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Enhancing the nutritional profile and *in vitro* digestion properties of dual-protein extrudates by blending surimi with soybean flour

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ABSTRACT

Plant protein-based meat analogues often suffer from imbalanced amino acid pattern and poor protein digestibility. To address these drawbacks, this study adopted a dual-protein strategy by co-extruding surimi and soybean flour at ratios from 0:10 to 4:6. Analysis of nutritional composition, fatty acid/amino acid profiling, and *in vitro* digestion coupled with peptide identification were used to evaluate the changes of nutritional and digestive properties of dual-protein extrudates. While basic composition changed minimally, surimi incorporation diversified the fatty acid profiles: total unsaturated fatty acids decreased gradually, but the proportion of ω-3 polyunsaturated fatty acids (EPA and DHA) reached a maximum of 10.37%. Meanwhile, the amino acid profile was improved with essential-to-total amino acid (EAA/TAA) meeting FAO/WHO recommended standards (>40%) at ratios of 3:7 and 4:6, accompanied by progressive increases in essential amino acid index (EAAI) and biological value (BV). Most notably, surimi incorporation linearly enhanced protein digestibility from 79.33% to 86.12%, along with increased free amino acids, 14.50% more identified intestinal peptides, more low-molecular-weight and potential bioactive peptides. These findings demonstrate that co-extruding surimi with soybean flour is an effective strategy to achieve nutritional complementation and enhance protein digestibility, with an optimal processing window identified as ratios ≥3:7.

1. Introduction

1.1. Nutritional and digestion limitations of plant-based meat analogues

Plant-based meat analogues, chiefly produced by extruding of plant proteins (predominantly soybean protein), have attracted considerable interest for mimicking meat sensory attributes and offering potential health benefits (lower levels of saturated fatty acids and cholesterol) (Mosibo et al., 2022; Sha & Xiong, 2020). However, plant protein-based extrudates suffer from intrinsic nutritional shortcomings, including an imbalanced essential amino acids profile (e.g., methionine deficiency), essential to total amino acid (EAA/TAA) ratio often below the FAO/WHO recommended 40%, (Bohrer, 2019; Yang et al., 2023), and poor protein digestibility compared to meat due to structural and anti-nutritional factors (Xie, Cai, Huang, et al., 2022; Xie, Cai, Zhao, et al., 2022; Yang et al., 2023). Although nutritional fortification can address amino acid deficiencies in protein-based extrudates (Kyriakopoulou, Keppler, & van der Goot, 2021), enhancing protein digestibility remains a persistent challenge.

1.2. Potential of dual-protein strategy with surimi to cover limitations

The development of dual-protein foods, which strategically combine animal and plant proteins, presents a promising strategy to overcome these limitations. By aiming for nutritional complementarity, animal protein compensates for the incomplete amino acid profile and improves protein digestibility, while plant protein supplies dietary fibre and reduces overall cholesterol and saturated fatty acid content (Guyomarc'h

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et al., 2021; Chalupa-Krebzdak, Long, & Bohrer, 2018). Previous studies have demonstrated the nutritional and digestive benefits of dual-protein systems: blends like dairy with soy or pea improve amino acid balance and digestibility in specific food formats (Huang et al., 2019; Le Roux et al., 2020; Liu et al., 2019), and combinations such as soy, casein, and whey can stimulate muscle protein synthesis (Berrazaga et al., 2019). Among animal protein sources, freshwater fish protein, owing to its balanced amino acid profile, low cholesterol, measurable levels of polyunsaturated fatty acids (including EPA and DHA) and high digestibility, is a promising candidate for blending with plant proteins. However, studies of dual-protein extrudates combining fish and plant proteins have so far concentrated on texture and flavour, and a systematic evaluation of their nutritional and digestive properties remains lacking (Nisov et al., 2020; Zhang et al., 2022).

1.3. Research gap and study objectives

It is reasonable to assume that increasing the proportion of fish protein with favorable nutritional attributes would enhance the overall quality of plant protein-based extrudates. However, the scientific rationale for this study extends beyond merely confirming that expectation. First, pure fish protein or excessively high fish to plant protein ratios cannot be successfully extruded into shape due to processing constraints (Li et al., 2022; Zhang et al., 2022), so it is essential to define an "optimal window" that reconciles processability with nutritional performance. Second, improvements in the nutritional and digestive properties of the dual-protein system are not a simple additive consequence of the raw materials. Our previous research (Li et al., 2022) systematically elucidated the effects of varying surimi (processed from freshwater fish) to soybean flour (pressed and defatted) ratios (0:10, 1:9, 2:8, 3:7, and 4:6) on the macroscopic properties, microscopic fibrous structure, and flavour profiles of these dual-protein extrudates. We found that increasing the surimi proportion produced more fragmented microstructures in extrudates, which might have a positive impact on digestion (Li et al., 2022). We hypothesise that the gradient rise in surimi proportion (core compositional variation) primarily boosts extrudates' digestive performance by introducing fish protein with intrinsically high digestibility, a core advantage reinforced by the established fact of soybean anti-nutritional factor dilution via surimi incorporation. This improvement may also be secondarily attributed to mild matrix disintegration resulting from the fragmented microstructures driven by the aforementioned compositional changes. However, systematic research focusing on the integrated relationship between nutritional composition and digestive properties of dual-protein extrudates, especially regarding how raw material ratios modulate these properties, remains limited in both scope and depth.

Therefore, unlike our previous study which focused on the structure and flavour, this study uniquely focused on investigating the effects of varying surimi to soybean flour (0:10, 1:9, 2:8, 3:7, and 4:6) on the nutritional and digestive properties of dual-protein extrudates. Specifically, we integrated nutritional profiling, protein digestive analysis, and qualitative peptide identification to establish a comprehensive relationship with blend ratios: (1) Characterize the basic nutritional composition, fatty acid profiles, and amino acid patterns; (2) Determine *in vitro* protein digestibility, hydrolysis degree, and particle size changes during digestion; (3) Identify digestion-released peptides and predict their potential bioactivity. These analyses will not only uncover the underlying mechanistic basis of how surimi incorporation alters protein composition to modulate nutritional and digestion properties of dual-protein extrudates, but also indirectly verify the aforementioned hypothesis, thereby providing theoretical and empirical support for developing nutritionally balanced, highly digestible dual-protein extrudates.

2. Materials and methods

2.1. Materials

Frozen silver carp (*Hypophthalmichthys molitrix*) surimi, produced via pre-treatment, meat collection, washing (2 × clean water and 1 × salt water), dehydration, 5% sucrose as cryoprotectant, and plate quick-freezing, was sourced from Jingli Aquatic Food Co., Ltd. (Honghu, Hubei, China), Frozen surimi contained 75.62% moisture, 16.58% protein, and 0.51% fat. Soybean flour, obtained from the Specialized Cooperative of Chengdou Bean-products (Zaozhuang, Shandong, China), had a moisture content of 10.66%, protein content of 39.85% and fat content of 7.19%. Pepsin (P6887), trypsin (T7409) and bile salts used in the simulated digestion model, 18 amino acid mixed standard, 37 fatty acid methyl ester mixed standard, L-leucine (>99%), sodium dodecyl sulfate (SDS, electrophoretic grade), glycine (electrophoretic grade), tris(hydroxymethyl)aminomethane (Tris, electrophoretic grade) and triundecanoin were all purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, Mo, USA). The pre-stained protein marker and SDS-PAGE fast gel kit were purchased from Labgic technology Co., Ltd (Beijing, China). All other reagents used in this study were analytical grade.

2.2. Preparation of surimi-soybean dual-proteins extrudates

Dual-protein extrudates with varying surimi to soybean flour ratios were prepared following our previous study (Li et al., 2022). In brief, the broken frozen silver carp surimi and soybean flour were mixed in mass ratio of 0:10, 1:9, 2:8, 3:7 and 4:6 on a wet weight basis, and the moisture content of each mixture adjusted to 37% by spraying a calculated amount of deionized water (incorporated the intrinsic moisture contents of surimi and soybean flour into the calculation) onto the mixture while stirring. After spraying, the mixtures were homogenized in a blender at room temperature for 15 min to ensure uniform moisture distribution. Then, a single screw extruder (YYFS-90, Shandong Yuya Soybean Machinery Manufacturing Co., Ltd., Jinan, Shandong, China) with a one-piece gradual compression single screw and a length to diameter ratio of 25:1 was employed to produce the dual-proteins extrudates. The extruder was equipped with four independent heating zones and cooling devices arranged sequentially along the material advancing direction, and the extrusion temperature corresponding to each heating zone was 70 °C, 120 °C, 240 °C, and 250 °C, respectively. Besides, the screw speed of the extruder was 28 Hz, and the material entered the barrel under its own gravity and the rotation of the screw. Especially, the single-screw extruder did not utilize a separate die component. Instead, the extrudates were formed through the annular gap between the cylindrical tail of the single screw and the inner wall of the extruder chamber. Then, the extrudates were subsequently collected, vacuum-sealed in plastic bags, and stored at −20 °C for future testing.

2.3. Determination of basic nutritional components

The moisture content of extrudates was determined using the oven method in accordance with AOAC (930.15). The crude protein content was measured via the Kjeldahl method, following AOAC (984.13, N × 6.25). Crude fat content was determined using the Soxhlet extraction method according to AOAC (920.39). Ash content was determined through the muffle furnace method according to AOAC (942.05) (AOAC International, 2019).

The total sugar content (based on glucose equivalent) was determined using the salicylic acid method as described by Du et al. (2023). The crushed extrudates were hydrolyzed using 6 mol/L HCl in boiling water bath for 30 min. Once cooled, the solution's pH was neutralised, and the volume was adjusted accordingly. A suitable amount of this diluent was then combined with the DNS solution and heated in a

boiling water bath for 5 min. After cooling, the absorbance was measured at 540 nm. Concurrently, glucose standard solutions of varying concentrations were employed to create a standard curve, and the total sugar content was calculated based on glucose equivalent using this standard curve. The formula for calculating total sugar is as follows:

$$\text{Total sugar } \% = \frac{0.9 \times C \times V}{M \times 1000} \times 100\% \quad (1)$$

In the formula, C represents the concentration of glucose in the hydrolyzed extrudates calculated from the glucose standard curve, mg/mL. V was the constant volume of the colorimetric reaction solution, mL. M was the sample quality, g. 0.9 was the conversion factor for total sugar based on glucose.

2.4. Determination of fatty acid composition and content

The extraction, saponification and methylation of fat in extrudates were thoroughly conducted using acid hydrolysis, alkali saponification and boron trifluoride methylation according to the method described by Yang et al. (2023). Then, fatty acid methyl esters were measured by using a gas chromatograph (7890 B, Agilent Technologies, Santa Clara, CA, USA) with an HP-88 column (100 m × 0.25 mm × 0.2 μm). The injector temperature was set to 250 °C, with a carrier gas flow rate of 1.0 mL/min and a split ratio of 50:1. The column's heating programme involved maintaining 50 °C for 3 min, then increasing at 10 °C/min to 160 °C, and finally raising the temperature at 2 °C/min to 250 °C, where it was held for 5 min (An et al., 2022). 37 fatty acid methyl ester mixed standard was used for qualitative analysis, while triumdecanon served as an internal standard to determine the fatty acid content.

2.5. Determination of amino acid composition and content

Amino acid analysis of the extrudates was thoroughly conducted using acid hydrolysis, following the method outlined by Ge et al. (2021). Additionally, alkaline hydrolysis was employed to determine tryptophan content, as described by (Yang et al., 2023). For this, 50 mg of extrudates were subjected to hydrolysis by adding 0.8 mL of 4 mol/L NaOH solution and heating at 110 °C for 20 h under a nitrogen atmosphere. Then, the pH was then adjusted to neutral, and the mixture was centrifuged at 12,000 r/min to obtain the supernatant for analysis. The composition and content of amino acid were determined using an automatic amino acid analyzer (A300, MembraPure GmbH, Berlin, Germany) and quantified via an external standard method.

The nutritional evaluation of essential amino acids was performed utilising the amino acid score (AAS), chemical score (CS), essential amino acid index (EAAI), and biological value (BV) calculations, following the methodology outlined by Liu et al. (2023). The formulas are as follows.

$$AAS = \frac{A_i}{A_{S1}} \quad (2)$$

$$CS = \frac{A_i}{A_{S2}} \quad (3)$$

$$EAAI = \sqrt[n]{\frac{Thr}{Thr_{s2}} \times \frac{Val}{Val_{s2}} \times \frac{(Met + Cys)}{(Met + Cys)_{s2}} \cdots \times \frac{Lys}{Lys_{s2}}} \quad (4)$$

$$BV = 1.09 \times EAAI - 11.7 \quad (5)$$

A_i represents the content of essential amino acids in the extrudates, g/100 g. A_{S1} refers to the reference value of the essential amino acids in the FAO/WHO model, g/100 g. A_{S2} is the content of essential amino acids in the protein model of eggs, g/100 g. n represents the number of essential amino acids used for calculation.

2.6. In vitro static simulation digestion

The dual-protein extrudates were subjected to *in vitro* static simulated digestion of proteins based on the INFOGEST method (Brodkorb et al., 2019). Simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared in accordance with INFOGEST guidelines. For the oral digestion phase, approximately 2.5 g of dual-protein extrudates (2 mm × 2 mm pieces) was combined with deionized water to achieve a total mixture mass of 5 g, ensuring a consistent protein content of 0.8 g across all samples in the digestion test. Subsequently, 5 mL of SSF (excluding salivary amylase) was added, and the mixture was homogenized at 10,000 r/min and 12,000 r/min for 1 min each, with a 1 min interval, to mimic the mastication process (Yang et al., 2023). In the gastric digestion phase, 10 mL of preheated SGF containing pepsin (final enzyme activity of 2000 U/mL) was incorporated, and the pH was adjusted to 3.0. The mixture was then placed in a constant temperature water bath shaker at 150 rpm for 2 h at 37 °C. Upon completion of gastric digestion, the pH was immediately adjusted to 7.5 using 1 mol/L NaOH. For the gastrointestinal digestion phase, 20 mL of preheated SIF with trypsin (final enzyme activity of 100 U/mL) and bile salts (final concentration of 10 mmol/L) was added, adjusting the pH to 7.0. The mixture was again placed in a constant temperature water bath shaker at 150 rpm for 2 h at 37 °C. Finally, the gastric and gastrointestinal digestion products were all heated at 95 °C for 5 min to inactivate the enzymes and centrifuged at 4000 r/min for 20 min to separate the supernatant and precipitate, then stored at −20 °C for subsequent analysis.

2.7. Determination of protein digestibility

The protein digestibility of dual-protein extrudates was measured using the method described by Wang et al. (2018). The precipitates from the gastric and gastrointestinal digestion phase were dried to a constant weight in an oven set at 50 °C. Subsequently, the protein content of the extrudates, both pre- and post-digestion, was determined using the Kjeldahl method. Protein digestibility was then calculated by the difference in protein content before and after digestion, The formulas are as follows:

$$\text{Protein digestibility } \% = \left(1 - \frac{W_2 \times V_2}{W_1 \times V_1}\right) \times 100\% \quad (6)$$

W_1 represents the weight of extrudate before digestion, g. V_1 represents the protein content of extrudate before digestion, %. W_2 represents the weight of the precipitate of the extrudates after digestion, g. V_2 represents the protein content of precipitate of extrudate after digestion, %.

2.8. Determination of total free amino groups concentration

The total free amino groups concentration in digestive supernatant of extrudates was determined using the ortho-phthalaldehyde (OPA) method to further assess the degree of protein hydrolysis (Sousa et al., 2023). In the presence of 1,4-dithiothreitol, OPA reacted with free amino groups to form a yellow compound, whose absorbance was measured at 340 nm. The concentration of free amino groups was calculated using a standard curve derived from L-leucine standard solutions of varying mass concentrations, prepared in a 10 mmol/L phosphate buffer. Results were expressed as L-leucine equivalents in mg/mL.

2.9. Determination of particle size

The particle size distribution of undigested (oral digestion precipitate) and digested samples (gastric and gastrointestinal digestion precipitates) of the extrudates were measured by using a Mastersizer 3000 (Malvern Instruments Co., Worcestershire, UK). The measurement

parameters were as follows: water served as the dispersant with a refractive index of 1.330, while the sample had a refractive index of 1.540, and the obscuration range was 10%-20%. Data processing was conducted using Malvern Mastersizer software to acquire particle size information. Dx4,3 represents the volume-average particle size.

2.10. Sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was employed to examine the protein profiles of both undigested and digested extrudates (supernatant from gastric and gastrointestinal digestion). For the undigested extrudates, proteins were extracted using 5% SDS in PBS. The protein concentration of all samples was determined by the Lowry method and adjusted to 2 mg/mL with PBS. Subsequently, each sample was combined with a reducing protein loading buffer (5 ×) in the correct ratio and heated at 95 °C for 5 min. Thereafter, 10 μL of each sample and 5 μL of protein marker were loaded into the wells of SDS-PAGE gels (comprising a 4% stacking gel and a 12% resolving gel). The gels were run in 500 mL of Tri-Gly running buffer, initially at 80 V for 30 min, followed by 120 V until the bromophenol blue indicator at the leading edge reached the bottom. Finally, the gel was stained and decolourised, and images were captured using an image scanner.

2.11. Analysis of free amino acids in digestive supernatant

The free amino acids content was measured using the method described by Li et al. (2024). Initially, the digested supernatants were centrifuged at 10,000 r/min for 10 min. Subsequently, a 5% sulfosalicylic acid solution was combined with the supernatant in a 1:4 (v/v) ratio and maintained at 4 °C for 1 h to precipitate the protein. Following this, the mixtures underwent centrifugation at 15,000 r/min for 10 min, and the supernatant was centrifuged once more. Finally, the supernatants were filtered through a 0.22 μm aqueous filter membrane and used to determine the free amino acid content with an automatic amino acid analyser (A300, MembraPure GmbH, Berlin, Germany).

2.12. Identification of peptide in digestion products by LC-MS/MS

Three extrudates with blending ratios of 0:10, 2:8, and 4:6 were chosen as representative low-, medium-, and high-level samples. Peptides in their gastric and gastrointestinal digestion products were isolated and identified by LC-MS/MS. Initially, all samples were filtered through 0.22 μm membranes and processed with a 10 kDa ultrafiltration tube before being loaded onto a trap column for enrichment and desalting. Subsequently, the peptides were separated using an UltiMate 3000 ultra-high performance liquid chromatography (UHPLC) system with a C18 analytical column (75 μm inner diameter, 3 μm resin, 25 cm long) at a flow rate of 300 nL/min. The gradient elution program was as follows: 0-5 min, 5% mobile phase B (98% ACN, 0.1% formic acid) and 95% mobile phase A (2% ACN, 0.1% formic acid); 5-45 min, linear increase of mobile phase B from 5% to 25%; 45-50 min, increase from 25% to 35%; 50-52 min, increase from 35% to 80%; 52-54 min, maintained at 80% B; 54-60 min, returned to 5% B (Zhao et al., 2020). Furthermore, the peptides separated by UHPLC were ionized via an electrospray ionization (ESI) source and then introduced into a Q-Exactive HF X tandem mass spectrometer for detection in data-dependent acquisition (DDA) mode. The key MS parameters were set as follows: ion spray voltage, 1.9 kV; full MS scan range, 350-1500 m/z at a resolution of 60, 000; MS/MS scan starting at m/z 100 with a resolution of 15,000. Peptide fragmentation was achieved via higher-energy collisional dissociation (HCD), and fragment ions were detected in the Orbitrap analyzer. The dynamic exclusion time was set to 30 s. Automatic gain control (AGC) targets were 3e6 for full MS and 1e5 for MS/MS. Finally, the raw MS/MS data were processed for database searching (UniProt *Hypophthalmichthys molitrix* and *Glycine* max protein, http://www.uniprot.org/) using Peak 8 software. The search parameters were configured as follows: no enzyme digestion; fixed modification; carboxyamidomethylation (C); variable modification: oxidation (M); precursor ion mass tolerance, ±15 ppm; fragment ion mass tolerance, ±0.02 Da. Only unique peptide sequences (with duplicates removed) were retained for subsequent analysis.

Venn diagrams were generated using the Venny 2.1 online tool (https://bioinfogp.cnb.csic.es/tools/venny/) to visualize the overlap and unique peptides among different treatment groups. The identification of 'unique' peptides for each group was based strictly on their presence (detected) or absence (not detected) in the respective dataset. PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/) was used to predict the potential bioactivity of the identified peptides based on sequence features.

2.13. Statistical analysis

All experiments were conducted in technical triplicate. Data were processed and plotted using Origin 2018 (Origin-Lab, Northampton, MA, USA), and results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA) after checking normality and homogeneity of variance using the SPSS software (Ver. 26, IBM Corporation, Armonk, New York, USA). Significant differences among groups were determined by Duncan's multiple range test, with a significance level of $P < 0.05$.

3. Results and discussion

3.1. Basic nutritional components analysis

Surimi incorporation will change the basic nutritional profile of dual-protein extrudates by altering key components like moisture, protein, fat, total sugar, and ash, which are fundamental indicators for evaluating the nutritional value of foods. As illustrated in Table 1 (All data of crude protein, crude fat, total sugar and ash are presented on a dry basis.), the ratio of surimi to soybean flour significantly impacted the basic nutritional composition of dual-protein extrudates. The moisture content of these extrudates ranged from 31.03% to 32.74%, classifying them as low-moisture extrusion products. An increase in the proportion of surimi resulted in a decrease in moisture content. This is attributed to the higher viscosity of mixtures with more surimi, which elevated extrusion pressure and enhanced the pressure drop upon exit, thus promoting moisture evaporation (Guo et al., 2020).

On a dry weight basis, the crude protein content of extrudates with the increasing surimi proportion showed no significant differences among all formulations, ranging from 45.70% to 46.76%. The average crude protein content across all five extrudates was 31.45% (wet basis), comparable to conventional cooked meat and higher than typical plant-based meat products (Yang et al., 2023). The crude fat content of the extrudates decreased significantly as the proportion of surimi increased, particularly when the surimi to soybean flour ratio was 4:6, resulting in a 13.70% reduction in crude fat content. This reduction was mainly due to the inherent difference in fat content between the two raw materials: soybean flour contained 7.06% crude fat, whereas frozen surimi contained only 0.48%. Similarly, the total sugar and ash contents (dry basis) decreased significantly with higher surimi proportion, with total sugar falling from 29.24% to 25.57% (a 12.51% drop) and ash declining from 4.74% to 4.14% (a 12.66% drop). Surimi contains minimal carbohydrates except for ~5% sucrose added as a cryoprotectant, whereas soybean flour is abundant in carbohydrates, which accounts for the total sugar reduction, and the ash decrease directly reflects the inherent ash difference between surimi and soybean flour.

3.2. Fatty acid analysis

Varying surimi to soybean flour ratios will reshape the fatty acid

Table 1
Basic nutritional composition (%, dry basis) of dual-protein extrudates with different surimi to soybean flour ratios ($P < 0.05$).

Surimi to soybean flour ratios	Moisture	Crude protein	Crude Fat	Total Sugar	Ash
0:10	32.74 ± 0.24^{a}	45.86 ± 0.59^{a}	7.81 ± 0.01^{a}	29.24 ± 1.02^{a}	4.74 ± 0.19^{a}
1:9	31.82 ± 0.39^{ab}	45.75 ± 1.13^{a}	7.57 ± 0.01^{a}	28.56 ± 0.70^{a}	4.59 ± 0.17^{a}
2:8	31.27 ± 0.29^{ab}	45.69 ± 1.01^{a}	7.32 ± 0.22^{b}	27.78 ± 0.70^{ab}	4.44 ± 0.04^{ab}
3:7	31.42 ± 0.37^{ab}	46.74 ± 0.16^{a}	7.11 ± 0.10^{b}	26.50 ± 0.96^{b}	4.29 ± 0.15^{bc}
4:6	31.03 ± 0.71^{b}	46.11 ± 0.75^{a}	6.74 ± 0.02^{c}	25.57 ± 0.43^{c}	4.14 ± 0.05^{c}

Note: Different letters in the same column mean significant differences ($P < 0.05$).

profile of dual-protein extrudates. Table 2 highlights the significant differences in the fatty acid composition and content of dual-protein extrudates with varying surimi to soybean flour ratios. In extrudates without surimi, the primary fatty acids were C18:2n6 (56.32%), C18:1 (16.33%), and C16:0 (13.60%). Notably, unsaturated fatty acids (UFA) constituted 81.57%, with polyunsaturated fatty acids (PUFA) making up 64.97%. However, C20:4n6, EPA, and DHA were absent, and the sole ω-3 PUFA present was C18:3n3, comprising 8.64%.

As the proportion of surimi increased, the levels of fatty acids, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and total fatty acids (TFA) gradually declined. This trend was due to surimi's significantly lower crude fat content compared to soybean flour. Notably, when the surimi to soybean flour ratio was 4:6, the content of C18:2n6 in the extrudate decreased most significantly by 555 mg/100 g (for a 100 g serving, 555 mg per serving), and the PUFA proportion fell to 62.61%. This was primarily because C18:2n6 is the dominant fatty acid in soybean lipids (Bukowski & Goslee, 2024), whereas silver carp surimi lipids mainly consist of C18:1 and C18:3n3, with a relatively lower proportion of C18:2n6 (An et al., 2022). Conversely, the levels of C20:4n6, EPA, and DHA increased markedly with higher surimi proportions, and the proportion of ω-3 PUFAs peaked at 10.37% at the 4:6 surimi to soybean flour ratio.

Although increasing the proportion of surimi reduced the UFA content in the dual-protein extrudates, the incorporation of C20:4n6, particularly EPA and DHA, which are ω-3 PUFAs that play an important role in cholesterol reduction and brain development (Zirpoli et al., 2020), provided specific nutritional benefits for dual-protein extrudates. Adding an appropriate amount of fish oil (a by-product of freshwater fish surimi processing) could be explored as a future research direction, aiming to compensate for the reduced total UFA content while further increasing the proportion of high-nutrition ω-3 PUFAs. Meanwhile, the potential lipid oxidation risk of ω-3 PUFAs should be considered, and antioxidant strategies or protective packaging will be explored in subsequent studies to improve the storage stability of extrudates.

Table 2
Fatty acid composition (mg/100 g) of dual-protein extrudates with different surimi to soybean flour ratios ($P < 0.05$).

Fatty acid	0:10	1:9	2:8	3:7	4:6
C14:0	8.32 ± 0.02^{c}	10.70 ± 0.30^{bc}	11.80 ± 0.00^{b}	12.15 ± 0.05^{b}	16.85 ± 0.15^{a}
C15:0	2.04 ± 0.07^{d}	6.01 ± 0.27^{c}	7.38 ± 0.25^{c}	9.47 ± 0.11^{b}	11.70 ± 0.40^{a}
C16:0	576.00 ± 2.00^{a}	562.50 ± 1.50^{a}	553.00 ± 2.00^{ab}	541.50 ± 4.50^{b}	527.50 ± 3.50^{c}
C18:0	164.50 ± 2.50^{a}	158.00 ± 1.00^{a}	152.50 ± 0.50^{a}	144.50 ± 2.50^{b}	142.00 ± 4.00^{b}
C20:0	13.40 ± 0.20^{a}	12.80 ± 0.30^{a}	11.05 ± 0.15^{b}	10.40 ± 0.20^{b}	9.85 ± 0.35^{b}
C22:0	16.00 ± 0.20^{a}	15.90 ± 0.10^{a}	15.30 ± 0.10^{a}	13.70 ± 0.30^{b}	13.15 ± 0.05^{b}
C16:1	5.38 ± 1.32^{e}	8.67 ± 0.26^{d}	11.65 ± 0.45^{c}	15.60 ± 0.60^{b}	22.70 ± 1.10^{a}
C18:1	691.50 ± 3.50^{a}	688.50 ± 4.50^{a}	579.00 ± 7.00^{ab}	560.50 ± 2.50^{b}	548.00 ± 4.00^{c}
C20:1	5.49 ± 0.13^{c}	6.37 ± 0.04^{b}	6.46 ± 0.05^{ab}	6.69 ± 0.11^{a}	7.15 ± 0.07^{a}
C18:2n6	2385 ± 25^{a}	2310 ± 20^{a}	2120 ± 40^{b}	1905 ± 45^{c}	1830 ± 10^{d}
C18:3n3	366.50 ± 4.50^{a}	359.00 ± 3.00^{a}	352.50 ± 1.50^{ab}	344.00 ± 4.00^{b}	337.00 ± 1.00^{b}
C20:4n6	/	/	3.87 ± 0.05^{c}	5.47 ± 0.21^{b}	8.19 ± 0.16^{a}
EPA	/	3.26 ± 0.08^{d}	6.66 ± 0.07^{c}	11.20 ± 0.4^{b}	15.05 ± 0.25^{a}
DHA	/	/	6.05 ± 0.25^{c}	8.45 ± 0.65^{b}	12.85 ± 0.55^{a}
SFA	780.26	765.91	751.03	731.72	721.05
MUFA	702.37	703.54	597.11	582.79	577.85
PUFA	2751.50	2672.26	2489.07	2274.12	2203.10
TFA	4234.13	4141.70	3837.21	3588.62	3518.81

Note: SFA, the total amount of saturated fatty acids; MUFA, the total amount of monounsaturated fatty acid; PUFA, the total amount of polyunsaturated fatty acid; TFA, the total amount of fatty acids;/, not detected. Different letters in the same line mean significant differences ($P < 0.05$).

3.3. Amino acid analysis

Adding surimi will change the amino acid composition and content of extrudates, which play a crucial role in determining the nutritional quality of proteins in foods. As shown in Table 3, the extrudate made solely from soybean flour exhibited high concentrations of Glu, Asp, and Lys, but low levels of Trp and Met. Met was identified as the first limiting amino acid in soybean protein. Moreover, the ratios of essential amino acids to total amino acids (EAA/TAA) and essential to non-essential amino acids (EAA/NEAA) were 36.92% and 58.54%, respectively, both falling short of the FAO/WHO recommended standards for ideal edible protein (EAA/TAA >40%, EAA/NEAA >60%), indicating the protein nutritional quality of the extrudate without surimi was deficient. This observation aligned with previous research findings, which highlighted deficiencies in the amino acid profiles of plant-based meat analogues (Liu et al., 2023).

Animal proteins generally contain more essential amino acids (EAAs) and a more balanced amino acid profile than plant proteins (Yang et al., 2023). As the proportion of surimi increased, the TAA content remained relatively stable, while the content of the limiting amino acid (methionine), EAAs, EAA/TAA and EAA/NEAA ratios gradually increased. Notably, when the surimi to soybean flour ratios were 3:7 and 4:6, both the EAA/TAA and EAA/NEAA ratios met the recommended standards for ideal edible protein by FAO/WHO (EAA/TAA >40%, EAA/NEAA >60%). This indicated that increasing the proportion of surimi enhanced the amino acid profile of the dual-protein extrudates, thereby improving their nutritional value.

Subsequent evaluations involved calculating the AAS, CS, EAAI, and BV values of amino acids, which are the protein quality evaluation indicators recommended by FAO/WHO (Table 4). The results indicated that the AAS for most essential amino acids showed an upward trend and surpassed 100 in all extrudates, aligning with the FAO/WHO recommended standards pattern. Val and Leu with lower AAS values were the primary limiting amino acids, but both increased progressively with a higher proportion of surimi. According to the CS values, all amino acids except Lys and Met + Cys scored below 100, highlighting a discrepancy with the standard egg protein pattern, where Val and Trp were the

Table 3
Amino acid composition (g/100 g) of dual-protein extrudates with different surimi to soybean flour ratios ($P < 0.05$).

Amino acid	0:10	1:9	2:8	3:7	4:6
Asp	3.43 ± 0.09^{a}	3.37 ± 0.05^{a}	3.28 ± 0.03^{ab}	3.10 ± 0.01^{b}	2.97 ± 0.11^{c}
Thr*	1.12 ± 0.01^{b}	1.10 ± 0.02^{b}	1.15 ± 0.03^{ab}	1.21 ± 0.02^{a}	1.20 ± 0.02^{a}
Ser	1.44 ± 0.03^{a}	1.36 ± 0.03^{a}	1.40 ± 0.03^{a}	1.45 ± 0.02^{a}	1.43 ± 0.05^{a}
Glu	5.83 ± 0.06^{a}	5.76 ± 0.04^{ab}	5.69 ± 0.10^{ab}	5.51 ± 0.02^{b}	5.42 ± 0.09^{c}
Gly	1.16 ± 0.02^{b}	1.15 ± 0.06^{b}	1.19 ± 0.01^{b}	1.31 ± 0.01^{a}	1.33 ± 0.01^{a}
Ala	1.27 ± 0.01^{a}	1.25 ± 0.04^{ab}	1.28 ± 0.04^{a}	1.36 ± 0.02^{a}	1.35 ± 0.06^{a}
Cys	1.88 ± 0.04^{a}	1.78 ± 0.05^{a}	1.59 ± 0.04^{b}	1.44 ± 0.02^{bc}	1.37 ± 0.03^{c}
Val*	1.32 ± 0.03^{c}	1.33 ± 0.03^{c}	1.38 ± 0.00^{c}	1.47 ± 0.03^{b}	1.57 ± 0.06^{a}
Met*	0.39 ± 0.03^{b}	0.39 ± 0.02^{b}	0.44 ± 0.02^{ab}	0.46 ± 0.02^{a}	0.49 ± 0.03^{a}
lle*	1.24 ± 0.02^{b}	1.24 ± 0.01^{b}	1.26 ± 0.00^{b}	1.41 ± 0.03^{a}	1.48 ± 0.01^{a}
Leu*	2.14 ± 0.03^{b}	2.14 ± 0.02^{b}	2.18 ± 0.03^{b}	2.35 ± 0.02^{a}	2.40 ± 0.02^{a}
Tyr	0.92 ± 0.03^{a}	0.94 ± 0.02^{a}	1.00 ± 0.02^{a}	0.98 ± 0.01^{a}	0.93 ± 0.04^{a}
Phe*	1.38 ± 0.01^{c}	1.37 ± 0.00^{c}	1.46 ± 0.04^{b}	1.59 ± 0.03^{a}	1.65 ± 0.06^{a}
Trp*	0.51 ± 0.05^{a}	0.52 ± 0.01^{a}	0.51 ± 0.01^{a}	0.54 ± 0.01^{a}	0.52 ± 0.01^{a}
His	1.98 ± 0.03^{b}	2.00 ± 0.03^{b}	2.05 ± 0.04^{ab}	2.11 ± 0.04^{a}	2.10 ± 0.01^{a}
Lys*	3.13 ± 0.06^{c}	3.17 ± 0.04^{c}	3.29 ± 0.03^{b}	3.38 ± 0.03^{ab}	3.44 ± 0.07^{a}
Arg	0.71 ± 0.01^{c}	0.71 ± 0.00^{c}	0.75 ± 0.02^{b}	0.77 ± 0.02^{ab}	0.85 ± 0.02^{a}
Pro	1.15 ± 0.03^{a}	1.07 ± 0.01^{a}	1.16 ± 0.07^{a}	1.12 ± 0.03^{a}	1.11 ± 0.02^{a}
EAA	11.21	11.25	11.58	12.38	12.73
NEAA	19.74	19.36	19.36	19.12	18.83
TAA	30.95	30.61	30.94	31.5	31.56
EAA/TAA (%)	36.21	36.75	37.44	39.31	40.32
EAA/NEAA (%)	56.76	58.11	59.84	64.77	67.57

Notes: *, essential amino acid; EAA, the total amount of essential amino acids; NEAA, the total amount of non-essential amino acids; TAA, the total amount of amino acids; Different letters in the same column mean significant differences ($P < 0.05$).

primary limiting amino acids, but both increased progressively. Additionally, a higher EAAI value suggests a higher quality of food proteins, while a higher BV is associated with improved post-digestive protein utilization (Liu et al., 2023). The results in Table 4 showed that both the EAAI and BV values in the nutritional evaluation of proteins in extrudates increased progressively with a higher surimi ratio, confirming that surimi incorporation enhanced the overall protein nutritional quality and bioavailability of dual-protein extrudates.

3.4. *In vitro digestion characteristics of protein in extrudates*

Conventional digestion assays (including protein digestibility, free amino group concentration, particle size distribution, SDS-PAGE and free amino acids content) were first used to characterize the phenotypic changes in digestibility of dual-protein extrudates with different surimi ratios. Meanwhile, LC-MS/MS-based qualitative peptide identification was further employed to qualitatively interpret the enhanced digestibility at a preliminary molecular level and predict the functional potential of digestive products via bioactive peptide screening, which goes beyond the phenotypic detection of standard digestion assays.

3.4.1. *Protein digestibility and free amino concentration analysis*

Protein digestibility of extrudates, widely regarded as a primary metric for evaluating protein bioavailability, will undergo obvious changes with the addition of surimi. Fig. 1A indicated that increasing the ratio of surimi to soybean flour simultaneously enhanced both gastric and gastrointestinal protein digestibility of dual-protein extrudates. Both protein digestibility peaked at a surimi to soybean flour ratio of 4:6, with gastric digestibility rising from 52.13% to 59.19% and gastrointestinal digestibility increasing from 79.33% to 86.12%. Furthermore, the total free amino group concentration serves as another crucial indicator of protein digestibility. As the surimi to soybean flour ratio increased, there was a significant rise in the total free amino group concentration in the gastric and gastrointestinal digestive supernatant of extrudates (Fig. 1B), indicating easier protein digestion. The improved protein digestibility and total free amino group concentration in extrudates with higher surimi to soybean flour ratios can be primarily attributed to changes in protein composition, as animal proteins are generally more digestible than plant proteins (Yang et al., 2023; Zhou et al., 2021). Besides, as discussed in our previous research (Li et al., 2022), the microscopic structural fractures observed in extrudates with higher surimi to soybean flour ratios (exceeding 2:8) may be consistent with enhanced enzymatic access for protein hydrolysis, and this structural feature may provide a potential secondary explanation for the improved protein digestibility.

Notably, the protein digestibility results were obtained based on an *in vitro* static digestion model (INFOGEST), which lacks gastrointestinal dynamics (e.g., peristaltic movement and real-time pH fluctuations) and may not fully reflect the *in vivo* digestion process. Future *in vitro* dynamic digestion model and *in vivo* studies are needed to address this limitation.

3.4.2. *Particle size analysis*

Particle size reduction during digestion directly reflects higher digestibility, and surimi proportion will influence this process. As demonstrated in Fig. 1C and F, the particle sizes of the undigested

Table 4
Nutritional evaluation of essential amino acids for dual-protein extrudates with different surimi to soybean flour ratios.

Surimi to soybean flour ratios	0:10		1:9		2:8		3:7		4:6	
Amino acid	AAS	CS	AAS	CS	AAS	CS	AAS	CS	AAS	CS
Thr	116.70	72.36	113.14	70.15	117.52	72.86	121.21	75.15	121.22	75.16
Val	99.23	57.50	99.07	57.41	102.11	59.17	106.24	61.57	114.45	66.33
lle	125.73	60.96	123.62	59.94	125.28	60.74	136.91	66.38	144.95	70.28
Leu	104.96	78.72	103.86	77.89	104.85	78.64	111.03	83.27	114.35	85.76
Lys	177.89	158.43	178.14	158.65	183.36	163.31	184.90	164.68	189.78	169.03
Trp	123.70	58.21	128.12	60.29	121.30	57.08	130.57	61.45	123.82	58.27
Met + Cys	263.18	129.67	256.84	126.54	238.63	117.57	218.85	107.83	215.47	106.16
Phe + Tyr	143.20	74.46	142.29	73.99	149.91	77.95	153.51	79.83	155.72	80.97
EAAI		80.58		80.04		80.03		82.88		84.27
BV		76.13		75.54		75.53		78.64		80.16

Notes: AAS, amino acid score; CS, chemical score; EAAI, essential amino acid index; BV, biological value.

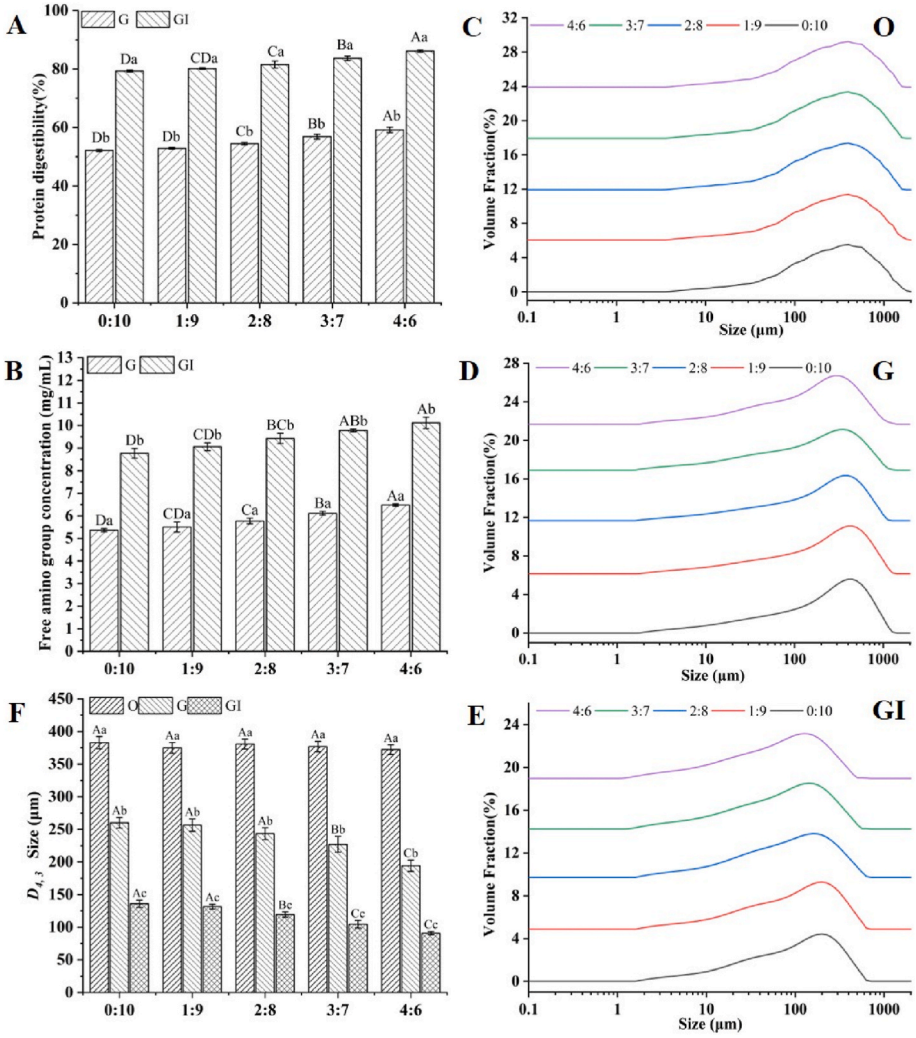

Fig. 1. Protein digestibility (A), free amino group concentration (B), particle size distribution (C-E) and average particle size (F) of dual-protein extrudates with different surimi to soybean flour ratios (0:10, 1:9, 2:8, 3:7 and 4:6) at each digestion phase. C: Oral digested sample (O); D: Gastric digested sample (G); E: Gastrointestinal digested sample (GI). Different capital letters indicate significant differences among different samples in the same digestion stage ($P < 0.05$); different lowercase letters indicate significant differences of the same sample between different digestion stages ($P < 0.05$).

extrudates (precipitate of oral digestion) were nearly identical, with an average diameter of 373.36 μm. This uniformity was achieved by using consistent pre-treatment methods to remove variations caused by simulated oral chewing, allowing a more focused examination of protein digestion in the gastrointestinal phase. As shown in Fig. 1C–F, a substantial decrease in the particle size of digestive precipitate was observed from the gastric to gastrointestinal digestion across all extrudates. At the same digestive stage, the average particle size of digested precipitate decreased progressively as the surimi proportion increased. Notably, when the surimi to soybean flour ratio increased from 0:10 to 4:6, the average particle size of precipitates in both gastric and gastrointestinal stages showed the most notable reductions, decreasing from 260.21 μm to 194.30 μm (a 25.33% decrease) and from 136.01 μm to 90.82 μm (a 33.22% decrease), respectively. This effect is mainly due to changes in protein composition and a reduction in polysaccharides and dietary fibre from soybean flour, which are known to hinder effective protease action. And, the microscopic structural fractures observed in extrudates with a higher surimi content may be conducive to protease penetration and access to digestible sites. As previous studies have shown that enzyme penetration is generally limited to a thin layer within 2 mm of the sample surface (Luo et al., 2017), this structural characteristic is consistent with the enzymatic hydrolysis rule and may provide more favorable conditions for the interaction between enzymes and substrates. Conversely, the progressive reduction in particle size contributed to the enhanced protein digestibility observed in Section 3.4.1, as smaller particles would provide a larger surface area for protease action during gastric and gastrointestinal digestion.

3.4.3. *SDS-PAGE analysis*

SDS-PAGE analysis was conducted for a preliminary and qualitative observation of the molecular weight variation of proteins in dual-protein extrudates at different digestion stages (Fig. 2). It was observed that undigested extrudates (Fig. 2 A) lacked high molecular weight protein bands, facilitating subsequent digestion. The extrudate composed solely of soybean flour exhibited typical soy protein bands, primarily corresponding to 7 S (α′, α, and β subunits) and 11 S (acidic A and basic B polypeptides) globulins (Liu et al., 2008). Actin (AC) and tropomyosin (TM) bands were only present in extrudates with higher surimi content, while the myosin heavy chain of high molecular weight was absent. After gastric digestion (Fig. 2B), protein bands exceeding 50 kDa vanished in all extrudates. However, new, unidentified bands were detected in the 40 kDa, 30 kDa, 25 kDa, and 10 kDa molecular weight ranges for all groups. And, the intensities of these new bands differed among extrudates with varying surimi proportion, indicating that the protein hydrolysis patterns were influenced by the surimi to soybean flour ratio. After intestinal digestion (Fig. 2C), all samples exhibited bands around

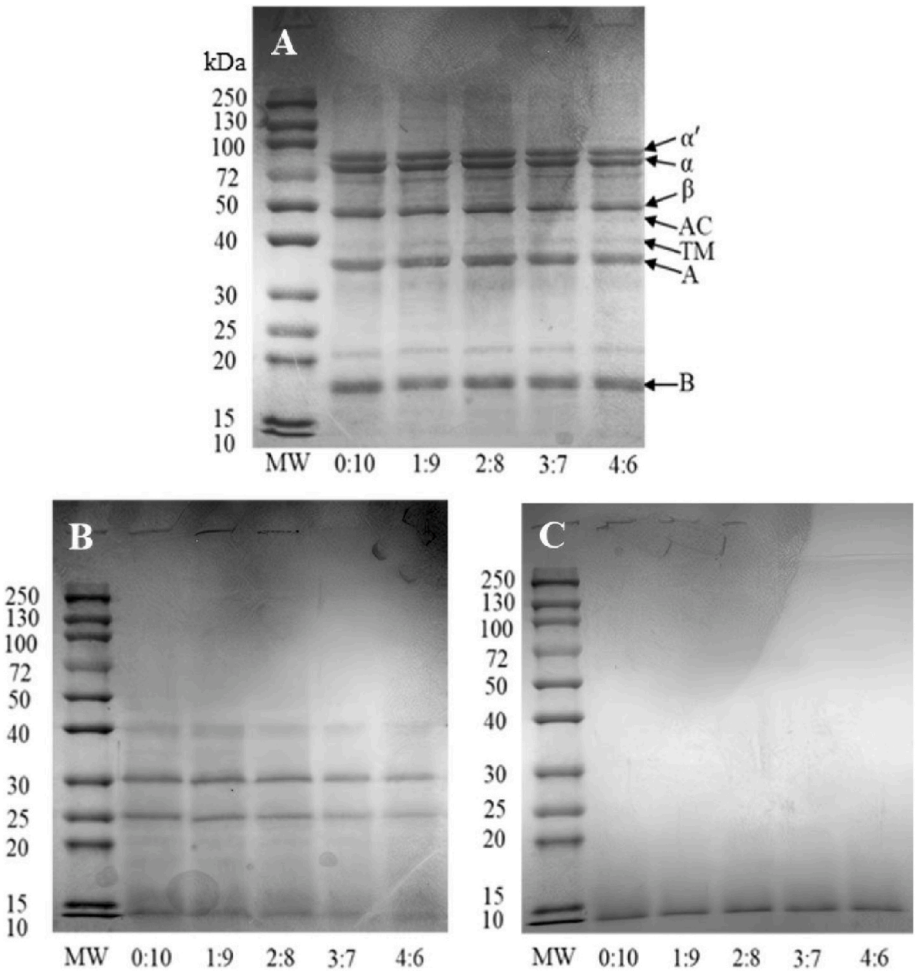

Fig. 2. SDS-PAGE profile of proteins in dual-protein extrudates with different surimi to soybean flour ratios (0:10, 1:9, 2:8, 3:7 and 4:6) at each digestion phase. A: Undigested sample; B: Gastric digested sample; C: Gastrointestinal digested sample. Lane 1-6 (from left to right) are Marker (MW), 0:10, 1:9, 2:8, 3:7, 4:6, respectively.

10 kDa, with no notable visual differences in intensity, indicating that trypsin further hydrolyzed the peptides into smaller peptides and amino acids (Chen et al., 2020). Further analysis of the amino acid composition in the digestive supernatant is necessary to elucidate the impact of surimi to soybean flour ratios on the protein digestibility of extrudates.

3.4.4. *Free amino acids analysis*

It is well established that a higher yield of free amino acids following gastrointestinal digestion, coupled with a more complete amino acid profile, indicates higher protein digestibility and nutritional quality of the food (Hernandez-Olivas et al., 2021). The change of surimi to soybean flour ratios will greatly alter the release and composition of free amino acids in the digestion products of extrudates. As demonstrated in Table S1, when compared with undigested extrudate (see Tables 3 and 0:10), the main amino acids in the digestive supernatant of extrudate containing pure soybean flour were Glu, His, Lys, and Asp. The increased proportion of basic amino acids (Lys, His) can be attributed to the preferential action of digestive enzymes on these residues (Ao & Li, 2013). Furthermore, the EAA/TAA (35.34%, 36.16%) and EAA/NEAA (54.66%, 56.65%) ratios in the gastric and gastrointestinal digestive fluids of extrudate with pure soybean flour were both below the FAO/WHO recommended standards, further confirming the inadequacy of soy protein's amino acid composition.

As the proportion of surimi increased, there was a consistent upward trend in EAA, NEAA, and TFA levels in both the gastric and gastrointestinal digestive supernatant of the extrudate. Furthermore, when the surimi to soybean ratio exceeded 2:8, the EAA/TAA and EAA/NEAA values for amino acids in the digestive fluids largely met the FAO/WHO requirements. This indicates that increasing the proportion of surimi enhances the nutritional qualities of extrudates during *in vitro* simulated digestion. This improvement is due to the inherently higher digestibility of fish protein, along with the previously observed more fragmented microstructure and reduced particle size during digestion, which together improve enzymatic accessibility and efficiency. The increased release of free amino acids with higher surimi proportion thus reflects the combined effects of enhanced protein digestibility and increased substrate surface area resulting from particle size reduction.

3.4.5. *Qualitative peptide identification and analysis*

To further qualitatively interpret the molecular basis of the enhanced protein digestibility revealed by conventional digestion assays (e.g., increased digestibility values, reduced particle size, elevated free amino acid concentration), and to comprehensively characterize the hydrolysis products beyond macroscopic indicators. Three extrudates with blending ratios of 0:10, 2:8, and 4:6 based on the preceding analysis of nutritional composition and digestive properties were subjected to LC-MS/MS-based qualitative peptide identification for peptide profiling in digestive products. The reason was 0:10 served as the pure soybean control, 2:8 was the key ratio where amino acid profiles began to approach FAO/WHO standards, and 4:6 (highest surimi proportion) exhibited the optimal protein digestibility. These ratios would comprehensively reflect the gradient effect of surimi proportion on peptide

release, providing representative data for in-depth analysis of digestion properties. The results focused on peptide number, molecular weight distribution, and differential peptide analysis (Peptides raw data are presented in Tables S2–7).

As illustrated in Fig. 3A, both the digestion phase and the surimi to soybean flour ratios affected the number of identified peptides. The number of identified peptides increased markedly from gastric to gastrointestinal digestion across all extrudates, indicating that trypsin further hydrolyzed proteins and peptides beyond those digested in the stomach, producing more small-molecule peptide segments. With the increase in surimi to soybean flour ratios (from 0:10 to 4:6), the number of identified peptides in the gastric and gastrointestinal digestion supernatant of extrudates increased from 11265 to 11870 (a 5.37% increase) and from 13732 to 15723 (a 14.50% increase), respectively, showing a consistent upward trend. This finding suggested that increasing the proportion of surimi in the raw materials enhanced the protein digestibility of extrudates, particularly generating more peptides during the gastrointestinal digestion phase, which aligned with the observed trend in changes to the total free amino acid concentration.

Subsequent analysis of molecular weight distribution was performed on the peptide fragments identified in the gastric and gastrointestinal supernatant of extrudates with varying surimi to soybean flour ratios (Fig. 3B–C). The findings revealed that the peptides predominantly ranged from 500 to 2500 Da, with the highest concentration between 1000 and 1500 Da. Following gastric digestion, there was a notable rise in low molecular weight peptides (500-1500 Da) as the proportion of surimi increased. Initially, peptides in the 1500-2500 Da range increased but later declined, while those above 2500 Da consistently decreased. During further gastrointestinal digestion, the number of peptides in the 500-2000 Da range rose obviously compared to the gastric stage, with the most substantial increase in the 1000-1500 Da range. This increase was more pronounced with higher surimi proportions, whereas peptides over 2000 Da diminished. This indicated that a higher proportion of surimi promoted the hydrolysis of proteins in extrudates into smaller peptides.

Venn diagrams were employed to aid in comparing and visualising the peptides identified in the gastric and gastrointestinal digestion supernatants of extrudates, where unique peptides were defined by their exclusive presence in one sample group. As shown in Fig. 3D–E, 2028, 1825, and 2605 unique peptides were identified in three extrudates (surimi to soybean flour ratios at 0:10, 2:8, and 4:6) after gastric digestion, accounting for 11.8%, 10.6%, and 15.1% of the total peptides, respectively, showing an initial slight decrease followed by an obvious increase. Additionally, 6794 peptides were common across the three

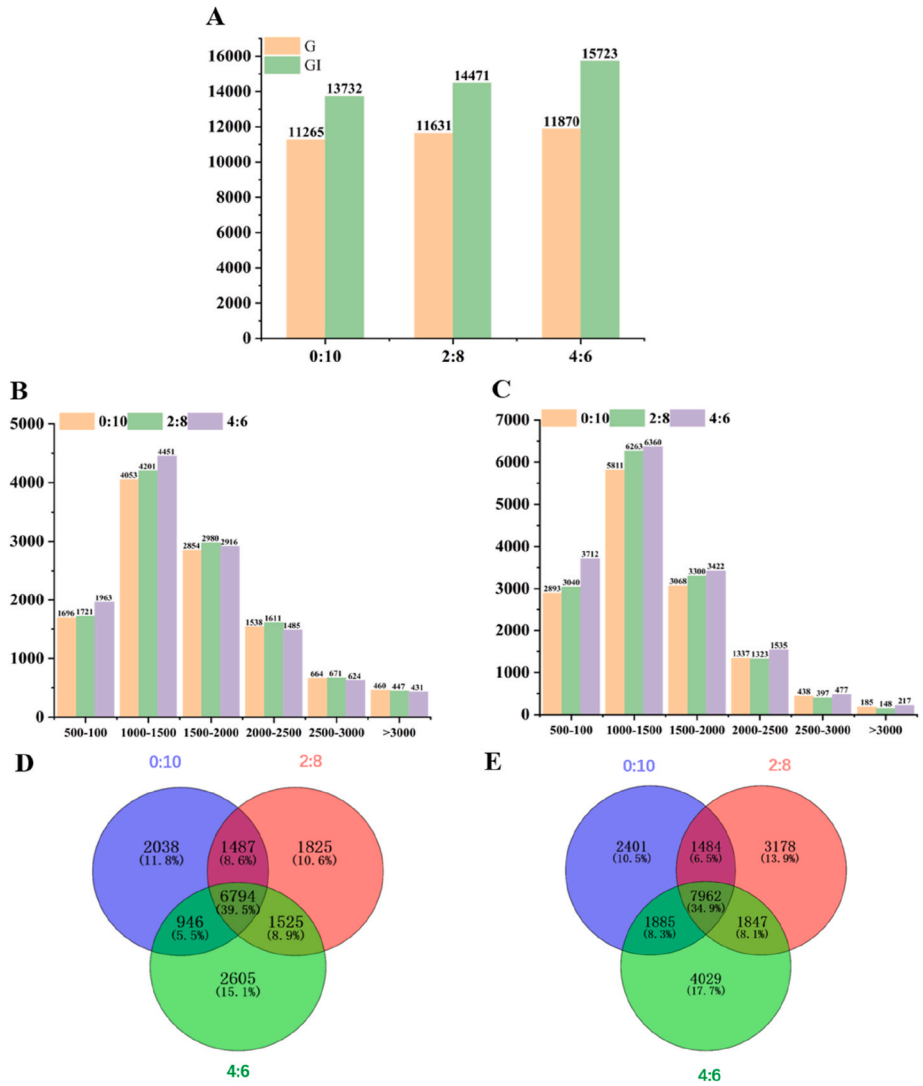

Fig. 3. Composition of the identified peptides in digestive supernatant of dual-protein extrudates with surimi to soybean flour ratios at 0:10, 2:8 and 4:6. A: Numbers of the identified peptides; B: Molecular weight distribution of peptides in the gastric digestion phases. C: Molecular weight distribution of peptides in the gastrointestinal digestion phase; D: Variance analysis of peptides in the gastric digestion phases; E: Variance analysis of peptides in the gastrointestinal digestion phases.

extrudates, constituting 39.5% of the total. In the gastrointestinal digestion phase, the number of common peptides rose slightly to 7962, though their proportion decreased to 34.9%. Meanwhile, the number of unique peptides further increased to 2401, 3178, and 4029, representing 10.5%, 13.9%, and 17.7%, respectively, showing a consistent upward trend with the rising proportion of surimi.

The comprehensive analysis of the number of identified peptides, along with their molecular weight distribution and variance, revealed that increasing the proportion of surimi led to the release of a greater number of smaller molecular weight peptides and a higher proportion of differential peptides in extrudates, thereby enhancing protein digestibility. This effect associated with improved digestion may primarily stem from the synergistic effect of changing in protein composition (Li et al., 2022; Yang et al., 2023) and structure (He et al., 2018; Li et al., 2022) of extrudates caused by the increased surimi proportion. These changes might enhance accessibility to proteolytic cleavage sites and generated unique peptides that would not appear when digesting any single protein alone (Luo et al., 2017).

3.4.6. *Potential bioactive peptide prediction*

Small molecular peptides, released from proteins following gastrointestinal digestion, have been reported to potentially exhibit bioactivities such as antioxidation and blood pressure reduction (Sayd et al., 2016). Conventional digestion assays can only characterize digestibility phenotypes but cannot evaluate the functional potential of digestive products, while this qualitative identification for peptide profiling analysis helps fill this gap. As previously indicated, increasing the proportion of surimi alters the protein composition of extrudates, generating a greater variety of unique peptides, which may fundamentally change the potency and diversity of potential bioactivities exhibited by the digestive peptides. PeptideRanker was employed to predict the potential bioactivity of identified peptides (solely based on sequence features) in the digestive supernatant of extrudates with surimi to soybean flour ratios of 0:10, 2:8, and 4:6, with peptides scoring above 0.5 defined as peptides with predicted bioactivity (Wang et al., 2022).

During the gastric phase, the number of peptides with predicted bioactivity and their proportion to the total identified peptides showed only minor changes as the surimi to soybean flour ratio increased from 0:10 to 2:8. However, at the 4:6 ratio, the number of peptides with predicted bioactivity rose from 1462 to 1625, with the proportion increasing from 12.98% to 13.69% (Fig. 4A). A similar upward trend was observed in the subsequent gastrointestinal phase, where the number of peptides with predicted bioactivity increased from 1787 to 2560, and their proportion rose from 13.01% to 16.28%, indicating a more notable upward trend (Fig. 4B). These findings suggest that increasing the surimi proportion, particularly at higher levels, enhances the release of peptides with predicted bioactivity during *in vitro* simulated gastrointestinal digestion, which will provide preliminary clues for exploring the bioactive peptide resources of dual-protein extrudates. This result aligns with previous research indicating that real meat is more likely to produce peptides with predicted bioactivity compared to plant-based meat analogues (Xie, Cai, Zhao, et al., 2022).

It should be noted that the bioactive potential of peptides was only predicted using PeptideRanker based on sequence features, with no *in vitro/in vivo* functional validation. The actual physiological effects of these predicted bioactive peptides require further verification through cell-based assays or animal experiments.

4. Conclusion

This study establishes a comprehensive link between the surimi to soybean ratio and the nutritional-digestive profile of dual-protein extrudates, with three key findings: First, incorporating silver carp surimi effectively improved the nutritional quality of soybean protein-based extrudates, including enriching ω-3 PUFAs (EPA and DHA) in lipid profiles and achieving FAO/WHO-recommended amino acid balance (EAA/TAA >40%) via amino acid complementarity. Second, increased surimi proportion linearly enhanced protein digestibility, reduced digesta particle size, and promoted the release of more low-molecular-weight and potential bioactive peptides, thereby improving protein bioavailability and functional potential. Third, a practical "optimal window" (surimi to soybean flour ratio ≥3:7) was defined, which successfully balanced processability with significant improvements in nutrition and digestibility. The surimi-soybean dual-protein strategy provides a viable technical pathway for upgrading plant-based meat analogues. Consequently, the soybean-surimi dual-protein strategy offers a viable pathway for developing the next generation of meat analogues with balanced nutrition and high absorbability, specifically for soybean protein-based extrudates.

Notably, this study has limitations: the findings rely on an *in vitro* static digestion model lacking gastrointestinal dynamics, and bioactive peptide predictions require *in vitro/in vivo* validation; additionally, the optimal ratio is only applicable to the tested raw materials and serves as a reference for other protein blends. Future research should address the limitations of the *in vitro* static digestion model, validate the improved bioavailability and metabolic benefits via *in vitro/in vivo* assays, inform other protein blends, and include sensory evaluations for market application.

CRediT authorship contribution statement

Xiaodong Li: Writing – original draft, Methodology, Investigation, Data curation. **Huihui Dai:** Methodology, Data curation. **Yanhong Bai:** Visualization, Supervision, Conceptualization. **Boning Mao:** Writing – review & editing, Visualization. **Xiaoying Luo:** Methodology, Data curation. **Lovedeep Kaur:** Writing – review & editing, Visualization,

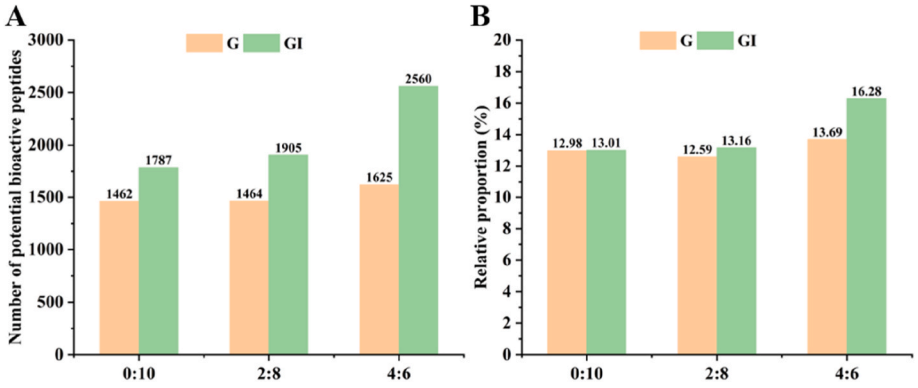

Fig. 4. Numbers (A) and proportions (B) of potentially bioactive peptides in digestive supernatant of dual-protein extrudates (with surimi to soybean flour ratios at 0:10, 2:8 and 4:6) at gastric (G) and gastrointestinal (GI) digestion phase.

Conceptualization. **Tao Yin:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Shanbai Xiong:** Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously.

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Appendix. ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2026.119246.

Data availability

Data will be made available on request.

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