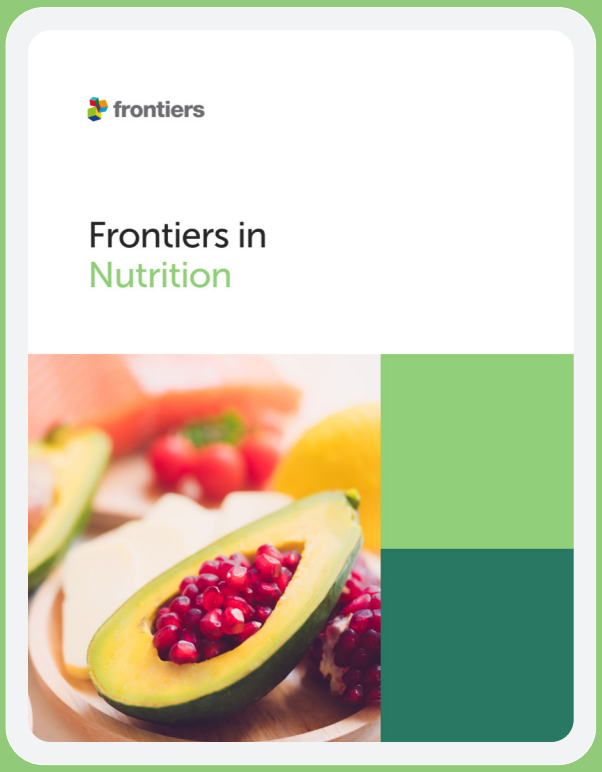

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