New Insights into the Antimicrobial Properties of Hydrolysates and Peptide Fractions Derived from Chia Seed (*Salvia hispanica* L.)

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Abstract

Bioactive peptides derived from chia (*Salvia hispanica*) seed with antioxidant, antihypertensive, and anti-inflammatory activities have been well documented; however, few studies describe the antimicrobial properties of these peptides, which is of great interest not only in the prevention of food-borne diseases but also food spoilage. The aim of this study was to generate chia seed peptides using microwave-assisted hydrolysis with sequential (alcalase + flavourzyme) enzymes (AF-MW), fractionate them into 3–10 and < 3 kDa fractions, and evaluate their potential antimicrobial activity towards *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes*. Overall, the peptide fraction < 3 kDa showed higher antimicrobial activity than both chia seed hydrolysate and peptide fraction 3–10 kDa. Furthermore, the < 3 kDa fraction showed remarkable increase in membrane permeability of *E. coli* (71.49% crystal violet uptake) and *L. monocytogenes* (80.10% crystal violet uptake). These peptides caused a significant extension in the lag phase, decreases in the maximum growth, and growth rate in the bacteria and promoted multiple indentations (transmembrane tunnels), membrane wrinkling, and pronounced deformations in the integrity of the bacterial cell membranes. Finally, a select group of peptides in the AF-MW < 3 kDa fraction contained 16 sequences with cationic and hydrophobic character, with seven of them sharing the exact same sequence (GDVIAIR) and eight of them having the amino acid K as either N- or C-terminal or both. In conclusion, our results indicate that bioactive peptides obtained from chia seed proteins by microwave and enzymatic hydrolysis could be employed as antimicrobial agents in foods and therapeutic applications.

Keywords Chia seed hydrolysates · Microwave treatment · Bioactive peptides · Antimicrobial activity

Introduction

In recent years, chia (*Salvia hispanica*) seeds have drawn attention due to their high nutritional quality and bioactive properties [1–3]. Chia seed is rich in protein (20–22%), lipids (30–35%), carbohydrates (25–41%), and crude fiber (18–30%) [1], making it a good source of essential and non-essential amino acids, ω-3 fatty acids, phytosterols, and dietary fiber [2, 3].

Studies have reported that hydrolysates of chia seed proteins possess diverse biological activities such as free radical-scavenging, inhibition of angiotensin-converting enzyme, and inhibition of pro-inflammatory mediators, among others [4–6]. These food-derived peptides could exert a protective effect against the development of chronic diseases, because of their potential antioxidant, antihypertensive, and anti-inflammatory activities exhibited both in vitro and in vivo models [7, 8]. However, little attention has been paid to other important bioactivities such as their antimicrobial properties, which is of great interest not only in the prevention of food-borne diseases but also food spoilage. Research suggests that antimicrobial peptides have potential therapeutic value in the treatment of infections caused by microorganisms that have developed resistance to commonly used antibiotics. Antimicrobial peptides have low tendency for bacterial resistance as they tend to quickly destroy bacterial cells through peptide-membrane interactions. Since the target of the peptides is the cell membrane, bacterial membrane redesign becomes a costly metabolic solution, which can also take long periods of time for

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most microbial species [9, 10]. Another application of these peptides could be to obtain bioactive edible films for food packaging, which by being in food contact, could exert a controlled release of the bioactive peptides towards the food surface [11].

There are few studies on the antimicrobial activities of protein hydrolysates derived from chia seeds [12, 13], which have reported contradictory results. Chia seed protein hydrolysates showed antimicrobial properties against *Staphylococcus aureus* [12], whereas another study stated that none of the chia seed protein hydrolysates exhibited antimicrobial activity [13]. Recently, our research group demonstrated that microwave (MW)-assisted hydrolysis with alcalase and flavourzyme improved bioactivity and functionality of chia seed protein hydrolysates compared to conventional hydrolysis methods [6]. MW-assisted hydrolysis has been applied to increase a protein's susceptibility to hydrolysis, leading to the improvement of bioactivities, and to impart a decreased allergenicity of the resulting hydrolysates. For instance, the application of proteolysis in combination with MW enhanced the hydrolysis rate of proteins and improved peptide bioactivity in dairy whey proteins [14], fish frame protein [15], and cricket protein [16].

With this in mind, the objective of this study was to evaluate the effect of microwave-assisted enzymatic hydrolysis on the antimicrobial properties of hydrolysates and peptide fractions from chia seeds.

Material and Methods

Materials and Reagents

Chia seeds were obtained from Healthworks (Black chia seed, Scottsdale, AZ, USA). Alcalase (E.C. 3.4.21.62), flavourzyme (E.C. 232-752-2), and Mueller Hinton Broth were purchased from Sigma Aldrich (St. Luis, MO, USA). MacConkey agar with sorbitol, modified Oxford agar, and XLT4 agar were obtained from Difco™ (BD Diagnostics, Sparks, MD, USA). All chemicals used were reagent grade.

Preparation of Chia Seed Protein Hydrolysates and Their Peptide Fractions

The hydrolysates of chia seed protein were obtained according to Urbizo-Reyes et al. [6]. First, chia seed mucilage was extracted using a combined approach with ultrasound treatment and vacuum-assisted filtration. Briefly, seeds were hydrated in distilled water (1:20 ratio by weight) for 24 h (at 4 °C) and after that were pre-heated to 55 ± 2 °C, followed by sonication at a 75% power input using an ultrasonic cell disruptor (Sonifier® Branson S-150D Danbury, CT, USA). Subsequently, defatted chia seed meal (22.5 mg protein/mL)

was hydrolyzed using single (alcalase, A) or sequential (alcalase followed by flavourzyme, AF) enzymatic processes, and under conventional-water bath (WB) or microwave-assisted (MW) hydrolysis (using a microwave-accelerated reaction system [MDS, MARS-Xpress/230/60, CEM Corporation, USA]). Samples A-WB and A-MW were hydrolyzed for 1 h with 3% (*w/w*) Alcalase®. For sequential hydrolysis, different times were used; due to the high efficiency of microwave-assisted hydrolysis, the time was cut down by half to obtain similar degree of hydrolysis [6]. For AF-MW, the reaction was initiated with 2% (*w/w*) of Alcalase® for 45 min followed by addition of 2% (*w/w*) of Flavourzyme® for an additional 45 min. For AF-WB, the reaction was developed using 2% (*w/w*) of Alcalase® for 90 min followed by 2% (*w/w*) of Flavourzyme® for another 90 min. Hydrolysis was terminated by heating to 95 ± 3 °C for 15 min. Subsequently, the hydrolysates were fractionated by ultrafiltration using a 10 kDa cutoff membrane, and the 10 kDa permeated were further fractionated using a 3 kDa cutoff membrane. Next, chia seed hydrolysate (CH) and the fractions were sterilized with a 0.22-μm pore size syringe filter. Protein concentration was determined for all peptide fractions by the bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Pierce Biotechnology Inc., Rockford, IL, USA), using bovine serum albumin as standard. The protein concentration of each sample was adjusted to 0.5 mg/mL using phosphate-buffered saline solution (PBS, 0.5 mM, pH 7.2). All samples were immediately stored in the dark at 4 °C until used.

Bacterial Culture

Gram-negative bacteria, *Escherichia coli* O157:H7 B6-914, *E. coli* ATCC 25922, *Salmonella enterica* serovar Typhimurium K1028, and Gram-positive bacteria, *Listeria monocytogenes* 10403S and *Listeria innocua* ATCC 33090, were used in this study. These bacteria were selected as target microorganisms because of their potential incidence in foods, their involvement in numerous foodborne disease outbreaks, or because they are used for antimicrobial susceptibility testing according to the Clinical and Laboratory Standards Institute (Wayne, PA, USA). The bacterial strains were provided from the culture collection in the Food Safety Lab of the Food Science Department at Purdue University (West Lafayette, IN, USA). The ATCC bacterial strains were obtained from American Type Culture Collection (10801 University Boulevard Manassas, VA 20110 USA). Stock cultures were first inoculated in specific and/or selective growth agar (MacConkey agar with sorbitol for *E. coli*, modified Oxford agar for *Listeria*, and XLT4 for *Salmonella*), allowing 12–24 h of growth depending on the strain. Single pure colonies of each microorganism were transferred to sterile Mueller Hinton Broth (MHB) and cultured in a temperature-

controlled orbital shaker at 37 °C and 160 rpm for 24 h. Prior to use, the cultures were subjected to two successive subcultures in MHB (14 h each).

Evaluation of Antimicrobial Activity

The antimicrobial activity of the CH and peptide fractions was evaluated using a microplate assay according to Dasari et al. [17]. Namely, a 14-h-old culture of each target bacteria (*E. coli* O157:H7 B6-914, *E. coli* ATCC 25922, *S. enterica* serovar Typhimurium K1028, *L. monocytogenes* 10403S, and *L. innocua* ATCC 33090) was washed twice with sterile PBS and resuspended with MHB (Sigma-Aldrich) to an optical density of 0.6 at 600 nm (ca. 10^7–10^8 CFU/mL). Then, 20 μL of either peptide fraction (CH, 3–19 kDa and < 3 kDa peptide fractions) or PBS (control) was mixed with 20 μL of each target bacteria and 160 μL of MHB in a sterile 96-microwell plate during 24 h at 37 °C. The optical density at 600 nm was recorded at the end of the incubation period. The percent of bacterial inhibition was calculated with following the equation:

$$(\%) \text{ Inhibition}: \left(\left[OD_{control} - OD_{sample}\right]/OD_{control}\right) \times 100$$

where $OD_{control}$ and OD_{sample} represent the optical density of the control and samples, respectively. Each sample was assayed in triplicate.

Among the CH and peptide fractions (< 3 kDa and 3–10 kDa), those peptide fractions with the highest antimicrobial activity were further selected to assess the effect of the peptide fraction on bacteria growth kinetic parameters and on membrane permeability of selected bacteria strains.

Effect of Peptide Fraction < 3 kDa on Bacteria Growth Kinetic Parameters

E. coli ATCC 25922, *S. enterica* serovar Typhimurium K1028, and *L. monocytogenes* 10403S were selected to explore the effect of peptide fraction < 3 kDa on bacteria growth kinetic parameters, because this peptide fraction showed the highest antimicrobial activity, with bacteria inhibition values > 60%. The experiment was performed under the same conditions described before using a microplate assay. The optical density at 600 nm was recorded every hour for 24 h. The growth curves were constructed after sample blank subtraction, containing media and peptide sample, from the OD reading. The growth kinetic parameters that were estimated included the growth rate (OD/h), lag time (h), and the maximum population density (OD) at the stationary phase, with the mechanistic modeling technique of Baranyi and RobertsBaranyi [18] (D-model, excel add-in DMFit v.3.5, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom).

Effect of Peptide Fraction < 3 kDa on Membrane Permeability

The alteration of the membrane permeability was evaluated using the crystal violet assay [19, 20]. The experiment was performed under the same conditions and strains described in the previous section using a microplate assay. Briefly, after 24 h of incubation, the untreated cells (control) and peptide-treated cells were collected by centrifugation, washed twice with PBS, and re-suspended in a crystal violet solution (2 μg/mL) and incubated during 30 min at 37 °C. Finally, the supernatant was collected by centrifugation and the OD was measured at 600 nm. The OD value of crystal violet solution was considered as 100%. The percentage of crystal violet uptake was expressed as follows:

%uptake of crystal violet

$$= (OD \text{ of sample}/OD \text{ crystal violet solution}) \times 100$$

Then, within the < 3 kDa fraction, those peptides that showed the highest effect on the growth parameters and percentage of crystal violet uptake were selected to assess their effect on bacterial cell wall integrity and to identify the possible peptides with bioactivity.

Effect of < 3 kDa Peptide Fraction on Bacterial Cell Wall Integrity by Scanning Electron Microscopy

The < 3 kDa fraction with the highest antimicrobial activity was selected to assess its effect on bacterial cell integrity. Untreated cells (control) and peptide-treated cells were visualized using a FEI NOVA nanoSEM field emission scanning electron microscope (FEI Company, Hillsboro, Oregon). Briefly, cells (1 mL at $OD_{600nm} = 0.5$) were centrifuged in order to remove the growth media and resuspended with glutaraldehyde (2.5%) for 1 h at room temperature. A drop of fixed cells in suspension was allowed to settle onto round poly-L-lysine-coated glass coverslips for 15 min. After, cells were rinsed three times with 0.1 M cacodylate buffer for 5 min each. Next, the cells were post-fixed with 1% osmium tetroxide at room temperature for 1 h and rinsed three times with distilled water for 5 min each. After washing, the samples were dehydrated in increasing concentrations of ethanol (50%, 75%, 95%, ≥ 99.5% *v/v*) for 5 min each. Finally, the dehydrated samples were immersed in hexamethyldisilazane (HMDS) three times (ethanol/HMDS 1:1, 100%, 100%) for 15 min each and were allowed to air-dry at room temperature overnight. The dried cells were mounted on to a SEM sample stub, sputter-coating with platinum and placed into the argon-vacuum chamber for 60 s at 45 °C.

Characterization and In Silico Antimicrobial Analysis of Select < 3 kDa Peptides

The identification of the < 3 kDa peptide fraction with the highest antimicrobial activity was carried out by liquid chromatography–mass spectrometry (LC-MS/MS) technique in the Proteomics Core facility at the Indiana University School of Medicine (Indianapolis, Indiana). Each identified peptide was analyzed using the *i*AMPpred in order tool to predict their antimicrobial potential score (AntimPS) based on their amino acid sequences (http://cabgrid.res.in:8080/amppred/server.php). The *i*AMPpred tool estimates the predicted antimicrobial activity score of a peptide in terms of probability with values that vary from 0 to 1. Values near 1 indicate peptides with high probability to present antimicrobial activity. Therefore, if the probabilities are > 0.5 in each case, the sequence is said to be predicted as antimicrobial (http://cabgrid.res.in:8080/amppred/img/User_Manual.pdf). Then, the peptides with AntimPS > 0.5 were selected in order to characterize their biochemical parameters using ExPASy ProtParam tool (https://web.expasy.org/protparam) that included the theoretical isoelectric point (pI), charge, aliphatic index, and grand average of hydropathy (GRAVY). In the present work, the prediction results were interpreted with the following proposed cutoff values for selection of probable activities: AntimPS > 0.84 as high probability to present antimicrobial activity, 0.67 > AntimPS < 0.83 as intermediate probability to present antimicrobial activity, and 0.5 > AntimPS < 0.67 as low probability to present antimicrobial activity.

Statistical Analysis

Each experiment was repeated three times, and all tests were run in triplicate for each experiment. The statistical analysis of experimental data was made using ANOVA followed by Tukey's test. Differences were considered to be significant when $p < 0.05$. All analyses were performed using the NCSS software version 2007 (NCSS Statistical software, Kaysville, UT, USA).

Results

Antimicrobial Activity

Our results (Table 1) showed that the peptide fraction < 3 kDa (at 0.5 mg/mL protein) exhibited higher ($P < 0.05$) antimicrobial activity than both the chia seed hydrolysate (CH) and the peptide fraction of 3–10 kDa. Overall, CH showed < 51% inhibition for all bacterial strains. Conversely, the peptide fraction

Table 1 Antimicrobial activity (expressed as % inhibition) of chia hydrolysates and molecular weight peptide fractions (0.5 mg/mL) against different bacteria strains

Sample code	Peptide fraction		
	CH % Inhibition	< 3 kDa	3–10 kDa
Escherichia coli ATCC 25922			
A-WB	24.73 ± 0.35 a	34.98 ± 0.95 a	36.84 ± 4.96 a
A-MW	14.25 ± 6.83 b	19.72 ± 9.8 b	18.49 ± 4.49 b
AF-WB	31.68 ± 0.17 c	39.39 ± 1.31 a	40.47 ± 2.15 a
AF-MW	46.43 ± 1.82 d	61.93 ± 5.10 c	42.07 ± 1.45 a
Escherichia coli O157:H7			
A-WB	20.91 ± 4.05 a	49.95 ± 1.61 a	27.56 ± 2.92 a
A-MW	40.72 ± 4.79 b	13.55 ± 3.17 b	29.03 ± 4.14 a
AF-WB	32.23 ± 2.72 c	24.03 ± 4.31 c	36.46 ± 1.01 b
AF-MW	21.02 ± 0.95 a	56.65 ± 1.50 d	32.72 ± 7.65 b
Salmonella enterica serovar Typhimurium K1028			
A-WB	29.94 ± 2.41 a	46.44 ± 5.01 a	9.15 ± 0.76 a
A-MW	28.50 ± 0.23 a	43.50 ± 1.35 a	40.45 ± 2.87 b
AF-WB	36.93 ± 0.92 b	47.98 ± 5.47 a	24.56 ± 3.64 c
AF-MW	45.56 ± 1.89 c	65.05 ± 3.376 b	62.32 ± 2.17 d
Listeria monocytogenes 10403S			
A-WB	28.69 ± 1.63 a	37.31 ± 3.69 a	31.32 ± 2.21 a
A-MW	35.80 ± 7.87 b	37.34 ± 4.66 a	47.94 ± 3.34 b
AF-WB	40.72 ± 2.68 c	51.68 ± 2.58 b	37.70 ± 6.87 a
AF-MW	50.84 ± 4.62 d	60.97 ± 3.85 c	67.19 ± 2.55 c
Listeria innocua ATCC 33090			
A-WB	28.94 ± 3.78 a	50.85 ± 1.87 a	23.02 ± 0.32 a
A-MW	25.86 ± 2.81 a	56.46 ± 2.26 ab	31.62 ± 1.89 b
AF-WB	41.01 ± 2.65 c	60.46 ± 1.04 b	37.83 ± 2.14 c
AF-MW	35.69 ± 4.48 d	51.73 ± 4.48 a	28.89 ± 2.86 b

Values are mean ± standard deviation of triplicate determinations. Different lowercase letters indicate statistical difference among treatments (columns) for each bacteria. CH: chia seed hydrolysate, < 3 kDa and 3–10 kDa indicate the molecular weight fractions, A-WB: chia seed protein hydrolyzed by alcalase enzyme using water bath heating method, A-MW: chia seed protein hydrolyzed by alcalase enzyme using microwave-assisted hydrolysis, AF-WB: chia seed protein hydrolyzed sequentially by alcalase and flavourzyme enzymes using water bath heating method, AF-MW: chia seed protein hydrolyzed sequentially by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis. The control of each bacteria did not exhibit any antimicrobial activity (data not shown)

3–10 kDa showed > 50% of bacterial inhibition on *S. enterica* serovar Typhimurium K1028, and *L. monocytogenes* 10403S, while the peptide fraction < 3 kDa showed > 50% of bacterial inhibition on all bacterial strains. Among the < 3 kDa fractions, those derived from the microwave-assisted hydrolysis using alcalase and flavourzyme (AF-MW) had the highest antimicrobial activity against *E. coli* ATCC 25922 (61.93% inhibition), *S. enterica* serovar Typhimurium K1028 (65.05% inhibition), and *L. monocytogenes* 10403S (60.97% inhibition).

Growth Kinetic Parameters

Based on our results, the < 3 kDa peptide fraction from all chia seed hydrolysate treatments was further selected to evaluate its effect on bacteria growth parameters such as the growth rate (OD/h), lag time (h) and the maximum population density at the stationary phase (OD), and on membrane permeability. Coefficients of determination ($R^2 = 0.96$–0.99) indicated that the model was able to describe microbial growth accurately ($P < 0.05$). Overall, the results show a significant ($P < 0.05$) extension in lag phase, decreases in the maximum growth and the growth rate in the peptide treatments compared to the control (Table 2). Particularly, the peptide fractions obtained by sequential hydrolysis (AF-WB and AF-MW) showed remarkable decrease on the growth rate and maximum growth on the three bacterial strains tested. Our results (Table 3) confirmed that the < 3 kDa peptide fractions obtained by sequential enzymatic hydrolysis (AF-WB and AF-MW) have the major antimicrobial activity on the three bacterial strains tested (*E. coli* ATCC 25922, *S. enterica* serovar Typhimurium K1028, and *L. monocytogenes* 10403S). Particularly, the peptide fraction obtained by the AF-MW treatment was the one that showed remarkable increases in membrane permeability on *E. coli* ATCC 25922 (71.49% crystal violet uptake) and

Table 2 Effects of < 3 kDa peptide fraction (0.5 mg/mL) on growth parameters of bacterial strains

Treatment	Lag (h)	Growth rate (O.D./h)	Y max (O.D.)	R^2
Escherichia coli ATCC 25922				
Control	3.71 a	0.19 a	1.04 a	0.98
A-WB	3.84 a	0.14 b	0.64 b	0.97
A-MW	5.50 b	0.15 b	0.73 b	0.97
AF-WB	5.51 b	0.22 a	0.60 b	0.99
AF-MW	4.44 c	0.09 c	0.37 c	0.96
Salmonella enterica serovar Typhimurium K1028				
Control	2.27 a	0.08 a	0.62 a	0.96
A-WB	6.25 b	0.16 b	0.34 b	0.97
A-MW	4.04 c	0.08 a	0.33 b	0.96
AF-WB	3.55 ab	0.07 a	0.32 b	0.97
AF-MW	5.25 c	0.05 a	0.21 c	0.96
Listeria monocytogenes 10403S				
Control	4.50 a	0.10 a	1.37 a	0.98
A-WB	4.95 b	0.10 a	0.98 b	0.99
A-MW	5.95 c	0.24 b	0.91 b	0.96
AF-WB	5.80 c	0.07 c	0.83 c	0.99
AF-MW	3.94 d	0.06 c	0.70 d	0.99

Values are mean ± standard deviation of triplicate determinations. Different lowercase letters (in columns) indicate statistical difference among treatments for each bacteria. Sample codes are defined in Table 1. Control = bacteria treated with PBS instead of peptide fractions

L. monocytogenes 10403S (80.10% crystal violet uptake). Because of their highest antimicrobial activity, within the < 3 kDa fractions, the peptides obtained from the microwave-assisted sequential enzymatic hydrolysis (AF-MW) treatment were selected in order to study their effect on bacteria cell wall integrity using scanning electron microscopy (SEM) and subjected to liquid chromatography–mass spectrometry in order to identify the possible peptides involved in bioactivity through an in silico analysis.

Cell Wall Integrity

The SEM image of untreated cells of selected control bacteria showed uniform structures with a smooth surface and with intact morphology. In contrast, in the peptide-treated cells, multiple indentations (transmembrane tunnels), membrane wrinkling, and pronounced deformations (Fig. 2a) were observed. In addition, some cells suffered lysis (membrane rupture and collapsed structure) of the outer membrane integrity, thus they collapsed due to the loss of the intracellular content (Fig. 2b, c).

In Silico Analysis of Identified Peptides

We identified close to 1700 peptide sequences in the AF-MW < 3 kDa peptide fraction by LC-MS/MS (data not shown). For the in silico analysis, these peptide sequences were analyzed for their antimicrobial potential score (AntimPS). The AntimPS was determined using the iAMPpred tool, and those peptides with AntimPS > 0.5 were selected for their biochemical parameters characterized by ExPASy ProtParam tool. A total of 279 sequences had AntimPS > 0.5 and are disclosed in the supplementary data (Table S1).

In this context, the biochemical characterization of theses peptides was done calculating their charge (net charge), hydrophobicity (grand average of hydropathy [GRAVY]), isoelectric point (theoretical), and the thermostability (aliphatic index) (Supplementary data Table S1). Overall, according to the bioinformatic studies, 25 peptides have an AntimPS > 0.84, which suggested that they have high probability to present antimicrobial activity. Our results with these peptides revealed that 67% (16 peptides) are cationic peptides due to their positive charge (+ 1 to + 2), and 63% (15 peptides) are hydrophobic according to their positive GRAVY index (0.078 to 1.429). Additionally, these peptides showed a wide theoretical isoelectric point in the range of 5.52 to 9.75, of which 67% (16 peptides) showed an isoelectric point above 8. Their aliphatic index showed values in the range of 24.19 to 222.86, of which 100% (24 peptides) showed thermostability according to their positive values of aliphatic index. In addition, 66 peptides have an AntimPS between 0.67 and 0.83 and 189 between 0.5 and 0.67, suggesting an intermediate and low probability to present antimicrobial activity, respectively.

Table 3 Percentages of crystal violet uptake by bacterial strains after the treatment with < 3 kDa peptide fractions (0.5 mg/mL)

Sample code	*Escherichia coli* ATCC 25922	*Salmonella enterica* serovar Typhimurium K1028	*Listeria monocytogenes* 10403S
A-WB	29.76 ± 3.50 a	55.65 ± 1.97 a	64.40 ± 1.29 a
A-MW	41.42 ± 0.31 b	66.07 ± 4.65 ab	54.63 ± 0.94 b
AF-WB	45.47 ± 5.23 b	73.09 ± 1.86 b	62.50 ± 1.25 a
AF-MW	71.49 ± 0.81 c	61.07 ± 7.40 ab	80.10 ± 7.68 c
Control	16.90 ± 1.44 d	20.35 ± 1.46 c	20.35 ± 1.6 d

Values are mean ± standard deviation of triplicate determinations. Different lowercase letters indicate statistical difference among treatments for each bacteria. Sample codes are defined in Table 1. Control = bacteria treated with PBS instead of peptide fractions

Of the 279 sequences obtained from the AF-MW < 3 kDa fraction, 16 peptide sequences were determined to have the best AntimPS and cationic charge (Table 4). Of the 16 sequences, 7 of them have the specific peptide sequence of GDVIAIR and 8 of them have the amino acid K as either N- or C-terminal or both.

Discussion

The current study was aimed to assess the antimicrobial properties of hydrolysates and peptide fractions from chia seeds. Our data indicated that the peptide fraction < 3 k\Da was the most effective fraction than both chia seed hydrolysate and peptide fraction 3–10 kDa. These results could be due to the lower molecular weight peptides that have an important role in

antimicrobial activity [21]. Even though the antimicrobial activity of low molecular peptides is not entirely well understood, it has been reported that their smaller size may facilitate their access to target sites on the bacterial surface [22]. Peptides can also translocate into the bacterial cell, via a "Trojan-horse" like mechanism, interfering with a series of cellular processes and metabolic functions of bacterial strains [23]. A study demonstrated that a hydrolyzed fraction derived from goat milk caseins with low molecular weight (< 3 kDa) showed the highest antimicrobial activity than those exhibited by the 3–5 or 5–10 kDa fractions [24]. Similarly, milk-derived peptides (< 3 kDa) obtained from fermented milks with specific *Lactobacillus plantarum* strains exhibited higher, or at least equal, to the 3–10 kDa peptide fraction against diverse bacterial strains. Tomita et al. [22] found that the antimicrobial potency of the lactoferrin hydrolysate fraction (< 6 kDa)

Table 4 Select peptide sequences derived from the AF-MW treatment < 3 kDa fraction, based on charge and highest antimicrobial potential score

No.	Peptide sequence	Antimicrobial potential score	Theoretical pI	Charge	Aliphatic index	GRAVY
1	KADVPGLKK	0.980	9.70	+ 2	86.67	− 0.822
2	KGDVIAIR	0.944	8.75	+ 1	146.25	0.338
3	LKQGDVIAIR	0.933	8.75	+ 1	24.19	0.300
4	GNIFRGL	0.931	9.75	+ 1	111.43	0.329
5	FQKGDVIAIR	0.926	8.75	+ 1	117.00	0.200
6	KGLVLPF	0.923	8.75	+ 1	152.86	1.243
7	GDKFIQVVNHK	0.907	8.60	+ 1	88.18	− 0.564
8	IGTPGKGIL	0.900	8.75	+ 1	130.00	0.600
9	QKGDVIAIR	0.892	8.75	+ 1	130.00	− 0.089
10	KQGDVIAIR	0.891	8.75	+ 1	130.00	− 0.089
11	QKGDVIAIRA	0.891	8.75	+ 1	127.00	0.100
12	KQGDVIAIR	0.891	8.75	+ 1	130.00	− 0.089
13	DVPGLKK	0.890	8.59	+ 1	97.14	− 0.757
14	GGVLPGIK	0.850	8.75	+ 1	133.75	0.725
15	HGPIKLH	0.845	8.76	+ 1	111.43	− 0.571
16	NFAVVKQ	0.842	8.75	+ 1	97.14	0.300

AF-MW: chia seed protein hydrolyzed sequentially by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis

showed an 8-fold higher inhibition than the undigested lactoferrin (80 kDa). These studies are consistent and support the results of the present study; however, more studies are needed to clarify the relationship between the molecular weight of the peptide and their antimicrobial activity.

In related works, protein hydrolysates derived from other plant seeds have exhibited antimicrobial activity against different bacterial strains. Song et al. [25] investigated the antimicrobial activity of different peptide fractions (F I-V) derived from cottonseed protein hydrolysates against *E. coli* ATCC 25922 with values in the range of 30 to 80% inhibition at 2 mg/mL protein. Authors observed that with the increase in positively charged peptide content as well as the lower content in negatively charged amino acids (from F–I to F-IV), the antimicrobial activity against *E. coli* increased. Because procedures for evaluating antimicrobial activity may vary, such as the way to express the results (e.g., % of survival population, % bacterial inhibition, inhibition zone diameter), comparison of results from different studies is as difficult as it is to distinguish only one mechanism or compound involved in the antimicrobial activity.

Kobus-Cisowska et al. [26] reported that different amaranth and quinoa protein hydrolysates, generated using different enzymes (Bromelain, Chymotrypsin, and Protease), showed a zone of inhibition ranging from 14 to 20 mm and 16 to 20 mm, respectively, against two Gram positive (*S. aureus* ATCC 15923 and *L. monocytogenes* ATCC 7644) and three Gram negative (*S. typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, and *E. coli* ATCC 35218) bacteria. In a similar study, Sonawane et al. [27] reported the antimicrobial activity of wood apple (*Limonia acidissima*) and watermelon (*Citrullus lanatus*) protein hydrolysates (at different protein concentration ranging from 50 to 300 mg/mL) against different bacteria strains (*E. coli* ATCC 8739, *S. typhi* ATCC 6539, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 13883). Their results showed that wood apple hydrolysates showed relatively larger inhibition zones (6–21 mm) as compared to watermelon hydrolysates (0–16 mm) against *S. typhi*, *Ps. aeruginosa*, *E. coli*, and *Kl. pneumoniae*. In addition, the antimicrobial activity observed was dose-dependent. On the other hand, there are few studies concerning the antimicrobial activity of protein hydrolysates derived from chia seeds, which have reported contradictory results. For example, chia seed protein hydrolysates (10 mg/mL) showed good antimicrobial properties against *S. aureus* (inhibition zones with diameter > 14 mm) and in lesser extent against *E. coli* (inhibition zones with diameter < 10 mm) [12], whereas, in another study, disk inhibition was not observed for chia protein hydrolysates (50 mg/mL) towards *E. coli*, *S. typhi*, *Shigella flexneri*, *Kl. pneumoniae*, *S. aureus*, *Bacillus subtilis*, and *Streptococcus agalactae* [13]. These differences observed may be attributed to diverse factors including the enzymatic hydrolysis

parameters used to obtain the peptides (e.g., enzyme to substrate ratio, hydrolysis time, single or sequential enzyme use, and incubation temperature), methods used for the determination of antimicrobial properties (e.g., agar disk diffusion, microtiter plate-based assay), and the peptide concentration.

Furthermore, our results indicate that peptides present in the < 3 kDa peptide fraction from chia seed hydrolysates have the ability to inhibit bacteria growth. Scientific literature shows that the antimicrobial mechanism of action of peptides is dependent on diverse factors such as the amino acid composition as well as their structure and physicochemical characteristics [28, 29]. Also, other studies have reported that antimicrobial peptides may act by inhibiting DNA, RNA, and protein biosynthesis or by establishing an electrostatic bonding between the peptide and bacterial membrane components that disturb the membrane permeability [30–32]. In this context, given that the lag phase takes place before cellular division occurs, our results indicate that the bacteria are struggling in adapting to the system conditions (new environment with the peptides); therefore, their growth rate is disturbed and the inhibition of the maximum growth occurs (Fig. 1). This extension may be due to the impairment of cell membrane functionality by inhibiting the absorption of nutrients or by affecting some key enzymes or membrane proteins essential for cellular multiplication. Additionally, the metabolic mechanism could have been slowed down because the efficiency of biomass production from available resources has been compromised. Conversely, the alteration in membrane permeability was observed through crystal violet uptake, because this compound displays weak penetration of the outer membrane, but on the contrary, it has been found to penetrate cells with impaired cell membranes [20].

In contrast, the data collected on the morphology of the strains visualized by SEM suggest that the peptides contained in the fraction < 3 kDa do exert antibacterial activity acting as membranolytic agents, but it is possible that other molecular mechanisms of action involving one or more intracellular targets are also implicated. However, our results show that the dominant antimicrobial mechanism of the peptides is the alteration of membrane permeability. This could be a result of establishing electrostatic bonding between the peptide and the components in the bacteria membrane, forming pores or transient transmembrane channels that were seen by SEM after the treatment with < 3 kDa peptide fraction obtained from AF-MW. Also, it was observed that the peptides in the AF-MW < 3 kDa fraction have different modes of action against Gram-negative and Gram-positive bacteria, which is associated with the capacity of these peptides to penetrate inside the cytoplasm of the microorganisms, as well as with the differences in their membrane structure (Fig. 2). Gram-positive bacteria (e.g., *E. coli*) have cell wall mainly composed of a thick layer of peptidoglycan, whereas Gram-negative bacteria (e.g., *Listeria*) have a layer of lipopolysaccharide at the external
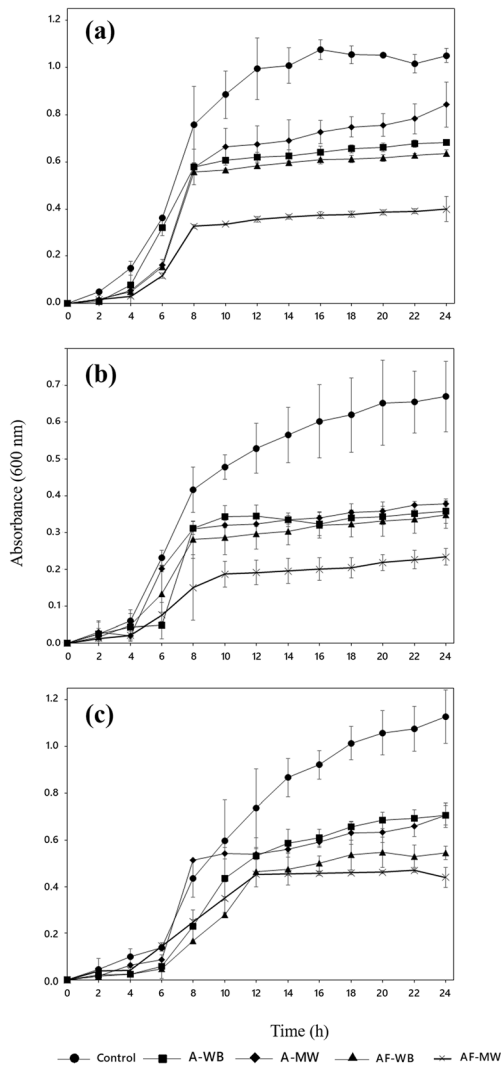
Fig. 1 Effect of peptide fraction < 3 kDa (0.5 mg/mL) from chia seed hydrolysate treatments on bacteria growth of **a** *Escherichia coli* ATCC 25922, **b** *Salmonella enterica* serovar Typhimurium K1028, and **c** *Listeria monocytogenes* 10403S. Sample codes are defined in Table 1. Control = bacteria treated with PBS instead of peptide fractions
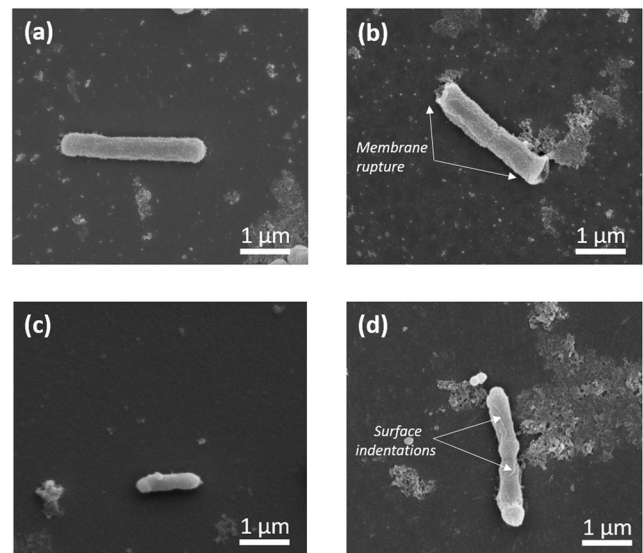

Fig. 2 SEM micrographs of untreated (**a** and **c**) and treated (**b** and **d**) cells of *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* 10403S, respectively. Cells were treated with <3 kDa peptide fraction (0.5 mg/mL) obtained from the AF-MW treatment. Arrows indicate the possible damage. AF-MW: chia seed protein hydrolyzed sequentially by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis

surface followed by a thin layer of peptidoglycan. However, no apparent relationship was observed between the antimicrobial activity of the peptides tested and the bacterial membrane structure. Then, our results suggest that the antimicrobial activity of peptides tested in the present study has different modes of action with broad spectrum against Gram-positive and Gram-negative strains, which are related to the ability of these peptides to interact with bacterial membrane, which depends upon its structure and the membrane target, and/or with their ability to disrupt the metabolism of the bacteria. Similar observations were reported using bioactive peptides derived from half-fin Anchovy (*Setipinna taty*) against *E. coli* [33], garlic against *E. coli* and *S. aureus* [34], and fermented milk beverage (kefir) against *E. coli* and *S. aureus* [35], where the peptides induced deformation in cell morphology, and

destruction of the cell integrity via irreversible membrane damage, of various pathogens.

On the other hand, there are several properties characteristic to antimicrobial peptides that are related mainly to their charge and hydrophobicity. The identified peptides showed cationic and hydrophobic character, as well as termostability. In this regard, in general, antimicrobial peptides have a positive charge, which provides binding specificity to the negatively charged bacterial membranes through electrostatic interaction. Additionally, their hydrophobic nature promotes their penetration into the hydrophobic core of the bacterial membrane in order to destabilize the bilayer and/or promote the cell depolarization [36]. Other authors indicate that antimicrobial peptides have an isoelectric point close to 8–12 [37], and this is also an important factor that affects the solubility of the peptides [38]. Finally, it has been reported that antimicrobial peptides tend to be more thermostable than native protein in general [39]. These last two factors could allow their application in a wide variety of food products as ingredients or incorporated into bioactive films. Besides, of the total identified sequences, seven of them have the specific peptide sequence of GDVIAIR and eight of them have the amino acid K as either N- or C-terminal or both. These observations indicate a potential role of this particular sequence on the antimicrobial effect observed for this particular peptide fraction (< 3 kDa, AF-MW). It has been reported that the amino acid K plays an important role in antimicrobial activity. A study by Misawa [40] evaluated the role of K in rationally designed and synthetized K-based peptides with the same amino acid composition (containing also the amino acids L and A), but with

different distribution of the K residues. The authors found that in the sequences in which the K residues were located at N-terminal position or on one side of the helical structure, showed potent and broad antimicrobial activity towards different Gram-positive and Gram-negative bacterial strains. In a similar study on frog skin peptides, Shang [41] found that by substituting neutral and non-polar amino acid residues on the hydrophilic face of the α-helix (i.e., Temporin-1CEb) by five or six K, the antimicrobial potency increased approximately 10-fold to 40-fold in the resulting eight temporin-1CEb analog peptides.

In related works, Beaulieu et al. [42] identified peptide sequences (TITLDVEPSDTIDGVK, ISGLIYEETR, MALSSLPR, ILVLQSNQIR, ISAILPSR, IGNGGELPR, LPDAALNR, EAESSLTGGNGCAK, and QVHPDTGISK) obtained from macroalgae (*Saccharina longicruris*) peptide fractions that showed antimicrobial activity against *S. aureus*. In particular, they observed that when the peptides were evaluated individually for their antimicrobial properties, they did not exhibit bioactivity; however, when mixed, the activity was significantly improved, indicating a synergistic interaction among the peptides. Bearing this in mind, our data obtained by the in silico analysis suggest that the peptides, mainly with cationic and hydrophobic character, contained in the < 3 kDa fraction obtained by the AF-MW treatment might possess antimicrobial activity and they could also act synergistically. However, further mechanistic studies must be pursued in order to clarify the relationship for the structure-antimicrobial activity of these peptides. Studies with individual peptides (either purified or synthetized) are required in order to determine the potential specific participation of each peptide on the total antimicrobial properties of the peptide fraction from which they derived, as well as their possible interactions (e.g., synergistic, antagonistic, or additive interactions).

Additionally, in vitro and in vivo studies should be performed to evaluate the impact of chia seed peptides on gut microbiota populations. This future direction is important because the microbiome plays an important role in maintaining intestinal and systemic health; therefore, if the gut microbiota is positively altered using bioactive peptides, these will constitute a new peptide-based approach for modulating and in the preservation of a healthy microbiome [43].

Conclusion

The present study reported the antimicrobial activity of chia seed protein hydrolysates. We observed that the < 3 kDa peptide fraction had greater antimicrobial activity than chia seed hydrolysate or the 3–10 kDa fractions. Particularly, the < 3 kDa fraction obtained from microwave-assisted enzymatic

hydrolysis (AF-MW) had the highest antimicrobial activities (> 60% inhibition) in most bacterial strains. These peptides caused a significant extension in lag phase, a decrease in the maximum growth and growth rate of the strains treated, and promoted multiple indentations (transmembrane tunnels), membrane wrinkling, and pronounced deformations in the integrity of the bacterial cell membranes. In addition, peptides tested in the present study have different modes of action with broad spectrum against Gram-positive and Gram-negative strains, which are related with the ability of these peptides to interact with the bacterial membrane, and/or with their ability to disrupt the metabolism of the bacteria. Finally, the identified peptides in < 3 kDa AF-MW fraction contained 16 sequences with cationic and hydrophobic character, with seven of them sharing the exact same sequence (GDVIAIR) as well as eight of them having the amino acid K as either N- or C-terminal or both. Our results demonstrated that the sequential hydrolysis with alcalase and flavourzyme, particularly those obtained with microwave-assisted hydrolysis, improved the antimicrobial activity of chia seed protein hydrolysates, suggesting that these peptides have potential to be used not only in the prevention of food-borne diseases but also food spoilage. However, further studies are needed to determine their precise mechanism of action, to clarify the relationship with peptide structure-activity, and to validate the biological activities in food (e.g., microbial challenge study, product shelf-life study) and in vivo models.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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