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ANTIOXIDANT POTENTIAL OF PROTEIN HYDROLYSATES FROM POULTRY BY-PRODUCTS OBTAINED BY MICROBIAL FERMENTATION

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Abstract

Protein hydrolysates and bioactive peptides are promising components with antioxidant properties. This study aimed to evaluate the antioxidant activity of protein hydrolysates obtained from microbial fermentation of broiler chicken gizzards using a concentration of bifidobacteria and propionic acid bacteria incorporated into whey. The total antioxidant capacity was determined by the FRAP method, the antiradical activity was determined using the DPPH assay with the detection of the IC_{50} index to assess the antioxidant potential. The results showed that the FRAP antioxidant activity of the experimental hydrolysate sample obtained by fermentation using bifidobacteria was 30 % lower than that of other samples. However, this sample exhibited the greatest free radical scavenging effect, with an IC_{50} of 1.363 mg/g. The content of free amino acids and peptides was also determined by UHPLC combined with mass spectrometry. The properties of peptides were identified by the in silico method using the BioPep and PeptideRanker databases. The research results showed an increase in the content of free amino acids in hydrolysates during microbial fermentation. The content of a bioactive peptide with antioxidant properties — VW, as well as several peptides with potentially high antioxidant properties, was revealed. The results obtained show the prospects for obtaining protein hydrolysates from poultry by-products by their microbial fermentation, as well as the need for further deeper studies of peptides with potential antioxidant properties.

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Introduction

A major challenge facing the food industry is the need to extend the shelf life of foods susceptible to oxidative spoilage. Oxidative spoilage is a complex process that degrades the sensory characteristics of products (including taste, aroma, and texture) and reduces nutritional value, which can negatively impact consumer health. Furthermore, toxic metabolites can accumulate during this process, which also poses potential health risks. On an industrial scale, it is often simpler and more cost-effective to use synthetic preservatives and antioxidants, as these substances effectively slow oxidation processes and extend the shelf life of products. However, in light of increasing consumer demands for food quality and safety, manufacturers are increasingly forced to rethink their approaches and shift their focus to natural food additives [1,2]. Natural antioxidants, in particular, play a key role in slowing the rate of oxidative reactions, including lipid oxidation, which in turn leads to a reduction in hydrogen peroxide and free radical levels in foods [3–5]. In recent years, active peptides, which have high potential as natural antioxidants, have attracted particular attention from researchers [6,7].

These biologically active components not only help extend the shelf life and enhance the stability of food products, but also possess a number of additional beneficial properties. This makes them particularly attractive for use in diets and in industrial food processing settings [8,9].

However, obtaining pure active peptides requires complex technological approaches to extraction and purification, which significantly increases their final cost. At the same time, numerous studies confirm the antioxidant properties of protein hydrolysates containing antioxidant peptides obtained from various types of protein raw materials. Other advantages of protein hydrolysates compared to purified peptides have been noted, such as the formation of oligopeptides as a result of absorption [10,11]. In recent years, research on protein hydrolysates containing bioactive peptides as functional and technological additives for food production has attracted the attention of food scientists all over the world [12,13]. Many studies corroborate the potential of using protein hydrolysates containing antioxidant peptides as food preservatives. The effectiveness of protein hydrolysates in reducing fat oxidation in food systems has been experimentally proven. Studies of various

food products containing carp hydrolysates have shown reductions in free fatty acids, peroxide value, and malondialdehyde levels, which generally contributes to improved shelf life of high-fat food systems [14,15].

Peptides have a lower molecular weight than proteins, and therefore are easily digestible and more bioavailable, and exhibit higher biological activity. Protein cleavage is carried out due to hydrolysis caused by the action of catalysts (acids, alkalis or enzymes). As a result, the cleavage products of the protein molecule are sequentially formed — first polypeptides, peptides, and then amino acids [16].

Enzymatic protein hydrolysis is the most widely used process to produce bioactive peptides, which are important components in a variety of fields, from medicine to the food industry [16–18].

This method offers a gentler and more environmentally friendly approach compared to traditional chemical processing of protein raw materials. Unlike chemical methods, which often involve the use of toxic reagents and can have a negative impact on the environment, enzymatic hydrolysis utilizes natural enzymes, minimizing the risk of harming the ecosystem.

However, modern research points to the significant advantages and promising potential of microbial fermentation, particularly using bacteria with a well-developed proteolytic system [19–21].

Certain microbial strains not only can effectively break down proteins into peptides but also have an additional impact on the quality of the final product. Microbial fermentation can facilitate the formation of new bioactive peptides with unique functional properties and also influence the amino acid composition, which can improve the nutritional properties of the resulting product [22,23]. Microbial fermentation enables the precise engineering of peptides with targeted functions, including antimicrobial and antioxidant activity, by harnessing bacterial metabolism. To fully realize this potential, further research is needed to refine the fermentation conditions.

Numerous studies highlight the significant potential of meat raw materials for producing protein hydrolysates and bioactive peptides, which can serve as functional components in diets, contributing to improved health [24,25].

With the active participation of microbial endo- and exopeptidases, protein proteolysis occurs, leading to the release and accumulation of bioactive peptides in the substrate. Under specific conditions, microbial fermentation produces significant quantities of short peptides and free amino acids, which have multiple beneficial effects on the body [26].

In this case, the activity of peptides as a whole depends on the sequence of amino acid residues, electronic properties and the degree of hydrophobicity. Scientific publications have noted a wider range of biological activity of protein hydrolysates obtained from several food proteins [27], as well as during the enzymatic processing of by-products [28].

Transforming animal and poultry by-products into protein hydrolysates offers a dual benefit: unlocking a high-value protein source and reducing agricultural waste. These products, often perceived as waste, actually contain significant amounts of amino acids and bioactive molecules, making them an important component in the development of new processing technologies and applications in the food and feed industries. Research shows that hydrolysates obtained from liver and heart exhibit not only high antioxidant activity but also pronounced antimicrobial activity [11]. This opens up new opportunities for their use as functional ingredients in various products, helping to extend shelf life and improve consumer safety. Activation of these hydrolysates can significantly improve the organoleptic and nutritional characteristics of final products, making them an important element in sustainable development strategies for the agricultural industry and processing [14]. Therefore, the development of technologies aimed at the rational and efficient use of by-products obtained from farm animals and poultry is an important scientific task with practical significance.

This process takes into account the interests of both producers seeking to optimize their resources and reduce losses, and consumers interested in high-quality and healthy products [11,29].

Protein hydrolysates containing bioactive peptides exhibit a diverse range of functional and physiological effects that impact both the human body and food systems. In terms of their effects on the human body, these hydrolysates exhibit a significant variety of properties, including immunomodulatory, anticancer, antihypertensive, antioxidant, anti-inflammatory, mineral-binding, opioid, antilipid, anti-aging, and osteoprotective effects [16–18,24,26,28]. Each of these effects is based on specific mechanisms of interaction between bioactive peptides and cellular receptors, enzymes, and other molecules in the body, allowing hydrolysates to be used as functional supplements for the prevention and treatment of various diseases.

The activity of protein hydrolysates in food systems is also worth noting. They exhibit antioxidant and antimicrobial properties, making them useful for improving food preservation and extending their shelf life.

Their antioxidant activity helps neutralize free radicals, which in turn prevents oxidative processes in food materials, preventing their spoilage. Antimicrobial properties help combat pathogenic microflora, ensuring the safety and quality of food products. By virtue of their bioactive peptides, protein hydrolysates offer dual utility: they provide documented health benefits while enhancing functional properties, paving the way for their adoption in nutraceutical, pharmaceutical, and next-generation food products [8,13,30,31].

The main goal of this study is to identify the antioxidant properties of protein hydrolysates obtained by microbial fermentation of broiler chicken stomachs with the addition of bifidobacteria and propionic acid bacteria concentrates in whey.

Objects and methods

Objects

This study characterized protein hydrolysates (PH) derived from gizzards of ROSS-308 broiler chickens (41 days old) via microbial fermentation. Hydrolysis was conducted in a soft cheese whey medium under three conditions: with a concentrate of *Bifidobacterium longum* B379M (PH-B), with a concentrate of *Propionibacterium freudenreichii shermanii* KM 186 (PH-P), and a control without bacterial addition (PH-C). The bacterial concentrates (Propionix, Moscow, Russia) had concentrations of viable bacterial cells of 10^{11}–10^{12} CFU/cm³ (bifidobacteria) and 10^{10}–10^{11} CFU/cm³ (propionibacteria). Optimized fermentation parameters presented in a previous study were used to obtain hydrolysates [32]. The hydrolysate production technology is shown in Figure 1.

Determination of antioxidant activity of protein hydrolysate

The Ferric-reducing antioxidant power (FRAP) assay of hydrolysate samples was determined in ethanol extracts. In order to prepare sample extracts, a sample was mixed with 96 % ethanol in a ratio of 1:15 (g:ml), homogenized using an automatic homogenizer S10 (Stegler, China) for 2 min at 8000 rpm, infused for 60 min at 22 ± 2 °C and filtered through a paper pleated filter.

The total antioxidant capacity of alcohol extracts was determined by the FRAP method on an SF-2000 spectrophotometer (OKB Spektr, Russia) in accordance with the procedure [33]. In order to prepare the FRAP reagent, 0.3 M acetate buffer (pH 3.6), was mixed with 10 mM solution of the photometric reagent TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (Acros Organics, China), by dissolving it in 40 mM hydrochloric acid and 20 mM aqueous iron (III) chloride (PanReac AppliChem, Spain) in ratios of 10:1:1, respectively. In order to measure the FRAP assay of the extract, 1.45 ml of freshly prepared FRAP reagent and 50 µl of the sample diluted with distilled water depending on the activity, or distilled water for measuring the control sample, were added to the tube. The reaction mixture was incubated for 30 min at 37 °C in the dark, after which the optical density was recorded at a wavelength of 594 nm. The FRAP assay of the samples was calculated according to the calibration curve ($R2 = 0.9987$), which was constructed using quercetin (Sigma-Aldrich, India) in the concentration range of 140 µM — 300 µM, and expressed in µmol-equiv. quercetin/g sample.

The DPPH radical scavenging activity (RSA) of the protein hydrolysates was determined according to [34]. Briefly, 0.5 g of each sample was subjected to extraction in 20 cm³ of 95 % ethanol for six hours at 20 °C. Subsequently, 1 ml of the extract was combined with 1 cm³ of an ethanolic DPPH solution and left to react in the dark for 30 minutes. The absorbance of the resulting mixture was measured at 517 nm using a Jenway 6404 UV/Vis spectrophotometer (Jenway, UK).

The radical scavenging efficiency (RSA) was calculated as a percentage using the formula:

$$RSA_{DPPH} = \left[1 - \frac{A_i - A_k}{A_0}\right] \cdot 100\%, \quad (1)$$

where: A_k is the value for test sample solution mixed with DPPH solution; A_i is the value for test sample solution mixed with 95 % ethanol; A_0 is the value for *DPPH* solution mixed with 95 % ethanol.

The IC_{50} value was also determined. This indicator characterizes the concentration of the substance that binds 50 % of the formed DPPH radicals. The IC_{50} value was determined from a calibration plot of RSA_{DPPH} values (%) for various concentrations of protein hydrolysates (from 0 to 0.05 mg/ml).

Determination of free amino acids in protein hydrolysates

Free amino acids were determined using liquid chromatography on an Agilent 1260 Infinity LC system (Agilent Technologies, USA). Samples were prepared by liquid extraction of the hydrolysate in a 20 % trichloroacetic acid solution, and the resulting homogenate was then adjusted to pH 2.2 using acidified saline buffer. The mixture was then centrifuged BKC-TL4IV (Biobase, China) (20 minutes at

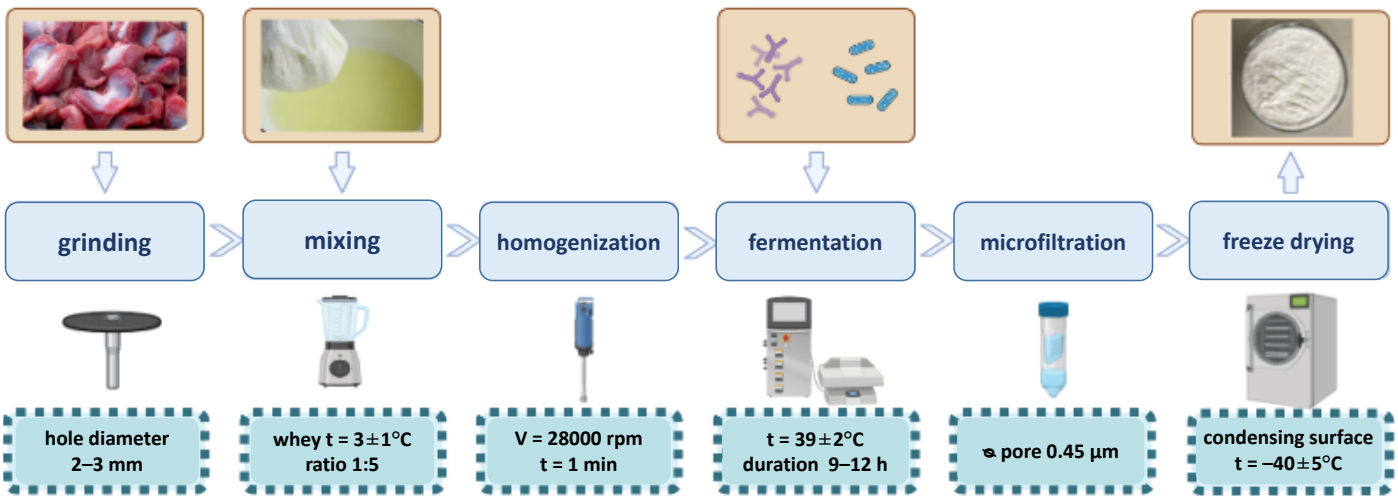

Figure 1. Technology flow chart for protein hydrolysate production (Created with BioRender.com)

4 °C with an RCF of 10,000 × g), and the supernatant formed after centrifugation was filtered into a vial. Chromatographic separation was performed on a C18 PA column (3.5 µm, 4.6×150 mm, ZORBAX) using a mixture of acetonitrile, methanol, and water in a ratio of 45:45:10 as mobile phase A and mobile phase B consisting of Na_2HPO_2 (1.42 g) and $Na_2B_2O_2$ (2.1 g), with pH 8.2. Orthophthalaldehyde for primary amino acids and 9-fluoromethyl chloroformate for secondary amino acids were used as derivatizing agents. Amino acid standards from Sigma Aldrich were used.

The content of free amino acids was expressed as milligrams per 100 g of liquid hydrolysates.

UPLC-ESI-Q-TOF-MS analysis and sequencing of biopeptides

The peptide separation was conducted on an Agilent Technologies 1290 Infinity UHPLC system [35,36], configured with an AdvanceBio Peptide Mapping column (2.1×250 mm, 2.7 µm) and a ZORBAX Extend-C18 guard column. The mobile phases consisted of 0.1% formic acid in H_2O (A) and acetonitrile (B), delivered at a flow rate of 0.2 mL/min with a 10 µL injection volume. A 184-minute linear gradient was employed: 2% B for 5 min, ramping to 43% B over 165 min, then to 100% B over 1 min, holding at 100% B for 6 min, and finally returning to 2% B over 7 min.

The UHPLC system was coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer equipped with a DuoJet Stream ESI source operating in positive ion mode. Key source parameters were: capillary voltage, 4000 V; nozzle voltage, 500 V; drying gas, 13 L/min at 325 °C; and nebulizer pressure, 35 psi. The ion funnel settings were 150 V (high-pressure) and 65 V (low-pressure). Data was acquired in full-scan MS (150–2100 m/z) and data-dependent MS/MS modes. The mass axis was calibrated internally using purine and HP0921. Nitrogen served as the collision gas.

Analytes were identified based on mass fragmentation using MSDIAL software (v.5.1) [36,37], applying mass accuracies of 0.01 Da for MS1 and 0.05 Da for MS2, which yielded over 300 compounds from the aqueous and ethanolic hydrolysate extracts. Individual peptide quantification was performed against a Leytragin® standard curve (1–1000 ng/mL) [38], with results expressed as mg Leytragin equivalents per 100 g of hydrolysate.

Quantification of the major peptides was performed using Leytragin® calibration curves, with a regression coefficient >0.990. The identified peptides were analyzed using the BioPep [39] and PeptideRanker [40] databases.

Statistical analysis

The experimental data were derived from five replicate samples, with each replicate subjected to three analytical measurements. The results are expressed as the mean value of the five replicates plus or minus the standard deviation. To assess statistical significance, the data were subjected to one-way ANOVA and Tukey's honest significant difference (HSD) test, implemented via a publicly accessible web application [41], using a significance threshold of $p \leq 0.05$.

Results and discussion

Antioxidant activity of protein hydrolysate

When assessing the antioxidant activity of protein hydrolysates, the following methods are mainly used: DPPH• radical absorption and iron-reducing antioxidant capacity (FRAP).

Methods for determining the antioxidant activity of hydrolysates (peptides) based on electron transfer include the ability of antioxidants to reduce iron and the activity of scavenging DPPH radicals [42]. FRAP analysis shows the reducing potential of antioxidant compounds through interaction with the ferric complex and tripyridyltriazine. According to Wong et al. [43], the antioxidant activity of peptides is mediated by functional groups — including phenolic hydroxyl, sulfhydryl, and imidazole — which directly scavenge free radicals and chelate prooxidant metal ions, thereby terminating oxidative chain reactions.

The results presented in Table 1 showed that the antioxidant activity of the FRAP hydrolysate sample obtained by fermentation with propionic acid bacteria was not statistically different from the control hydrolysate sample (PH-C). However, the level of antioxidant activity in the hydrolysate sample obtained by fermentation with bifidobacteria was approximately 30% lower than that of the other samples.

The results show (Table 1) that the sample of the hydrolysate fermented with bifidobacteria at a concentration of 1.363 mg/g has the greatest radical scavenging effect.

The calculated IC_{50} value for the DPPH peptide EPEVLR of 2.03 mg/ml in the studies of Lin et al. (2025) [44] was comparable to the values for the commercial antioxidant glutathione, which allowed the authors to conclude that it has significant antioxidant efficacy.

Table 1. Antioxidant properties of protein hydrolysates

Sample	FRAP, µmol-equiv. quercetin/g	DPPH, IC50 mg/g
PH-C	235 ± 7.02a	2.994 ± 0.015b
PH-P	242 ± 5.87a	1.597 ± 0.011b
PH-B	190 ± 4.8a	1.363 ± 0.009b

Values are means ± SEM, $n = 5$ per treatment group. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the TUKEY test

Most antioxidant peptides contain from 4 to 16 amino acids and have a molecular weight of about 400–2000 Da [45]. It was noted that low molecular weight peptides have higher electron-donating properties, therefore the FRAP of peptides decreases with increasing molecular weight. The experimental results confirm that the ultrafiltered sheep placenta peptide fraction < 3 kDa exhibited the highest FRAP activity [46].

In this case, the type of amino acid plays an important role: aromatic amino acids neutralize radicals by donating protons; hydrophobic amino acids can increase the amount

of peptides at the water-lipid interface and scavenge free radicals from the lipid phase; carbonyl and amino groups in the side chain of acidic amino acids act as metal ion chelators [47].

High antioxidant activity was found in quinoa peptides consisting of amino acid residues such as arginine, histidine, aspartate, glycine, and glutamate [48].

Hydrolysate samples with a large number of short-chain peptides have a higher reducing capacity due to better contact of proton-donor groups with the metal.

The results obtained by Liu et al. [45] showed that as the meat matured and the amount of small peptides increases, FRAP activity increases. The authors attribute the reducing capacity of antioxidant peptides to their more accessible structure, where exposed functional groups of amino acid residues can readily react with oxidizing agents [12].

The antioxidant activity of hydrolysates may be due to changes in protein structure that occur as a result of enzymatic hydrolysis. The ability of amino acids to interact with oxidants leads to changes in their structure and, consequently, to changes in the properties of residual groups.

Free amino acids in protein hydrolysates

The bioavailability and nutritional value of the human diet depend on the presence of free amino acids, which are absorbed directly in the small intestine, unlike native proteins. Their breakdown occurs through enzymatic hydrolysis in the digestive tract. This property ensures their rapid participation in protein synthesis and other metabolic functions in the body.

Analysis of free amino acids revealed an increase in their content in both the control and experimental hydrolysate samples after fermentation (Figure 2).

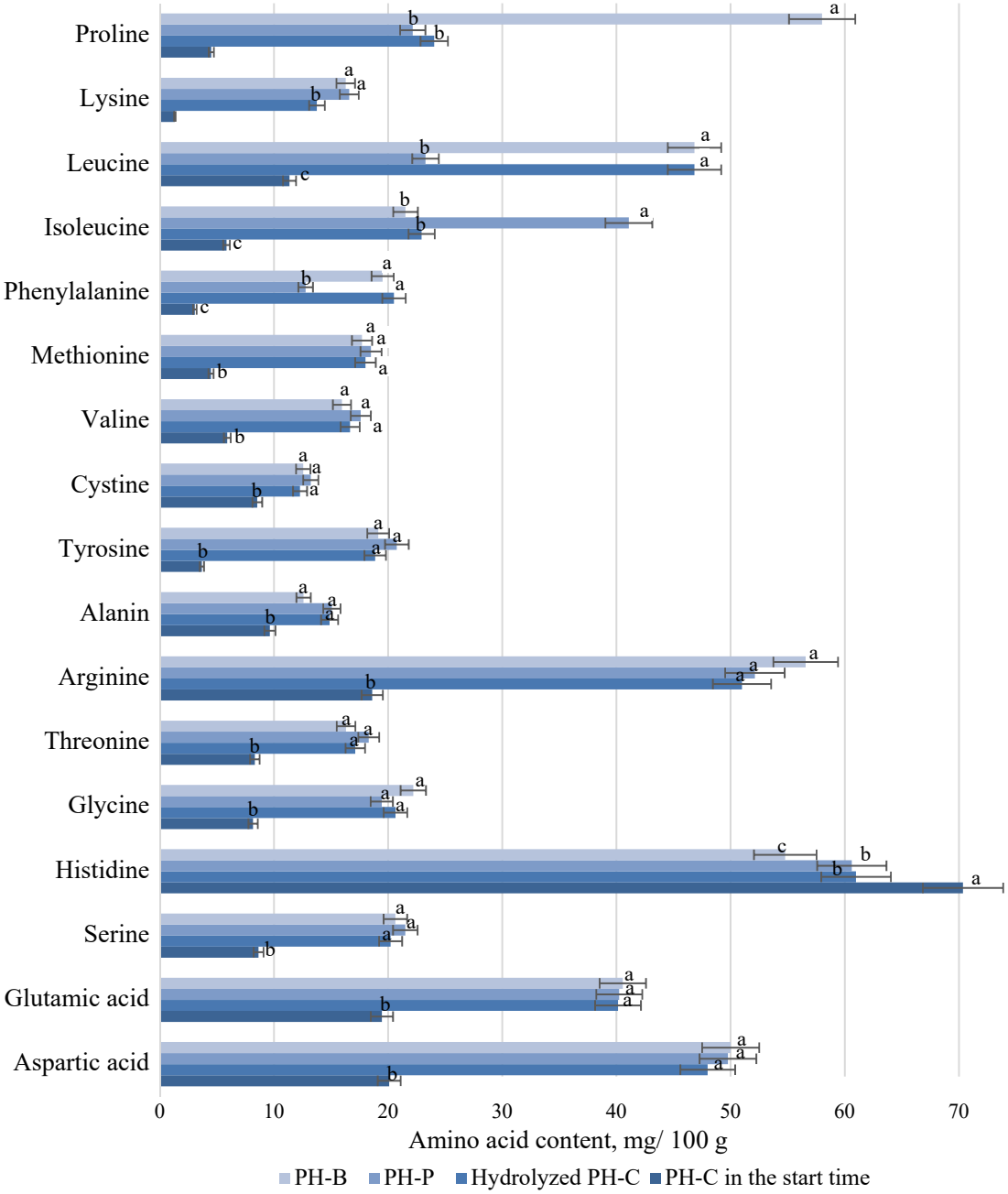

Figure 2. The content of free amino acids in protein hydrolysates. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the TUKEY test ($n = 5$).

It is particularly noteworthy that in the hydrolysates from the gizzard of broiler chickens fermented using propionic acid bacteria and bifidobacteria, a significant increase in the level of essential amino acids was recorded compared to the control sample at the initial stage (from 212.14 mg/100 g to 463.15 mg/100 g and 501.31 mg/100 g, respectively). In the experimental samples, a decrease in the histidine content by 12–23 % relative to the initial values was also observed. Significant differences in the amino acid content were also noted in all experimental samples compared to the control sample before fermentation. The presented results demonstrate that the bacteria used exhibit proteolytic activity against proteins of the muscular and connective tissue of the gizzard. The effective action of exopeptidases on proteins is noteworthy. The significant accumulation of individual free amino acids confirms that probiotic microorganisms produce them during metabolism. Based on the findings in [49], the abundant free amino acids — tyrosine, methionine, and lysine — are likely key contributors to the observed antioxidant activity in the studied hydrolysates.

Fermentation of black soldier fly larvae paste by *L. paracasei* was shown by Zhang et al. [29] to markedly enhance the free amino acid profile, with levels of serine, valine, isoleucine, aspartic acid, glutamic acid, and histidine increasing by over 100 % compared to the control. Furthermore, over 90 % of the resulting small peptides were rich in hydrophobic amino acids, a feature linked to their antioxidant potential [29]. This aligns with the established principle that antioxidant capacity in hydrolysates arises from a synergy of hydrophobic and hydrophilic residues [50].

Specifically, the presence of key hydrophobic amino acids, such as proline, alanine, valine, leucine, and isoleucine, is a critical design consideration for synthetic antioxidant peptides [51].

The antioxidant contribution is further amplified as amino acids, such as tyrosine, methionine, proline, histidine, lysine, and tryptophan, are intrinsically bioactive, exerting antioxidant effects both within peptide sequences and in their free form [52].

According to our research, a significant accumulation of the amino acid proline was observed in the hydrolysate obtained by fermentation of by-products by bifidobacteria. It is also worth noting that the hydrolysate obtained by fermenting with propionic acid bacteria contained approximately twice as much isoleucine after 12 hours of fermentation compared to the control.

Sulfur-containing amino acids, which can act as antioxidants on their own, are particularly noteworthy. Therefore, peptides containing these amino acids in very short chains may possess high antioxidant potential. Cysteine has proton-donating properties, while essential amino acids can chelate metal ions [53].

During the fermentation process, the hydrolysates we obtained released a significant amount of sulfur-containing amino acids, which may also have influenced the enhancement of the antioxidant properties of the hydrolysates.

According to Wang et al. [54], the antioxidant potency of the VKVGNEF and MEAPPHI peptides stems from their abundance of hydrophobic amino acids — such as proline, valine, and methionine. These residues are strategically distributed along the peptide chains and are notably positioned at the terminal ends, a configuration that enhances their activity. The mechanism for effective free radical scavenging is primarily mediated by phenylalanine at the C-terminus of VKVGNEF, which scavenges oxidative chains via hydrogen atom transfer.

Many authors have studied the effect of the amino acid composition of hydrolysates and peptides on the antioxidant activity and the mechanisms of such action (Table 2).

High antioxidant activity was demonstrated in soy hydrolysates against oil oxidation during several frying cycles of chips. The authors attribute the oxidative stability of palm kernel oil after 8 and 12 frying cycles to its low content of hydroperoxides, carbonyl and volatile compounds, unique structural features, and high short-chain peptide

Table 2. Mechanisms of manifestation of antioxidant properties of amino acids

Amino acid	Mechanism of action	Source
histidine	scavenges free radicals through the imidazole ring	[55]
proline	due to the low ionization potential of the pyrrolidine ring, as a proton/hydrogen donor, it can quench singlet oxygen	[56]
cysteine, lysine, histidine, methionine, tryptophan, and tyrosine	effectively scavenge free radicals	[57]
sulfur-containing amino acids (cysteine and methionine)	due to the easy oxidation of sulfhydryl groups by free radicals, they protect normal cells from damage	[58]
cysteine, histidine, aspartic acid, and glutamic acid	promote metal chelation as an important part of free radical inhibition	[56,59]
hydrophobic amino acids	could easily pass through the cell membrane lipid bilayer to destroy a reactive oxygen species in cells	[60–63]
	might increase the affinity and reactivity of peptides to the cell membranes and contribute to the accessibility of peptides to lipid-soluble reactive oxygen species to terminate lipid peroxidation	[64]
aromatic amino acids (tryptophan, tyrosine, histidine, and phenylalanine)	phenolic, indole and imidazole groups act as hydrogen radical donors for electron-deficient free radicals	[64–66]

content. The mechanism for this activity of the hydrolysate during soy fermentation with pepsin was revealed by analyzing the results of SDS-PAGE and tandem mass spectrometry. Furthermore, the scientists note the high content of amino acids with electron-donating capacity — tryptophan, histidine, and methionine [67].

UPLC-ESI-Q-TOF-MS analysis and sequencing of biopeptides

As a result of determining the peptides (Figure 3) using high-performance liquid chromatography combined with mass spectrometry, it was established that their total number in hydrolysates is more than 300.

In silico studies play a crucial role in assessing the activity of protein hydrolysates, providing powerful tools for analyzing and predicting their functional properties at the molecular level.

These approaches, based on computational models and algorithms, allow researchers to process and interpret large volumes of bioinformatics data, making them indispensable in modern scientific research. One of the key aspects of using *in silico* methods is the ability to rapidly screen and identify potential bioactive peptides derived from protein sources. Combining molecular modeling, structural biology, and computational analysis enables effective prediction of the activities of such peptides, including antioxidant, antimicrobial, and anti-inflammatory properties.

This significantly accelerates the discovery and development of new functional supplements based on protein hydrolysates, which is relevant in the face of growing consumer demand for products with improved nutritional properties.

In order to identify bioactive peptides, as well as to predict their biological activity *in silico*, the BioPep database was used, as recommended by many scientists [68,69].

The PeptideRanker system is recommended for assessing the probability of peptide activity by conditionally assigning scores from 0 to 1, where "1" and "0" represent the highest and lowest probability, respectively [40].

Peptide profiling (Table 3) revealed a diverse range of physiologically active sequences.

Notably, the antioxidant peptide VW was present at a significantly elevated concentration in the PH-P sample relative to the control.

We also detected several other peptides with putative antioxidant functions, including HHY, SQLPLHR, GHHS, PTHHFHVALL, and AVHHMVW, although these were found in statistically insignificant quantities (BioPep database). Furthermore, analysis using the PeptideRanker database (Table 4) identified additional high-potential bioactive peptides, among which PHHSSASCCLW, PPHM, and HGVCWIY were also predicted to possess antioxidant activity.

Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the TUKEY test.

Table 3. Characterization of the identified peptides

Sequence	Activity (according to BioPep)	Content, mg/100 g PH		
		PH-B	PH-C	PH-P
TR	dipeptidyl peptidase IV inhibitor	0.2 ± 0.01^{e}	0.5 ± 0.01^{d}	9.0 ± 0.03^{d}
SY	ACE inhibitor dipeptidyl peptidase IV inhibitor	10.6 ± 0.03^{b}	20.3 ± 0.08^{b}	9.8 ± 0.06^{c}
VW	ACE inhibitor Antioxidative dipeptidyl peptidase IV inhibitor alpha-glucosidase inhibitor	1.4 ± 0.01^{c}	7.5 ± 0.02^{c}	20.1 ± 0.17^{b}
PPP	ACE inhibitor	16.1 ± 0.11^{a}	15.2 ± 0.09^{a}	35.7 ± 0.26^{a}
SW	dipeptidyl peptidase IV inhibitor	1.1 ± 0.03^{d}	3.0 ± 0.07^{d}	7.2 ± 0.07^{e}

Values are means ± SEM, $n = 5$ per treatment group. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the TUKEY test.

Table 4. Peptides with high potential biological activity according to the PeptideRanker database

Sequence	Probability that the peptide will be active (according to PeptideRanker)	Content, mg/100 g PH		
		PH-B	PH-C	PH-P
KEPPPGM	0.752229	$0,8 \pm 0.01^{e}$	$4,2 \pm 0.01^{e}$	$1,7 \pm 0.01^{e}$
HGVCWIY	0.759981	$6,2 \pm 0.02^{c}$	$0,2 \pm 0.01^{e}$	$4,1 \pm 0.01^{e}$
PGTHPLLVF	0.761792	$3,4 \pm 0.02^{c}$	$0,7 \pm 0.01^{e}$	$0,2 \pm 0.01^{e}$
SGAPM	0.770648	$157,0 \pm 1.31^{a}$	$10,4 \pm 1.29^{a}$	$14,4 \pm 0.02^{c}$
PAVVSCLPGPL	0.771864	$8,5 \pm 0.02^{c}$	$0,4 \pm 0.01^{e}$	$6,2 \pm 0.01^{e}$
PPPGV	0.776567	$0,2 \pm 0.01^{e}$	$6,2 \pm 0.02^{c}$	$0,4 \pm 0.01^{e}$
HGSPGHGWVL	0.780265	$0,2 \pm 0.01^{e}$	$11,5 \pm 0.02^{c}$	$6,8 \pm 0.02^{c}$
GRGHIWGQM	0.782054	$0,2 \pm 0.01^{e}$	$12,6 \pm 0.02^{c}$	$0,2 \pm 0.01^{e}$
PHHSSASCCLW	0.785109	$16,0 \pm 0.02^{c}$	$0,1 \pm 0.01^{e}$	$10,1 \pm 0.01^{e}$
ICIMAPIAF	0.786922	$0,2 \pm 0.01^{e}$	$4,3 \pm 0.02^{c}$	$24,8 \pm 0.06^{c}$
VGICIYCL	0.791613	$0,4 \pm 0.01^{e}$	$8,2 \pm 0.02^{c}$	$0,4 \pm 0.01^{e}$
VICFFSVW	0.823681	$6,2 \pm 0.02^{c}$	$0,1 \pm 0.01^{e}$	$4,1 \pm 0.01^{e}$
GLGGAWAF	0.83727	$0,1 \pm 0.01^{e}$	$3,1 \pm 0.02^{c}$	$0,4 \pm 0.01^{e}$
KVPPPRPPL	0.837789	$3,5 \pm 0.02^{c}$	$0,1 \pm 0.01^{e}$	$0,2 \pm 0.01^{e}$
GSAPCPG	0.860877	$6,4 \pm 0.02^{c}$	$14,1 \pm 0.02^{c}$	$0,1 \pm 0.01^{e}$
PGGPGPAM	0.872244	$0,1 \pm 0.01^{e}$	$2,2 \pm 0.01^{c}$	$0,1 \pm 0.01^{e}$
IHPF	0.888252	$0,2 \pm 0.01^{e}$	$4,9 \pm 0.02^{c}$	$0,6 \pm 0.01^{e}$
PPHM	0.899505	$9,6 \pm 0.02^{c}$	$1,8 \pm 0.01^{e}$	$0,1 \pm 0.01^{e}$
PCSIF	0.899527	$7,5 \pm 0.02^{c}$	$16,5 \pm 0.09^{b}$	$0,2 \pm 0.01^{e}$
GCTF	0.906178	$12,3 \pm 0.02^{c}$	$3,6 \pm 0.02^{c}$	$10,3 \pm 0.02^{e}$
QPPQPALAGLVF	0.906993	$0,8 \pm 0.01^{e}$	$3,5 \pm 0.01^{b}$	$15,8 \pm 0.02^{c}$
VAPWIMM	0.927034	$12,6 \pm 0.09^{b}$	$0,1 \pm 0.01^{e}$	$0,2 \pm 0.01^{e}$
PFGAFCNVW	0.93261	$6,4 \pm 0.02^{c}$	$0,2 \pm 0.01^{e}$	$0,2 \pm 0.01^{e}$
IVCWLPAF	0.937182	$0,1 \pm 0.01^{e}$	$10,3 \pm 0.02^{c}$	$0,1 \pm 0.01^{e}$
GGPPPPPPHPG	0.960782	$8,6 \pm 0.02^{c}$	$0,2 \pm 0.01^{e}$	$6,1 \pm 0.01^{e}$
PPPHPFPVALL	0.961163	$12,1 \pm 0.02^{c}$	$0,2 \pm 0.01^{e}$	$0,2 \pm 0.01^{e}$
GFPFPGIHW	0.975569	$0,1 \pm 0.01^{e}$	$10,1 \pm 0.02^{c}$	$1,9 \pm 0.01^{e}$

Values are means ± SEM, $n = 5$ per treatment group.

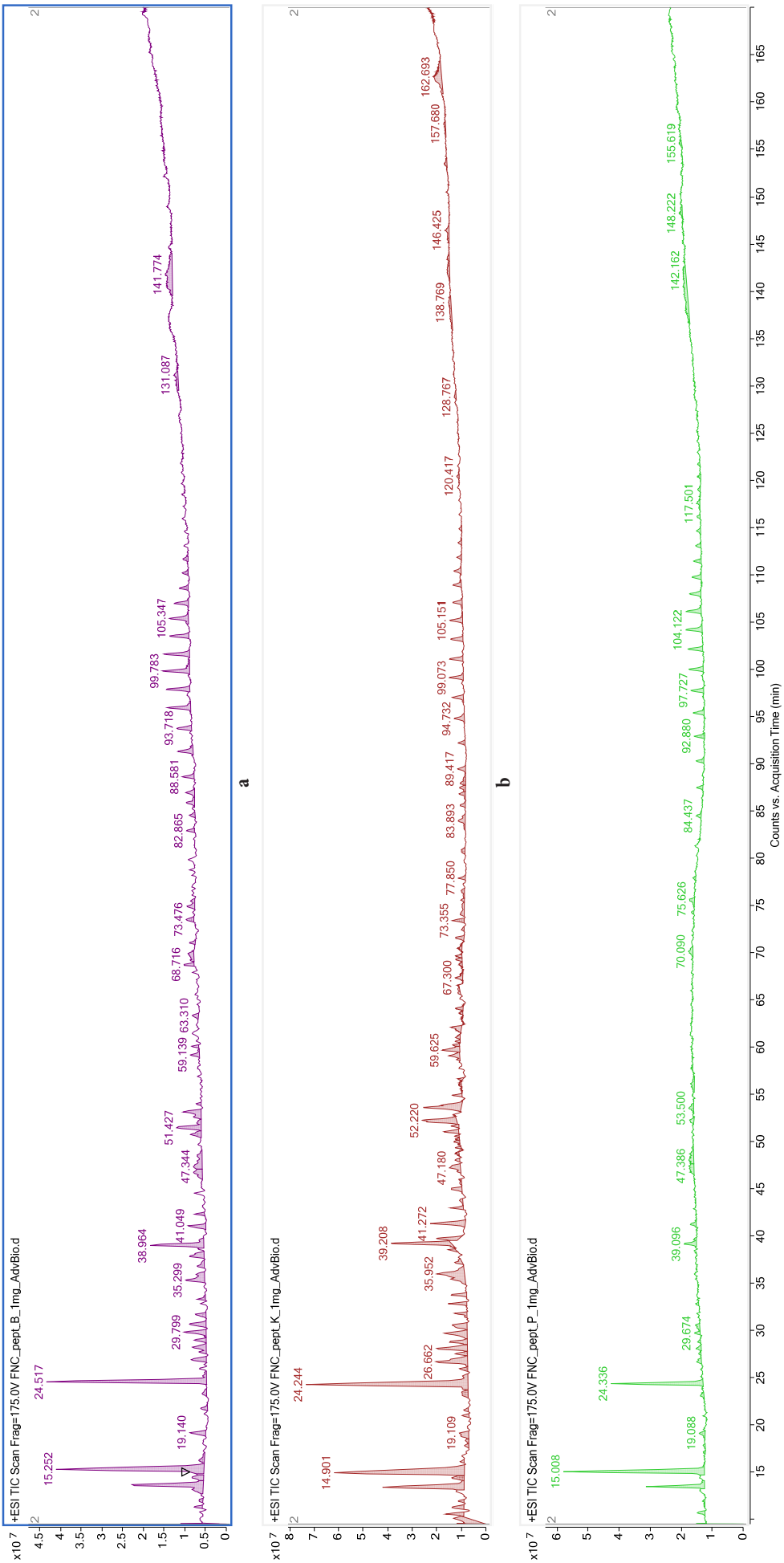

Figure 3. Chromatograms obtained during the research of protein hydrolysates: a) PH-B, b) PH-C, c) PH-P

The hydrolysate obtained by fermentation of raw materials with bifidobacteria contained the lowest content of a peptide with measured antioxidant activity. However, it contains a large number of peptides with high potential antioxidant activity; which, apparently, affects the formation of a high antiradical activity of DPPH (76.5 % for the 1 % protein hydrolysate solution).

Li et al. [70] determined that H, P, C, Y, W, F, and M are involved in the prevention of lipid peroxidation [70] and in the transfer of electrons and protons. The authors attributed the enhanced DPPH radical scavenging activity in the 5–10 kDa fraction to a high proportion of hydrophobic residues, specifically aliphatic (Val, Ile, Leu) and aromatic (Phe, Tyr) amino acids, a finding that aligns with our data. We further posit that leucine and proline within the active peptides facilitate interactions with radical species through hydrophobic forces. This is consistent with prior research [58], which has documented the broad bioactivity of tryptophan-containing peptides.

These peptides possess antioxidant activity, allowing them to protect cells from oxidative stress, and also act as angiotensin-converting enzyme (ACE) inhibitors, which may help regulate blood pressure. Furthermore, they exhibit antidiabetic properties, helping to normalize blood sugar levels and improve metabolism. Thus, tryptophan-containing peptides represent a promising group of compounds for the development of functional foods and therapeutic agents. Peptides with tryptophan at the C-terminus, identified in experimental samples of hydrolysates, have a high capacity to reduce trivalent iron ions.

Numerous researchers in food science and biochemistry have identified bioactive peptides with antioxidant properties obtained through whey hydrolysis, confirming the high value of this source as an ingredient for the development of functional foods [28,59].

These studies open new horizons in understanding the mechanisms of antioxidant action and their potential use in the human diet. Furthermore, modern scientific research is actively pursuing studies aimed at analyzing the bioactive peptides formed in raw meat during natural maturation and fermentation, which can significantly improve their nutritional properties and functionality in food products [71,72].

Significant research focus is being directed toward the byproducts of enzymatic hydrolysis, presenting a promising route for valorizing resources that were once considered inaccessible [42,73].

Concurrently, microbial fermentation of livestock and poultry organs is emerging as a key area of interest. This approach has proven effective, as demonstrated by the fermentation of broiler chicken stomachs to generate a protein hydrolysate containing peptides with both documented and potential antioxidant properties. These results highlight the importance and need for further research aimed at identifying and thoroughly characterizing the discovered peptides. A detailed analysis of their properties could facilitate the development of new functional additives that could improve human health and extend the shelf life of food products, a significant step toward creating a nutritious and safe diet.

Conclusion

The results of studies conducted to evaluate the properties of protein hydrolysates obtained by microbial fermentation demonstrate a high level of antioxidant activity in these compounds.

The results showed that the FRAP antioxidant activity of the experimental hydrolysate sample obtained by fermentation using bifidobacteria was 30 % lower than that of other samples. However, this sample exhibited the greatest free radical scavenging effect, with an IC_{50} of 1.363 mg/g.

A significant accumulation of the amino acid proline was observed in the hydrolysate obtained by fermentation of by-products by bifidobacteria. It is also worth noting that the hydrolysate obtained by fermenting with propionic acid bacteria contained approximately twice as much isoleucine after 12 hours of fermentation compared to the control.

During the fermentation process, the hydrolysates we obtained released a significant amount of sulfur-containing amino acids, which may also have influenced the enhancement of the antioxidant properties of the hydrolysates.

These data are consistent with the results of peptide analysis, highlighting the link between the fermentation process and the properties of the resulting hydrolysates. The microbial species used demonstrated pronounced proteolytic activity, which can be characterized by the significant accumulation of free amino acids and peptides in the resulting hydrolysates.

During the analysis of the hydrolysates, peptides with established antioxidant activity and peptides with potential antioxidant properties were identified, opening new horizons for further research. These findings validate the scientific potential for refining the production and application of these peptides.

Consequently, the study confirms microbial fermentation as an effective method for generating protein hydrolysates and underscores the necessity of further characterizing the detected peptides. Such research is a significant contribution to food science, paving the way for novel functional products with enhanced antioxidant activity and superior nutritional profiles, thus creating new opportunities for safe, healthy food ingredients.

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