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Antimicrobial peptides from free-range chicken egg white prepared ultrasound

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Abstract

Antimicrobial peptides are biomolecules that have great potential for pharmaceutical application, as they are considered to be natural alternatives for combating multi-resistant agents to traditionally marketed drugs. The objective of this research was to prospect for new antimicrobial peptides obtained from free-range egg white proteins that had been pre-treated by ultrasonic sonication, and subjected to the hydrolytic action of commercial trypsin. The maximum percentage of 25.90% proteolysis was found from the application of sonication pre-treatment of egg white for 16 minutes and 29 seconds, followed by enzymatic hydrolysis for 128 minutes and 35 seconds. The peptides (< 3 kDa) were evaluated for their antimicrobial action against six bacterial strains. These tests presented a minimum inhibitory concentration of 1.56 mg.mL^{-1} for the strains *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, and *Salmonella typhimurium* 31194; while for the strains *Listeria monocytogenes* ATCC 19117, *Escherichia coli* ATCC 25922, and *Serratia marcescens* ATCC 13880 the value of minimum bactericidal concentration was 0.78 mg.mL^{-1}. Furthermore, a minimum bactericidal concentration of 0.78 mg.mL^{-1} was observed for all strains, being considered equivalent to the minimum inhibitory concentration. The study of cell death kinetics revealed excellent antimicrobial action in less than 120 minutes for all bacterial strains evaluated. 10 were identified and considered significant for the present study. These characteristics have not yet been reported in scientific studies, nor are they present in databases related to proteins and peptides. Given these results, this protein substrate has a high potential for use in the prospecting of antimicrobial products.

Keywords: antimicrobial peptides; free-range eggs; protein hydrolysis; sonication; high frequency.

Practical Application: The bioactive peptides derived from egg white proteins identified in this study have high potential for practical application as antimicrobial products that can be used to mitigate infectious processes and to control/inhibit bacterial growth in vitro, since they were active in inhibiting growth under the conditions evaluated. Thus, new studies can be conducted for the formulation of antimicrobial pharmaceutical ingredients.

1 INTRODUCTION

The World Health Organization reports the growing number of pathogenic microorganisms that are multi-resistant to the most commonly used drugs in antimicrobial therapies. The development of new classes of antimicrobial agents is necessary, especially those of natural origin, such as bioactive peptides derived from food proteins. These molecules can combat a wide variety of microbial agents (Ikhimiukor & Okeke, 2023; Zharkova et al., 2019).

Traditionally, biotechnological processes utilize enzymatic proteolysis to identify bioactive peptides. However, new technologies are being applied prior to enzymatic action to modify protein structures, enhancing enzymatic accessibility to sites that were previously inaccessible to catalysis. This approach enables the production of bioactive peptides with unique amino acid sequences (Acquah et al., 2019; Gao et al., 2023; Zhu et al., 2018).

Antimicrobial peptides (AMPs) are protein fragments containing 12–37 amino acids in their structural chain. These molecules generally have amphipathic structural characteristics and cationic electrical charges, facilitating interactions with bacterial cell membranes and intracellular components. Their mechanisms of action include cell disruption and alterations in macromolecule synthesis and metabolism, making them valuable allies in combating microbial infections (Ageitos et al., 2017; Jia et al., 2019).

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Industrial chicken eggs are commonly exploited due to their low production cost and high protein content. However, free-range chicken eggs have gained interest because their production process involves free-range chickens raised without exposure to antimicrobial and antiparasitic drugs, among other substances, a method referred to as "green production" (Bestman & Bikker-Ouwejan, 2020). This product has been explored as a technological resource for identifying bioactive peptides, including those with antimicrobial properties (Pfalzgraff et al., 2018; Rai et al., 2023).

Therefore, the present work aimed to identify antimicrobial peptides from the hydrolysis of free-range egg white proteins, previously subjected to ultrasonic wave treatment.

1.1 Relevance of the work

This study is due to the process of obtaining peptides with a molecular weight of less than 3 kDa from the enzymatic proteolysis of free-range egg whites pretreated by ultrasonic sonication. We show the optimization of the enzymatic hydrolysis of free-range chicken egg white protein using trypsin for the release of antibacterial peptides. In addition, the ultrafiltration technique proved to be effective in separating low-molecular-weight peptides, which were submitted to mass spectrometry sequencing analyses, as being evaluated for antimicrobial potential against *Bacillus cereus*, *Enterococcus faecalis*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and *Serratia marcescens*.

2 MATERIALS AND METHODS

2.1 Obtaining the raw material

Free-range chicken eggs were obtained from free-range chicken producers in the city of Garanhuns, located in the southern rural region of the state of Pernambuco (Latitude: –8.89074, Longitude: –36.4966). Collection was carried out immediately after laying, and the eggs were sanitized by immersion in a 2% chlorhexidine antiseptic solution for 30 seconds. They were then stored in thermal boxes under refrigeration (± 4 °C) and transported to the laboratory for subsequent analyses.

2.2 Solubilization of free-range egg white

Initially, the egg white and yolk were manually separated. The free-range egg white was then solubilized in a 0.05 M sodium phosphate buffer solution (pH 8.0), with a final protein substrate proportion of 13.5% (v/v) of the total volume.

2.3 Enzyme solution

Commercial trypsin (E.C. 3.4.21.4 – Sigma-Aldrich®) was used for enzymatic hydrolysis under optimal conditions specified by the manufacturer (37 °C and pH 8.0). The assays were conducted in 250 mL Erlenmeyer flasks, each containing 50 mL of protein substrate, with an enzyme-to-substrate ratio of 1:50 (v/v). Each assay had an average enzyme concentration of 150 $U.mL^{-1}$.

2.4 Protein hydrolysis

Protein hydrolysis was evaluated using a completely randomized block experimental design, consisting of the application of an enzyme-to-substrate ratio, four ultrasonic sonication cycles, and four enzymatic hydrolysis cycles (1x4x4). The experimental condition that resulted in the highest hydrolysis percentage was applied as the central point in the rotated central composite design (RCCD) to maximize protein hydrolysis under experimental conditions. The RCCD design matrix was based on five levels (–1.44, –1, 0, + 1, and + 1.44) for each independent variable (sonication time and hydrolysis time).

2.5 Degree of hydrolysis

The degree of hydrolysis was determined according to the methodology proposed by Adler-Nissen (1979) using 2,4,6-trinitrobenzenesulfonic acid (TNBS). For the reaction, 250 µL (2 $mg.mL^{-1}$) of protein hydrolysate samples were incubated with 2 mL of 0.2125 M sodium phosphate buffer (pH 8.2) and 2 mL of 0.1% TNBS (v/v) diluted in ultrapure water. The reaction was carried out at 50 °C for 60 minutes in the absence of light. After this period, 4 mL of 0.1 M HCl was added to stop the reaction. The reaction mixture was kept at room temperature for 30 minutes, protected from light. The absorbance of the test samples was then analyzed in a spectrophotometer at 340 nm. For the total hydrolysate test, 2 mg of the protein substrate was solubilized in 4 mL of 6 M HCl, and the sample was dried in an oven at 110 °C for 24 h. Furthermore, the degree of hydrolysis of the protein substrate (2 $mg.mL^{-1}$) that was not subjected to enzymatic hydrolysis was quantified. The calibration curve was performed with L-leucine as a standard amino acid, with concentrations ranging from 0.25 to 10 mM. The percentage of hydrolysis was calculated according to Equation 1:

$$Degree\ of\ hydrolysis\ (\%) = \frac{A_{N2} - A_{N1}}{N_{pb}}\ x\ 100 \qquad (1)$$

Where:

AN2 is the amount of α-amino acid released during hydrolysis;

AN1 is the amount of α-amino acids in the original protein substrate solubilized in the working buffer; and

Npb is the total number of amino groups in the protein substrate (total hydrolysate).

2.6 Fractionation of protein hydrolysate

Fractionation of the protein hydrolysate by molecular mass range was carried out through ultrafiltration with cut-off membranes of 100, 50, 30, 10, and 3 kDa porosity, using Amicon® Ultra-15 (Millipore Ireland Ltd, Tullagreen, Ireland). The protein hydrolysate was sequentially centrifuged at 4,000 x *g* for 40 minutes at 4 °C. The permeate fraction smaller than 3 kDa was lyophilized and stored at –18 °C for further analyses.

2.7 Peptide identification and bioinformatic analysis

The peptides were reconstituted in 0.1% formic acid and 2% acetonitrile (final concentration of 0.25 μg.μL^{-1}), and 5 μL was injected for separation using the nanoAcquity UPLC® system (Waters GmbH, Eschborn, Germany). The peptides were retained in a C18 2G-V/MTrap column, Symmetry®, 180 μm × 20 mm, particle size 5 μm (Waters GmbH, Eschborn, Germany), for 3 min at a flow rate of 7 μL.min-1. Separation was performed using a C18 BEH 130 column, 100 μm × 100 mm, particle size 1.7 μm (Waters GmbH, Eschborn, Germany). The mobile phase consisted of a gradient of solvent A (0.1% formic acid) and solvent B (99.9% acetonitrile and 0.1% formic acid), at a flow rate of 0.4 μL.min–1 and a column temperature of 40 °C. The nanoAcquity UPLC system was coupled to a Bruker Daltonics MaXis 3G UHR-ESI-Q-TOF mass spectrometer (Bremen, Germany) equipped with a CaptiveSpray source (Bremen, Germany). The mass spectrometer was operated with a capillary voltage of 3,000 V, a desolvation temperature of 150 °C, a drying gas flow of 3 L.min^{-1}, and an ionization source in positive mode.

Spectra were obtained using MassLynx software (version v.4.1). The raw data files were converted using Mascot Distiller software (version 2.5; Matrix Science, London, United Kingdom) and compared against the *Gallus gallus* database (18,113 sequences; 10,113,287 residues) using BiotoolsTM software (version 3.0) combined with MASCOT version 2.5 (Matrix Science, London, United Kingdom).

The following search parameters were used: fixed modification = cysteine carbamidomethylation; variable modification = methionine oxidation; number of lost cleavages = 2; enzyme used = trypsin; peptide mass tolerance = ± 20 ppm; fragment mass tolerance = ± 0.6 Da; and significance threshold = $p < .05$. Peptide scores greater than 21 (in the Uniprot database) were considered significant ($p < .05$). Identified proteins were categorized based on their biological processes, molecular functions, and cellular components using the Blast2GO bioinformatic tool (http://www.blast2go.org).

Each significant peptide was analyzed in the Biopep-UVM (https://biochema.uwm.edu.pI/biopep-uwm/) and NCBI (https://ncbi.nlm.nih.gov/) databases. Additionally, relevant scientific literature was reviewed in major databases.

2.8 Antibacterial activity

2.8.1 Minimum inhibitory concentration

The test was based on microplate turbidity assessment (Clinical and Laboratory Standards Institute [CLSI], 1999). The strains *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19117, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 31194, and *Serratia marcescens* ATCC 13880 were previously cultivated in Mueller-Hinton broth for 12 hours at 37 °C. The absorbance of the bacterial suspensions was adjusted to 0.1 OD on a spectrophotometer, corresponding to 0.5 on the McFarland scale (Biochrom Libra S6®, Cambridge, United Kingdom). The tests consisted of mixing 100 μL of peptide solution at different concentrations (6.25–0.39 mg.mL^{-1}), 90 μL of Mueller-Hilton broth, and 10 μL of the standardized bacterial suspension. The negative control for bacterial growth inhibition consisted of 100 μL of sterile water, while the positive control was the commercial antimicrobial chloramphenicol (50 μg.mL^{-1}). All assays were performed in sterile, flat-bottom, 96-well polystyrene microplates (Corning®) and conducted in triplicate. The percentage of bacterial growth inhibition was calculated using Equation 2:

$$Growth\ inhibition\ (\%) = \frac{O.D_{Negative\ control} - O.D_{sample}}{O.D_{Negative\ control}}\ x\ 100 \quad (2)$$

Where:

O.DNegative control corresponds to the optical density of the control with microbial growth;

O.Dsample corresponds to the optical density of the assay sample containing the peptide solution.

2.8.2 Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined according to the method described in protocol M26-A (CLSI, 1999). For this, 100 μL from wells treated with peptides in the minimum inhibitory concentration (MIC) assay that showed no visible bacterial growth, along with the immediately lower concentration that showed microbial growth, were inoculated onto Mueller-Hinton agar and incubated at 37 °C for 24 hours to determine the number of colony-forming units (CFU). The MBC was defined as the concentration at which the colony count was equal to or less than 10 CFU.mL^{-1}.

2.8.3 Cell death kinetics

The cell death kinetics assay was performed to determine the efficiency of peptides with molecular mass < 3 kDa in eradicating bacterial strains. The test followed the method proposed by Singh et al. (2020). Bacterial strains were incubated with different peptide concentrations for 0, 10, 20, 30, 60, and 120 minutes. At each time point, 100 μL of bacterial suspension was inoculated onto Mueller-Hinton agar and incubated at 37 °C for 24 hours, after which the number of CFUs was estimated. All experiments were carried out in triplicate.

2.9 Statistical analyzes

All experiments were conducted in triplicate. Significant differences ($p < .05$) were identified by the Tukey mean comparison method using the statistical software SISVAR 5.7 (Ferreira, 2014) and Statistic 10.

3 RESULTS AND DISCUSSION

3.1 Hydrolysis of free-range egg white proteins

Trypsin was effective in hydrolyzing free-range chicken egg white proteins, with hydrolysis percentages ranging between 1.79 ± 0.38% and 25.39 ± 0.75%, as shown in Table 1.

Sonication for 15 minutes combined with 120 minutes of hydrolysis resulted in the highest degree of hydrolysis (25.39 ± 0.75%), which was statistically significant according to Tukey's test ($p < .05$).

Knežević-Jugović et al. (2012), Stefanović et al. (2014), and Stefanović et al. (2017) also evaluated the percentage of hydrolysis at different times during ultrasonic sonication (40 kHz) and hydrolytic action. Despite differences in proteolytic enzymes, the hydrolytic behavior was similar, with peak hydrolysis occurring at 15 minutes of sonication, varying only in enzymatic action duration and hydrolysis percentages.

Stefanović et al. (2014) also applied sonication at 40 kHz and hydrolyzed chicken egg white proteins using a combination of alcalase, neutrase, and papain, followed by a second hydrolysis step with flavorezime, achieving a maximum hydrolysis degree of 31.44 ± 0.25%.

In the present study, sonication for 30 minutes resulted in a reduction in hydrolysis percentage, with an even more pronounced decreased at 60 minutes. Knežević-Jugović et al. (2012) investigated the effects of prolonged exposure of protein substrates to sound waves at 40 kHz and reported negative impacts on hydrolysis. These authors suggested that sonication beyond 30 minutes induces structural alterations in the protein matrix, leading to protein aggregation that hinders enzymatic catalysis.

Overall, the findings highlight the need to employ more elaborate statistical processes to determine the optimal interaction between sonication and hydrolysis time, as these variables directly affect the degree of hydrolysis. The tests designed based on RCCD showed variations in hydrolysis percentage based on the specific treatment conditions, as shown in Table 2.

Under the conditions established in the experimental design matrix (RCCD), the degree of hydrolysis ranged from 8.79 ± 0.39% to 25.20 ± 0.75%, emphasizing the importance of statistical planning in bioprocesses. From the data obtained for the response variable (degree of hydrolysis), the significance of the model was evaluated by analysis of variance (ANOVA) and presented in Table 3. The statistical model demonstrated a quality fit with an $R^2 = 0.94067$, indicating that the adjusted model explained 94.06% of the total variation in the degree of hydrolysis. In this way, the aforementioned model can reliably predict the degree of hydrolysis within the studied experimental conditions.

Table 2. Degree of hydrolysis of free-range egg white proteins in the Rotated Composite Central Design by the action of trypsin obtained through the interaction between different sonication times and enzymatic hydrolysis with concentration E:S = 1:50.

Test	Sonication time	Hydrolysis time	Hydrolysis percentage
1	7min30s	60min	10.67 ± 0.52^{C}
2	7min30s	180min	12.08 ± 0.32^{C}
3	22min30s	60min	12.90 ± 0.82^{C}
4	22min30s	180min	13.13 ± 0.64^{C}
5	4min39s	120min	11.49 ± 0.62^{C}
6	26min1s	120min	17.12 ± 0.37^{B}
7	15min	35min14s	8.79 ± 0.39^{D}
8	15min	205min5s	17.12 ± 0.53^{B}
9	15min	120min	24.03 ± 1.00^{A}
10	15min	120min	25.20 ± 0.75^{A}
11	15min	120min	23.02 ± 0.71^{A}
12	15min	120min	24.00 ± 0.99^{A}

Equal capital letters (hydrolysis percentage) indicate that there are no statistical differences between treatments ($p < .05$).

Table 1. Degree of hydrolysis of free-range egg white proteins by the action of trypsin obtained through the interaction between different sonication times and enzymatic hydrolysis with concentration E:S = 1:50.

Sonication (minutes)	Enzymatic hydrolysis (minutes)			
	0	**60**	**120**	**240**
0	1.79 ± 0.38^{Ba}	15.92 ± 0.57^{Aa}	18.93 ± 0.87^{Ab}	16.32 ± 1.15^{Aab}
15	$1.8\ 2 \pm 0.74^{Da}$	19.04 ± 0.99^{Ba}	25.39 ± 0.75^{Aa}	6.50 ± 0.41^{Cc}
30	2.73 ± 0.42^{Ba}	12.83 ± 0.64^{Ab}	13.30 ± 0.98^{Ac}	12.60 ± 1.53^{Ab}
60	3.97 ± 0.47^{Ba}	9.31 ± 0.38^{Ab}	10.02 ± 0.85^{Ac}	11.42 ± 1.11^{Ab}

Equal capital letters in rows (enzymatic hydrolysis), and equal lowercase letters in columns (sonication) indicate that there are no statistical differences between treatments ($p < .05$).

Table 3. Analysis of variance of the degree of hydrolysis of egg white proteins in the Rotated Composite Central Design by the action of trypsin obtained through the interaction between different times of sonication and enzymatic hydrolysis with the concentration E:S = 1:50

Factor	Sum square	Degree of freedom	Quadratic mean	*fcal*	*p-value*
(1) Sonication (L)	19.2960	1	19.2960	24.2649	.0160*
Sonication (Q)	173.5707	1	173.5707	218.2662	.0006*
(2) Hydrolysis (L)	22.5719	1	22.5719	28.3843	.0129*
Hydrolysis (Q)	222.6998	1	222.6998	280.0463	.0004*
1L and 2L	0.2689	1	0.2689	0.3381	.6017
Lack of fit	21.0021	3	7.0007	8.8034	.0535
Pure error	2.3857	3	0.7952		
Total	394.1689	11			

*Statistically significant ($p < .05$).

The maximum yield for the degree of hydrolysis of egg white proteins was determined by analyzing the response surfaces and the values predicted by the statistical model used. The predicted results showed that the maximum percentage of the degree of hydrolysis would be obtained under the following conditions: sonication for 16 minutes and 29 seconds, and the enzymatic hydrolysis for 128 minutes and 35 seconds, with a maximum predicted hydrolysis value of 27.23%.

The response surface graphic presented in Figure 1 shows the optimal levels of hydrolysis. Additional experiments were carried out under maximum conditions aiming to validate the statistical method as a reproducible model. The maximum percentage levels obtained for the degree of hydrolysis were on average 27.40 ± 0.10%, indicating the validation of the model and the existence of an optimal point.

Statistical modeling methods applied to bioprocesses help reduce the total number of experiments, consequently minimizing execution time and resulting in financial savings. These methods also contribute to increased product yield, especially when optimal points are determined for each studied variable (Jiang et al., 2018; Uluko et al., 2013; Zhou et al., 2016).

3.2 Identification of peptides

The sample of peptides with < 3 kDa, originating from the enzymatic hydrolysis of free-range chicken egg white, was analyzed by mass spectrometry. This analysis revealed 10 distinct peptide sequences, containing between 8 and 14 amino acid residues. These sequences, with a score greater than 22 in the Uniprot database, were considered significant ($p < .05$) for the present study, according to data presented in Table 4.

Regarding the size of the peptide chain, as previously mentioned, the peptides sequenced in this study ranged between 10 and 16 amino acids. Ramazi et al. (2022) and Oliveira et al. (2023) highlighted that the size, type, and arrangement of amino acids in the peptide chain influence antimicrobial action. Shorter peptides, particularly those with 2 to 20

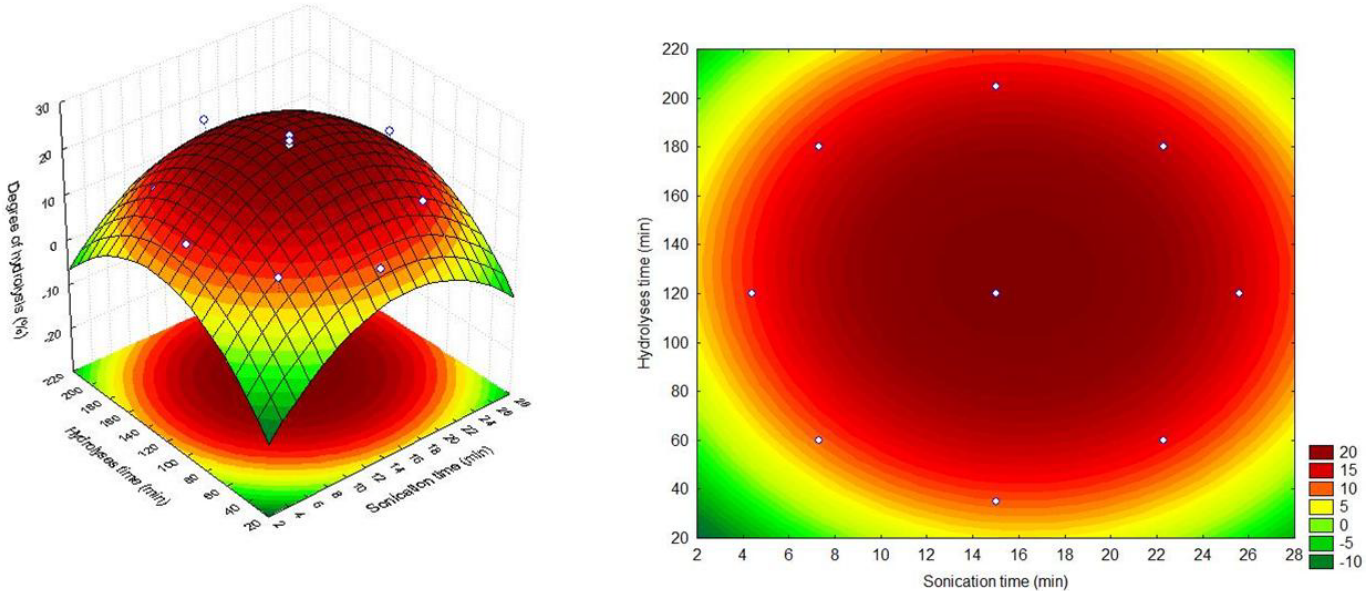

Figure 1. Response surface and contour curve for the percentage degree of hydrolysis of free-range egg white proteins, generated through the interaction between different sonication times and different enzymatic hydrolysis times.

Table 4. Peptides from egg white with molecular mass less than 3 kDa identified by mass spectrometry.

Peptide sequence	Peptide position	Experimental mass (Da)	Calculated mass (Da)	Peptide score (%)[a]
FESCHETGECLE	(120–129)	1220.45	1220.44	25.72
FPSRPAPLPPK-	(838–847)	1058.61	1058.62	26.84
LPQPHSGASYAIYLH	(344–356)	1402.69	1402.69	28.14
LTIQYLVQLG	(468–475)	976.55	976.56	25.31
FHCEHVSSLQRYDALL	(222–235)	1713.77	1713.79	25.06
LIVKAGVDMVFLY*	(136–146)	1206.66	1206.67	24.37
LSVLRAAPALS	(336–344)	896.53	896.54	23.64
LSAQARVAELE	(581–589)	943.52	943.51	23.42
LQMAAEPISARQPALR*	(157–170)	1497.76	1497.76	22.34

[a]The score is a measure of the statistical significance of a match (Mascot); * + Oxidation (M).

amino acids, are known to exhibit higher antimicrobial activity (Hioki et al., 2014).

A search for the peptide sequences in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) revealed that these particular sequences have not yet been reported in the literature. However, other peptide sequences from egg white proteins have been documented for their antimicrobial properties.

Pimchan et al. (2023) identified a new antimicrobial peptide (AMP) (KGGDLGLFEPTL), with high bactericidal action against the Staphylococcus aureus strain, with a MIC of 2 mM. Ma et al. (2020) identified the AMP AGLAPYKLKPIA, with a molecular mass of 1,240.76 Da, derived from egg-transferrin, with an MIC between 2 and 128 μg.mL^{-1}.

The microbial action of peptides is primarily influenced by factors such as electrical charge and hydrophobicity, which play a crucial role in determining the interaction between peptides and bacterial cell membranes (Oliveira et al., 2023). AMPs are commonly made up of disulfide bonds, cysteine, glycine, and lysine (Wang, 2023).

In general, basic residues such as arginine, lysine, and histidine confer positive charge to peptides, while tyrosine, valine, leucine, and isoleucine confer hydrophobicity (Zouhir et al., 2016). In the present study, both peptide sequences containing amino acids associated with hydrophobicity and a positive electrical charge were identified.

3.3 Antibacterial activity

All bacterial strains tested in the present study were sensitive to all concentrations (6.25–0.39 mg.mL^{-1}) of peptides with molecular mass < 3 kDa, derived from the enzymatic hydrolysis of free-range chicken egg white proteins. MIC to inhibit 100% of bacterial growth was found to be 1.56 mg.mL^{-1} for *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, and *Salmonella typhimurium* ATCC 31194. For the strains *Listeria monocytogenes* ATCC 19117, *Escherichia coli* ATCC 25922, and *Serratia marcescens* ATCC 13880, MIC was 0.78 mg.mL^{-1}, as presented in Table 5.

At a concentration of 0.78 mg.mL^{-1}, bacterial growth inhibition greater than 90% was observed for *B. cereus*, *E. faecalis*, and *S. typhimurium*, with values of 98.5 ± 0.6%; 94.9 ± 0.5%, and 93.2 ± 0.6%, respectively. At a concentration of 0.39 mg.mL^{-1}, inhibition above 90% was observed for *L. monocytogenes*, *E. coli*, and *S. marcescens*, with inhibition values of 95.0 ± 0.5%; 96.1 ± 1.6%, and 92.4 ± 0.6%, respectively.

Given the positive results, the promising nature of the peptides obtained in the present study for combating infectious agents that cause deleterious effects on human and animal health is demonstrated. Additionally, these findings underscore the importance of scientific studies focused on the prospecting of antimicrobial substances of natural origin.

Studies on the antimicrobial activity of peptides generally focus on substances with a molecular mass < 3 kDa, as peptides with low molecular mass have shown higher antibacterial power compared to those with high molecular mass (Abdel-Hamid et al., 2016).

The prospecting of antibacterial peptides for pathogen control presents positive characteristics, as these substances commonly exhibit broad-spectrum bactericidal and/or bacteriostatic activity. Furthermore, these bioactive compounds rarely induce microbial resistance and have a high capacity for synergistic action with commercially available antibiotics (Sila et al., 2014; Wang et al., 2023).

The antibacterial potential depends on the interaction between the peptides and the microbial cell wall. Electrostatic characteristics and the amphipathic structure facilitate interaction with the components of the microbial cell membrane (Ageitos et al., 2017), with electrical charge being the most relevant factor (Glinel et al., 2012). Strains of *E. coli* and *Salmonella* sp. are considered the most common foodborne contaminants and pose significant public health risks due to the potential for food poisoning outbreaks (Waseem et al., 2018). Some studies have identified the antimicrobial potential of peptides prospected from egg white proteins against *Escherichia coli*, *Leuconostoc mesenteroides*, and *Staphylococcus carnosus*, with MIC values of 0.01 mg.mL^{-1} (Bhat et al., 2015; Memarpoor-Yazdi et al., 2012).

Table 5. Minimum inhibitory concentration of peptides with molecular mass < 3 kDa against Gram-positive, and Gram-negative strains.

	Antibacterial activity (%)					
Conc. (mg.mL^{-1})	***B. cereus* ATCC 11778**	***E. faecalis* ATCC 29212**	***L. monocytogenes* ATCC 19117**	***E. coli* ATCC 25922**	***S. typhimurium* ATCC 31194**	***S. marcescens* ATCC 13880**
6.25	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}
3.12	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}
1.56	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}
0.78	98.5 ± 0.6^{A}	94.9 ± 0.5^{B}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	93.2 ± 0.6^{B}	100.0 ± 0.0^{A}
0.39	82.9 ± 1.0^{B}	81.3 ± 1.5^{C}	95.0 ± 0.5^{B}	96.1 ± 1.6^{B}	88.0 ± 0.9^{C}	92.4 ± 0.6^{B}

Equal capital letters in a column indicate that there are no statistical differences between treatments for ($p < .05$).

Singh et al. (2020) employed α-amino acid sulfonation and lipidification techniques to prospect semi-synthetic peptides. The authors report that some techniques can improve antimicrobial activity. In this case, the test was performed only on a methicillin-resistant *Staphylococcus aureus* strain, with an MIC of 0.001 mg.mL^{-1}.

Righetto et al. (2023) prospectively synthesized a hydrophobic peptide, Fmoc-Plantaricin149. This AMP showed a wide variation in MIC values against 60 bacterial strains. MICs between 0.001–0.128 mg.mL^{-1} were observed for Gram-positive strains and 0.016–0.512 mg.mL^{-1} for Gram-negative strains.

Regarding the activity of the MBC, the lowest concentration demonstrated the ability to promote bacterial cell death was 0.78 mg.mL^{-1} for all strains tested (Table 6). In general, the pool of peptides < 3 kDa used in the present study demonstrated similar results for MIC and MBC.

Cochet et al. (2021) reported the antimicrobial activity of two peptides with molecular masses < 10 kDa (FVPPVQR and GDPSAWSWGAEAHS) from chicken eggs against the strain of *Salmonella enterica* serovar Enteritidis. The MBC value was 100 µg.mL^{-1} and resulted in complete cell death.

Venkateswarulu et al. (2019), studied the prospecting of AMPs from *Saccharomyces boulardii* and found MBC values of 0.5, 0.06, and 0.02 mg.mL^{-1} for *Agrobacterium tumefaciens*, *Helicobacter pylori*, and *Klebsiella pneumoniae*, respectively, demonstrating the versatility of antimicrobial peptide prospecting.

While Alsaggar et al. (2022) observed values between 0.015 and 0.020 mM for the *Escherichia coli* and *Listeria monocytogenes* strains from a structurally modified peptide with an amino acid position corresponding to GVKFAKRFWRFAKKAFKRFEK.

The study of cell death kinetics is crucial in evaluating the antimicrobial potency of peptides. The data presented in Figure 2 demonstrate the potent action of the AMPs prospected in the present study, which were able to promote favorable cell death kinetics, since, in 120 minutes of exposure to the peptide pool, there is a significant decrease concerning the negative control.

Antimicrobial peptides can disrupt the bacterial cell wall and/or interact with cellular targets that interfere with metabolic processes essential for bacterial survival, such as the synthesis of genetic material, and proteins, among other processes (Gupta et al., 2023).

Disruptive mechanisms tend to drastically reduce the period necessary for microbial cell death since the interaction of these AMPs with the bacterial membrane already promotes cell death in the latency phase. On the other hand, non-disruptive ones require microorganisms to go through the adaptation phase and begin the exponential phase so that they can significantly interfere with bacterial metabolism (Gupta et al., 2023; Snoussi et al., 2018).

Prada-Prada et al. (2020) studied the effect of the Ib-M1 peptide (EWGRRMMGRGPGRRMMEWWR-NH2) on the *E. coli* O157:H7 strain and identified a 99.9% reduction in bacterial growth in the number of < 3 CFU.mL^{-1}, only after 8 hours of exposure at 2xMIC and after 24 hours for 1xMIC. In this study, the authors indicate that the long action time of the peptide may be related to the non-disruptive mechanism, or even the dual action (disruptive and non-disruptive).

4 CONCLUSION

The enzymatic hydrolysis of free-range chicken egg white proteins through the action of the trypsin enzyme presents a good technological alternative for the generation of antimicrobial peptides. From statistical studies, it was possible to optimize the hydrolysis process and reach the maximum level for the conditions analyzed. The peptides with a mass < 3 kDa prospected in the present study can promote the death of bacterial strains with equivalent MIC and MBC, and from the study of cell death kinetics, the potent antimicrobial action of the peptides was verified due to the short time necessary for effective bacterial killing.

Table 6. Minimum bactericidal concentration of peptides with molecular mass < 3 kDa against Gram-positive and Gram-negative strains.

	Antibacterial activity (CFU.mL^{-1})					
Conc. (mg.mL^{-1})	***B. cereus* ATCC 11778**	***E. faecalis* ATCC 29212**	***L. monocytogenes* ATCC 19117**	***E. coli* ATCC 25922**	***S. typhimurium* ATCC 31194**	***S. marcescens* ATCC 13880**
6.25	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{C}
3.12	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{C}
1.56	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{C}
0.78	4.0 ± 1.0^{B}	7.0 ± 1.7^{B}	9.0 ± 0.6^{B}	6.0 ± 3.5^{B}	5.0 ± 0.6^{B}	7.0 ± 0.0^{B}
0.39	24.0 ± 4.5^{A}	27.0 ± 0.6^{A}	32.0 ± 6.6^{A}	18.0 ± 3.5^{A}	30.0 ± 0.6^{A}	34.0 ± 4.0^{A}

Equal capital letters in a column indicate that there are no statistical differences between treatments for ($p < .05$).

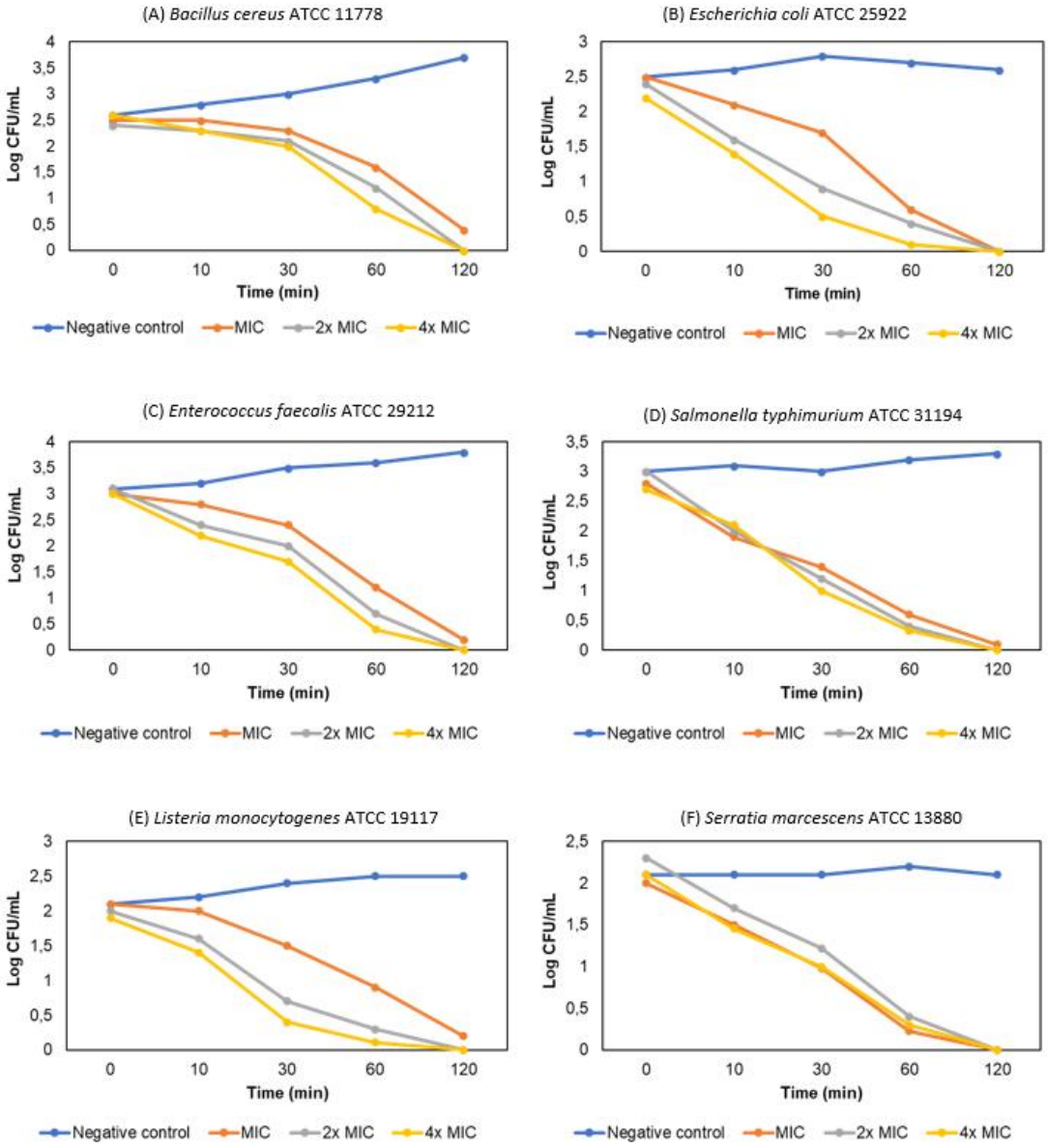

Figure 2. Time kinetics of bacterial cell death of peptides < 3 kDa from egg white proteins. **(A)** *Bacillus cereus* ATCC 11778, **(B)** *Escherichia coli* ATCC 25922, **(C)** *Enterococcus faecalis* ATCC 29212, **(D)** *Salmonella typhimurium* ATCC 31194, **(E)** *Listeria monocytogenes* ATCC 19117 and **(F)** *Serratia marcescens* ATCC 13880.

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