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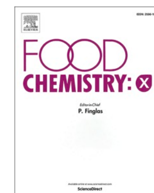

Fermented blend from sunflower seed press-cake and bovine sweet whey: Protein breakdown during *in vitro* gastrointestinal digestion

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ABSTRACT

Sustainable food production implements circular economic system, valuing side streams and minimizing waste. This study was aimed to develop a new food by fermenting a blend of dehulled sunflower seed protein powder (SSPP) and reconstituted bovine sweet whey powder (RSWP). Blends were inoculated with *Lactococcus lactis* B12 alone or in association with *Saccharomyces cerevisiae* L12, and fermentation proceeded until reaching pH 4.8. After *in vitro* static gastrointestinal digestion, RSWP and SSPP proteins were highly proteolyzed and the soluble nitrogen content was 69–71% of total nitrogen. In digests, 42–75 unique peptides were identified, and most of them weighed 500–1000 Da. Free amino acids accounted for 202–228 mg/g protein in digests. Few bioactive peptides derived from RSWP were identified. These findings demonstrated strong degradability of RSWP and SSPP proteins during digestion and shed light on nutritional properties exploitable for food applications of the developed fermented blend.

1. Introduction

So far, side streams from the food supply chain are still underexploited for human consumption, even though some of them contain interesting levels of nutrients. Often, they are intended for animal feed or to produce fertilisers, energy and other materials (Hagman, 2023). However, the valorisation of food side streams through their use as new ingredients or products is fundamental to support the implementation of a circular economy system and more sustainable food production (HLPE, 2014). In this scenario, plant by-products, such as those derived from oilseed production, are not only cost-effective raw materials, but can also serve as suitable sources of dietary fibres and proteins (Arrutia et al., 2020; Hoehnel et al., 2022). Furthermore, the demand for plant-based proteins among consumers increased due to a growing awareness of the impact of personal choices on the environment, health and animal welfare (Hertzler et al., 2020). The food industry can capitalize on this trend by formulating innovative foods based on alternative protein sources to meet new needs. In this context, approximately 36% of the mass remaining after sunflower oil extraction is converted into a solid seed press cake. This side stream is derived from the third largest seed oil production globally, after soybean and rapeseed (USDA, n.d.). Sunflower seeds contain approximately 21% of proteins consisting of 40–90% salt-soluble globulins or helianthinins and 10–30% water-soluble albumins (Arrutia et al., 2020). Helianthinins are oligomers with high molecular weight (11S globulins), which may dissociate to either lower molecular weight oligomers (7S) or monomers (2S—3S) (Ivanova et al., 2013). According to Hadidi et al. (2024), protein content in the derived dehulled sunflower seed press (SSPP) cake can reach 50% (*w*/w), or even more. On the other hand, plant proteins show generally a lower digestibility and may lack essential amino acids when compared to animal counterparts, and particularly sunflower proteins lack lysine (Conde et al., 2005). In addition, various industrial oil extraction methods can influence nutritional properties of proteins (Ivanova et al., 2013). Overall, *in vitro* gastrointestinal digestion protocols have resulted in an estimated protein digestibility-corrected amino acid score (PDCAAS) below 0.60 (Alexandrino et al., 2017; Petrusán et al., 2016).

A strategy for improving the nutritional value of plant-based proteins is to combine them other protein sources (Petrusán et al., 2016). This

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approach also helps to reduce food waste by blending side streams containing proteins with complementary amino acid composition. In this regard, bovine sweet whey contains proteins with high biological value, and it is a valuable source of dietary essential amino acids. It represents the liquid side stream that remains after renneting of milk and separation of curd during cheesemaking and, although it has various uses in human and animal nutrition, it is estimated that 50% of this side stream is wasted (Pires et al., 2021).

Recently, there has been significant interest in fermenting plant materials to create more sustainable foods with improved functional and sensory properties and long shelf life. This is achieved through organic acid and/or ethanol production by lactic acid bacteria and yeasts, which creates a hostile environment that limits or inhibits the growth of harmful or spoilage bacteria and moulds. Moreover, they have often been associated with several beneficial effects, including synthesis of vitamins, removal of antinutrients, enhancement of protein digestibility, and their potential use as probiotics (Şanlier et al., 2019).

Previous studies have enhanced the safety and technological properties of blends produced from sunflower seed proteins and bovine sweet whey through the utilisation of fermentation with co-cultures of the *L. lactis B12* strain and the *Kluyveromyces lactis* L2 strain (Mangieri et al., 2022; Raak et al., 2023). This approach proved to be effective in establishing an unfavourable environment for proliferation of pathogens, spoilage bacteria, and moulds upon a reduction in the blend pH value to 4.8 within 24 h and the production of ethanol. Based on these findings, in the present work we designed and prepared a fermented blend of SSPP and reconstituted bovine sweet whey powder (RSWP) inoculated with L. *lactis* B12 and *S. cerevisiae* L12 strain, alone or as a coculture. This yeast strain was selected over the *K. lactis* L2 strain for its higher pleasantness, as indicated by sensory evaluations in preliminary trials (personal communication by Sophie Morejón Caraballo, PhD).

The purpose of this work was to evaluate some nutritional properties of this food by studying the degree of protein breakdown during *in vitro* gastrointestinal digestion. To this aim, the release of soluble nitrogen with a molecular weight < 3 kDa, the peptidomic and amino acid profiles of the digested blends were studied. Moreover, the release of potential bioactive peptides (BAP) in the digested blends was assessed.

2. Materials and methods

2.1. Preparation of the fermented blend

The blend was prepared by using a commercial SSPP (Schalk Mühle, Kalsdorf bei Ilz, Austria) and a commercial powder of bovine sweet whey (Tolnatej zrt Szekszárd – Hungary). The SWP was used after reconstitution (6%, *w/v*) in demineralized water, while the SSPP was previously subjected to a washing step in demineralized water at a ratio of 1:10 (*w/v*) by stirring 10 min at 480 rpm at room temperature. This preliminary washing aimed to partially debitter the SSPP by removing polyphenols. After centrifugation at 8000*g* for 30 min at 10 °C, the pellet was recovered. The SSPP pellet and the RSWP were mixed at 60:40 mass ratio, corresponding to 22.5% and 2.4% solids in the final blend, respectively. The blend was kneaded in a planetary machine (Bosch mod. CNUM5ST) at speed 4 (considered as "fast") for 10 min and then it was sterilized in autoclave at 121 °C for 15 min and promptly cooled to room temperature. One blend aliquot (sample A) was inoculated with fresh cultures of *Lactococcus lactis* B12 and *Saccharomyces cerevisiae* L12 at a starting concentration of 6.0 log CFU/g and 5.0 log CFU/g, respectively. Another aliquot (sample B) was inoculated (6.0 log CFU/g) only with L. *lactis* B12. Finally, a non-inoculated aliquot served as a control (sample C). Fresh cultures of L. *lactis* B12 and *S. cerevisiae* L12 were prepared according to Raak et al. (2023). Samples A and B were incubated under static conditions at 26 °C, and fermentation was stopped when a pH value of 4.8 was reached, by quickly refrigerating at 4 °C. Control sample C was acidified before sterilization with L-lactic acid (80% solution, Merck, Darmstadt, Germany) to achieve the same pH value. Microbial counts were carried out following standard protocols of decimal dilutions of the sample and plating in specific growth media for each investigated group (Raak et al., 2023). The samples were then stored at −20 °C. The flowchart of the production of the fermented blend is reported in Fig. 1.

2.2. Static in vitro gastrointestinal digestion

The fermented blends and the control sample were digested according the INFOGEST protocol (Brodkorb et al., 2019). In the present study, 2.5 g of each sample were mixed with 2.5 mL of simulated salivary fluid (SSF) and 75 U mL^{-1} SSF of salivary α-amylase incubating at 37 °C for 2 min. Then 5 mL of simulated gastric fluid (SGF) and porcine pepsin (2000 U mL^{-1} SGF) were added into the mixtures. The gastric digestion was carried out at 37 °C for 2 h at pH 3.0, adjusted with 2 N HCl. Following the gastric digestion, 10 mL of simulated intestinal fluid (SIF) and bile salts (10 mM) were added to the gastric digestate. Porcine trypsin (200 U mL^{-1} SDF), bovine chymotrypsin (50 U mL^{-1} SIF), pancreatic amylase (200 U mL^{-1} SIF), porcine intestinal lipase (4000 U mL^{-1} SIF) and co-lipase (2:1 ratio) were used as enzymes for the intestinal digestion. The intestinal phase of gastrointestinal digestion took place at 37 °C for 2 h and at a pH of 7.0. The digests were frozen at −20 °C until analyses. A digestion blank (enzymes, bile salts and simulated digestive fluids in absence of substrate/blend) was also prepared. All enzymes used in this experiment were from Merck.

2.3. Determination of soluble nitrogen content

To assess protein breakdown, the soluble nitrogen (SN) fraction with a molecular weight < 3 kDa was measured before and at the end of the *in vitro* gastrointestinal digestion of blends. To this purpose, the samples were ultrafiltered using a stirred ultrafiltration (UF) cell equipped with a regenerated cellulose membrane (Amicon, Merck) with a molecular weight cut-off of 3 kDa. The digested samples were directly ultrafiltered through the membrane, while the undigested counterparts were suspended in water (1:10, *w/v*) prior to UF. The protein breakdown (as % of total N) was calculated by Eq. (1):

$$N_T(\%) = \frac{N_{SGID} - N_B - N_{SB}}{N_T} \bullet 100 \quad (1)$$

where: N_{SGID}, N content of the UF (3 kDa) permeate of the samples after gastrointestinal digestion; N_B, N content of the UF (3 kDa) permeate of digestion blank (enzymes, bile salts and simulated digestive fluids) after gastrointestinal digestion; N_{SB}, N content of UF (3 kDa) permeate of the samples before gastrointestinal digestion; N_T, total N content of the samples.

The N content of each fraction was determined by Kjeldahl method.

2.4. SDS-page

The SDS-PAGE was performed on the (non-)digested samples using 12% polyacrylamide gels. Before analysis, both non-digested and digested samples were diluted with Tricine Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 2% β-mercaptoethanol. After denaturation at 95 °C for 5 min, 10 μL of the diluted samples and 5 μL of the ladder (Protein Marker VI, PanReac, Castellar del Valles, Spain) were loaded, and the gel was run in TRIS/Glycine/SDS Running Buffer (Bio-Rad Laboratories) on a Mini vertical electrophoresis unit (SE250, Hoefer, Holliston, MA, USA) at a constant voltage of 85/100 V. Gel was subsequently stained with Coomassie brilliant Blue R-250 (Merck).

2.5. Determination of free amino acids by UPLC/HR-MS/MS

In vitro digests of the blends were analysed to assess the free amino acid content. Specifically, after centrifugation at 10000*g* for 10 min the

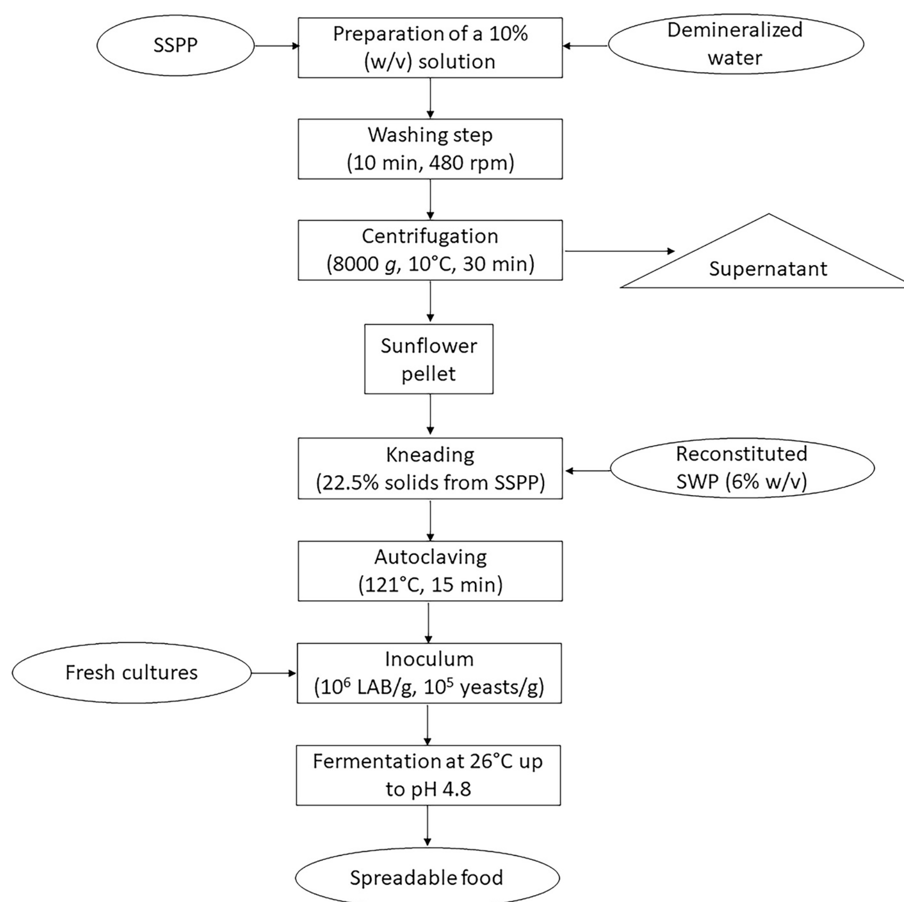

Fig. 1. Flowchart of the production process of the fermented blend.

supernatants were filtered through a 3 kDa MWCO Omega polyethersulfone UF membrane in a Nanosep Advance device (Pall, Port Washington, NY, USA). Free amino acids were quantified on an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Q Exactive high-resolution mass spectrometer (HR-MS) (Thermo Fisher Scientific, Waltham, MA, USA). An aliquot of 10 μL was diluted 1:9 with eluent A and separated on an Accucore 150 Amide HILIC column (150 × 2.1 mm, 2.6 μm) (Thermo Fisher Scientific) maintained at 30 °*C. mobile* phase A consisted of acetonitrile-ammonium formate buffer at pH 2.8 (90:10 *v/*v) and mobile phase B water-ammonium formate buffer at pH 2.8 (90:10 v/v). The final ammonium formate concentration in both mobile phases was 20 mM. For UPLC separation, a linear elution gradient (0%-to-15% of eluent B in 8 min) was applied at a flow rate of 0.4 mL/min, followed by a column wash and re-equilibration (run-to-run time 20 min). The UPLC eluate was analysed by HR-MS on a Q Exactive instrument interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The ion source and interface conditions were: spray voltage 2500 V (positive polarity); probe heater temperature 280 °C; ion transfer tube temperature 350 °C; S-lens RF level 50; sheath gas pressure 35 psig; auxiliary gas pressure 15 psig. Mass spectra were acquired over the *m/z* range 50–500 in positive polarity in a Full MS mode. The resolution was set at 70000. The automatic gain control (AGC) target was 3×10^6, and the maximum ion injection time was 200 ms. Amino acid identification and quantification were conducted using the Xcalibur software (v3.0, Thermo Fisher Scientific). Peak areas were calculated from extracted ion chromatograms of each protonated amino acid ion, with 5-ppm mass tolerance. Analyses were performed in triplicate, and the results were expressed as mg amino acid/g protein. Serial dilutions of single amino acid were prepared as external standards (5-point calibration curve).

2.6. *Determination of peptides by UPLC/HR-MS/MS*

To identify peptides, 5 μL of digested and 10 kDa-ultrafiltered [10 kDa MWCO Omega polyethersulfone UF membrane in a Nanosep Advance device (Pall)] samples were separated on an Aeris PEPTIDE XB-C18 column (150 × 2.1 mm, 1.7 μm) (Phenomenex, Torrance, CA, USA) maintained at 50 °C on an Acquity UPLC chromatographic system (Waters). The eluents were: 0.1% (*v*/v) formic acid (FA) in MilliQ-treated water (solvent A) and 0.1% (v/v) FA in acetonitrile (solvent B). For UPLC separation, a linear elution gradient was applied (2% to 55% of solvent B in 35 min) at a flow rate of 0.3 mL/min. The LC eluate was analysed by HR-MS/MS on a Q Exactive instrument (Thermo Fisher Scientific) interfaced through a HESI-II probe for electrospray ionization (Thermo Fisher Scientific). The ion source and interface conditions were set according to Cattaneo et al. (2020). The LC eluate was analysed by MS using full scan and data dependent tandem MS analysis (ddMS2). Mass spectra were acquired over *m/z* range 100–1500; the ten most intense (Top 10) $1+-8+$ charged ions detected in each spectrum underwent HCD fragmentation. The resolution was set at 70000 and 17,500 for full scan and ddMS2 scan types, respectively. The AGC targets were 1×10^5, and maximum ion injection times were 110 ms. Peptide sequences from sunflower proteins were identified from MS/MS spectra using SequestHT algorithm against the protein databases of *Helianthus annus* L. (UniProt taxon ID 4232) and *Bos taurus* (UniProt taxon ID 9913). Automatic peak detection was performed with a setting of signal-to-noise ratio of 4. A non-specific enzyme cleavage pattern was defined, and 12 missed cleavage sites (maximum allowed by the algorithm) were set. Phosphorylation of serine and threonine, deamidation of asparagine, glutamine and arginine, oxidation of methionine and cyclisation of an N-terminal glutamine to pyro-glutamic acid were selected as dynamic

modifications. Mass error tolerance for precursor ions was 5 ppm and for fragment ions was 0.02 Da. A strict false discovery rate of peptide identification was set (FDR = 0.01). Two digests (obtained in two consecutive days) were used for the UPLC/HR-MS/MS analysis by performing double injections of each. Acquisitions were processed with Proteome Discoverer 1.4 software (Thermo Fisher Scientific) upon merging the four analyses data outputs, to obtain multiconsensus reports with an absolute number of peptides that was found in each sample.

2.7. Identification of bioactive peptides

To identify BAP in the digested samples, the sequences of the peptides detected through UPLC/HR-MS/MS were searched against the Biologically Active Peptide Sequences database (BIOPEP: https://biochemia.uwm.edu.pl/biopep/peptide_data.php) (Minkiewicz et al., 2019) and the Milk Bioactive Peptide database (MBP: http://mbpdb.nws.oregonstate.edu) (Nielsen et al., 2017; updated 16th of April 2024). The search was performed by setting the following parameters: search by "sequence" for BIOPEP database and "100" for similarity threshold, "identity" for amino acid scoring matrix and "yes" for getting extra output for MBP database. To expand the search for potential BAP from sunflower proteins, a sunflower BAP database was created upon a literature survey, as detailed in Supplementary Material.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to infer the existence of differences among the mean values, utilising the StatGraphics Plus 5.1 program (Statgraphics Technologies, The Plains, VI, USA). Subsequently, the post-hoc Tukey's test was conducted with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Preparation of fermented blend from SSPP and RSWP and evaluation of its protein breakdown

The use of plant proteins increased in recent years due to their positive effects, provided the essential amino acid balance is maintained and the intake is in line with current dietary recommendations (Hertzler et al., 2020). For these reasons, it is fundamental to assess the protein degradability since vegetable proteins may display low digestibility beside lacking some essential amino acids (Santos-Hernández et al., 2020).

The entire process for manufacturing the fermented blend is shown in Fig. 1. The gross composition of the blend was calculated from the labels of SSPP and SWP provided by the commercial suppliers, and it was as follows (%, *w*/w): 1.9 fat, 2.9 carbohydrates, 5.9 fiber, 13.9 proteins. An aliquot of blend was inoculated with L. *lactis* B12 and *S. cerevisiae* L12 (sample A). Another aliquot (sample B) was inoculated only with L. *lactis* B12 strain, whereas a non-inoculated aliquot acidified to pH 4.8 with lactic acid before sterilization served as a control (sample C). The fermentation time to reach pH of 4.8 was approximately 24 h for both samples. After fermentation, the lactococci count in sample A increased from 6.0 log CFU/g to 9.1 log CFU/g, while that of yeasts raised from 5.0 log CFU/g to 7.1 log CFU/g. In sample B, the lactococci count reached 8.9 log CFU/g. As expected, the counts of the lactococci and yeasts in non-inoculated blend (sample C) were below the detection limit (<2.0 log CFU/g).

All three samples were then submitted to static *in vitro* gastrointestinal digestion according to the protocol proposed by Brodkorb et al. (2019). At the end of digestion, the protein breakdown was evaluated by determining the content of soluble nitrogen ($N < 3$ kDa) (Table 1).

In non-digested blends, this content accounted for 2.8–3.4% of total N, whereas after digestion it largely increased, reaching 69–71% of total N in all samples. As a matter of fact, no detectable proteolysis was ascribable to fermentative activities of L. *lactis* B12 or *S. cerevisiae* L12.

Table 1
Soluble nitrogen content (%) of blends A, B and C (average values of 3 analyses) before and after static *in vitro* gastrointestinal digestion. Lower-case letters indicate significant ($p < 0.05$) differences among non-digested and digested samples.

	Blend		
	A	B	C
Non-digested	$2.8^a \pm 0.4$	$3.3^a \pm 0.3$	$3.4^a \pm 0.3$
Digested	$70.0^b \pm 1.2$	$69.1^b \pm 0.9$	$70.6^b \pm 2.2$

The blends A, B and C were also submitted to SDS-PAGE before and after the gastric and intestinal phases of *in vitro* digestion. The electrophoretic patterns of non-digested blends showed the presence of bands corresponding in size to the most abundant proteins of SSPP (11S-helianthinin and 2S-helianthinin) (Fig. 2). Electrophoretic bands potentially attributable to whey proteins were faint or unclear, given both their low amount in blends and the adopted staining procedure. The fermentative step did not discriminate fermented samples A and B from the control based on the revealed electrophoretic patterns. Consequently, neither L. *lactis* B12 nor *S. cerevisiae* L12 showed proteolytic activity in the adopted experimental conditions and in this growth medium, as already observed by Raak et al. (2023). Although the L. *lactis* presents proteolytic systems on the cell wall (Guillot et al., 2016), the availability of free amino acids derived from the sunflower component of the blend (Arrutia et al., 2020) was likely sufficient to prevent significant proteolysis.

After the gastric phase, only smeared bands below 20 kDa were observed, indicating a notable protein breakdown upon pepsin attack. At the end of the intestinal phase, no electrophoretic bands were detected in all digests, confirming that a strong protein breakdown occurred upon the complete *in vitro* gastrointestinal digestion of blends.

3.2. Free amino acid profile

Generally, milk proteins show higher true amino acid digestibility (TAAD) than plant counterparts (Rutherfurd & Moughan, 1998). Nonetheless, few research studies are available dealing with the degradability of proteins from SSPP and the release of amino acids upon gastrointestinal digestion. The fermented and digested blends were analysed by UPLC/HR-MS/MS to assess their free amino acid content. At the end of digestion, the total amount of free amino acids was similar in the three studied samples (Table 2).

Conde et al. (2005) examined the protein composition of the residual sunflower cake after oil extraction, highlighting that the amino acid composition met the standard requirements (FAO - Food and Nutrition Paper 92, 2011), except for the lysine content. This outcome was confirmed by other authors for sunflower proteins derived from kernels, oil cakes, seeds or meal (Nenova & Drumeva, 2012; Zhang et al., 2023).

After static *in vitro* gastrointestinal digestion, proteolysis performed by digestive enzymes of SSPP and RSWP proteins accounted for an important release of free amino acids. In this regard, Salgado et al. (2012) firstly observed a high *in vitro* degradation of protein isolates from sunflower press cake with a value over 95% when compared to casein as a reference (100%). However, the digestibility was calculated using a potentiometric method after a multienzyme treatment, which differed from the *in vitro* digestion protocol (Brodkorb et al., 2019) adopted in the present work. In contrast, Petrusán et al. (2016) reported a low digestibility value of sunflower proteins (0.37), expressed as PDCAAS, based on results dating back to the last century. Recently, the *in vitro* digestibility of various sunflower protein isolates was estimated to range from 91% to 95%, although another different digestion protocol was used (Alexandrino et al., 2017). The related calculated values of PDCAAS also varied from 0.52 to 0.59.

In the digests of blends, the free lysine content was <2.0 mg/g protein and, therefore, it accounted for about 10% or 3% of the total

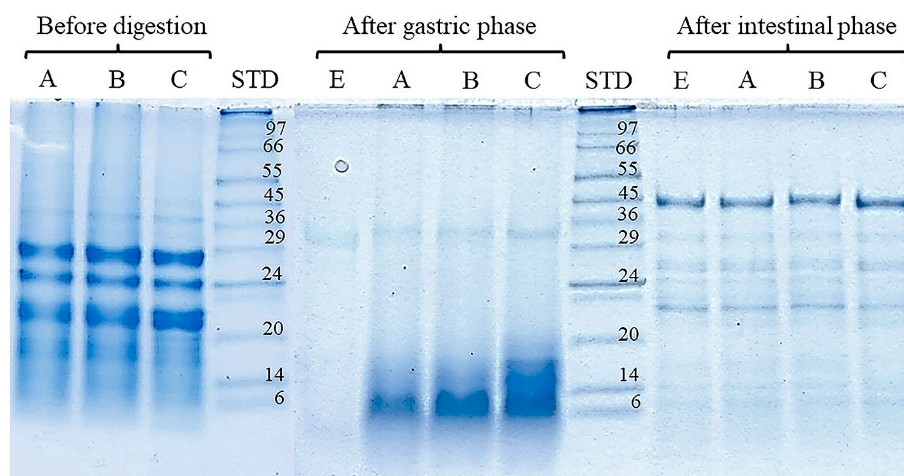

Fig. 2. SDS-PAGE profiles of blends A, B and C before *in vitro* static gastrointestinal digestion, after gastric and after intestinal phases; E: gastric and intestinal enzymes blank; STD: protein ladder (reference band sizes are reported in kDa).

Table 2
Free amino acid content (mg/g protein) of *in vitro* digested A, B and C blends (average values of 3 analyses); nq: not quantifiable, <0.1 mg/g protein. Values in rows followed by different superscript letter differ significantly ($p \leq 0.05$).

	Blend		
Amino acid	A	B	C
Trp	12.0 ± 0.2	14.6 ± 0.2	13.0 ± 0.3
Phe	38.3 ± 0.3	42.3 ± 0.5	39.4 ± 0.4
Leu	51.0 ± 0.7	58.8 ± 0.8	53.2 ± 0.4
Ile	10.2 ± 1.0	12.5 ± 0.6	12.0 ± 0.2
GABA	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Met	9.7 ± 0.1	8.3 ± 0.1	11.3 ± 0.3
Tyr	17.1 ± 0.2	11.8 ± 0.1	18.2 ± 0.2
Val	9.9 ± 0.1	12.6 ± 0.3	12.0 ± 0.1
Pro	0.8 ± 0.0	1.5 ± 0.0	1.4 ± 0.0
Ala	1.6 ± 0.2	3.8 ± 0.3	3.7 ± 0.1
Thr	0.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.0
Gly	0.5 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
Glu	1.1 ± 0.0	0.6 ± 0.1	0.4 ± 0.1
Ser	1.1 ± 0.1	0.6 ± 0.1	0.5 ± 0.2
Gln	34.3 ± 0.5	40.0 ± 0.5	38.4 ± 0.3
Asn	0.4 ± 0.2	1.9 ± 0.2	2.3 ± 0.0
Asp	0.9 ± 0.0	0.8 ± 0.1	0.7 ± 0.0
Arg	9.0 ± 0.8	12.7 ± 0.4	13.1 ± 0.2
His	0.1 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
Orn	nq	0.2 ± 0.0	0.1 ± 0.0
Lys	2.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.1
Cys/Cys-Cys	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
Total	201.7 ± 0.9[a]	227.7 ± 0.4[c]	224.3 ± 0.8[b]

lysine releasable from complete digestion of one gram of SSPP or RSWP proteins, respectively, considering their aminoacidic composition (Farkye & Shah, 2015; Ivanova et al., 2013). According to San Juan and Villamide (2001), the TAAD of lysine is strongly affected by the sunflower seed processing during oil extraction. The same authors observed a minor decrease for TAAD of branched-chain amino acids. In the present work, we recorded that the released mean amount of Leu, Ile and Val accounted for about 18% of the total amount of branched-chain amino acids releasable per gram of SSPP and RSWP proteins. Interestingly, a higher relative release (about 34%) was observed for the aromatic species (Phe, Trp and Tyr), which were present in blend digests at 68.9 ± 0.5 mg/g, on average. The free sulfur-containing amino acids were almost represented by methionine (mean 9.8 mg/g protein). San Juan and Villamide (2001) observed a stronger TAAD decrease for cystine as compared to that of methionine after sunflower seed processing. Rutherfurd and Moughan (1998) found TAAD of methionine and lysine to be 2 and 1.3 times higher, respectively, in dairy proteins than in soy proteins.

Overall, the strong breakdown observed during the gastrointestinal digestion support the high digestibility of protein of SSPP and RSWP even in blends. This feature is paramount because simultaneous and strong protein degradation allows the release of amino acids that complement the biological value of the two groups of proteins.

3.3. Peptidomics

Fig. 3 shows the number of unique peptides detected in the gastric and intestinal digests of fermented blends and in the control sample, according to their parent proteins. Overall, 354, 350 and 255 peptides were revealed in the gastric digest of blends A, B and C, respectively. More than a half of the identified peptides in all digests originated from 11S helianthinin, the most abundant globulin in sunflower seeds (Arrutia et al., 2020). About 25% of the unique sunflower peptides detected in the gastric digest originated from Heat Shock Protein Class I, 2S Seed Storage Protein and Albumin-8. Despite the lower content of whey proteins in the blend, β-lactoglobulin was a relevant source of peptides in digested samples (Fig. 3). After the gastric phase, the higher number of peptides released from this protein in fermented samples might be attributed to a moderate proteolytic activity of L. *lactis* during fermentation of blends (Kazemi et al., 2018). This difference disappeared at the end of the digestion due to the activity of the intestinal enzymes. At the end of gastrointestinal digestion, the number of detected peptides decreased to 42, 75 and 47 in digest of blends A, B and C, respectively (Fig. 3). Again, most of peptides were released from 11S helianthinin, while peptides from whey proteins persisted in digests. The presence of peptides derived from casein fractions in blend digest can be attributed to the presence of casein (sub)micelles in the bovine sweet whey that are not retained in the coagulum during cheesemaking. Moreover, presence of peptides from β- and κ-caseins relates to the hydrolysis of proteose peptones (derived from the plasminolysis of β-casein) and glycomacropeptide originating from rennet coagulation of cheese milk (Wada & Lönnerdal, 2015).

The distribution of peptides according to MW is shown in Fig. 4. For all samples, about 75% of the peptides, released after the gastric phase, were within the MW range 500–1500 Da, *i.e.* on average 4–13 amino acids long. After the intestinal phase, peptides of 500–1000 Da mainly generated, representing 60–80% of total peptides, and species above 2500 Da did not occur (Fig. 4). These findings account for an important breakdown of blend proteins due to the activity of intestinal enzymes. It should be remembered that the peptidome search engine used in the present work could not recognize sequences shorter than 4 amino acids. This feature also supports the small number of peptides revealed in the

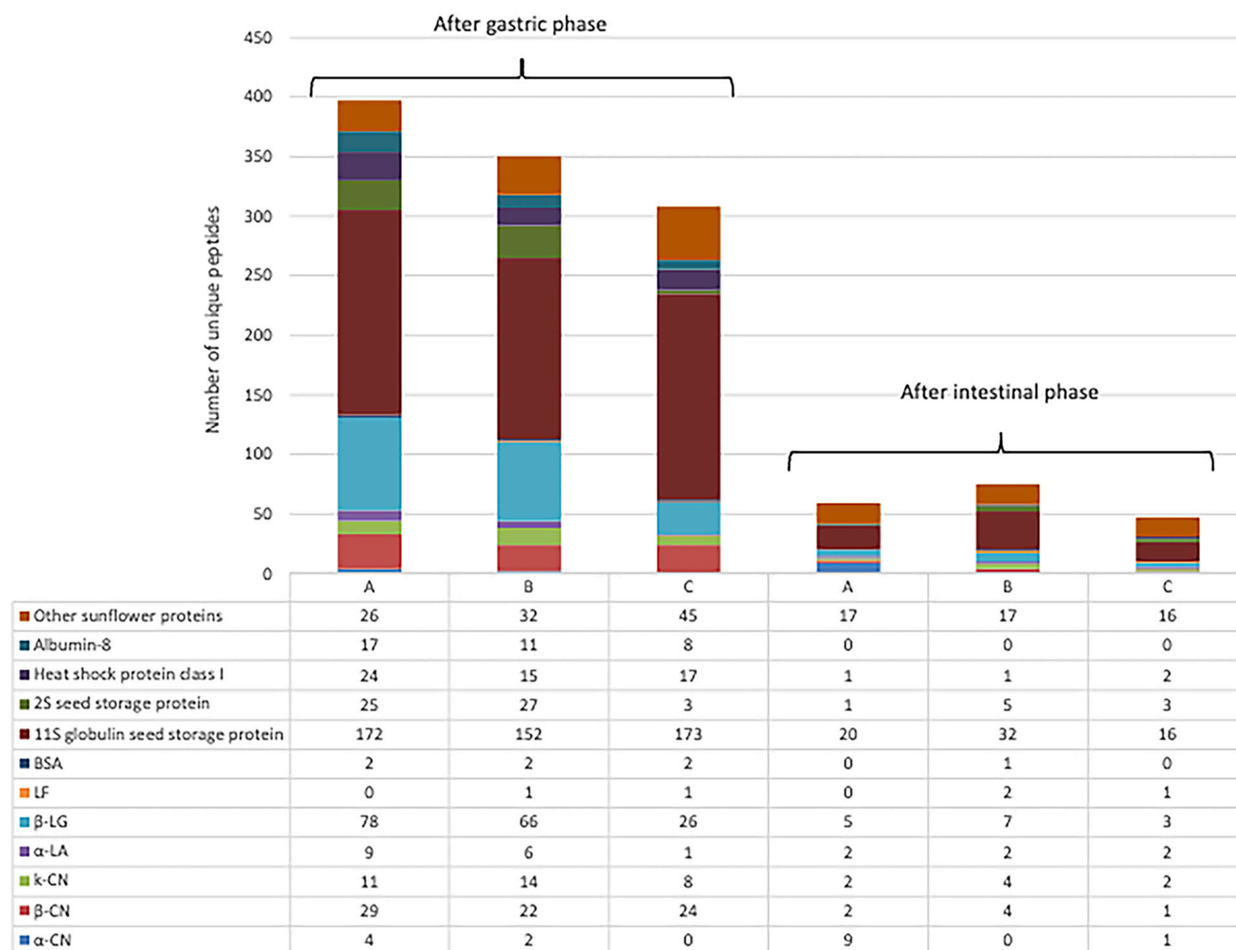

Fig. 3. Number and parent proteins of unique peptides revealed in digests of A, B and C blends, after gastric and intestinal phases of *in vitro* digestion.

intestinal digests of blends.

While plethora of research studies on BAP derived from bovine whey proteins are available, little information is present on bioactive species from sunflower seeds. Particularly, biological activities that have been associated with specific peptides derived from sunflower proteins are: antioxidant, angiotensin-converting enzyme inhibition, anti-inflammatory and immunomodulating) as well as antimicrobial (Dabbour et al., 2019; Dabbour et al., 2020; Megías et al., 2009; Taha et al., 2013; Velliquette et al., 2020; Zhao et al., 2017). Most of this evidence comes from *in vitro* studies, and only a few peptides have been tested with cell cultures or animal models. Moreover, an *in vitro* digestion simulation was not often used, which raises questions whether these peptides are produced *in vivo*. In a recent study by Tonolo et al. (2024), isolated sunflower proteins were subjected to *in vitro* static gastrointestinal digestion according to the same protocol used in this work. The peptide fraction obtained from the digestion was tested *in vivo* both on zebrafish larvae and on Caco-2 cells. Some individual peptides were selected *in silico* based on their possible antioxidant properties, and then they were synthesized and tested alone on Caco-2 cells. It emerged that both the peptide fraction resulting from digestion and the synthetic peptides (DVAMPVPK, VETGVIKPG, TTHTNPPPEAE, LTHPQHQQQGPSTG and PADVTPEEKPEV) showed antioxidant capacity. The peptide PADVTPEEKPEV also inhibited the expression of pro-inflammatory cytokines and the activation of NF-kB pathway.

To identify BAP, we searched the peptide sequences detected in the digested blend samples against the databases BIOPEP and MBP (as reported in Materials and Methods). Furthermore, an archive of sunflower protein sequences was set up by identifying 71 scientific papers (Supplementary Materials). Seven articles were selected based on their reported amino acid sequences of sunflower proteins, which were deemed useful for aligning purposes. In this work, only four peptides released upon digestion of caseins present in RSWP were reported as BAP. In detail, the peptides VYPFPGPI and EAMAPK from β-casein were reported as ACE- and prolyl endopeptidase-inhibitors and antimicrobials, respectively (Asano et al., 1992; Pepe et al., 2016). ACE-inhibitor and antithrombotic activities have been suggested for the κ-casein peptide MAIPPK (Hayes et al., 2007). Finally, the peptide YLGYLEQLLR from α_{S1}-casein has been described to elicit antianxiety function (Wada & Lönnerdal, 2015). Conversely, the search against databases did not result in any BAP from the SSPP proteins.

4. Conclusions

The results of the present study indicate that proteins in fermented blend of SSPP and RSWP underwent considerable breakdown during *in vitro* gastrointestinal digestion. Most of nitrogen in digested blend was present as soluble compounds with molecular weight below 3 kDa. Moreover, peptides of 500–1000 Da mainly generated in digests, and approximately 20% of blend protein broke down to free amino acids. Although no BAP was identified from SSPP, it cannot be excluded that peptides with yet unidentified biological properties may be present. Indeed, the search for sunflower BAP is still in its infancy, and data coming mainly from *in vitro* studies and non-harmonized digestion protocols are available in literature.

Overall, the obtained results highlight a strong degradability of SSPP proteins shedding light on nutritional traits to be exploited for its use as food or ingredient in food formulations. Based on this, the fermented blend developed in this work appears as an interesting nutritional

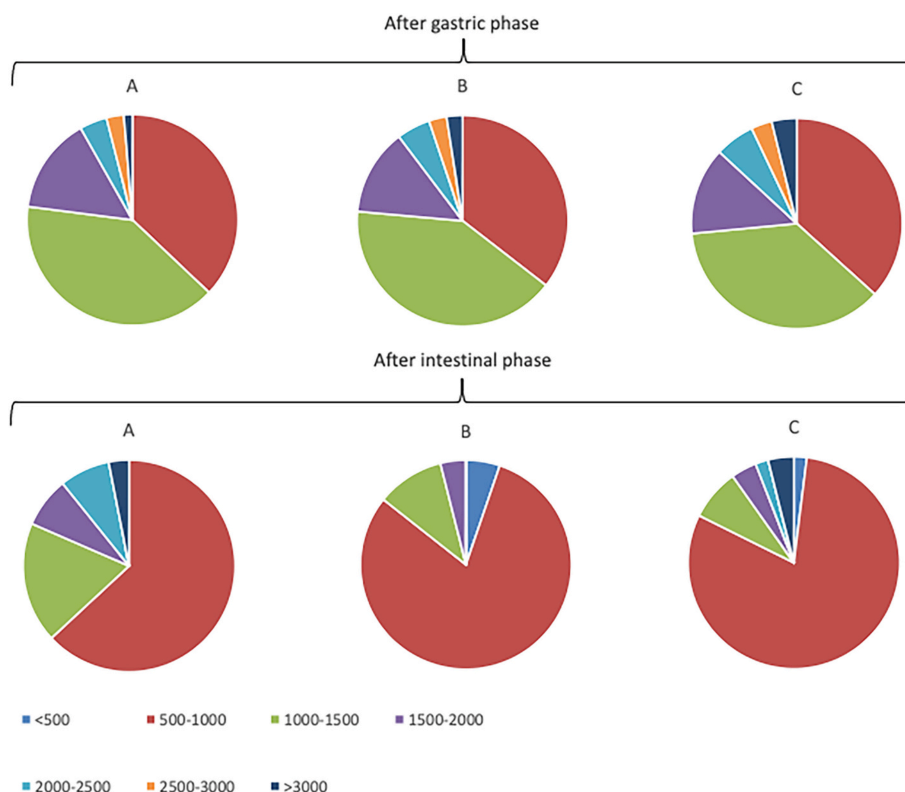

Fig. 4. Molecular weight distribution of peptides detected in the gastric and intestinal *in vitro* digests of A, B and C blends; values are expressed in Da.

product and a technological approach to increase the circularity of food supply chains by valorising sunflower and dairy side streams.

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CRediT authorship contribution statement

Sofia Mendo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Irene Da Costa:** Writing – original draft, Investigation, Formal analysis, Data curation. **Stefano Cattaneo:** Formal analysis, Data curation. **Fabio Masotti:** Formal analysis, Data curation. **Milda Stuknytė:** Writing – review & editing, Formal analysis, Data curation. **Ivano De Noni:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Roberto Foschino:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101745.

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