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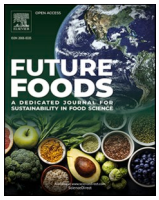

Bioprocessing of food industry surplus to obtain novel food ingredients enriched in bioactive peptides

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ABSTRACT

The food processing industries are responsible for the generation of substantial quantities of protein-rich waste and by-products posing significant environmental and economic challenges. However, their valorization through enzymatic hydrolysis, to produce biologically active peptides, offers a sustainable, circular approach with potential applications in novel functional foods.

In this study, aiming at releasing bioactive peptides, several food grade carbohydrases and proteases, alone or in combination, were used to treat brewers' spent grain (BSG), wasted bread (WB), soy okara (SOK), grape pomace (GP), and wine lees (WL). The results demonstrated that, due to their strong endopeptidase activity, Alcalase® and Neutrase® significantly enhanced peptide content in BSG, WB, and SOK, with increases reaching up to 22-fold, while Veron PS exhibited comparatively weaker effects. The radical scavenging activity markedly improved, particularly in WB after treatment with Alcalase® (up to 150 %). Whereas other residues were found to have higher (up to +20 %) antihypertensive activity compared to untreated samples. UHPLC/HR-MS^2 in conjunction with *in silico* tools led to the identification of numerous peptide sequences that possess significant antioxidant and antihypertensive properties. Moreover, short epitopes (e.g., PFP, PPP, PLL, GLFL), typical of bioactive peptides, were recurrently identified.

As proof-of-concept, the enzymatically treated ingredients exhibiting the highest bioactivity were incorporated into a novel functional food prototype, a "granola", with a nutritionally relevant profile, rich in fibres and protein, and potential multi-faceted health benefits, since the antioxidant and ACE-inhibitory properties were retained after baking.

1. Introduction

The global food industry faces a significant challenge in managing the vast quantities of waste and by-products generated during food processing, which contribute to environmental pollution and loss of valuable resources. For this reason, there is a growing interest in valorizing food residues through sustainable and innovative approaches that align with the principles of the circular economy (Sarker et al., 2024; Zhou et al., 2023a). One promising strategy involves the use of enzymatic hydrolysis which offers a sustainable and cost-effective approach to waste management. This approach has been shown to reduce environmental impact and promote a circular economy (Bilal and Iqbal, 2019). Enzymes exhibit superior reaction rates and efficiency under mild conditions in comparison to microorganisms (Chinea et al., 2025). They have a variety of applications, related to food industry waste and by-products, including i) the production of biofuels and other biomaterials, ii) the pre-treatment of waste for improved bioremediation processes like composting or anaerobic digestion (Bilal and Iqbal, 2019),

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but most of all iii) the breakdown of complex macromolecules like proteins, into smaller ones (e.g., bioactive peptides) (Wang and Qi, 2024).

The health-promoting properties of bioactive peptides (antioxidant and antihypertensive activities among others) make them attractive candidates for incorporation into functional foods. Antioxidant peptides can neutralize free radicals, reducing oxidative stress, while antihypertensive peptides can inhibit angiotensin-converting enzyme (ACE), contributing to blood pressure regulation (Zhou et al., 2023a). Building on this knowledge, this study aimed at exploring the potential of various surpluses of the food industry (brewers' spent grains, wasted bread, soy okara, wine lees and grape pomace) as sources of bioactive peptides, using food grade enzymes commonly used in the food industry.

Brewers' spent grain (BSG) is the main by-product of beer production, accounting for up to 85 % of brewery waste. On average, every 100 L of produced beer generates 20 kg of BSG. Specifically, it consists of the outer layers of the barley kernel and is obtained as a result of the malting and mashing process (Jaeger et al., 2021). The bread wasted (WB) throughout its entire life cycle, from production to consumption, including distribution, is considerable. In fact, it is estimated that hundreds of tons of bread are wasted every day worldwide (Melikoglu and Webb, 2013). Soy okara (SOK), on the other hand, is the insoluble part of soybeans remaining after the production of plant-based drinks, following their grinding and filtration. World production of okara is approximately 1.4 billion tons, with Asian countries such as Japan, China and Singapore being the largest producers (Kamble and Rani, 2020). Grape pomace (GP), together with wine lees (WL), are the main waste products of the wine industry. GP, consisting of grape skins, seeds, and possibly stems, can represent up to 30 % of the weight of the grapes (Beres et al., 2017), whereas WL consists of yeast debris and other insoluble particles that accumulate at the bottom of fermenters (De Iseppi et al., 2020). Considering that more than 50 million tons of grapes are produced and used for winemaking worldwide each year (20 million tons in Europe alone) (Beres et al., 2017), the scale of the problem is clear and the search for strategies to exploit it is necessary. Hence, BSG, WB, SOK, GP and WL, which are rich in proteins and thus might serve as good sources of bioactive peptides, were selected as they represent some of the most significant residues of the food industries. Therefore, their valorization in a circular economy framework is necessary in order to achieve sustainability.

In this study, several enzymatic treatments were set-up. The degree of hydrolysis was monitored and the release of potentially bioactive peptides further evaluated through *in vitro* assays. Advanced peptide profiling techniques were used, coupling liquid chromatography followed by mass spectrometry with *in silico* bioactivity prediction tools. Finally, as proof-of-concept, the enzymatically treated ingredients exhibiting the highest bioactivity were incorporated into a novel functional food prototype, specifically an innovative "granola", with a nutritionally relevant profile and potential multi-faceted health benefits.

2. Materials and methods

2.1. Raw materials, enzymes and microorganisms

The proximal composition of BSG, WB, GP, WL and SOK used for this study is reported in Table S1. BSG, kindly provided by Peroni brewery (Bari, Italy), was obtained from the production of a lager beer brewed with barley malt (70 %) and maize (30 %) and did not contain spent yeast; whereas WB consisted of white bread cuttings, from sandwiches production and were kindly provided by Valle Fiorita Ltd (Ostuni, Italy). GP and WL were provided by a wine company located in Bolzano (Italy), whereas SOK was kindly provided by Unigrà S.p.a. (Conselice, Italy). To facilitate their storage, matrices (BSG, WB, GP and SOK) were dried in a ventilated oven at 50 °C for approx. 5 h and finely ground with a laboratory mill (IKA A11 basis, Werke, Germany).

Aiming at hydrolyzing the fiber and protein component of the by-products employed, several enzymatic preparations were used. Depol^{TM} 761P (Biocatalysts, Chicago, IL, United States), a preparation derived from *Bacillus subtilis* having xylanase activity (14,670 nkat/g) was used to treat BSG, as previously reported (Verni et al., 2020a). Cellulases and hemicellulases (Biocatalysts, Chicago, IL, United States) were used to hydrolyze 1,4-β-D-glycosidic bonds in cellulose and hemicellulose, respectively. Among proteases, Veron PS (227 U_{Hb}/g, where 1 U_{Hb}/g corresponds to the release of 1 μmol/min of tyrosine from hemoglobin at 37 °C and pH 5.0) obtained from *Aspergillus oryzae* (AB Enzymes GmbH, Darmstadt, Germany) was used because in previous studies was able to release antimicrobial peptides (Rizzello et al., 2015; 2017). Neutrase® (Novozymes, Denmark), an endopeptidase (1.5 AU/g) derived from *Bacillus amyloliquefaciens* and Alcalase® 2.4 L FG (Novozymes, Denmark), a commercial liquid preparation of serine endopeptidase (EC. 3.4.21.62) extracted from *Bacillus licheniformis* (mainly subtilisin A; 27.3 kDa), which hydrolyses amino esters, were also used because were able to release several bioactive peptides (Tacias-Pascacio et al., 2020).

For the antimicrobial activity assays, *Staphylococcus aureus* DSM799, *Bacillus cereus* DSM31, and *Listeria monocytogenes* DS15675, purchased from DSMZ (Leibniz Institute DSMZ, Braunschweig, Germany), were used. *L. monocytogenes* was routinely propagated in Luria Bertani (LB, Oxoid Ltd., Basingstoke, Hampshire, UK) at 37 °C, whereas *S. aureus* and *B. cereus* in Tryptone Soya Yeast Extract (TSYE, Merck, Milan, Italy) at 37 and 30 °C, respectively. Also, *Penicillium roqueforti* DPPMAF1, belonging to the Culture Collection of the Environmental biology Department (Sapienza University of Rome) and propagated in Potato Dextrose Agar (PDA, Oxoid Ltd.), was used.

2.2. Enzymatic treatment

All by-products were added of distilled water at different ratios depending on the matrix considered. A ratio 20:80 (by-products:water) was used for GP and BSG, whereas a ratio 10:90 was used for SOK and WB. Due to the starchy nature of SOK and WB, which led to the formation of a sludge at 20:80 ratio, a higher water content was chosen for these matrices. Wine lees were used as such, without adding water, as they were already liquid and the drying processes would have led to excessive oxidation. Then, the pH of each sample was corrected to a value of 5, with a solution of 0.1 M NaOH or 5 M HCl, in order to make the pH uniform between the conditions and to allow the functioning of all the enzymes within the optimal pH range.

Food grade enzymes, specifically chosen based on the chemical composition of the by-products and their activity, were used individually or in combination according to the scheme reported in Table 1. Enzymes were added at the concentrations recommended by the manufacturer: cellulase (10 mg/100 g), hemicellulase (10 mg/100 g), xylanase (10 mg/100 g), Veron PS (40 mg/100 g), Alcalase® (0.5 % v/w) and Neutrase® (0.5 % v/w). All samples were then incubated at 50 °C for 6 h. For each matrix an untreated control was prepared and used as reference, a total of 36 conditions were considered.

A FiveEasy FP20 pHmeter (Mettler Toledo, USA), equipped with a food penetration probe was used to monitor the pH. After incubation, the mixtures were centrifuged (10,000 × g for 15 min at 4 °C) to separate the non-hydrolyzed solid residues and the supernatant was recovered for further analysis. The degree of protein hydrolysis was monitored. Peptides concentration was determined by the *o*-phtaldialdehyde method (Church et al., 1983) whereas total free amino acids (TFAA) were analyzed using the Cadmium-Ninhydrin method (Doi et al., 1981).

2.3. Screening for functional potential

2.3.1. Antimicrobial activity

The supernatant from all thirty-six conditions, obtained as described above, was tested for its ability to inhibit gastrointestinal pathogens and

Table 1
List of experimental theses and description of the enzymatic treatment used.

Sample	Treatment
BSG	Untreated brewers' spent grain
BSG X	Brewers' spent grain treated with xylanase
BSG A	Brewers' spent grain treated with Alcalase®
BSG VP	Brewers' spent grain treated with Veron PS
BSG N	Brewers' spent grain treated with Neutrase®
BSG A + X	Brewers' spent grain treated with xylanase and Alcalase®
BSG VP+X	Brewers' spent grain treated with xylanase and Veron PS
BSG N + X	Brewers' spent grain treated with xylanase and Neutrase®
WB	Untreated wasted bread
WB A	Wasted bread treated with Alcalase®
WB VP	Wasted bread treated with Veron PS
WB N	Wasted bread treated with Neutrase®
GP	Untreated grape pomace
GP C + E	Grape pomace treated with cellulases and hemicellulases
GP A	Grape pomace treated with Alcalase®
GP VP	Grape pomace treated with Veron PS
GP N	Grape pomace treated with Neutrase®
GP A + C + E	Grape pomace treated with cellulases, hemicellulases and Alcalase®
GP VP + C + E	Grape pomace treated with cellulases, hemicellulases and Veron PS
GP N + C + E	Grape pomace treated with cellulases, hemicellulases and Neutrase®
WL	Untreated wine lees
WL C + E	Wine lees treated with cellulases and hemicellulases
WL A	Wine lees treated with Alcalase®
WL VP	Wine lees treated with Veron PS
WL N	Wine lees treated with Neutrase®
WL A + C + E	Wine lees treated with cellulases, hemicellulases and Alcalase®
WL VP + C + E	Wine lees treated with cellulases, hemicellulases and Veron PS
WL N + C + E	Wine lees treated with cellulases, hemicellulases and Neutrase®
SOK	Untreated soy okara
SOK C + E	Soy okara treated with cellulases and hemicellulases
SOK A	Soy okara treated with Alcalase®
SOK VP	Soy okara treated with Veron PS
SOK N	Soy okara treated with Neutrase®
SOK A + C + E	Soy okara treated with cellulases, hemicellulases and Alcalase®
SOK VP + C + E	Soy okara treated with cellulases, hemicellulases and Veron PS
SOK N + C + E	Soy okara treated with cellulases, hemicellulases and Neutrase®

spoiling fungi of the baking industry.

The inhibition of potential gastrointestinal pathogens was tested using a well diffusion assay, as described by Schillinger and Lucke (1989). Assays were carried out using different soft agar media (5 ml), either LB or TSYE depending on the strain, overlaid on 15 ml of agar-H_2O (2 %, wt/vol). Indicator strains were inoculated at 4 log cfu/ml. 5-mm diameter wells were cut into the agar plates and 50 µl of supernatant were placed in each well. Plates were then stored at 4 °C for 4 h to allow radial diffusion of the antimicrobial substance, after which they were incubated at 30 or 37 °C for 48 h and examined for zones of inhibition. Fifty microliters of sterile water and chloramphenicol (final concentration 0.1 g/l) were used as negative and positive controls, respectively.

The hyphal radial growth rate of *P. roqueforti* was also assessed. The supernatants were sterilized by filtration on 0.22 µm membrane filters (Millipore Corporation, Bedford, MA, USA) and added (30 %, vol/vol, final concentration) to sterilized PDA in Petri plates (90 mm diameter). Control plates contained PDA alone. The assay was carried out by placing a 3-mm diameter plug of growing mycelium onto the center of Petri dishes containing the culture medium. Plates were then incubated aerobically at 25 °C. The radial growth of the mycelium (colony diameter, mm) was measured 7 days after inoculation. Each data point represents the mean of at least four measurements. The percentage of growth inhibition was calculated from mean values as follows: Percentage of inhibition = [(mycelial growth under control conditions − mycelial growth in the presence of the supernatant)/mycelial growth under control conditions] × 100.

2.3.2. ACE-inhibitory and antioxidant activities

The analysis of ACE (angiotensin-I converting enzyme) inhibitory activity was carried out by using the ACE KIT-WST (Dojindo Molecular Technologies Inc., Japan), in accordance with the manufacturer's instructions (Dojindo Molecular Technologies Inc., Japan).

The supernatants were also used to assess the antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonate)) radicals. The free radical scavenging activity on DPPH was determined according to the method reported by Yu et al. (2003) and expressed as follows: DPPH scavenging activity (%) = [(blank absorbance—sample absorbance)/blank absorbance] × 100. The value of absorbance was compared with 75 ppm butylated hydroxytoluene (BHT), which was used as the antioxidant reference.

DPPH radical scavenging activity was also determined on methanolic extracts which, compared to water-soluble extracts, can extract less polar substances like phenolic compounds. Briefly, 5 g of each dried sample were mixed with 50 mL of 80 % methanol to obtain methanolic extracts (ME). The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4600 × *g* for 20 min. ME were further purged with nitrogen stream and stored at ca. 4 °C before analysis. The assay was performed according to the method reported by Yu et al. (2003), and the activity expressed as reported above.

ABTS scavenging capacity was measured using the Antioxidant Assay Kit CSO790 (Sigma Chemical Co.), following the manufacturer's instruction. Trolox (6-hydroxy 2,4,7,8-tetramethylchroman-2-carboxylic acid) was used as standard. The scavenging activity was expressed as Trolox equivalent.

2.4. Peptide extraction and analysis

2.4.1. Ultra-High performance liquid chromatography high-resolution tandem mass spectrometry (UHPLC/HR-MS^2)

In order to separate the active peptide's fraction, the supernatants from samples showing the highest ACE-inhibitory and antioxidant activities were subjected to ultrafiltration (molecular weight cut-off <3 kDa) as previously described by Trossolo et al. (2025), with some modifications. In detail, 10 mL of the sample were placed in a Vivaspin®20 column, 3000 MWCO-PES (Sartorius, Italy), and subjected to centrifugation at 4000 × g for 40 min. The obtained low molecular weight water soluble extract (LMW-WSE) was used for next investigations.

Peptides in LMW-WSE were identified by UHPLC/HR-MS^2 (UHPLC Ultimate 3000; Thermo Scientific, San Jose, CA, USA; Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Zorbax SB-C18 Reversed-phase, 2.1 × 50 mm, 1.8 µm particle size, Agilent Technologies, Santa Clara, CA, USA), as previously described by Martini et al. (2020).

The MS data were first transformed in a .mgf file and then processed with the MASCOT software (Matrix Science, Boston, MA, USA) for peptide sequencing and identification. The parameters used for the identification process were: enzyme, none; peptide mass tolerance, ±5 ppm; fragment mass tolerance, ±0.1 Da; variable modification, Deamidation (NQ), oxidation (M) and phosphorylation (ST); the maximal number of post-translational modifications permitted in a single peptide, 1. Only peptides identified with a significance threshold of $p < 0.05$ were considered in the analysis.

2.4.2. Bioactive peptides in silico identification

The peptide sequences identified via Orbitrap were first analyzed by using the PeptideRanker tool by Bioware (Mooney et al., 2012) that predicts the likelihood of bioactivity in peptides using an advanced N-to-1 neural network model (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/help/peptideranker/help.php). Sequences with ≥0.8 value of the highest probability of bioactivity were further analyzed software using the following *in silico* tools: i) *AHTpin* to assess the antihypertensive potential of the peptides identified in BSG N + X,

WB VP, and SOK VP (Kumar et al., 2015); and ii) Antioxidative Peptide Predictor AnOxPP (https://www.sciencedirect.com/science/article/pii/S0010482523000562) for the screening of potential antioxidant peptides from the sample WB A, a tool based on BiLSTM neural network and optimized amino acid descriptors (SDPZ27) to predict the bioactivity .

2.5. Formulation of a functional food

2.5.1. Production of granola snacks

Dried by-products, among those having the most promising bioactivities, before (Granola Ct) and after (Granola Enz) the enzymatic treatment, were used as ingredients (Table 2) to produce granola snacks as proposed by Limongelli et al. (2023) with some modifications. Specifically, a syrup was prepared by heating water, molasses and oil in a pot, then all the dry ingredients were added to the syrup and mixed with a spatula. Finally, the granola was evenly distributed on a baking tray and baked in an oven for 20 min at 160 °C.

2.5. Characterization of granola snacks

The proteins (total nitrogen × 5.7), lipids, moisture, total dietary fiber, and ash of granola snacks were determined according to the Approved Methods of the American Association of Cereal Chemists 46–11.02, 30–10.01, 44–01.01, 32–05.01, and 08–01.01 (AACC, 2010). Available carbohydrates were calculated as follows: [100 − (proteins + lipids + ash + total dietary fiber)]. Samples activity water (a_w) was measured using a Humimeter RH2 (Schaller Messtechnik, Austria).

Water/salt-soluble extracts of the snacks were prepared according to the method described by Weiss et al. (1993) and used to determine the scavenging activity on DPPH and ABTS radicals as described above, as well as ACE-inhibitory activity.

Sensory analysis of granola snacks was performed by a trained panel group composed of fifteen assessors (6 male and 9 females, mean age: 34 years, range: 25–48 years). The enrolled panelists, who did not suffer from any food intolerances or allergies, received information on the objectives of this study and provided written informed consent. The sensory attributes, scored on a scale from 0 to 10 (with 10 the highest score), were discussed with the assessors during the introductory 2h-training session. Sensory evaluations were carried out following the independent method of the "Sensory analysis - Methodology - Flavour Profile" methods (ISO 6564–1985) with some modification. In details, the library of the Environmental Biology Department of the Sapienza University of Rome (Italy) was used instead of cabinets as previously proposed by Elia (2011). Roughly 15 g of granola snacks, as homogeneous as possible among panelists, were served in a randomized order 2 h after the baking step. A glass of water was drunk by the panelists between samples.

Table 2
Recipes of granola snacks produced with by-products, before (Granola Ct) and after (Granola Enz) the enzymatic treatment. Ingredients are expressed as g/100 g of the total mixture before baking.

Ingredients	Granola Ct	Granola Enz
Water	25	25
Seed oil	5	5
Molasses	17	17
BSG	6	-
BSG *N* + *X*	-	6
WB	12	-
WB A	-	6
WB VP	-	6
SOK	6	-
SOK VP	-	6
Puffed spelt	7	7
Almonds	8.5	8.5
Hazelnuts	8.5	8.5
GP	5	5

2.6. Statistical analysis

Analyses were carried out in triplicate for each batch of samples. Data underwent one-way ANOVA, and pair comparison of treatment means was performed by applying Tukey's procedure at $p < 0.05$, using the statistical software Statistica 12.5 (StatSoft Inc., Tulsa, OK, USA). Violin plots of DPPH radical scavenging activity and single gradient heatmap of 3-mer epitopes in identified peptides were obtained using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Evaluation of the hydrolysis degree

The effect of enzymatic treatments on the by-products was evaluated by quantifying protein and peptides. The protein content of the matrices differed among matrices and ranging roughly from 35 to 120 mg/g (Fig. 1A). More specifically, protein concentration was 49.4 ± 3.92, 86.3 ± 1.96, 35.2 ± 2.29, 97.1 ± 8.61, and 118.4 ± 0.98 mg/g d.w. for BSG, WB, GP, WL and SOK, respectively. The treatment of BSG with xylanase and proteases (Alcalase® and Neutrase®), either alone or combined, led to significant ($p < 0.05$) increases (up to 3-fold). On the contrary, the use of Veron PS alone did not significantly modify protein content ($p > 0.05$). When wasted bread was treated with proteolytic enzymes, on average a 27 % increase was observed compared to WB. Treatments with cellulases and hemicellulases, alone or coupled with proteases yielded different results according to the matrix. More precisely, in WL-treated samples no differences were observed for protein content compared to the control, whereas for grape pomace, carbohydrases rather than proteases, resulted in protein increases up to 2.7-fold higher than GP. For soy okara, instead, all treatments, except for the combined use of Alcalase®, cellulases and hemicellulases (SOK A + C + E), significantly increased protein content (Fig. 1A).

Peptide concentration was also evaluated in the matrices and ranged from 3 to 33 mg/g d.w., with WB and WL having the lowest and highest values, respectively (Fig. 1B). A clearer trend among treatments was observed for peptides, namely, Alcalase® and Neutrase® were able to generate the greatest increases (from 6- to 22-fold) in brewers' spent grain, wasted bread and soy okara, whereas VeronPS was the least effective. In grape pomace and wine lees, although significant ($p < 0.05$) increases were observed after treatment, differences were less pronounced (Fig. 1B).

3.2. Antimicrobial activity

The effect of the enzymatic treatments on the release of potentially antimicrobial peptides was evaluated using food pathogenic bacteria and *P. roqueforti*. None of the matrices, either before or after enzymatic treatment, showed the ability to inhibit the growth of *S. aureus, B. cereus*, and L. *monocytogenes*, used as indicators. Whereas the growth of *P. roqueforti*, although different among matrices, was partly inhibited. BSG, WB, GP, WL and SOK showed antifungal activity of 53 ± 3, 72 ± 1, 37 ± 3, 68 ± 6, and 56 ± 1 %, respectively. However, except for a few conditions, treatments did not result in increased inhibition. Indeed, only BSG N and BSG N + X showed higher antifungal activity than the control (68 and 76 %).

3.3. Antioxidant activity

DPPH radical scavenging activity of the matrices before and after treatment, was assessed on both aqueous and methanolic extracts (Fig. 2). The antioxidant activity of BSG aqueous extracts was high even before the treatments (87 ± 2 %) and, except for the treatment with Veron PS (BSG VP), which did not improve it, all other approaches

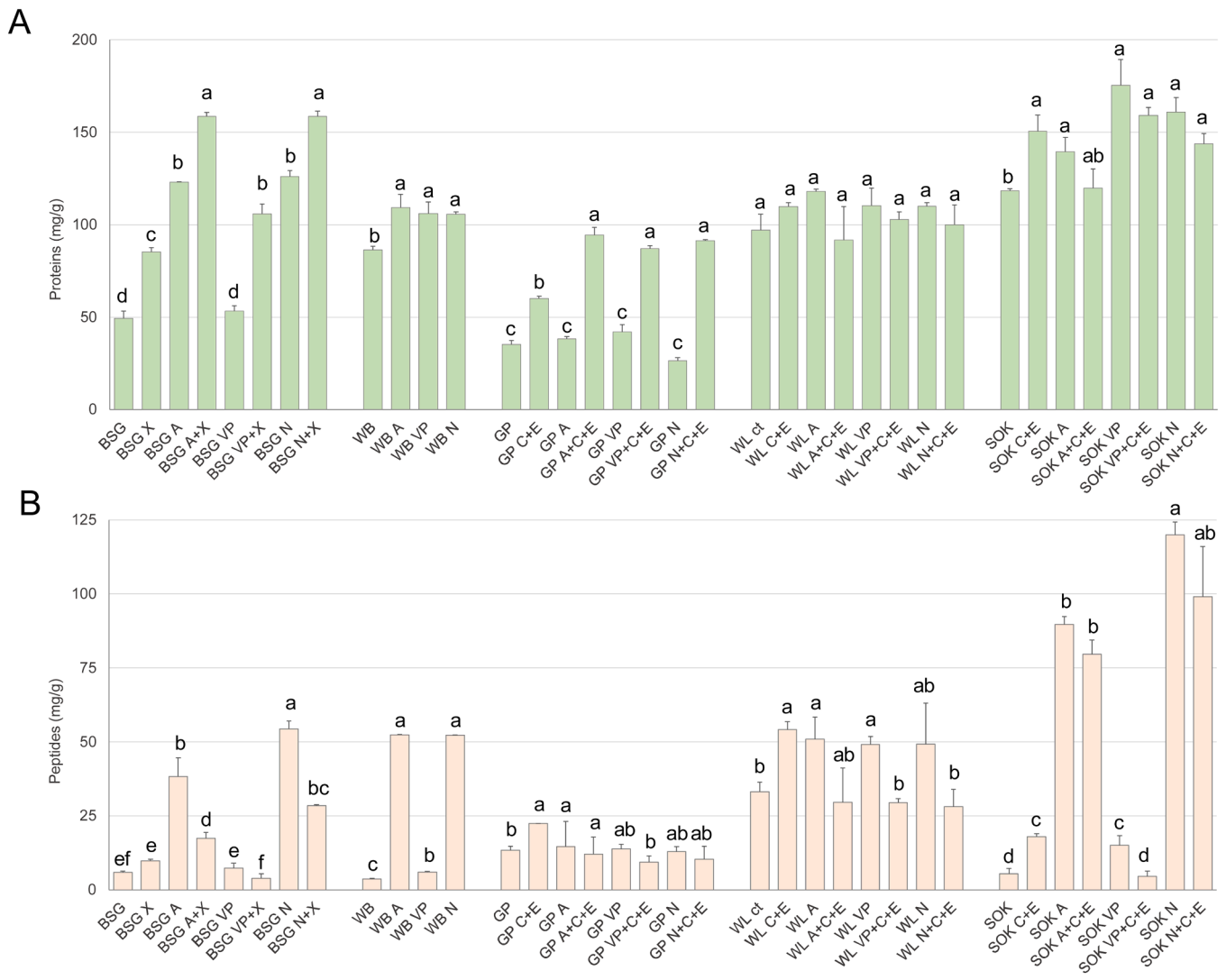

Fig. 1. Concentration of proteins (A) and peptides (B) in brewers' spent grain (BSG), wasted bread (WB), grape pomace (GP), wine lees (WL), and soy okara (SOK) untreated or treated with Alcalase® (A) Veron PS (VP), and Neutrase® (N) alone or combined with xylanase (X) and cellulase and hemicellulose (C+E). [a-f]Values within the same matrix, with different letters, differ significantly ($p < 0.05$).

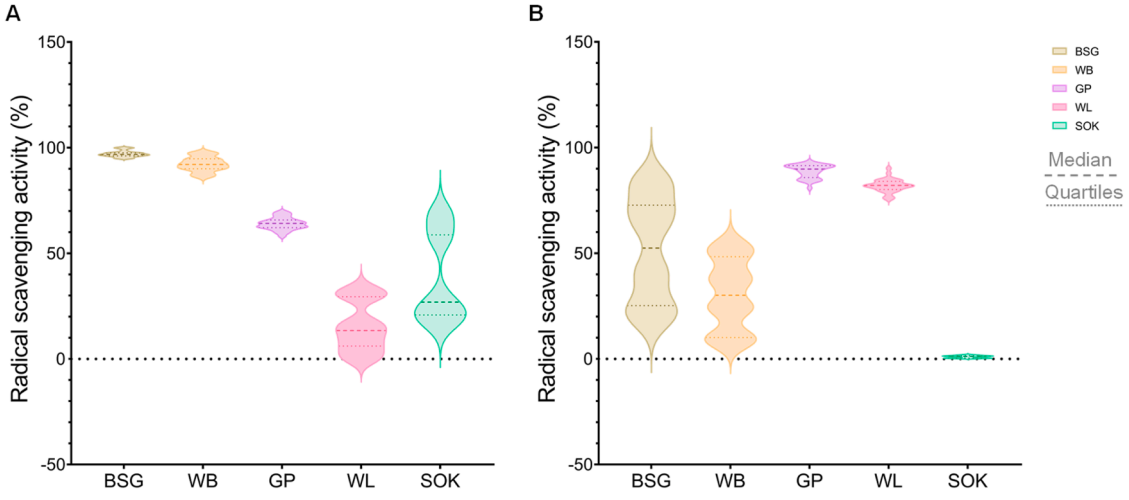

Fig. 2. Violin plots, representing the distribution of DPPH radical scavenging activity of water salt-soluble (A) and methanolic (B) extracts, of treated and untreated brewers' spent grain (BSG), wasted bread (WB), grape pomace (GP), wine lees (WL), and soy okara (SOK).

determined slight but significant increases ($p < 0.05$), reaching values up to 96 %. A similar trend to that of brewers' spent grain was observed for wasted bread and grape pomace samples (Fig. 2A). Interesting results were obtained for okara, where DPPH scavenging activity more than doubled in aqueous extracts when Alcalase® and Neutrase® were used alone or in combination with cellulases and hemicellulases, compared to SOK (23 ± 2 %). In contrast, aqueous extracts from wine lees subjected to the carbohydrases and proteases treatment (WL A + C + E, WL VP + *C* + *E*, WL N + C + E) showed significant increases (up to 30 %) in DPPH scavenging activity, compared to the control (Fig. 2A).

A different trend was observed for methanolic extract, for which the highest activity was found for GP- and WL-samples (Fig. 2B). Indeed, GP and WL were able to scavenge 89 ± 6 and 80 ± 2 % of the DPPH radical in the solution, but very little variations ($p > 0.05$) were observed between the control and enzymatic treated matrices. BSG and WB methanolic extracts had the lowest antioxidant activity before the treatment (37 ± 3 and 24 ± 5 %, respectively). In treated brewers' spent grain higher values were observed only when xylanase was used, alone or in combination with proteases (BSG X, BSG A + X, BSG VP + X, BSG N + X). Whereas, for WB, only treatment with Alcalase® and Neutrase® doubled DPPH radical scavenging activity. Neither the methanolic extracts of SOK nor the extracts after treatment showed antioxidant activity on DPPH (Fig. 2B).

The samples that showed the greatest increases in DPPH radical scavenging activity compared to controls (BSG X, BSG A + X, BSG VP + X, BSG N + X, WB A, GP VP, GP A + C + E, WL A + C + E, WL VP + C + E, WL N + C + E, SOK A, SOK N, SOK A + C + E, SOK N + C + E), were also tested for their ABTS radical scavenging potential. Values in a different order of magnitude were observed for all the matrices. Specifically, SOK, WB and BSG were the matrices with the lowest ABTS radical scavenging activity (0.11 ± 0.01, 0.36 ± 0.02, 0.58 ± 0.03 mmol Trolox eq./kg, respectively). WL showed significantly higher antioxidant activity (approx. 20 mmol Trolox eq./kg) compared to GP (approx. 1.6 mmol Trolox eq./kg) and the other matrices, but no significant differences ($p > 0.05$) were observed between controls and treated samples for wine lees, grape pomace, okara and brewers' spent grain. In contrast, a significant increase of 150 %, compared to WB, was observed when bread was treated with Alcalase®.

3.4. *ACE-inhibitory activity*

Overall, high values of ACE-inhibitory activity were found for all samples (Fig. 3). Indeed, before treatment, on average matrices had a 78 % ACE-inhibitory activity. Following enzymatic treatments applied to grape pomace and wine lees, no significant increases in ACE-inhibitory activity were found; rather, significant decreases were observed. All hydrolyzed wasted bread and okara had significantly higher ACE-inhibition than WB and SOK. Nevertheless, BSG N + X, WB VP and SOK VP showed the greatest ($p < 0.05$) increases compared to the controls, up to 20 %.

3.5. *Identification of bioactive peptides*

Aiming at identifying the peptides responsible for the bioactivity observed, the LMW-WSE obtained from BSG N+X, WB VP, SOK VP, and WB A were analyzed through liquid chromatography coupled with mass spectrometry (Figure S1). A total of 4203, 4077, 4004 and 5628 peptides were identified in BSG N + X, WB VP, SOK VP, and WB A, respectively. Peptides contained from 4 to 30 amino acid residues, but the highest abundance was reported for tetra and pentapeptides, especially in WB A and BSG N + X (Figure S2).

To support and validate the findings of the *in vitro* assays, predictive databases were employed to identify potential bioactive peptides. Hence, peptide sequences identified via Orbitrap were analysed using three different prediction tools: PeptideRanker, Antioxidative Peptide Predictor and AHTpin.

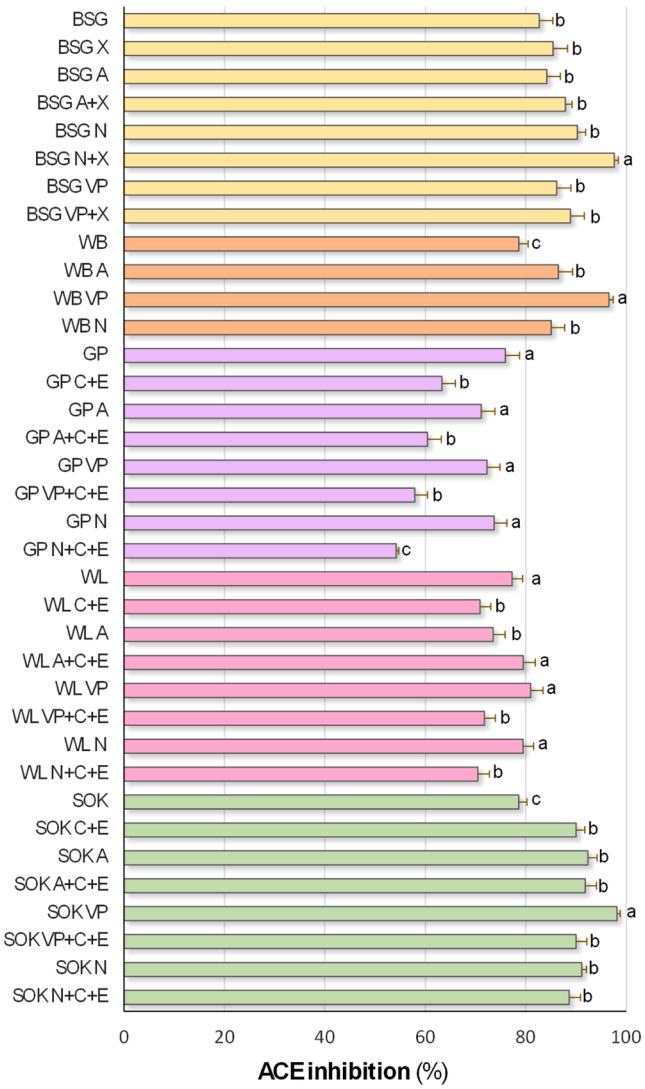

Fig. 3. ACE-inhibitory activity of brewers' spent grain (BSG), wasted bread (WB), grape pomace (GP), wine lees (WL), and soy okara (SOK) untreated or treated with Alcalase® (A) Veron PS (VP), and Neutrase® (N) alone or combined with xylanase (X) and cellulase and hemicellulose (C+E). [a-c]Values within the same matrix, with different letters, differ significantly ($p < 0.05$).

3.5.1. *Antioxidant peptides in WB A*

A total of 197 peptides derived from wasted bread hydrolysed with Alcalase® were predicted as antioxidant peptide, sowing a score greater than 0.75 (Qin et al., 2023). Among these, 39 peptides were assigned the maximum prediction score of 1.0 (Table S2). Peptide lengths ranged from 4 to 19 amino acid residues, with the majority concentrated between 5 and 7 residues: specifically, 29 peptides were 5 residues long, 40 were 6 residues, and 67 were 7 residues. The molecular mass of the peptides ranged from 435.5 Da to 2020.5 Da, with most peptides (123 out of 197) falling within the 434–753 Da range.

Hydrophobicity was calculated using the Kyte–Doolittle scale (GRAVY, Grand Average of Hydropathy) (Kyte and Doolittle, 1982) and ranged from −2.26 to 3.38. Most of the antioxidant sequences identified (140 peptides, 71 %) were hydrophobic, while only 57 peptides (29 %) were hydrophilic (Table S2).

Analysis of the 197 peptides with high predicted antioxidative activity revealed the presence of multiple recurrent short sequence motifs across the dataset. Among the tripeptides (3-mers), the most frequent epitopes embedded in peptides were PGA, GAL, PPF, and PAG (each occurring 12 times), followed by AGP (11 times), AGF, LFL, PPP (9

occurrences) and other hydrophobic-rich motifs such as GLF (Fig. 4). At the tetrapeptide (4-mer) level, PPPP was the most common motif (6 occurrences), while AGPP, GLFL, GPPF, and QQPF also appeared multiple times. For pentapeptides (5-mers), PPPPP emerged as the most repeated motif, followed by LGLFL, QAGPP, and AGPPF, each observed three or more times.

The recurrence of these short motifs highlights a clear enrichment in proline-rich sequences and hydrophobic amino acids such as leucine (L), phenylalanine (F), alanine (A), and glycine (G). The predominance of hydrophobic residues aligns with the overall physicochemical trend observed in the dataset, where approximately 71 % of peptides were classified as hydrophobic.

3.5.2. Antihypertensive peptides in BSG N+X, WB VP, and SOK VP

Antihypertensive peptides were identified using the AHTpin tool. Peptides with an SVM (distance of the peptide from the Support-Vector-Machine decision hyper-plane, Kumar et al., 2015) score > 1, were detected for each sample. Overall, the molecular mass range of these sequences varied from approximately 500 Da to 940 Da and the hydrophobicity values (based on the Kyte-Doolittle scale) ranged from about −2.3 to 1.7 (Table S3).

Most peptides ranged between 4 and 9 residues, with a clear predominance of short peptides (4–6 residues) in BSG N + X and WB VP, reflecting their lower molecular weights, while SOK VP contained a greater proportion of longer peptides (7–9 residues), consistent with its higher average molecular weights. Indeed, the distribution of peptides reveals that lower molecular weight peptides (below 700 Da) were more abundant, especially in the BSG N + X and WB VP, where the majority of peptides fell within the 500–650 Da range. In contrast, the SOK VP showed a broader molecular weight distribution, including a higher proportion of medium-sized peptides (700–850 Da). Regarding hydrophobicity, peptides with positive values (more hydrophobic) were highly frequently represented in all three samples, particularly in SOK VP.

When analysing all peptides from the three samples collectively, several recurring epitope motifs of lengths 3 and 4 amino acids were identified. Among the 3-mer motifs, those with a frequency greater than 3 included: PFP (11 occurrences), PLL (8), GPP, PPP and QQP (7), LLP, AGP (6), QPF, QPL, PLP, GAP, CCS, CSC (5), as well as others like FPL, FLP, CGS, and CSC (4 each) (Fig. 5). For 4-mers, only three motifs exceeded the threshold: QPFP, PQQP, and PLLP (4 occurrences each). No 5-mer motifs were found with a frequency above 3.

3.6. Granola characterization

The matrices that promoted the highest increase in antioxidant and ACE-inhibitory activities (BSG N + X, WB VP, WB A and SOK VP) were selected as ingredients to manufacture a functional granola (Granola Enz), which was compared with the same granola, obtained with untreated BSG, WB and SOK (Granola Ct). Moisture content and a_w of both samples after baking were around 12 % and 0.730, respectively, and did not show significant differences between Granola Enz and Granola Ct (Table 3). The granola made with enzymatically treated matrices showed higher ACE-inhibitory activity and greater DPPH and ABTS radical scavenging activity than the granola Ct (Table 3).

The two granola snacks were also subjected to a sensory analysis (Fig. 6). Overall, they were appreciated by panelists with general acceptability scores of 7.2 points. Panelists also positively evaluated the toasted and caramel notes as well as crunchy texture (Fig. 6). Only minor differences were observed between the two snacks, notably Granola Enz had a more persistent taste and was more savory than Granola Ct (5.38 ± 0.4 vs. 2.62 ± 0.1 points, respectively).

4. Discussion

The food industry produces large volumes of protein-rich residues that are often discarded or underexploited, contributing to pollution and economic inefficiencies (Zhou et al., 2023a). Valorizing them through bioprocessing technologies is increasingly recognized as a sustainable strategy to reduce environmental burden and enhance resource utilization (Siroli et al., 2022). Through bioprocessing techniques like enzymatic hydrolysis, these materials can be converted into peptide-rich matrices with bioactive potential (Wang and Qi, 2024). Bioprocessing technologies also offer scalability, cost-effectiveness, and environmental compatibility, supporting innovation in the development of clean-label, health-oriented food products (Ghinea et al., 2025). Hence, this study

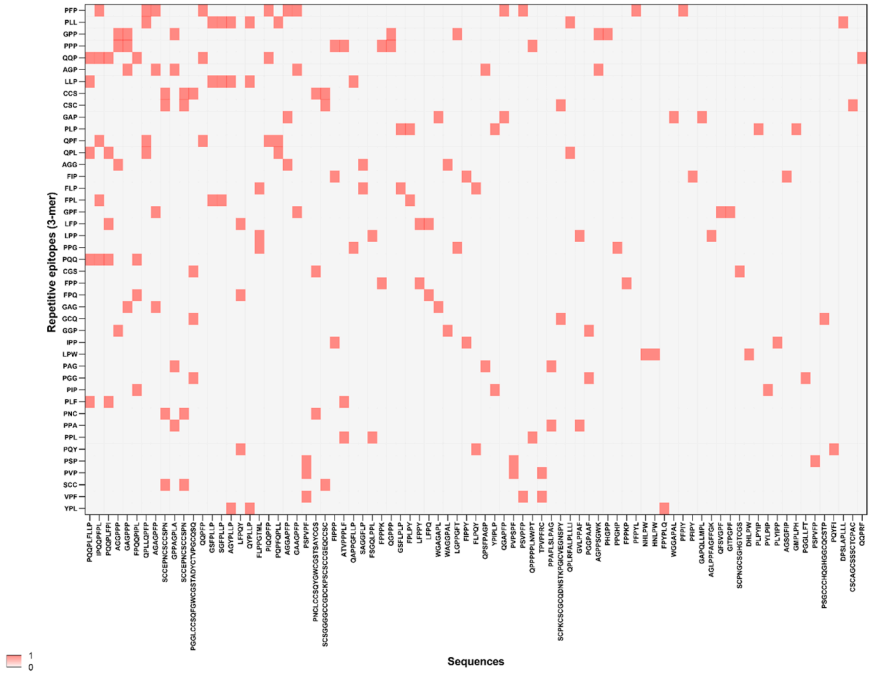

Fig. 4. Single gradient heatmap highlighting the presence of recurring 3-mer epitopes (red squares) across sequences of antioxidant peptides in WB A.

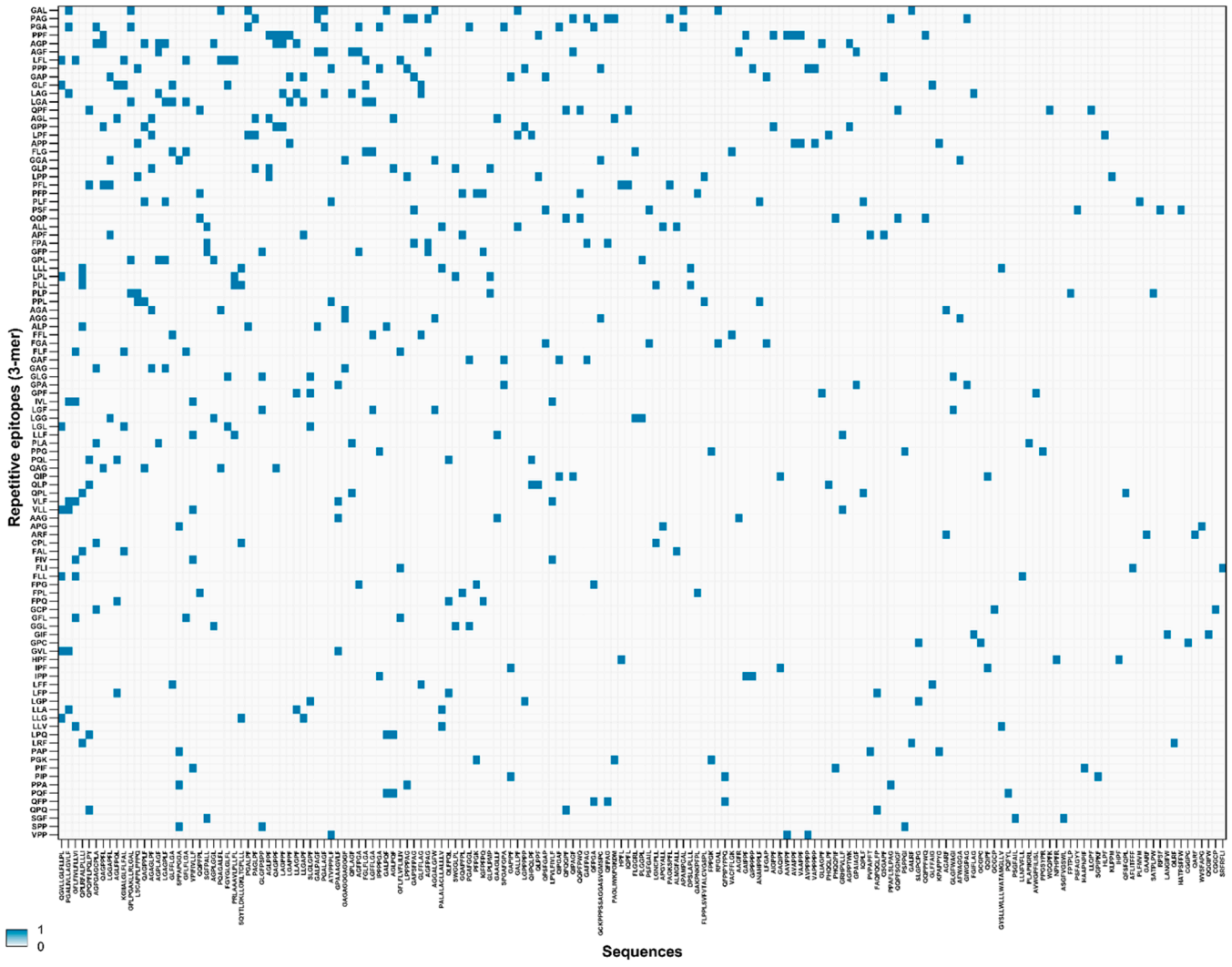

Fig. 5. Single gradient heatmap highlighting the presence of recurring 3-mer epitopes (blue squares) across sequences of antihypertensive peptides in BSG *N*+*X*, WB VP, and SOK VP.

Table 3
Nutritional and functional characterization of the granola snacks produced with by-products, before (Granola Ct) and after (Granola Enz) the enzymatic treatment.

	Granola Ct	Granola Enz
Fats (g/100 g)	12.4 ± 0.4[a]	12.1 ± 0.3[a]
Carbohydrates (g/100 g)	28.3 ± 0.9[a]	28.8 ± 0.6[a]
Fibers (g/100 g)	12.4 ± 0.2[a]	12.7 ± 0.4[a]
Proteins (g/100 g)	9.6 ± 0.1[a]	9.4 ± 0.2[a]
Energetic values (kcal/100 g)	187 ± 2[a]	185 ± 3[a]
Relative umidity (%)	12.5 ± 0.6[a]	11.6 ± 0.7[a]
Activity water	0.721 ± 0.015[a]	0.733 ± 0.006[a]
ACE-inhibitory activity (%)	79 ± 1.2[b]	91 ± 0.9[a]
DPPH radical scavenging activity (%)	71 ± 3[b]	82 ± 1[a]
ABTS radical scavenging activity (mmol Trolox eq/kg)	0.543 ± 0.018[b]	0.819 ± 0.022[a]

[a-b]Values within the same row, with different superscript letters, differ significantly ($p < 0.05$).

Fig. 6. Sensory analysis of granola snacks produced with by-products, before (Granola Ct) and after (Granola Enz) the enzymatic treatment.

aimed at valorizing food industry residues, through enzymatic treatments to generate bioactive peptides exhibiting health-promoting properties, thus making them promising ingredients in novel functional foods. The enzymes used are food grade enzymatic preparations commonly used in the food industry. They are far more economic than

lab-grade enzymes because they are produced at large industrial scales using cost-effective microbial sources and optimized fermentation processes. The high-volume production, combined with lower purity requirements and application in high-value food industries, makes food-grade enzymes very affordable (Kumar et al., 2024).

Due to the relatively limited availability of proteins, the set-up of the biotechnological process developed in this study included an initial treatment with enzymes that hydrolyze the fibrous component, specifically aimed at releasing protein material often bound to polysaccharides structures. Indeed, cell wall dissolution performed by pectinases and carbohydrases contributes to the regulated extrusion of protein from cells promoting cellular protein outflow (Khan et al., 2023). Specifically, for brewer's spent grain, a xylanase was used, which had already proven effective in releasing hydroxycinnamic acids and proteins from arabinoxylans in BSG (Verni et al., 2020a). Whereas for wine lees, grape pomace, and soy okara, due to the nature of the fibrous component, mainly consisting of cellulose and hemicellulose (Colletti et al., 2020; Nanni et al., 2021), cellulases and hemicellulases were used. The use of these hydrolytic enzymes alone led to an average increase of 46 % in solubilized proteins, with WL C + E and BSG X showing the lowest (13 %) and highest (73 %) increases, respectively. Similarly, treatment with xylanase or cellulases and hemicellulases resulted in an increase in peptide content of approximately 66 % for wine lees, grape pomace, brewers' spent grain, and up to three times higher than the control for soy okara, reaching up to 18 mg/g.

Moreover, since proteases increase protein output of carbohydrases by releasing proteins from the polysaccharide structure (Khan et al., 2023), the process set-up included the use of proteases, applied individually or through a two-step treatment in combination with fiber-hydrolyzing enzymes. Only in the case of wasted bread, which contains a low amount of fiber (also the lowest among by-products, Table S1), because it is the residue of white sandwich bread, carbohydrases were excluded from enzymatic treatments and only proteases were used. Depending on the proteases used, a significant ($p < 0.05$) increase in solubilized proteins was observed, especially when combined with carbohydrases. In general, a lower proteolytic activity of Veron PS, compared to Alcalase® and Neutrase®, was observed in brewers' spent grain, wasted bread and okara, both when used alone and in combination with xylanase or cellulases and hemicellulases. The combined use of proteolytic and fiber-hydrolyzing enzymes resulted in almost always significant decreases in peptide content compared to using the proteases alone (Fig. 1). It is possible that when the treatment involved a two-step enzymatic process, some of the proteins solubilized by the hydrolytic enzymes were further broken down into peptides and amino acids. In fact, although Alcalase® and Neutrase® are enzymatic preparations with mainly endopeptidase activity, an increase in free amino acids when used has already been reported in the literature for various food matrices, including legumes (Vogelsang-O'Dwyer et al., 2023), whey proteins (Ou et al., 2010), microalgae (Verni et al., 2021), and even insect flours (Verni et al., 2025; Zhu et al., 2020). Overall, the treatments were most effective in terms of proteolysis for okara, wasted bread, and spent grain (Fig. 1).

Nevertheless, since higher proteolytic activity does not necessarily translate to higher bioactivity, all matrices, before and after treatment, were characterized for the ability to inhibit the growth of fungi and pathogenic bacteria, as well as radical scavenging activity and ACE-inhibitory potential.

In general, inhibitory activity against *P. roqueforti* growth was found to be high for wine lees, most likely due to the high phenolic compounds content. Indeed, it has been shown that tannins, which wine lees are rich in, inhibit the germination of *Penicillium* spores and cause cell wall rupture (Zhu et al., 2019). The treatments did not result in increased antifungal activity for grape pomace, wasted bread, nor soy okara. Whereas the high antifungal activity of WB before treatments, is likely due to the presence of residual food preservatives such as propionates and sorbates, commonly used in the baking industry to extend the shelf-life of sandwich bread (Gerez et al., 2016; Verni et al., 2020b).

Antioxidant activity was evaluated as the percentage of DPPH radical scavenged, using methanolic extracts, which should primarily extract polyphenols, and aqueous extracts, which in addition to the more water-soluble phenolic components may contain antioxidant peptides (Fig. 2). Intuitively, the by-products that showed the highest antioxidant activity in methanolic extracts, with values above 80 %, were wine lees and grape pomace, which are known to be rich in anthocyanins and flavanols (Beres et al., 2017; De Iseppi et al., 2020) but their enzymatic treatments did not cause significant changes compared to the control. Aqueous extracts from GP and WL showed a similar trend as the methanolic ones, but with lower average values. The absence of differences is consistent with the biochemical characterization data, as GP and partly WL were the matrices for which the smallest increases in peptides were observed (Fig. 1). Methanolic extracts from spent grain, on the other hand, revealed differences between treatments. Although the greatest increases were found for the treatment involving the combined use of xylanase and protease. Probably, as already observed by Verni et al. (2020a), who developed a combined treatment with xylanase and lactic acid bacteria fermentation, xylanase enabled the release of phenolic acids and proteins, then the latter were hydrolyzed by lactic acid bacteria proteolytic system. Overall, matrices treated with Alcalase®, especially WB A, were the ones that mostly improved DPPH and ABTS radical scavenging activity. Indeed, the ability of Alcalase® to produce antioxidant peptides is well known and lies in the presence of sulfur-containing amino acids such as cysteine and methionine, aromatic amino acids, or histidine in the peptides it releases (Tacias-Pascacio et al., 2020).

The matrices, both before and after enzymatic treatment, were analyzed for their ability to inhibit ACE activity, which is considered a key mechanism in blood pressure-lowering effects (Cutrell et al., 2023). Studies have demonstrated that peptides derived from BSG (although microencapsulated) can lower systolic blood pressure in hypertensive rat models (Garzón et al., 2022) whereas soy-derived peptides have shown significant ACE-inhibitory activity (Daliri et al., 2019). In the condition of this study, specific enzymatic treatments successfully increased ACE-inhibitory activity of BSG, WB and SOK, whereas they did not influence that of GP and WL (Fig. 3), although previous studies found that enzymatic hydrolysis of grape pomace can produce peptides with significant ACE-inhibitory activity (Knuf et al., 2025).

Hence, aiming at identifying potential bioactive sequences, extracts of SOK VP, BSG N + X, WB VP, and WB A were selected for their higher ACE-inhibitory and antioxidant activities compared to untreated matrices and subjected to UHPLC/HR-MS^2 and *in silico* tools to predict their bioactivity. The identification of a very high number of sequences from wasted bread (WB A) hydrolysate with predicted antioxidative activity highlights the potential of agri-food byproducts as sources of bioactive compounds. Notably, 20 % of the peptides exhibited the maximum prediction score of 1.0, indicating high confidence in their antioxidative potential based on *in silico* predictions (Qin et al., 2023). The length distribution (4–19 amino acids, with a majority between 5 and 7 residues) aligns with previous findings that antioxidant peptides are typically short, often fewer than 10 residues, which may enhance their bioavailability and interaction with radical species (Udenigwe and Aluko, 2012). Moreover, short peptide sequences exhibit greater resistance to gastrointestinal degradation than longer peptides and are more likely to reach their targets *in vivo*. The molecular mass distribution of peptides, primarily between 0.43 - 0.75 kDa, further supports their classification as small peptides, which is consistent with previous reports demonstrating that low molecular weight peptides often display stronger radical scavenging activity due to greater access to reactive sites (Zou et al., 2016). A predominance of hydrophobic peptides was found, corroborating the well-known role of hydrophobic residues in enhancing antioxidant function, as they improve the interaction between peptides and lipid radicals or membranes (Elias et al., 2008; Rizzello et al., 2016).

Moreover, the amino acid sequences analysis revealed a marked

enrichment in proline-rich and hydrophobic sequences. Tripeptides such as PPP, PGA, GAL, and PPF, and tetrapeptides like PPPP, GLFL, and GPPF, were recurrent among the antioxidant peptides (Fig. 4). Particularly, proline-containing motifs (PPP, PPPP, PPPPP) were highly represented. Proline residues are known for conferring conformational rigidity and enhancing resistance to proteolysis, potentially contributing to improved peptide stability and prolonged antioxidative activity in biological systems (Wang et al., 2025). The presence of hydrophobic residues such as leucine, phenylalanine, alanine, and glycine within these motifs supports their contribution to antioxidant function via hydrophobic interactions and radical stabilization mechanisms (Sarmadi and Ismail, 2010).

Also, antihypertensive peptides identified in BSG N + X, WB VP, and SOK VP hydrolysates demonstrated predicted bioactivity based on SVM scoring using AHTpin. Peptides with an SVM score >1 were selected as candidates, consistent with thresholds adopted in recent literature for predicting ACE-inhibitory activity (Dai et al., 2023; Zhou et al., 2023b). These peptides exhibited molecular weights ranging from 0.50 to 0.94 kDa, and lengths from 4 to 9 residues. The distribution patterns suggest that hydrolysates from BSG N + X and WB VP contained a greater proportion of short peptides, whereas SOK VP featured longer peptides (7–9 residues). Such difference may influence *in vivo* behaviour, as shorter peptides are more likely to resist gastrointestinal degradation and be absorbed intact (Rizzello et al., 2016; Udenigwe and Aluko, 2012).

For antihypertensive peptides, a large presence of hydrophobic sequences across all samples, was found as well. Hydrophobic residues have been associated with increased ACE-inhibitory potential, as they enhance binding affinity to the hydrophobic pockets of the ACE active site (Miguel et al., 2004).

Motif analysis across the antihypertensive peptide datasets identified recurrent short sequence motifs such as PFP, PPP, PLL, and GPP, many of which are rich in proline (Fig. 5). The frequent occurrence of proline, glutamine, and leucine suggests a potential structural basis for ACE-inhibition, in line with established findings that ACE-inhibitory peptides often feature hydrophobic or cyclic residues at the C-terminal end, especially proline and phenylalanine (Wu et al., 2006). It can be also underlined that epitopes containing proline–proline, such as Val–Pro–-Pro (VPP) and Ile–Pro–Pro (IPP), are well-documented ACE-inhibitory and antihypertensive peptides from fermented milk products, with demonstrated blood pressure–lowering effects in animal models and clinical trials (Jäkälä and Vapaatalo, 2010).

Based on the results obtained, the potential application of the bioprocessed matrices as ingredients in functional foods, was assessed. In recent years, the consumption of functional foods, capable of providing health benefits and reducing disease risks, has increased. This trend is growing as people become more aware that a healthy and balanced diet plays a key role in the body's normal function and the maintenance of health (Fan et al., 2022). Therefore, a functional food similar to a granola was developed. Incorporating bioactive peptide-rich matrices obtained from food by-products into innovative foods not only adds economic value but also addresses sustainability goals, making it a strategic solution for future food systems. Therefore, the granola formulation included the highest possible content of by-products from the food industry. SOK VP, BSG N + X, WB VP, and WB A were selected because of their high ACE-inhibitory and antioxidant activities. Additionally, molasses, by-product of sugar production, were used in place of conventional sugar, whereas untreated pomace was added instead of the typical berries usually found in this type of product. The developed formulation, allowed for the production of a granola with a balanced nutritional profile (Table 3), which, according to EU Regulation No 1924/2006, can be labeled as "high in fiber" (as it contains more than 12 g of fiber per 100 g of product) and "high in protein" (as proteins account for more than 20 % of the food's energy value).

Since, given the distinct characteristics of many of the ingredients, it was not possible to compare the granola to commercial products, a version was produced using the same matrices without enzymatic treatment. Granola moisture content and activity water values, while slightly above average compared to such kind of food, are still in line with many commercial equivalents. Nevertheless, the values observed generally fall below microbial-growth thresholds ($a_w < 0.70–0.75$) that ensure microbiological stability during the product's shelf-life (Bauer et al., 2022). The granola was assessed for the functional activities observed in the biomasses (antioxidant and ACE-inhibitory) and, despite the thermal treatment, these activities were mostly retained. Indeed, Granola Enz showed significantly higher DPPH and ABTS radical scavenging activities compared to Granola Ct, as well as higher ACE-inhibitory activity. It is known that small peptides (<3 kDa) are generally heat-stable through typical cooking and can also withstand simulated gastro-intestinal digestion (Arnal et al., 2023). The experimental granola was also subjected to a descriptive sensory analysis, with general acceptability ratings of 7 out of 10 (Fig. 6). Overall, no major differences were noted between the two formulations, except for a more pronounced savory flavor in Granola Enz. This could likely be attributed to the peptides released during the enzymatic hydrolysis of the matrices, which are known to contribute to flavor, unlike native proteins. Nonetheless, it is important to note that bitterness, often attributed to bioactive peptides (Jiang et al., 2024), was only slightly detected in both granolas, and no differences were observed between the two formulations.

5. Conclusions

This study has successfully led to the identification of a series of biotechnological processes with the potential to achieve the functionalization of food industry residues, thereby addressing economic, environmental, and nutritional challenges. Specifically, among the 36 conditions tested, 4 proved to be highly promising, as they significantly enhanced the functional properties of the by-products, thereby promoting their application in novel functional foods. Several peptide sequences were identified as potential antihypertensive and antioxidant peptides, highlighting that the *in vitro* activities observed are due to their synergistic effect. Most of them were short, proline-rich, and hydrophobic peptides known for being heat-stable and able to withstand simulated gastro-intestinal digestion. This further underlines the nutritional and therapeutic relevance of these ingredients in food applications. Nevertheless, although *in silico* analyses are considered reliable to predict bioactivity, *in vivo* studies are often necessary to confirm it. Hence, future research will focus on validating the efficacy on human models (e.g., SHIME) assessing the effect of the granola on the gut microbiota for instance. Moreover, although the process itself can be easily scaled up because industries could use pre-existing equipment for the enzymatic treatments without incurring any additional costs, the availability of the residues can vary seasonally and geographically. However, this could also open up a new supply chain entirely dedicated to the repurposing of such matrices, guaranteeing temporal continuity and standardization.

Hence, by upcycling food industry residues into health-promoting ingredients, the approach developed in this study not only contributes to reducing their environmental burden but also supports the development of next-generation functional foods aligned with circular economy principles.

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Ethical statement - studies in humans and animals

While the primary experimental research did not involve human or animal subjects, the sensory analysis component was conducted with human participants and received ethical approval from the local health board of Lazio region (n. 0998/2024). Informed consent was obtained from all participants prior to their involvement, and appropriate protocols were followed to protect their rights and privacy.

CRediT authorship contribution statement

Federica Violetta Conti: Formal analysis. **Elisabetta Trossolo:** Visualization, Formal analysis. **Pasquale Filannino:** Writing – review & editing, Validation. **Rosalba Lanciotti:** Validation. **Francesca Patrignani:** Writing – review & editing, Validation. **Margherita D'Alessandro:** Formal analysis. **Marco Gobbetti:** Validation. **Raffaella Di Cagno:** Writing – review & editing, Validation, Resources. **Carlo Giuseppe Rizzello:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation. **Michela Verni:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Carlo Giuseppe Rizzello reports financial support was provided by Ministry of Education and Merit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100781.

Data availability

Data will be made available on request.

References

AACC, 2010. Approved Methods of the American Association of Cereal Chemistry, 11th ed. AACC, St. Paul, MN, USA.

Arnal, M., Gallego, M., Talens, P., Mora, L., 2023. Impact of thermal treatments and simulated gastrointestinal digestion on the α-amylase inhibitory activity of different legumes. Food Chem. 418, 135884. https://doi.org/10.1016/j.foodchem.2023.135884.

Bauer, A.S., Leppik, K., Galić, K., Anestopoulos, I., Panayiotidis, M.I., Agriopoulou, S., Krauter, V., 2022. Cereal and confectionary packaging: background, application and shelf-life extension. Foods 11 (5), 697. https://doi.org/10.3390/foods11050697.

Beres, C., Costa, G.N., Cabezudo, I., da Silva-James, N.K., Teles, A.S., Cruz, A.P., Freitas, S.P., 2017. Towards integral utilization of grape pomace from winemaking process: a review. Waste Manag. 68, 581–594. https://doi.org/10.1016/j.wasman.2017.07.017.

Bilal, M., Iqbal, H.M., 2019. Sustainable bioconversion of food waste into high-value products by immobilized enzymes to meet bio-economy challenges and opportunities–A review. Food Res. Int. 123, 226–240. https://doi.org/10.1016/j.foodres.2019.04.066.

Church, F.C., Swaisgood, H.E., Porter, D.H., Catignani, G.L., 1983. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66 (6), 1219–1227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2.

Colletti, A., Attrovio, A., Boffa, L., Mantegna, S., Cravotto, G., 2020. Valorisation of by-products from soybean (*Glycine* max (L.) Merr.) Processing. Molecules 25 (9), 2129. https://doi.org/10.3390/molecules25092129.

Cutrell, S., Alhomoud, I.S., Mehta, A., Talasaz, A.H., Van Tassell, B., Dixon, D.L., 2023. ACE-inhibitors in hypertension: a historical perspective and current insights. Curr. Hypertens. Rep. 25 (9), 243–250. https://doi.org/10.1007/s11906-023-01248-2.

Dai, H., He, M., Hu, G., Li, Z., Al-Romaima, A., Wu, Z., Qiu, M., 2023. Discovery of ACE inhibitory peptides derived from green coffee using in silico and in vitro methods. Foods 12 (18), 3480. https://doi.org/10.3390/foods12183480.

Daliri, E.B.M., Ofosu, F.K., Chelliah, R., Park, M.H., Kim, J.H., Oh, D.H., 2019. Development of a soy protein hydrolysate with an antihypertensive effect. Int. J. Mol. Sci. 20 (6), 1496. https://doi.org/10.3390/ijms20061496.

De Iseppi, A., Lomolino, G., Marangon, M., Curioni, A., 2020. Current and future strategies for wine yeast lees valorization. Food Res. Int. 137, 109352. https://doi.org/10.1016/j.foodres.2020.109352.

Doi, E., Shibata, D., Matoba, T., 1981. Modified colorimetric ninhydrin methods for peptidase assay. Anal. Biochem. 118 (1), 173–184. https://doi.org/10.1016/0003-2697(81)90175-5.

Elia, M., 2011. A procedure for sensory evaluation of bread: protocol developed by a trained panel. J. Sens. Stud. 26 (4), 269–277. https://doi.org/10.1111/j.1745-459X.2011.00342.x.

Elias, R.J., Kellerby, S.S., Decker, E.A., 2008. Antioxidant activity of proteins and peptides. Crit. Rev. Food Sci. Nutr. 48 (5), 430–441. https://doi.org/10.1080/10408390701425615.

Fan, H., Liu, H., Zhang, Y., Zhang, S., Liu, T., Wang, D., 2022. Review on plant-derived bioactive peptides: biological activities, mechanism of action and utilizations in food development. J. Future Foods. 2 (2), 143–159. https://doi.org/10.1016/j.jfutfo.2022.03.003.

Garzón, A.G., Ferreira, M.D.R., Cian, R.E., Oliva, M.E., D'Alessandro, M.E., Drago, S.R., 2022. Microencapsulated bioactive peptides from brewer's spent grain promotes antihypertensive and antidiabetogenic effects on a hypertensive and insulin-resistant rat model. J. Food Biochem. 46 (10), e14283. https://doi.org/10.1111/jfbc.14283.

Gerez, C.L., Bustos, A.Y., Font, G.M., 2016. Antifungal and antiochratoxigenic properties of chemical preservatives of bread. J. Food Technol Pres. 1, 6–10.

Ghinea, C., Ungureanu-Comăniță, E.D., Țăbuleac, R.M., Oprea, P.S., Coșbuc, E.D., Gavrilescu, M., 2025. Cost-benefit analysis of enzymatic hydrolysis alternatives for food waste management. Foods 14 (3), 488. https://doi.org/10.3390/foods14030488.

Jaeger, A., Zannini, E., Sahin, A.W., Arendt, E.K., 2021. Barley protein properties, extraction and applications, with a focus on brewers' spent grain protein. Foods 10 (6), 1389. https://doi.org/10.3390/foods10061389.

Jäkälä, P., Vapaatalo, H., 2010. Antihypertensive peptides from milk proteins. Pharmaceuticals 3 (1), 251–272. https://doi.org/10.3390/ph3010251.

Jiang, Y., Sun, J., Chandrapala, J., Majzoobi, M., Brennan, C., Zeng, X.A., Sun, B., 2024. Recent progress of food-derived bioactive peptides: extraction, purification, function, and encapsulation. Food Front. 5 (3), 1240–1264. https://doi.org/10.1002/fft2.383.

Kamble, D.B., Rani, S., 2020. Bioactive components, in vitro digestibility, microstructure and application of soybean residue (okara): a review. Legume Sci. 2 (1), e32. https://doi.org/10.1002/leg3.32.

Khan, Z.S., Amir, S., Sokač Cvetnić, T., Jurinjak Tušek, A., Benković, M., Jurina, T., Gajdoš Kljusurić, J., 2023. Sustainable isolation of bioactive compounds and proteins from plant-based food (and byproducts). Plants 12 (16), 2904. https://doi.org/10.3390/plants12162904.

Knuf, F., Caspers-Weiffenbach, R., Schieber, A., Fontana, A., 2025. Peptidomics profiling and biological activities of grape pomace protein hydrolysates. Food Chem. 463, 141032. https://doi.org/10.1016/j.foodchem.2024.141032.

Kumar, A., Dhiman, S., Krishan, B., Samtiya, M., Kumari, A., Pathak, N., Dhewa, T., 2024. Microbial enzymes and major applications in the food industry: a concise review. Food Prod. Proc. Nutr. 6 (1), 85. https://doi.org/10.1186/s43014-024-00261-5.

Kumar, R., Chaudhary, K., Singh Chauhan, J., Nagpal, G., Kumar, R., Sharma, M., Raghava, G.P., 2015. An in silico platform for predicting, screening and designing of antihypertensive peptides. Sci. Rep. 5 (1), 12512. https://doi.org/10.1038/srep12512.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157 (1), 105–132. https://doi.org/10.1016/0022-2836(82)90515-0.

Limongelli, R., Minervini, F., Calasso, M., 2023. Fermentation of pomegranate matrices with Hanseniaspora valbyensis to produce a novel food ingredient. LWT 180, 114687. https://doi.org/10.1016/j.lwt.2023.114687.

Martini, S., Conte, A., Tagliazucchi, D., 2020. Effect of ripening and in vitro digestion on the evolution and fate of bioactive peptides in Parmigiano-Reggiano cheese. Int. Dairy J. 105, 104668. https://doi.org/10.1016/j.idairyj.2020.104668.

Melikoglu, M., Webb, C., 2013. Use of waste bread to produce fermentation products. In: Kosseva, M., Webb, C. (Eds.), Food Industry Wastes: Assessment and Recuperation of Commodities. Elsevier BV, Amsterdam, Netherlands, pp. 63–76. https://doi.org/10.1016/B978-0-12-391921-2.00004-4.

Miguel, M., Recio, I., Gómez-Ruiz, J.A., Ramos, M., López-Fandiño, R., 2004. Angiotensin I–converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. J. Food Prot. 67 (9), 1914–1920. https://doi.org/10.4315/0362-028x-67.9.1914.

Mooney, C., Haslam, N.J., Pollastri, G., Shields, D.C., 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. PLoS One 7 (10), e45012. https://doi.org/10.1371/journal.pone.0045012.

Nanni, A., Parisi, M., Colonna, M., 2021. Wine by-products as raw materials for the production of biopolymers and of natural reinforcing fillers: a critical review. Polymers (Basel) 13 (3), 381. https://doi.org/10.3390/polym13030381.

Ou, K., Liu, Y., Zhang, L., Yang, X., Huang, Z., Nout, M.R., Liang, J., 2010. Effect of neutrase, alcalase, and papain hydrolysis of whey protein concentrates on iron uptake by Caco-2 cells. J. Agric. Food Chem. 58 (8), 4894–4900. https://doi.org/10.1021/jf100055y.

Qin, D., Jiao, L., Wang, R., Zhao, Y., Hao, Y., Liang, G., 2023. Prediction of antioxidant peptides using a quantitative structure− activity relationship predictor (AnOxPP)

based on bidirectional long short-term memory neural network and interpretable amino acid descriptors. Comput. Biol. Med. 154, 106591. https://doi.org/10.1016/j.compbiomed.2023.106591.

Rizzello, C.G., Lavecchia, A., Gramaglia, V., Gobbetti, M., 2015. Long-term fungal inhibition by Pisum sativum flour hydrolysate during storage of wheat flour bread. Appl. Environ. Microbiol. 81 (12), 4195–4206. https://doi.org/10.1128/AEM.04088-14.

Rizzello, C.G., Tagliazucchi, D., Babini, E., Rutella, G.S., Saa, D.L.T., Gianotti, A., 2016. Bioactive peptides from vegetable food matrices: research trends and novel biotechnologies for synthesis and recovery. J. Funct. Foods 27, 549–569. https://doi.org/10.1016/j.jff.2016.09.023.

Rizzello, C.G., Verni, M., Bordignon, S., Gramaglia, V., Gobbetti, M., 2017. Hydrolysate from a mixture of legume flours with antifungal activity as an ingredient for prolonging the shelf-life of wheat bread. Food Microbiol. 64, 72–82. https://doi.org/10.1016/j.fm.2016.12.003.

Sarker, A., Ahmmed, R., Ahsan, S.M., Rana, J., Ghosh, M.K., Nandi, R., 2024. A comprehensive review of food waste valorization for the sustainable management of global food waste. Sus. Food Technol. 2 (1), 48–69. https://doi.org/10.1039/D3FB00156C.

Sarmadi, B.H., Ismail, A., 2010. Antioxidative peptides from food proteins: a review. Peptides 31 (10), 1949–1956. https://doi.org/10.1016/j.peptides.2010.06.020.

Schillinger, U., Lucke, F.K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55 (8), 1901–1906. https://doi.org/10.1128/aem.55.8.1901-1906.1989.

Siroli, L., Giordani, B., Rossi, S., Gottardi, D., McMahon, H., Augustyniak, A., Lanciotti, R., 2022. Antioxidant and functional features of pre-fermented ingredients obtained by the fermentation of milling by-products. Fermentation 8 (12), 722. https://doi.org/10.3390/fermentation8120722.

Tacias-Pascacio, V.G., Morellon-Sterling, R., Siar, E.H., Tavano, O., Berenguer-Murcia, A., Fernandez-Lafuente, R., 2020. Use of Alcalase in the production of bioactive peptides: a review. Int. J Biol. Macromol. 165, 2143–2196. https://doi.org/10.1016/j.ijbiomac.2020.10.060.

Trossolo, E., Tlais, A.Z.A., Tonini, S., Filannino, P., Gobbetti, M., Di Cagno, R., 2025. Fermentation of a wine pomace and microalgae blend to synergistically enhance the functional value of protein-and polyphenol-rich matrices. Food Res. Int. 202, 115785. https://doi.org/10.1016/j.foodres.2025.115785.

Udenigwe, C.C., Aluko, R.E., 2012. Food protein-derived bioactive peptides: production, processing, and potential health benefits. J. Food Sci. 77 (1), R11–R24. https://doi.org/10.1111/j.1750-3841.2011.02455.x.

Verni, M., Pontonio, E., Krona, A., Jacob, S., Pinto, D., Rinaldi, F., Rizzello, C.G., 2020a. Bioprocessing of brewers' spent grain enhances its antioxidant activity: characterization of phenolic compounds and bioactive peptides. Front. Microbiol. 11, 1831. https://doi.org/10.3389/fmicb.2020.01831.

Verni, M., Minisci, A., Convertino, S., Nionelli, L., Rizzello, C.G., 2020b. Wasted bread as substrate for the cultivation of starters for the food industry. Front. Microbiol. 11, 293. https://doi.org/10.3389/fmicb.2020.00293.

Verni, M., Dingeo, C., Rizzello, C.G., Pontonio, E., 2021. Lactic acid bacteria fermentation and endopeptidase treatment improve the functional and nutritional features of *Arthrospira platensis*. Front. Microbiol. 12, 744437. https://doi.org/10.3389/fmicb.2021.744437.

Verni, M., Squeo, G., Perri, G., Demarinis, C., Rizzello, C.G., Caponio, F., Pontonio, E., 2025. Optimizing *Tenebrio molitor* powder as ingredient in breadmaking: impact of enzymatic hydrolysis on dough techno-functional properties and bread quality. Fut. Foods, 100665. https://doi.org/10.1016/j.fufo.2025.100665.

Vogelsang-O'Dwyer, M., Sahin, A.W., Bot, F., O'Mahony, J.A., Bez, J., Arendt, E.K., Zannini, E., 2023. Enzymatic hydrolysis of lentil protein concentrate for modification of physicochemical and techno-functional properties. Eur. Food Res. Technol. 249 (3), 573–586. https://doi.org/10.1007/s00217-022-04152-2.

Wang, Y., Qi, H., 2024. Waste to wealth: bioprocessing methods for the conversion of food byproducts into value-added products: a mini-review. Curr. Opin. Food Sci. 101215. https://doi.org/10.1016/j.cofs.2024.101215.

Wang, C., Udenigwe, C.C., Zheng, L., Zhao, M., 2025. Bioactivities, preparation, and bioavailability of food-derived proline-containing peptides. Trends Food Sci. Technol. 155, 104776. https://doi.org/10.1016/j.tifs.2024.104776.

Weiss, W., Vogelmeier, C., Görg, A., 1993. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. Electrophoresis 14, 805–816. https://doi.org/10.1002/elps.11501401126.

Wu, J., Aluko, R.E., Nakai, S., 2006. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure− activity relationship study of di- and tripeptides. J. Agric. food Chem. 54 (3), 732–738. https://doi.org/10.1021/jf051263l.

Yu, L., Perret, J., Harris, M., Wilson, J., Haley, S., 2003. Antioxidant properties of bran extracts from "Akron" wheat grown at different locations. J. Agric. food Chem. 51 (6), 1566–1570. https://doi.org/10.1021/jf020950z.

Zhou, J., Li, D., Zhang, X., Liu, C., Chen, Y., 2023a. Valorization of protein-rich waste and its application. Sci. Total Environ. 901, 166141. https://doi.org/10.1016/j.scitotenv.2023.166141.

Zhou, L., Mendez, R.L., Kwon, J.Y., 2023b. In silico prospecting for novel bioactive peptides from seafoods: a case study on pacific oyster (*Crassostrea gigas*). Molecules 28 (2), 651. https://doi.org/10.3390/molecules28020651.

Zhu, C., Lei, M., Andargie, M., Zeng, J., Li, J., 2019. Antifungal activity and mechanism of action of tannic acid against Penicillium digitatum. Physiol. Mol. Plant Pathol. 107, 46–50. https://doi.org/10.1016/j.pmpp.2019.04.009.

Zhu, D., Huang, X., Tu, F., Wang, C., Yang, F., 2020. Preparation, antioxidant activity evaluation, and identification of antioxidant peptide from black soldier fly (*Hermetia illucens* L.) larvae. J. Food Bioch. 44 (5), e13186. https://doi.org/10.1111/jfbc.13186.

Zou, T.B., He, T.P., Li, H.B., Tang, H.W., Xia, E.Q., 2016. The structure-activity relationship of the antioxidant peptides from natural proteins. Molecules 21 (1), 72. https://doi.org/10.3390/molecules21010072.