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Bromelain-catalyzed hydrolysis of fish and poultry by-products: a sustainable approach to biopeptide production

Sandra Borges [a,*], Tânia C.F. Ribas [a], Maria Leonor Castro [a], Débora Campos [b], Maria João Mota [c,d], André Almeida [e,f], Manuela Pintado [a]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal
[b] AgroGrIN Tech – Molecule Message Unipessoal Lda, Porto, Portugal
[c] SORGAL - Sociedade de Óleos e Rações, S.A., São João de Ovar, Portugal
[d] SAVINOR - Sociedade Avícola do Norte S.A., Trofa, Portugal
[e] SEBOL - Comércio e Indústria do Sebo, S.A., Loures, Portugal
[f] I.T.S. – Indústria Transformadora de Subprodutos S.A, Loures, Portugal

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ABSTRACT

This study aimed to develop bioactive protein hydrolysates from fish and poultry by-products using bromelain, an enzyme obtained from pineapple by-products, with potential applications in food and biotechnology. The global rise in protein-rich food consumption has led to increased generation of animal by-products from the meat and fish industries. These by-products are rich in protein, making them suitable for producing bioactive compounds. A multifactorial design was employed to optimize the hydrolysis process, evaluating key parameters such as enzyme-to-substrate (E/S) ratio and hydrolysis time. The optimal conditions were identified as 1.3 % (E/S) for 240 min for fish by-products, and 2.0 % (E/S) for 180 min for poultry by-products. These conditions were scaled up to produce fish (FH) and poultry (PH) hydrolysates.

The hydrolysates showed high protein content (70.8 ± 0.1 % for FH and 56.3 ± 1.0 % for PH), potent antioxidant activity (ORAC: 249.02 ± 4.71 μmol eq. Trolox/g for FH and 144.41 ± 11.62 μmol eq. Trolox/g for PH), and notable ACE inhibitory effects (IC_{50}: 693 ± 12 μg protein/mL for FH and 1585 ± 6 μg protein/mL for PH).

Furthermore, hydrolysates promoted probiotic strains' growth, with 1 % (w/v) FH or PH showing comparable performance to conventional culture media in supporting *Lactobacillus casei* 01 ($p > 0.05$). These results highlight the potential of bromelain-derived hydrolysates as sustainable, cost-effective alternatives to traditional nitrogen sources in functional fermented food or microbiological culture media.

This study contributes to value creation from agro-industrial by-products and aligns with global sustainability initiatives by promoting circular bioeconomy approaches.

1. Introduction

In recent years, the global consumption of protein-rich foods has risen significantly. In consequence, the livestock industry, slaughterhouses and fishing industry generate large amounts of low-value by-products daily, posing both economic and environmental challenges. Utilizing these by-products in the production chain could significantly reduce animal waste. These by-products include animal parts (e.g. viscera, bonds) or entire carcasses that are not suitable for direct human consumption [1]. For example, fish by-products consist of carcasses, heads, viscera, skin, and bones [2], while poultry by-products include blood, bones, meat trimmings, skin, adipose matter, feet, cranial components and visceral organs [3]. Reducing food loss and waste has gained significant global attention and plays a key role in the 2030 Agenda for Sustainable Development. Target 12.3 of the United Nations' Sustainable Development Goals (SDGs) sets an ambitious objective to cut global food loss and waste by 50 % by 2030 [4]. Animal by-products are typically processed into ingredients for animal nutrition or converted into organic fertilizers. However, research has shown that these by-products contain significant amounts of proteins, lipids, carbohydrates, and micronutrients (minerals and vitamins). This makes them a promising resource for extracting new compounds and functional

* Corresponding author.
E-mail address: sandraferreiraborges@gmail.com (S. Borges).

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ingredients, offering the potential for better utilization of existing protein resources in the diet [5].

The increasing awareness of the significance of high-quality proteins in the human diet has sparked scientific interest in discovering eco-friendly, sustainable, and health-promoting ingredients. Enzymatic hydrolysis enables the production of protein hydrolysates with a high degree of hydrolysis, leading to increased yield. This process promotes the release of smaller peptides, enhancing their biological properties [6]. For instance, fish hydrolysates (FHs) and poultry hydrolysates (PHs) have revealed antihypertensive activity via angiotensin-I converting enzyme (ACE) inhibitory mechanisms as well as antioxidant activity [2,7,8]. In fact, antioxidant peptides have the potential to mitigate oxidative stress in the human body, thereby decreasing the likelihood of developing degenerative disorders [9]. Research studies also revealed that protein hydrolysates can also stimulate probiotic growth [10]. Notably, protein hydrolysates derived from poultry bones and feathers by-products have proven to be especially effective in promoting probiotic growth [11]. Therefore, protein hydrolysates could serve as alternative nitrogen sources in microbiological culture media and also act as functional ingredients in the formulation of fermented foods with added health benefits.

In this sense, alcalase, pepsin, papain, and trypsin are usually employed in the extraction of bioactive peptides from animal protein sources [12]. While these enzymes are well-studied and widely applied, this study uniquely explores the use of bromelain, a less commonly used plant-derived enzyme, for hydrolyzing multiple animal by-products. Bromelain may interact with multiple substrates due to its unique binding sites, which enable it to catalyze a broad range of proteolytic reactions. Specific catalytic residues, including cysteine, histidine, and asparagine, are essential for its activity, as they contribute to the enzyme's ability to cleave peptide bonds and thereby enhance its proteolytic efficiency [13]. Bromelain is commonly used for the enzymatic hydrolysis of legume seed extracts [14,15]. Therefore, this work fills a gap by evaluating bromelain's effectiveness not only in peptide generation but also in supporting probiotic growth and functional food applications, aspects less addressed in previous studies.

Bromelain is an enzymatic complex found in plants of the Bromeliaceae family, primarily in *Ananas comosus*, and is made up of endopeptidases, carbohydrates and glycoproteins [16]. Although this enzymatic complex has been extracted from the whole fruit, which has a large environmental and economic impact, it can also be found in various parts of pineapple residues, such as stems and peels. Bromelain has been investigated for multiple applications in the food industry, including meat tenderization, anti-browning activity and in alcoholic fermentation processes [17]. Recent investigations have demonstrated that bromelain can also be used to hydrolyze animal by-products, such as pork liver [1,18] and fish skin [19,20]. However, studies using bromelain focus on limited substrates, and there is still scarce information on the functional application of hydrolysates derived from animal by-products using this enzyme. The current work contributes to filling this research void by producing and scaling up bromelain-derived hydrolysates from fish and poultry by-products, and evaluating their antioxidant and ACE-inhibitory activities, as well as their ability to support probiotic growth, demonstrating their dual potential as ingredients in microbial culture media and functional fermented food formulations.

2. Material and methods

2.1. Materials and reagents

2.1.1. Raw materials and enzyme

Fish by-products and poultry by-products were obtained from companies specializing in the collection of animal by-products, ETSA group (Loures, Portugal) and SAVINOR (Trofa, Portugal), respectively. Fish by-products supplied were classified as category 3, i.e., materials downgraded from food grade due to low commercial value (e.g., heads and fins of some species), breakage of the cold chain, or nearing the end of their shelf-life. The poultry by-products included various materials such as heads, viscera, blood, trimmings, bones, and skin. By-product samples were collected in bulk, homogenized and stored at −20 °C until further processing. Bromelain was derived from pineapple by-products and provided by AgroGrIN Tech (Porto, Portugal).

2.1.2. Reagents and chemicals

AAPH (Thermo Fisher Scientific, Waltham, MA, USA); ABTS (Thermo Fisher Scientific, Waltham, MA, USA); azocasein (Sigma-Aldrich, St. Louis, MO, USA); ABz-Gly-Phe(NO_2)-Pro (Bachem Feinchemikalien, Bubendorf, Switzerland); ACE (Sigma Chemical, MO, USA); fluorescein (Sigma-Aldrich, St. Louis, MO, USA); Glycerol (Sigma-Aldrich, St. Louis, MO, USA); HCl (Sigma-Aldrich, St. Louis, MO, USA); HNO_3 (VWR Chemicals, Leuven, Belgium); $K_2O_8S_2$ (Sigma-Aldrich, St. Louis, MO, USA); KCl (Sigma-Aldrich, St. Louis, MO, USA); L-leucine (Sigma-Aldrich, St. Louis, MO, USA); Man Rogosa Sharpe (MRS) agar (VWR Chemicals, Leuven, Belgium); MRS broth (Biokar Diagnostics, Allonne, France); NaCl (Sigma-Aldrich, St. Louis, MO, USA); NaH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA); NaOH (Sigma-Aldrich, St. Louis, MO, USA); o-Abz-Gly-p-Phe(NO_2)-Pro-OH (Bachem, Bubendorf, Switzerland); Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA); TNBS (Sigma-Aldrich, St. Louis, MO, USA); Tris base (Sigma-Aldrich, St. Louis, MO, USA); trichloroacetic acid ((Sigma-Aldrich, St. Louis, MO, USA) Trolox (Sigma-Aldrich, St. Louis, MO, USA); $ZnCl_2$ (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Analysis of raw materials and enzyme

2.2.1. Composition analysis of fish and poultry by-products

The proximate composition of fish and poultry by-products was analyzed according to standard methods established by the Association of Official Analytical Chemists [21]. Protein content was determined using the Kjeldahl method, applying a nitrogen-to-protein conversion factor of 6.25. Moisture was measured by drying the samples at 105 °C for 24 h, while ash content was quantified by incineration at 550 °C for 5 h.

2.2.2. Enzymatic activity of bromelain

Bromelain enzyme activity was assessed using azocasein as the substrate, following the method described by Borges *et al.* [2]. The assay was conducted at pH 7 and 37 °C, which correspond to the optimal conditions for bromelain activity as specified by AgroGrIN Tech, the enzyme supplier. Therefore, the enzyme was combined with 30 mM acetate buffer (pH 7) containing 1 % (w/v) azocasein and incubated at 37 °C for 60 min. The reaction was terminated by adding 10 % (w/v) trichloroacetic acid, followed by centrifugation at 8000 rpm for 5 min. The resulting supernatant was mixed with 1 M NaOH, and absorbance was measured at 440 nm. All measurements were performed in triplicate, and enzyme activity was reported in units per gram (U/g).

2.3. Fish and poultry by-products hydrolysis

2.3.1. Multifactorial optimization assay

A multifactorial assay was designed, envisaging the optimization of the hydrolysis procedure for poultry and fish by-products using bromelain (Fig. 1).

The poultry by-products were pre-treated with temperature and pressure (121 °C, and 15 psi for 15 min) before the hydrolysis process. Afterwards, the enzymatic hydrolysis was performed under the conditions for optimal bromelain activity [17]. Substrate material (poultry and fish by-products) was suspended in 30 mM acetate buffer (pH 7) at a ratio substrate to buffer of 1:10 (w/v). The hydrolysis process was conducted at 37 °C under continuous agitation (150 rpm). The hydrolysis optimization was conducted envisaging two variable factors, the

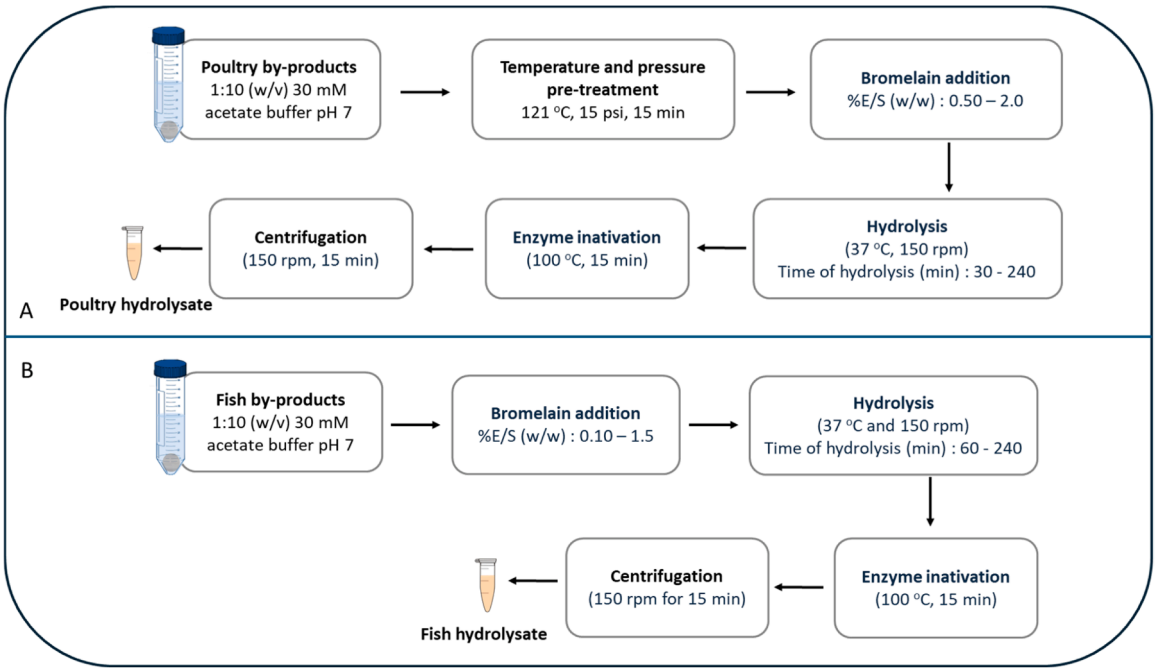

Fig. 1. Flow diagram of the hydrolysis process of poultry (A) and fish (B) by-products.

enzyme percentage, through the ratio enzyme/substrate (%E/S) masses, and the time of hydrolysis (Table 1). Considering the differences between fish and poultry proteins, particularly the higher content of connective tissue proteins such as collagen and elastin in poultry [22], which may hinder enzymatic breakdown, a higher enzyme concentration range was applied for the hydrolysis of poultry by-products. Furthermore, several studies have indicated that enzymatic activity is most effective during the early stages of the reaction, when the substrate is more accessible and enzyme–substrate interactions are more favorable [2,23]. Therefore, a maximum hydrolysis time of 240 min was selected to balance efficiency and feasibility for potential industrial applications. At the end of each determined time of hydrolysis, the samples were taken to monitor the progress of the enzymatic hydrolysis procedure. Then, at the specific sampling points the mixture was heated to 100 °C for 15 min to terminate the enzymatic activity. After enzyme inactivation, the mixtures were centrifuged (5000 rpm for 15 min) and the supernatant was collected. Hydrolysates were then stored at −20 °C for subsequent analysis. For the hydrolysates analysis, three responses were assessed, being those, the soluble protein content following the bicinchoninic acid (BCA) method, the degree of hydrolysis following trinitro-benzene-sulfonic acid (TNBS) method, and antioxidant activity through the 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay. The experimental design consisted of 20 individual randomized experiments, varying the two factors (%E/S, and time of hydrolysis) depicted on Table 1. Afterwards, for comparison purposes the three responses were evaluated intending hydrolysis optimization. This was achieved by evaluating the responses separately and together to reach the optimal conditions of hydrolysis for each substrate. Then, using Deringer's 'desirability' function [24], the optimized conditions (%(E/S) and time of hydrolysis) were determined for the hydrolysis of poultry and fish by-products using bromelain.

Table 1
Factors evaluated for the optimization of the poultry and fish by-products hydrolysis process using bromelain, and respective levels assessed. %E/S, ratio enzyme substrate in percentage.

Factor	Fish by-products			Poultry by-products		
	Low	Median	High	Low	Median	High
%E/S	0.10	0.80	1.50	0.50	1.25	2.0
Time of hydrolysis (min)	60	150	240	30	135	240

2.3.1.1. ° of hydrolysis. The TNBS assay for the measurement of DH was adapted from Hsu *et al.* [25] to a 96-well microplate assay. Total free amino groups and those in peptide bonds were assessed using acid hydrolysis at high temperature. For that, 10 mg of sample was hydrolyzed with 1 mL of a 6 mol/L HCl solution at 110 °C for 20 h. The hydrolysate was then neutralized (1 mL of a 6 mol/L NaOH solution). For calibration purposes, a 10 mmol/L-leucine stock solution was prepared and diluted in the range of 50 to 250 μmol/L. The 96-well microplate was prepared in duplicate for each standard/sample solution as follows: 50 μL of standard/sample solution, 125 μL of a 75 mmol/L sodium phosphate buffer (pH 8.2), and 50 μL of 0.025 % TNBS solution. After incubation of the microplate (1 h at 50 °C), the absorbance (λ = 340 nm) was measured using a Synergy H1 microplate reader (Microplate Reader Biotek Synergy H1, Santa Clara, CA, USA) with Gen5 Biotek software version 3.04. The DH was calculated through DH (%) = $(L_t - L_0)/(L_{max} - L_0)$*100, where L_t represents the concentration of amino groups released at a specific hydrolysis time, L_0 corresponds to the concentration of amino groups in the initial sample, and L_{max} denotes the maximum concentration of amino groups in the fish or poultry by-products.

2.3.1.2. Total soluble protein content. The soluble protein content was determined through the BCA methodology, using the Pierce BCA Protein Assay Kit. The method was performed following the kit guidelines as follows: bovine serum albumin (BSA) standards were prepared by proper dilution of the kit standard stock solution in the range of 25 to 2000 μg/mL; working BCA reagent solution was prepared by mixing the reagent A with Reagent B in the ratio 50:1, respectively. The 96-well microplate was prepared in duplicate for each standard/sample solution as follows: 25 μL of standard/sample solution and 200 μL of BCA reagent solution. The microplate was incubated 30 min at 37 °C, and then, the absorbance (λ = 562 nm) was measured using a Synergy H1 microplate reader.

2.3.1.3. Antioxidant activity. The antioxidant activity of FH and PH was assessed using the ABTS assay, as described by Castro *et al.* [26]. An

$ABTS^+$ stock solution was prepared by mixing two aqueous solutions, 7 mM ABTS and 2.44 mM $K_2O_8S_2$. This mixture was stirred for 16 h to generate the $ABTS^+$ radical cation. The working solution was then obtained by diluting the stock solution with water to reach an absorbance at 734 nm of 0.70 ± 0.02. In addition, a Trolox standard curve solution was prepared in the range of 25–175 μM. For the assay, 20 μL of each sample dilution, Trolox standard, or ultrapure water (as control), were pipetted into a 96-well microplate in triplicate, followed by the addition of 180 μL of $ABTS^+$ solution. After a 6 min incubation at 30 °C, absorbances were measured at 734 nm using a microplate reader. Antioxidant activity was expressed in μmol of Trolox equivalent per gram of dry weight (μmol eq. Trolox/g).

2.3.2. Laboratory scale-up

After the optimization process, the best hydrolysis conditions were chosen based on those that provided a higher degree of hydrolysis to proceed to a scale-up of the process for each substrate. The hydrolysis conditions were identified as 1.3 % (E/S) for 240 min for fish by-products, and 2.0 % (E/S) for 180 min for poultry by-products. The supernatants, rich in protein hydrolysates, were freeze-dried (Armfield SB4 model, Hampshire, England). The resultant hydrolysates obtained from fish and poultry by-products hydrolysis were used for a comprehensive characterization of their molecular and functional properties.

2.4. Protein hydrolysates characterization

2.4.1. Proximate composition

The proximate composition assessment was carried out following the same protocol as described in Section 2.2.1.

2.4.2. Minerals determination

2.4.2.1. Hydrolysates microwave-assisted digestion. Mineral determination was performed following the methodology described by Vieira *et al.* [27] with some modifications. Microwave-assisted digestion was performed in dried hydrolysates using a Mars One digestion system (CEM, USA) following the specific protocol provided by the manufacturer for the digestion of food samples. For that, 0.5 g of the sample was mixed with 10 mL of 65 % HNO_3 in a Teflon reaction vessel. Then, the vessels were kept open for 15 min before sealing to allow the predigestion of the hydrolysates. Afterwards, the digestion procedure was carried out with the following instructions: ramp time 20 min; hold time 15 min; temperature 210 °C, and the pressure of 800 psi. The resulting clear solutions were transferred to flasks and made up with ultrapure water to a final volume of 50 mL. Control digestion was made without samples.

2.4.2.2. Minerals quantifications – inductively coupled plasma - optical emission spectrometry. After hydrolysate digestion, minerals quantification (Na, Mg, K, Ca, Mn, Fe, Cu, P, Se, Cr, Mo, Ni, Co, B, and Al) in hydrolysates was carried out by inductively coupled plasma-optical emission spectrometry (Opima 7000 DV ICP-OES) following the manufacturer's instructions.

2.4.3. Hygroscopicity analysis

Hygroscopicity measurement was performed according to Juarez-Enriquez *et al.* [28]. Approximately 1 g of each sample was conditioned in an airtight glass container with a saturated potassium chloride solution (86 % relative humidity) at room temperature for one week. Weight gain was employed to classify powders according to the hygroscopicity category defined by Callahan *et al.* [29].

2.4.4. Molecular weight profile

High-Performance Size Exclusion Chromatography (HPSEC) was performed according to the method outlined by Fernandez Cunha *et al.* [30]. An Agilent AdvanceBio SEC column (Agilent Technologies, London, UK) with a 2.7 μm particle size, 130 Å pore size, and dimensions of 7.8 mm inner diameter × 300 mm length was used. The column was eluted under isocratic conditions using a phosphate buffer (0.15 M NaH_2PO_4, pH 7) at a flow rate of 1 mL/min. Samples (10 μL) were injected after being filtered through PTFE/L 0.22 μm filters. The analysis was carried out on a Waters 2690 system equipped with a photodiode array (PDA) detector (190–600 nm), and data were processed using Empower 3 software. Molecular weights (MW) of chromatogram peaks were determined using a calibration curve with the following protein standards: Ovalbumin (44,300 Da); Myoglobin (17,600 Da); Cytochrome C (12,327 Da); Aprotinin (6511 Da); Neurotensin (1672 Da); Angiotensin-II (1040 Da); Tyr-Phe dipeptide (328.4 Da); and L-tryptophan (204 Da).

2.5. Bioactivity of protein hydrolysates

Both protein hydrolysates (FH and PH) were characterized regarding their bioactivity, specifically antioxidant activity, anti-hypertensive activity, and their influence on probiotics' growth, as described below.

2.5.1. Antioxidant activity

The antioxidant activity of FH and PH was assessed using the following methods: the ABTS assay and the Oxygen Radical Absorbance Capacity (ORAC) assay.

The ABTS assay was performed according to subSection 2.3.1.3.

The ORAC assay was performed according to Dávalos *et al.* [31]. For that, a 75 mM PBS buffer was prepared by dissolving NaH_2PO_4 in ultrapure water and adjusting the pH to 7.44, using a monovalent strong base. A fluorescein solution was prepared at a concentration of 116.66 nM. The Trolox standard curve solution was made in the range of 10–80 μM. Finally, AAPH solution was prepared by dissolving it in PBS (13.018 mg/mL). Briefly for the assay procedure, 20 μL of sample (each dilution), Trolox, or solvent (PBS buffer) for the blank were pipetted in duplicate into each well of a 96-well plate followed by the addition of 120 μL of fluorescein solution. The reaction mixture was initially incubated at 37 °C for 10 min, followed by the addition of 60 μL of AAPH solution. Subsequently, the mixture was incubated for an additional 140 min at 37 °C, during which fluorescence was measured every minute using a microplate reader. Final ORAC-FL values were expressed as μmol eq. Trolox/g.

2.5.2. Anti-hypertensive activity

The inhibitory effect on ACE activity was assessed following the method described by Borges *et al.* [2]. The substrate used was o-Abz--Gly-p-Phe(NO_2)-Pro-OH, and the reaction was carried out in the presence of ACE. Firstly, ACE (EC 3.4.15.1, 5.1 U/mg) was diluted in 5 mL of a 50 % glycerol and ultra-pure water solution and subsequently diluted 1:24 in 150 mM Tris buffer (pH 8.3) containing 1 μM $ZnCl_2$ (final concentration of 42 mU/mL). Then, 40 μL of either ultrapure water or ACE working solution was adjusted to 80 μL by adding ultrapure water to the blank, control, or samples. The reaction was initiated by adding 160 μL of substrate solution (0.45 mM ABz-Gly-Phe(NO_2)-Pro) in 150 mM Tris buffer (pH 8.3) with 1.125 M NaCl. The reaction mixture was incubated at 37 °C, and fluorescence was measured during 30 min using a multi-detection microplate reader, with excitation and emission wavelengths of 350 nm and 420 nm, respectively. Non-linear fitting of the data was used to calculate the IC_{50} (the protein concentration required to inhibit 50 % of ACE activity). Each sample's ACE inhibitory activity was analyzed in duplicate.

2.6. Probiotic growth kinetics

The probiotic strains used in this study were *Lactobacillus casei* 01 (Chr. Hansen, Hørsholm, Denmark) and *Lacticaseibacillus rhamnosus* LGG ATCC 53,103 (ATCC, Manassas, USA). The bacteria were grown on MRS agar plates at 37 °C, for 48 h. Then, the probiotics were transferred to

MRS broth and incubated at 37 °C for 24 h. For the growth curves, commercial MRS and MRS without protein were used as positive and negative control, respectively. To substitute the traditional protein content (i.e., 2.5 % (w/v) of peptone and yeast extract) in MRS broth, 0.5, 1 and 2.5 % (w/v) of the hydrolysates were used. Briefly, 180 μL of media and 20 μL of the inoculum were pipetted into each well of a 96-well plate, with each condition tested in triplicate. The microplate was incubated at 37 °C in a microplate reader and the OD_{600} was measured every hour for 24 h. The preferred nitrogen source for each species tested was determined using the maximum specific growth rate (μ_{max}). The maximum specific growth rate, μ_{max}, was established by analyzing the slope of the exponential growth phase ($\mu_{max} = \Delta \ln (OD_{600})/\Delta t$, where t is time and expressed as h^{-1}) [32].

2.7. Statistical analysis

Statistical analysis was conducted using IBM® SPSS® Statistics 26. Data normality was assessed with the Shapiro–Wilk test. To evaluate differences among more than two groups, a one-way ANOVA was applied for normally distributed data with LSD post hoc test, or the Kruskal–Wallis test was used for non-normal distributions. For comparisons between two groups, the Student's *t*-test was utilized for normal distributions, and the Mann–Whitney test was employed for non-normal distributions. A significance level of 0.05 was set for all statistical tests.

3. Results and discussion

3.1. Analysis of raw materials and enzyme

The compositional analysis of fish and poultry by-products is crucial for the hydrolysis process as it provides essential information about the raw materials, such as protein content. This data is vital for optimizing bromelain-catalyzed enzymatic hydrolysis, ensuring process efficiency. Table 2 presents the proximate composition of fish and poultry by-products, with protein levels of 13.8 g and 16.7 g per 100 g, respectively. Similar findings have been reported in previous studies for fish [2,33] and chicken [34] by-products. This high protein content highlights the potential of these by-products as sources of protein hydrolysates and bioactive peptides. Various factors could significantly influence the composition of fish and poultry by-products, such as undigested feed in their gastrointestinal tracts, the proportions of different parts (such as heads, viscera, blood, trimmings, bones), and species variations [35].

The enzymatic activity of bromelain extracted from pineapple was 1003 ± 5 U/g, as determined using azocasein as a substrate. This result indicates a high proteolytic capacity, consistent with previous studies reporting bromelain activities in a similar range. For example, bromelain powders produced from pineapple peels and cores have shown enzymatic activities of 1179 and 694 gelatin digesting units (GDU)/g, respectively [36]. The hydrolysates produced through bromelain-mediated proteolysis have potential applications in the food industry as functional and bioactive ingredients, such as flavor enhancers, antioxidant and anti-hypertensive agents, as well as components in the formulation of culture media for microbial fermentation. The high enzymatic activity obtained in this study supports the suitability of this bromelain extract for such applications, particularly within sustainable approaches aimed at valorizing food processing by-products.

Table 2
Proximate composition of fish and poultry by-products.

	Fish by-products	Poultry by-products
Protein (%)	13.8 ± 0.7	16.7 ± 0.7
Ash (%)	4.1 ± 0.9	5.5 ± 0.6
Moisture (%)	64.5 ± 1.7	66.6 ± 0.1

3.2. Optimization assay

The enzymatic hydrolysis of poultry and fish by-products using bromelain was evaluated through the Deringer's 'desirability' function (Fig. 2). The experiment analyzed the antioxidant activity (ABTS), soluble protein content (BCA), and free amino groups (TNBS) within the system provided insights into the effects of varying experimental factors such as the E/S ratio and reaction time.

Regarding fish by-product hydrolysis (Fig. 2A), the model revealed that the optimal conditions to maximize antioxidant activity and protein content were an E/S % of 1.2 and a time of 240 min with the adjusted r^2 of 93.6 % and 80.6 %, respectively. Regarding the degree of hydrolysis, a similar response was observed at an E/S % of 1.5 and a time of 240 min ($r^2 = 93.0$ %). Overall, the experiments demonstrated that the E/S ratio and reaction time significantly influenced all three responses. The comprehensive analysis, across all three responses, provided maximal predicted values achieved with an E/S % of 1.3 % and reaction time of 240 min, maximizing antioxidant activity, protein content and degree of hydrolysis with maximum predicted values of 1068 μmol eq. Trolox/L, 4452 μg/mL, and 18.7 % for the mentioned responses, respectively. Regarding the hydrolysis of poultry by-products (Fig. 2B), for antioxidant activity and protein content, the model showed a similar response with maximal predicted values achieved with an E/S of 2.0 % and a reaction time of 240 min. With these conditions, the predicted values for antioxidant activity, total soluble protein and free amino groups were 1217 μmol eq. Trolox/L, 4053 μg/mL, and 18,729 μmol/L, respectively. However, the free amino groups quantification showed, with significant improvement, maximum predicted values with an E/S ratio of 2 % and a hydrolysis time of 180 min ($r^2 = 47.8$ %). The optimal conditions were set based on the free amino groups analysis with maximum predicted value, for this response, of 19,533 μmol/L.

Previous studies have demonstrated the effectiveness of bromelain in the hydrolysis of animal by-products, leading to the production of protein hydrolysates with improved solubility, digestibility, and bioactivity [37–39]. Nevertheless, data regarding fish and poultry by-products hydrolysis remain scarce. Therefore, these findings demonstrate that bromelain is a promising tool in the bioconversion of fish and poultry processing residues into functional protein hydrolysates. These hydrolysates can be further exploited as functional ingredients in food and pharmaceutical applications or as nitrogen sources in microbial culture media.

3.2.1. Scale-up

Considering the optimized conditions of hydrolysis stated in sub-Section 3.2, a validation of the conditions was performed for both substrates. Hydrolysis of poultry by-products was performed using a %E/S of 2 % and a time of hydrolysis of 2 h. Regarding the fish by-products, the hydrolysis was performed using a %E/S of 1.3 % and a time of hydrolysis of 4 h. The supernatant obtained in the hydrolysis process was evaluated in terms of the soluble protein content, the degree of hydrolysis, and antioxidant activity. The lyophilized hydrolysates that resulted from these processes were used for a comprehensive characterization of their molecular and functional properties.

3.3. Characterization of the poultry and fish by-products hydrolysates

3.3.1. Proximate composition and mineral content

The proximate composition of the scale-up produced dried hydrolysates, produced under optimal conditions for both by-products hydrolysis, was evaluated in terms of protein, moisture, and ash content, as summarized in Table 3. Regarding the protein content, the fish hydrolysate (FH) exhibited a significantly higher content (70.8 ± 0.1 %) when compared to the poultry hydrolysate (PH), which is 56.3 ± 1.0 %. The higher protein content observed in FH compared to the PH can be attributed to differences in protein solubility and enzymatic efficiency rather than the absolute protein content of the raw materials. Although

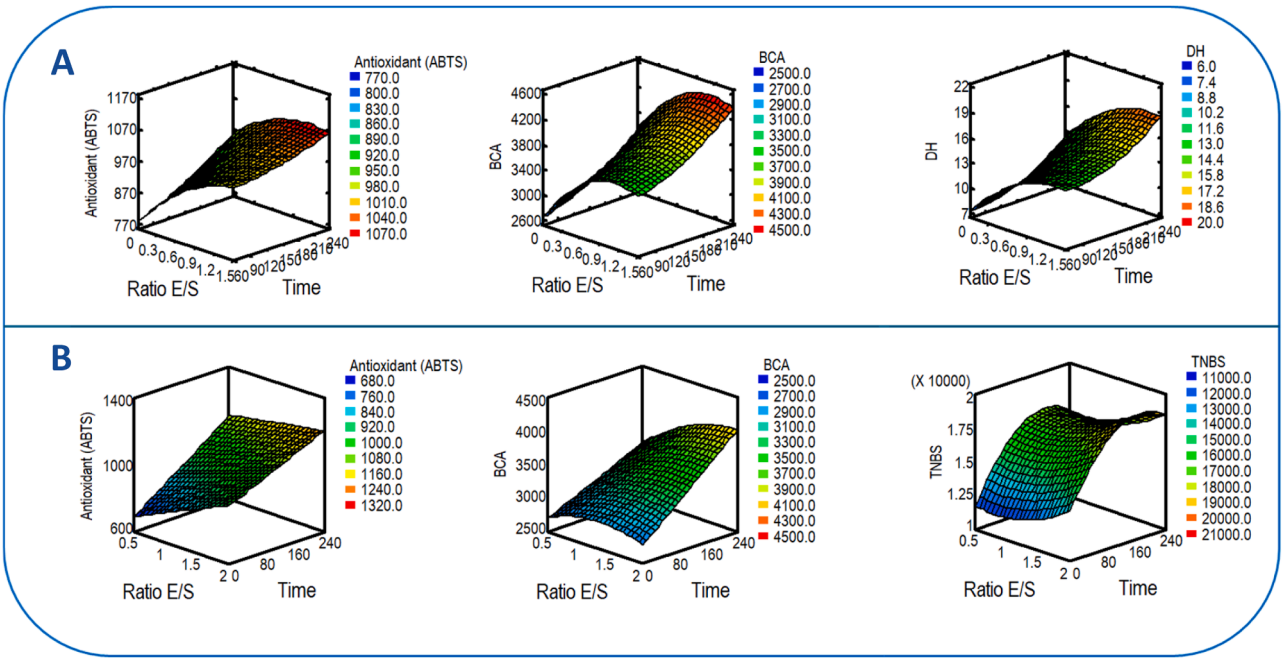

Fig. 2. Derringer's function for the optimization of the hydrolysis of fish (A) and poultry (B) by-products using bromelain. Predicted response surface using two factors %E/S (for fish by-products were 0.10, 0.80, and 1.50 %; for poultry by-products were 0.50, 1.25, and 2.00 %) and time of hydrolysis (for fish by-products were 60, 150, 240 min; for poultry by-products were 30, 135, and 240 min), with the evaluation of three responses (antioxidant activity, soluble protein content (BCA) and degree of hydrolysis (DH – TNBS)).

Table 3
Proximate composition and mineral content (mean ± SD) of FH and PH.

	FH	PH
Protein (g/100 g hydrolysate)	70.8 ± 0.1	56.3 ± 1.0
Moisture (g/100 g hydrolysate)	11.5 ± 0.3[a]	11.6 ± 0.5[a]
Ashes (g/100 g hydrolysate)	14.2 ± 0.2	17.0 ± 0.5
K (mg/100 g hydrolysate)	4116 ± 566	5079 ± 268
Na (mg/100 g hydrolysate)	1052 ± 11	1191 ± 78
P (mg/100 g hydrolysate)	570 ± 8	516 ± 22
Ca (mg/100 g hydrolysate)	109 ± 6	–
Mg (mg/100 g hydrolysate)	98.6 ± 0.8	47.2 ± 2.4
Cu (mg/100 g hydrolysate)	0.337 ± 0.043	0.120 ± 0.002
Se (mg/100 g hydrolysate)	0.193 ± 0.063	0.113 ± 0.002

[a] Same lowercase letters represent no significant difference ($p > 0.05$).

poultry generally has a higher overall protein content than fish, the solubility of these proteins during hydrolysis plays a crucial role in the final hydrolysate composition. Fish proteins, such as myofibrillar and sarcoplasmic proteins, are generally more soluble and susceptible to enzymatic hydrolysis due to their lower collagen content and less complex structural organization [40]. In contrast, poultry contains a higher proportion of connective tissue proteins like collagen and elastin, which are less soluble and consequently, more resistant to enzymatic breakdown [22,41]. This structural difference means that even if poultry has a higher initial protein content, a lower percentage may be efficiently hydrolyzed and solubilized into the hydrolysate. Additionally, the efficiency of enzymatic hydrolysis can vary depending on the specificity of the enzyme used. Bromelain is a cysteine protease that targets the peptide bond inside the protein molecule where they have a cysteine amino acid [15]. So, bromelain may be more effective in breaking down fish proteins due to their composition and structure, leading to a greater yield of soluble peptides in FH. Furthermore, FH presents high protein content, which aligns with reported values for fish protein hydrolysates produced with different enzymes, which typically range from 70.4 % to 88.7 % [42,43]. On the other hand, the protein content of PH was lower compared to other hydrolysates derived from poultry raw materials. For instance, Lindberg *et al.* [44] produced protein hydrolysates using alcalase and reported varying protein concentrations depending on the initial raw material, 88 % for turkey by-product and mechanically deboned turkey by-product, 85 % for chicken carcass and 84 % for mechanically deboned chicken by-product.

Both hydrolysates presented a similar moisture content, although PH presented a slightly higher content in ashes when compared with FH, which reflects the higher concentration of minerals in PH by-products, which completely solubilized in the hydrolysate. The variations in protein and ashes could influence the functional properties and potential applications of these hydrolysates in food or feed formulations.

The mineral contents were assessed and are illustrated in Table 3. The hydrolysates obtained from the scale-up processes exhibited distinct mineral compositions, with significant differences observed between them ($p < 0.05$). In both hydrolysates, the predominant minerals were potassium, sodium and phosphorus. When comparing both hydrolysates, FH demonstrated higher levels of magnesium, copper, and selenium than PH. Additionally, it should be highlighted that only FH contains calcium in its composition. This is likely due to the composition of the by-products used to produce the hydrolysate, for instance, the use of fish bones, scales, or other calcium-rich components in FH production increases the calcium content of the hydrolysate [45]. These minerals were also detected in tuna protein hydrolysate prepared from both dark and white meat using papain, showing low levels of potassium (26.89–31.92 mg/100 g) and sodium (27.04–35.21 mg/100 g), and high levels of phosphorus (1483–1657 mg/100 g) and calcium (593.84–626.34 mg/100 g) [46]. Protein hydrolysates of sardine waste also showed the presence of free essential nutrients (N, P, and K) as well as other micronutrients and oligo-elements as Ca, Mg, Cu, Zn, and Fe [47]. The presence of selenium and other trace minerals aligns with the nutritional value often reported for fish-derived products [45].

Mineral composition analyses in hydrolysates derived from poultry by-products remain limited in the current literature. Nevertheless, turkey meat hydrolysate demonstrated key minerals, including phosphorus (137.0 mg/100 g), calcium (10.3 mg/g), and iron (0.43 mg/g) [48].

3.3.2. Hygroscopicity analysis

The hygroscopicity values and their classification for both scale-up produced hydrolysates are summarized in Table 4. The FH exhibited a hygroscopicity of 1.5 %, classifying it as slightly hygroscopic. On the other hand, the PH showed a significantly higher hygroscopicity ($p < 0.05$) with the value of 3.1 %, thereby classified as moderately hygroscopic. In fact, it has been reported that the presence of larger peptides can reduce the hygroscopicity of a hydrolysate [49], which is in accordance with the molecular weight results discussed in Section 3.3.3. Peptides with lower MW possess a higher specific surface area and a greater proportion of polar groups. The presence of –OH and –NH_3 at both ends of the molecule increases the number of water-binding sites [50]. Similarly, the presence of substantial amounts of water-soluble ions, such as Na^+ and K^+, implicates a hygroscopic nature [51].

3.3.3. Molecular weight profile

The fish and poultry by-products hydrolyzed with bromelain in the scale-up process were analyzed for their molecular size profiles (Fig. 3). FH and PH contained peptides smaller than 10 kDa. However, FH also included small amounts of peptides with MW above 10 and 50 kDa, indicating variation in peptide size between samples. This difference may stem from bromelain's specificity as a cysteine protease, which targets glycyl, alanyl, and leucyl peptide bonds [15], potentially influencing the molecular size and bioactivity of the resulting hydrolysates. Low MW peptides are more biostable and can permeate the intestinal membrane more effectively compared to high MW peptides, facilitating cellular absorption and systemic distribution. This increased bioavailability enhances their potential for biological activities, including antioxidant and antihypertensive activity effects [2,52]. Consequently, low MW peptides are frequently linked with heightened bioactivity due to their efficient interactions with cellular and molecular targets [53].

3.4. Bioactivity of protein hydrolysates

3.4.1. Antioxidant activity

The antioxidant activity of the scale-up produced hydrolysates was evaluated using the ABTS and ORAC assays, and the results are presented in Table 5. For both methods, FH presented significantly higher antioxidant activity ($p < 0.05$). The obtained values were 47.28 and 249.02 μmol eq. Trolox/g for ABTS and ORAC, respectively, whereas for PH, the obtained values were 21.76 and 144.41 μmol eq. Trolox/g, respectively.

The MW of the peptides may affect their pathways to target sites, which can potentially enhance antioxidant activity within the body [52]. Peptides with low MW, especially those with proline at terminal residues or those rich in proline, have demonstrated antioxidant properties and evident resistance to breakdown by intestinal enzymes during transepithelial transport [54]. As previously observed, FH and PH contained a high proportion of peptides with MW below 10 kDa, which contributes to the antioxidant potential. According to Korczek *et al.* [55], fish by-product hydrolysates can have high antioxidant activity, and they are ideal ingredients for functional diets due to their ease of isolation and widespread availability.

The antioxidant activity is not only attributable to peptide size, but it is also recognized that the influence of amino acid composition and sequence. As demonstrated in the literature, the type, position, and characteristics of specific amino acid residues, such as hydrophobicity, aromaticity, and the presence of functional groups like sulfhydryl or hydroxyl, can significantly affect radical scavenging capacity and metal ion chelation. For instance, residues such as Trp, Tyr, His, and Cys have been repeatedly associated with enhanced antioxidant effects [56]. Moreover, specific peptide sequences have been identified with strong antioxidant potential, including Asp-Cys-Gly-Tyr and Asn-Tyr-Asp-Glu-Tyr from tilapia protein hydrolysates [57], and Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL) and Glu-Leu (EL) from other food-derived peptides [58]. These sequences often feature a combination of hydrophobic and aromatic residues, which contribute to electron or proton donation, radical stabilization, and improved solubility in lipid environments, factors that enhance their antioxidant efficacy. Therefore, understanding peptide sequence characteristics is crucial to fully elucidate structure-activity relationships in antioxidant peptides.

These hydrolysates from fish and poultry by-products are considered safer to employ in the food industry to prevent lipid oxidation, unlike synthetic antioxidants that can pose risks [59]. Moreover, dietary antioxidants from natural sources may play a valuable role in enhancing human health by boosting the body's antioxidant defenses. This is significant, as oxidative stress caused by reactive oxygen species (ROS) and free radicals has been closely associated with chronic diseases, including cancer, coronary heart disease, and Alzheimer's disease [60].

3.4.2. Anti-hypertensive activity

The anti-hypertensive activity of scale-up produced hydrolysates was evaluated using the *in vitro* method that measures the ACE inhibitory effect. The FH showed better ACE inhibitory activity (IC_{50} of 1739 μg hydrolysate/mL corresponding to 693 μg protein/mL) than PH (IC_{50} of 2772 μg hydrolysate/mL corresponding to 1585 of μg protein/mL). These findings align with previous research on hydrolysates produced using bromelain. Blue shark skin collagen hydrolysates and protein fractions from trevally (*Pseudocaranx* sp.) demonstrated IC_{50} values ranging from 1073 to 3340 μg protein/mL [19]. Chicken hydrolysates have also shown ACE inhibitory effects with IC_{50} values of 313 μg protein/mL [61].

Reports indicated that ACE inhibitory activity that low MW peptides (<1 kDa) has generally been associated with stronger antihypertensive potential [62]. However, variations in bioactivity between similarly sized peptides suggest that specific residues and their arrangement play

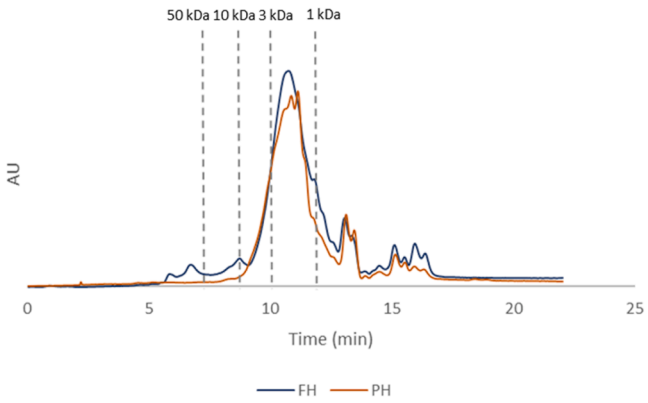

Fig. 3. Molecular weight distribution of FH and PH.

Table 5
Antioxidant activity values (mean ± SD) determined by the ABTS and ORAC assays for both hydrolysates.

Hydrolysate	ABTS (μmol eq. Trolox/g)	ORAC (μmol eq. Trolox/g)
FH	47.28 ± 5.12^a	249.02 ± 4.71^c
PH	21.76 ± 1.05^b	144.41 ± 11.62^d

[a,b,c,d]Different lowercase letters represent significant difference ($p < 0.05$).

Table 4
Hygroscopicity values (mean ± SD) and hygroscopicity category for both hydrolysates.

Hydrolysate	Hygroscopicity (%)	Classification
FH	1.5 ± 0.1^a	Slightly hygroscopic
PH	3.1 ± 0.1^b	Moderately hygroscopic

[a,b]Different lowercase letters represent significant difference ($p < 0.05$).

a critical role in activity. For instance, peptides such as Gly-Glu-Pro-Asp-Ala [63] and Val-Glu-Leu-Tyr-Pro [64], identified from fish protein hydrolysates, exhibit potent ACE inhibitory properties despite differences in chain length. The presence of hydrophobic, aromatic, or positively charged residues at the C- or N-terminal ends has been particularly associated with enhanced binding affinity to the ACE active site [62]. These findings highlight the complexity of structure-activity relationships in bioactive peptides and suggest that further studies are needed to deepen the understanding of the mechanisms underlying their biological functions.

Hypertension can lead to a variety of health problems, such as heart and kidney disease, arteriosclerosis, and stroke. Among the most extensively researched treatments for hypertension are anti-hypertensive peptides, also known as ACE inhibitors, which are derived from protein hydrolysates. ACE plays a key role in blood pressure regulation by converting angiotensin I to angiotensin II, a process that increases blood pressure. Bioactive peptides, especially those with low MW, have been shown to inhibit ACE, effectively reducing blood pressure and helping to prevent hypertension [52].

3.4.3. *Probiotic growth kinetics*

The growth of *L. casei* 01 and *L. rhamnosus* LGG with the studied hydrolysates produced at scale-up is shown in Figs. 4 and 5. For FH (Fig. 4A and 4B), it is evident that for *L. casei* 01, a concentration of 1 % (w/v) hydrolysate is enough to effectively replace the standard protein level of 2.5 % (w/v) peptone and yeast extract ($p > 0.05$), providing a viable alternative with a lower hydrolysate amount. Regarding *L. rhamnosus* LGG (Figs. 4C and 4D), FH supports the growth of this lactic acid bacterium, as evidenced by the growth curves (Fig. 4C). However, FH at 2.5 % (w/v) was insufficient to fully replace the standard protein amount without affecting probiotic growth, leading to a reduction in μ_{max} values ($p < 0.05$) (Fig. 4D). Vázquez *et al.* [65] also found that the growth with alternative media supplemented with fish protein hydrolysate was similar to or higher than the observed in commercial MRS. Concerning PH (Fig. 5A and 5B) and *L. casei* 01, 1 % (w/v) hydrolysate proved to be enough to successfully replace the usual amount of protein, with similar growth curves and no significant differences between μ_{max} values ($p > 0.05$). Lastly (Fig. 5C and 5D), for *L. rhamnosus* LGG, a higher protein concentration would be necessary to maintain optimal probiotic growth. Furthermore, hydrolysate concentrations of 2.5 and 1 % (w/v) produced similar μ_{max} values ($p > 0.05$). However, at 0.5 % (w/v), PH compromised *L. rhamnosus* LGG growth, decreasing both final OD_{600} and μ_{max} values ($p < 0.05$). Similar findings have been observed with poultry protein hydrolysates, indicating that they are a useful alternative to expensive supplements for *Lactobacillus* growth [11, 66]. It is important to note that in all cases, at least one of the tested hydrolysate concentrations allowed the probiotic strains to grow significantly better (μ_{max}) when compared to MRS medium lacking protein.

These results demonstrated the feasibility of using bromelain-generated protein hydrolysates from fish and poultry by-products to support the growth of probiotic strains, underscoring their potential as sustainable and cost-effective alternatives to conventional nitrogen sources. Both FH and PH proved effective in replacing standard components in culture media, making them promising candidates for use in microbiological applications and in the development of functional fermented foods.

4. Conclusions and future perspectives

The efficient utilization of animal by-products through enzymatic hydrolysis presents a promising avenue to address economic and environmental challenges posed by industrial waste. By transforming low-value by-products into high-value protein hydrolysates with bioactive properties, this strategy aligns with global efforts to reduce food loss and waste, as emphasized in the United Nations' Sustainable Development

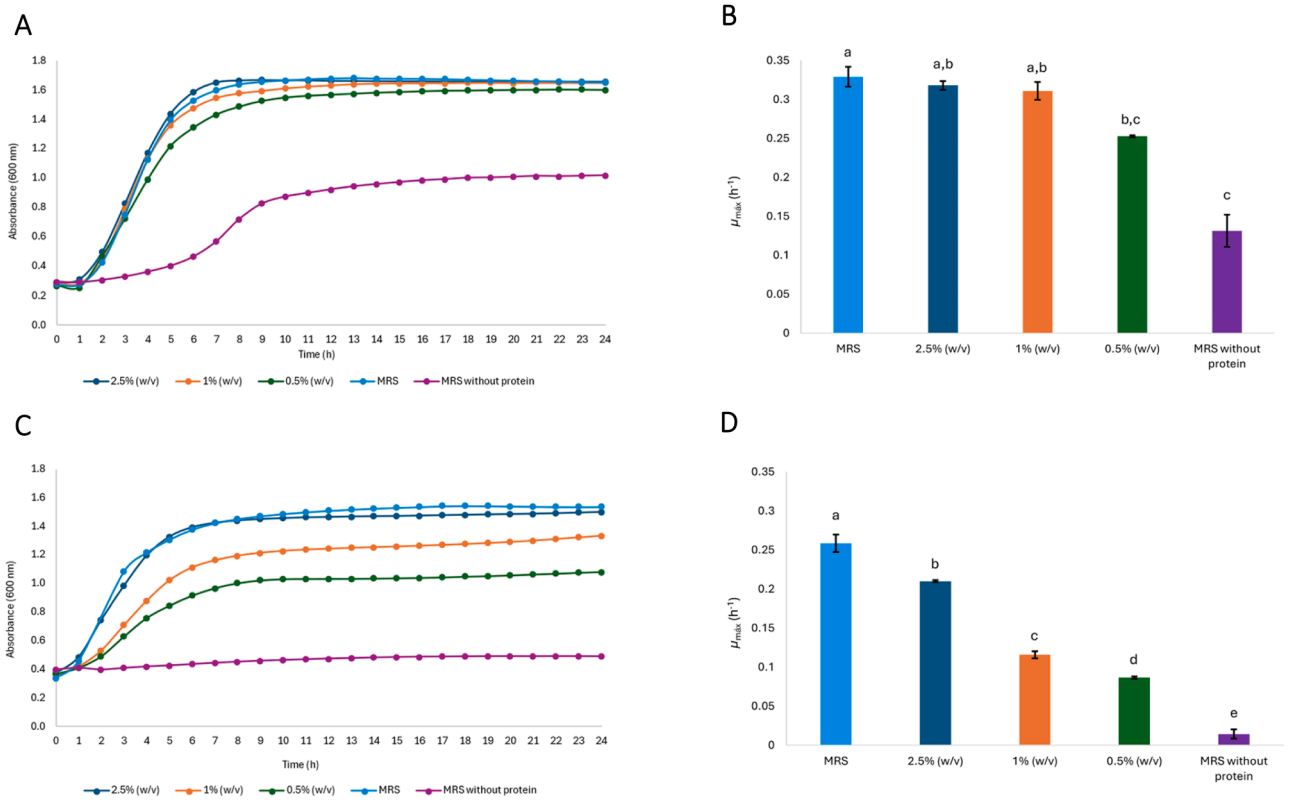

Fig. 4. (A,C) Growth analysis in FH of *L. casei* 01 and *L. rhamnosus* LGG during 24 h, respectively. (B,D) Maximum specific growth rates of *L. casei* 01 and *L. rhamnosus* LGG, respectively, in FH. ^{a,b,c,d,e} Different lowercase letters represent significant difference ($p < 0.05$).

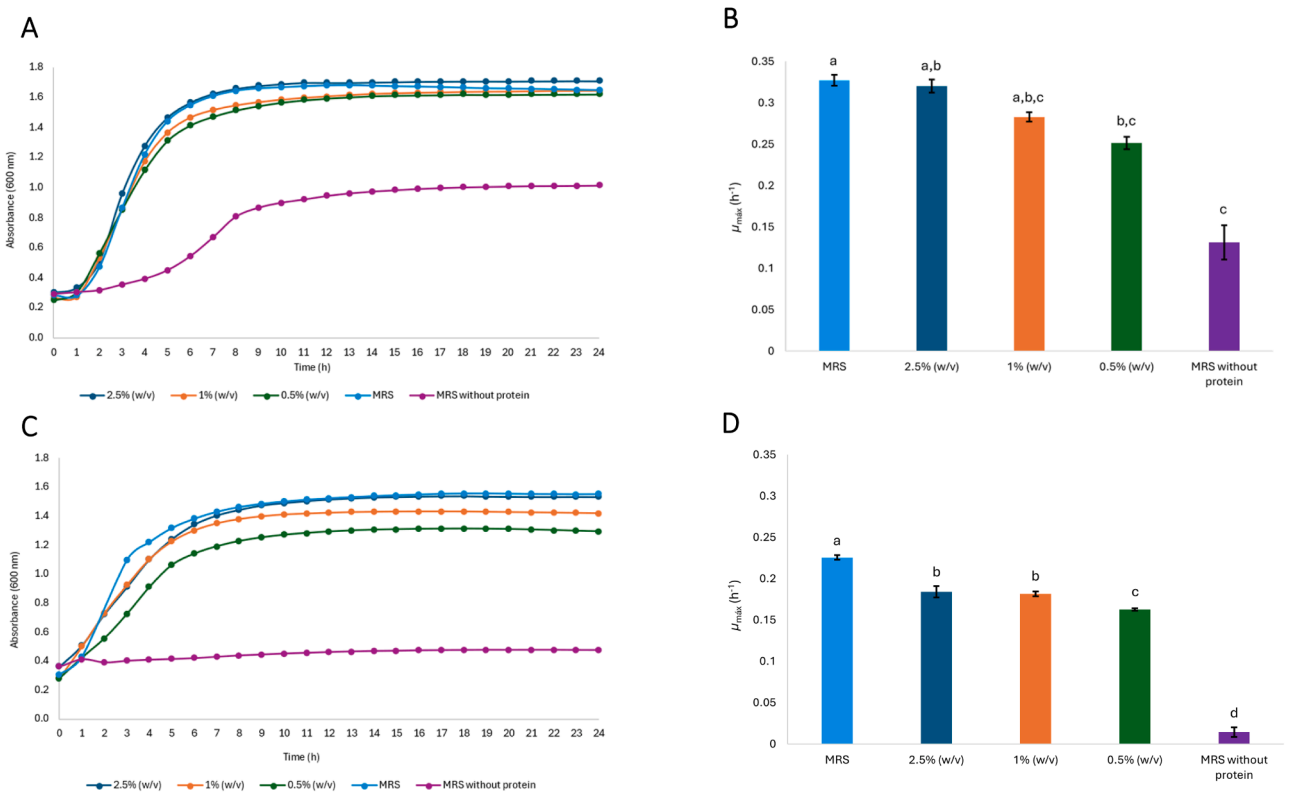

Fig. 5. (A,C) Growth analysis in PH of *L. casei* 01 and *L. rhamnosus* LGG during 24 h, respectively. (B,D) Maximum specific growth rates of *L. casei* 01 and *L. rhamnosus* LGG, respectively, in PH. ^{a,b,c,d} Different lowercase letters represent significant difference ($p < 0.05$).

Goals. In this study, factorial designs were utilized to identify the optimal combination of experimental factors for producing the most efficient hydrolysate from fish and poultry by-products. The application of bromelain demonstrated significant potential in hydrolyzing fish and poultry by-products, yielding biopeptides with antioxidant, antihypertensive, and probiotic-promoting activities. It is worth mentioning that the ability of the obtained hydrolysates to support the growth of probiotic strains underscores their potential application as alternative nitrogen sources in microbiological culture media, offering also an option for fermentation-based processes. These results support the research objective of developing functional and sustainable protein ingredients from underutilized animal by-products. Thus, bromelain demonstrated high efficiency in producing bioactive hydrolysates, showcasing its innovative potential for converting animal by-products into valuable ingredients. However, it should be noted that intrinsic batch-to-batch variability in the biochemical composition of animal by-products may affect the reproducibility of hydrolysis outcomes. This variability represents a relevant consideration for industrial applications.

Additionally, although the study demonstrated significant bioactivities, future research should focus on the identification and characterization of amino acid profiling and specific peptide sequences to clarify the structure-function relationships and further validate the functional potential of the hydrolysates. Applications of these hydrolysates in functional food formulations, including fermented products, should also be explored to assess their performance in real matrices.

This innovative method not only enhances the sustainability of protein production but also supports the development of eco-friendly and health-promoting ingredients, paving the way for a more sustainable and nutritious food system.

Data availability

Data will be available on request.

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CRediT authorship contribution statement

Sandra Borges: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Tânia C.F. Ribas:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Maria Leonor Castro:** Writing – original draft, Investigation, Formal analysis. **Débora Campos:** Writing – review & editing, Visualization, Validation, Project administration. **Maria João Mota:** Writing – review & editing, Visualization, Validation, Project administration. **André Almeida:** Writing – review & editing, Visualization, Validation, Project administration, Funding acquisition. **Manuela Pintado:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition.

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.

References

[1] P. Borrajo, M. Pateiro, M. Gagaoua, D. Franco, W. Zhang, J.M. Lorenzo, Evaluation of the antioxidant and antimicrobial activities of porcine liver protein hydrolysates obtained using alcalase, Bromelain Papain, Appl. Sci. 10 (7) (2020) 2290. Mar 27.

[2] S. Borges, J. Odila, G. Voss, R. Martins, A. Rosa, J.A. Couto, et al., Fish by-products: a source of enzymes to generate circular bioactive hydrolysates, Molecules 28 (3)

(2023) 1155. Jan 24Available from: https://www.mdpi.com/1420-3049/28/3/1155.
[3] M.G. Romero-Garay, E. Montalvo-González, C. Hernández-González, A. Soto-Domínguez, E.M. Becerra-Verdín, Lourdes De, M. García-Magaña, Bioactivity of peptides obtained from poultry by-products: a review, Food Chem. X. 13 (2022) 100181. Mar.
[4] N. Kaushik, E. Falch, R. Slizyte, A. Kumari, Hjellnes V Khushboo, et al., Valorization of fish processing by-products for protein hydrolysate recovery: opportunities, challenges and regulatory issues, Food Chem. 459 (2024) 140244. Nov.
[5] B. Alao, A. Falowo, A. Chulayo, V. Muchenje, The potential of animal by-products in food systems: production, prospects and challenges, Sustainability 9 (7) (2017 Jun 22) 1089.
[6] L. Mora, F. Toldrá, Advanced enzymatic hydrolysis of food proteins for the production of bioactive peptides, Curr. Opin. Food Sci. 49 (2023) 100973. Feb.
[7] B. Ozturk-Kerimoglu, A. Heres, L. Mora, F. Toldrá, Antioxidant peptides generated from chicken feet protein hydrolysates, J. Sci. Food Agric. 103 (14) (2023 Nov 7) 7207–7217.
[8] W. Wongngam, T. Mitani, S. Katayama, S. Nakamura, J. Yongsawatdigul, Production and characterization of chicken blood hydrolysate with antihypertensive properties, Poult. Sci. 99 (10) (2020) 5163–5174. Oct.
[9] Y. Zhu, K. Wang, X. Jia, C. Fu, H. Yu, Y. Wang, Antioxidant peptides, the guardian of life from oxidative stress, Med. Res. Rev. 44 (1) (2024) 275–364. Jan 25.
[10] P.P. Gao, H.Q. Liu, Z.W. Ye, Q.W. Zheng, Y. Zou, T. Wei, et al., The beneficial potential of protein hydrolysates as prebiotic for probiotics and its biological activity: a review, Crit. Rev. Food Sci. Nutr. (2023) 1–13. Oct 9.
[11] C. Lazzi, F. Meli, F. Lambertini, C. Bottesini, I. Nikolaev, M. Gatti, et al., Growth promotion of bifidobacterium and Lactobacillus species by proteinaceous hydrolysates derived from poultry processing leftovers, Int. J. Food Sci. Technol. 48 (2) (2013) 341–349. Feb 24.
[12] G.J. Fadimu, T.T. Le, H. Gill, A. Farahnaky, O.O. Olatunde, T. Truong, Enhancing the biological activities of food protein-derived peptides using non-thermal technologies: a review, Foods. 11 (13) (2022) 1823. Jun 21.
[13] M. Venetikidou, E. Lykartsi, T. Adamantidi, V. Prokopiou, A. Ofrydopoulou, S. Letsiou, et al., Proteolytic enzyme activities of bromelain, ficin, and papain from fruit by-products and potential applications in sustainable and functional cosmetics for skincare, Appl. Sci. 15 (5) (2025) 2637. Feb 28.
[14] K.Y. Ee, L.Y. Khoo, W.J. Ng, F.C. Wong, T.T. Chai, Effects of bromelain and trypsin hydrolysis on the phytochemical content, antioxidant activity, and antibacterial activity of roasted butterfly pea seeds, Processes 7 (8) (2019) 534. Aug 14.
[15] X. Xu, Y. Qiao, B. Shi, Dia VP, Alcalase and bromelain hydrolysis affected physicochemical and functional properties and biological activities of legume proteins, Food Struct. 27 (2021) 100178. Jan.
[16] I.C. Pereira, E.E. Sátiro Vieira, L.R. de Oliveira Torres, F.C. Carneiro da Silva, J. M. de Castro e Sousa, F.L Torres–Leal, Bromelain supplementation and inflammatory markers: a systematic review of clinical trials, Clin. Nutr. ESPEN. 55 (2023) 116–127. Jun.
[17] D.A. Campos, E.R. Coscueta, N.W. Valetti, L.M. Pastrana-Castro, J.A. Teixeira, G. A. Picó, et al., Optimization of bromelain isolation from pineapple byproducts by polysaccharide complex formation, Food Hydrocoll. 87 (2019) 792–804. Feb.
[18] M. López-Pedrouso, P. Borrajo, M. Pateiro, J.M. Lorenzo, D. Franco, Antioxidant activity and peptidomic analysis of porcine liver hydrolysates using alcalase, bromelain, flavourzyme and papain enzymes, Food Res. Int. 137 (2020) 109389. Nov.
[19] E.R. Coscueta, N. Cunha Fernandes, M.E. Brassesco, A. Rosa, A. Almeida, M. M Pintado, Turning discarded blue shark (Prionace glauca) skin into a valuable nutraceutical resource: an enzymatic collagen hydrolysate, Food Biosci. 60 (2024) 104472. Aug.
[20] E.R. Coscueta, M.E. Brassesco, M. Pintado, Collagen-based bioactive bromelain hydrolysate from salt-cured cod skin, Appl. Sci. 11 (18) (2021) 8538. Sep 14.
[21] AOAC, Official methods of analysis. Association of Official Analytical Chemists, 16th ed., Washington, DC, USA, 1995.
[22] Y. Zhang, M. Huang, X. Shao, F. Zhang, Z. Li, Y. Bai, et al., Insights into intramuscular connective tissue associated with wooden breast myopathy in fast-growing broiler chickens, Foods. 12 (12) (2023) 2375. Jun 15.
[23] T. Rocha Camargo, P. Ramos, J.M. Monserrat, C. Prentice, C.J.C. Fernandes, W. F. Zambuzzi, et al., Biological activities of the protein hydrolysate obtained from two fishes common in the fisheries bycatch, Food Chem. 342 (2021) 128361. Apr.
[24] B. Bourguignon, D.L. Massart, Simultaneous optimization of several chromatographic performance goals using Derringer's desirability function, J. Chromatogr. A 586 (1) (1991) 11–20. Nov.
[25] K.C. Hsu, Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product, Food Chem. 122 (1) (2010) 42–48. Sep.
[26] M.L. Castro, J. Azevedo-Silva, D. Valente, A. Machado, T. Ribeiro, J.P. Ferreira, et al., Elevating Skincare science: grape seed extract encapsulation for dermatological care, Molecules 29 (16) (2024) 3717. Aug 6.
[27] E.D.F. Vieira, D. Styles, S. Sousa, C. Santos, A.M. Gil, A.M. Gomes, et al., Nutritional, rheological, sensory characteristics and environmental impact of a yogurt-like dairy drink for children enriched with lupin flour, Int. J. Gastron. Food Sci. 30 (2022) 100617. Dec.
[28] E. Juarez-Enriquez, G.I. Olivas, P.B. Zamudio-Flores, E. Ortega-Rivas, S. Perez-Vega, D.R. Sepulveda, Effect of water content on the flowability of hygroscopic powders, J. Food Eng. 205 (2017) 12–17. Jul.
[29] J.C. Callahan, G.W. Cleary, M. Elefant, G. Kaplan, T. Kensler, R.A. Nash, Equilibrium moisture content of pharmaceutical excipients, Drug Dev. Ind. Pharm. 8 (3) (1982) 355–369. Jan 20.
[30] M. Fernandez Cunha, E.R. Coscueta, M.E. Brassesco, R. Marques, J. Neto, F. Almada, et al., Exploring bioactivities and peptide content of body mucus from the lusitanian toadfish Halobatrachus didactylus, Molecules 28 (18) (2023) 6458. Sep 6.
[31] A. Dávalos, C. Gómez-Cordovés, B. Bartolomé, Extending applicability of the oxygen radical absorbance capacity (ORAC−Fluorescein) assay, J. Agric. Food Chem. 52 (1) (2004) 48–54. Jan 14.
[32] F. Meli, C. Lazzi, E. Neviani, M. Gatti, Effect of protein hydrolizates on growth kinetics and aminopeptidase activities of some bifidobacterium species, Anaerobe 22 (2013) 130–133. Aug.
[33] C. Fuentes, S. Verdú, R. Grau, J.M. Barat, A. Fuentes, Impact of raw material and enzyme type on the physico-chemical and functional properties of fish by-products hydrolysates, LWT 201 (2024) 116247. Jun.
[34] P.N. Seong, S.H. Cho, K.M. Park, G.H. Kang, B.Y. Park, S.S. Moon, et al., Characterization of chicken by-products by mean of proximate and nutritional compositions, Korean J. Food Sci. Anim. Resour. 35 (2) (2015) 179–188. Apr 30.
[35] M. Nikoo, J.M. Regenstein, M. Yasemi, Protein hydrolysates from fishery processing by-products: production, characteristics, food applications, and challenges, Foods. 12 (24) (2023) 4470. Dec 13.
[36] P.G. Chiarelli, B. Martinez, T. Nakamura, K. Mis Solval, Enhancing bromelain recovery from pineapple by-products: a sustainable approach for value addition and waste reduction, Foods. 13 (4) (2024) 589. Feb 15.
[37] D. Lindberg, K.A. Kristoffersen, S.G. Wubshet, L.M.G. Hunnes, M. Dalsnes, K. R. Dankel, et al., Exploring effects of protease choice and protease combinations in enzymatic protein hydrolysis of poultry by-products, Molecules 26 (17) (2021) 5280. Aug 31.
[38] H.G. Kristinsson, B.A. Rasco, Fish protein hydrolysates: production, biochemical, and functional properties, Crit. Rev. Food Sci. Nutr. 40 (1) (2000) 43–81. Jan.
[39] M.C. Gómez-Guillén, B. Giménez, M.E. López-Caballero, M.P. Montero, Functional and bioactive properties of collagen and gelatin from alternative sources: a review, Food Hydrocoll. 25 (8) (2011) 1813–1827. Dec.
[40] A.T. Himonides, A.K.D. Taylor, A.J. Morris, A study of the enzymatic hydrolysis of fish frames using model systems, Food Nutr. Sci. 02 (06) (2011) 575–585.
[41] N. Kamaruzaman, M. Fauzi, S. Yusop, Characterization and toxicity evaluation of broiler skin elastin for potential functional biomaterial in tissue engineering, Polym. (Basel) 14 (5) (2022) 963. Feb 28.
[42] D.S. Akhila, P. Ashwath, K.G. Manjunatha, S.D. Akshay, V.K. Reddy Surasani, F. R. Sofi, et al., Seafood processing waste as a source of functional components: extraction and applications for various food and non-food systems, Trends. Food Sci. Technol. 145 (2024) 104348. Mar.
[43] A. Henriques, J.A. Vázquez, J. Valcarcel, R. Mendes, N.M. Bandarra, C. Pires, Characterization of protein hydrolysates from fish discards and by-products from the North-West Spain fishing fleet as potential sources of bioactive peptides, Mar. Drugs 19 (6) (2021) 338. Jun 13.
[44] D. Lindberg, K.A. Kristoffersen, H. de Vogel-van den Bosch, S.G. Wubshet, U. Böcker, A. Rieder, et al., Effects of poultry raw material variation and choice of protease on protein hydrolysate quality, Process. Biochem. 110 (2021) 85–93. Nov.
[45] B. de la Fuente, T. Aspevik, F.J. Barba, K. Kousoulaki, H. Berrada, Mineral bioaccessibility and antioxidant capacity of protein hydrolysates from salmon (Salmo salar) and mackerel (Scomber scombrus) backbones and heads, Mar. Drugs 21 (5) (2023) 294. May 11.
[46] Gopalan J., Mathew S., Dana E.B., Jinoy V.G., Saleena M. Nutritional evaluation of fish protein hydrolysate from Tuna, Thunnus albacares (Bonnaterre, 1788) [Internet]. Vol. 7, Print) International Journal of Life Sciences Research. Available from: https://www.researchgate.net/publication/338140424.
[47] F. Tayi, F.N.N.N. Metomo, Y. Essamlali, A. Akil, O. Amadine, S. Aboulhrouz, et al., Preparation and characterization of fish-derived protein hydrolysate and assessment of its effect on tomato and sorghum plants growth and productivity, Sustain. Chem. Pharm. 43 (2025) 101877. Feb.
[48] M.E.M. Pinto e Silva, I. Paton, M. Trigo, M.C.B.C. von Atzingen, C.S. Kira, E. I. Inomata, et al., Mineral and vitamin content of beef, chicken, and turkey hydrolysates mineral and vitamin content of protein hydrolysates, Quim. Nova 31 (1) (2008) 41–43.
[49] S.A. Hogan, D.J O'Callaghan, Moisture sorption and stickiness behaviour of hydrolysed whey protein/lactose powders, Dairy. Sci. Technol. 93 (4–5) (2013) 505–521. Jul 3.
[50] X. Peng, C. Liu, B. Wang, L. Kong, R. Wen, H. Zhang, et al., Hygroscopic properties of whey protein hydrolysates and their effects on water retention in pork patties during repeated freeze–thaw cycles, LWT 184 (2023) 114984. Jul.
[51] H. Zhang, W. Gu, Y.J. Li, M. Tang, Hygroscopic properties of sodium and potassium salts as related to saline mineral dusts and sea salt aerosols, J. Environ. Sci. 95 (2020) 65–72. Sep.
[52] A.A. Zaky, J. Simal-Gandara, J.B. Eun, J.H. Shim, Abd El-Aty AM. Bioactivities, applications, safety, and health benefits of bioactive peptides from food and by-products: a review, Front. Nutr. 8 (2022). Jan 20.
[53] I.U. Okagu, T.P.C. Ezeorba, E.C. Aham, R.N. Aguchem, R.N. Nechi, Recent findings on the cellular and molecular mechanisms of action of novel food-derived antihypertensive peptides, Food Chem. 4 (2022) 100078. Jul.
[54] S.M. Querobino, M.S. Costa, C. Alberto-Silva, Protective effects of distinct proline-rich oligopeptides from B. jararaca snake venom against oxidative stress-induced neurotoxicity, Toxicon. 167 (2019) 29–37. Sep.

[55] K. Korczek, J. Tkaczewska, W. Migdał, Antioxidant and antihypertensive protein hydrolysates in fish products - a review, Czech J. Food Sci. 36 (3) (2018) 195–207. Jun 30.
[56] X. Liu, Q. Hu, Y. Shen, Y. Wu, L. Gao, X. Xu, et al., Research progress on antioxidant peptides from fish by-products: purification, identification, and structure–Activity relationship, Metabolites 14 (10) (2024) 561. Oct 20.
[57] J. Fan, J. He, Y. Zhuang, L. Sun, Purification and identification of antioxidant peptides from enzymatic hydrolysates of Tilapia (Oreochromis niloticus) frame protein, Molecules 17 (11) (2012) 12836–12850. Nov 1.
[58] K. Suetsuna, H. Ukeda, H. Ochi, Isolation and characterization of free radical scavenging activities peptides derived from casein, J. Nutr. Biochem. 11 (3) (2000) 128–131. Mar.
[59] P.J. García-Moreno, I. Batista, C. Pires, N.M. Bandarra, F.J. Espejo-Carpio, A. Guadix, et al., Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species, Food Res. Int. 65 (2014) 469–476. Nov.
[60] C.B. Ahn, J.Y. Je, Y.S. Cho, Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis, Food Res. Int. 49 (1) (2012) 92–98. Nov.
[61] Y.H. Chen, Y.H. Liu, Y.H. Yang, H.H. Feng, C.T. Chang, C.C. Chen, Antihypertensive effect of an enzymatic hydrolysate of chicken essence residues, Food Sci. Technol. Res. 8 (2) (2002) 144–147.
[62] U.G. Y, I. Bhat, I. Karunasagar, B.S. M, Antihypertensive activity of fish protein hydrolysates and its peptides, Crit. Rev. Food Sci. Nutr. 59 (15) (2019) 2363–2374. Aug 22.
[63] X. Liu, M. Zhang, C. Zhang, C. Liu, Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from Rhopilema esculentum, Food Chem. 134 (4) (2012) 2134–2140. Oct.
[64] R. Balti, A. Bougatef, A. Sila, D. Guillochon, P. Dhulster, N. Nedjar-Arroume, Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (Sepia officinalis) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats, Food Chem. 170 (2015) 519–525. Mar.
[65] J.A. Vázquez, A. Meduíña, A.I. Durán, M. Nogueira, A. Fernández-Compás, R. I. Pérez-Martín, et al., Production of valuable compounds and bioactive metabolites from by-products of fish discards using chemical processing, enzymatic hydrolysis, and bacterial fermentation, Mar. Drugs 17 (3) (2019) 139. Feb 27.
[66] F. Meli, C. Lazzi, E. Neviani, M. Gatti, Effect of protein hydrolysates on growth kinetics and aminopeptidase activities of Lactobacillus, Curr. Microbiol. 68 (1) (2014) 82–87. Jan 29.