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Microscopic and metabolomics analysis of the anti-*Listeria* activity of natural and engineered cruzioseptins

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ABSTRACT

Listeria monocytogenes is a human opportunistic foodborne pathogen that produces life-threatening infections with a high mortality rate. The control of Listeria in the food production environment and effective clinical management of human listeriosis are challenging due to the emergence of antibiotic resistance. Hence we evaluate the *in vitro* anti-Listeria activity of two synthetic cruzioseptins reproducing their natural sequences CZS-9, and CZS-12, and one engineered sequence based on CZS-1, named [K4K15] CZS-1. The assessment of the *in vitro* potential of cruzioseptins, highlighted the promising antibacterial effect of [K4K15]CZS-1 in very low concentrations (0.91 μM) and its thermal stability at high-temperature conditions, is compatible with the food industry. Microscopic and metabolomic analyses suggest cruzioseptin induces anti-*Listeria* bioactivity through membrane disruption and changes in the intracellular metabolome. We also report that [K4K15]CZS-1 is not resistant to peptidases/proteases emphasizing a key advantage for their use as a food preservative. However, there is a need for further structural and functional optimisations for the potential clinical application as an antibiotic. In conclusion, [K4K15]CZS-1 stand out as membrane-active peptides with the ability to induce shifts in the bacteria metabolome and inspire the development of strategies for the prevention of *L. monocytogenes* emergence and dissemination.

1. Introduction

Listeriosis is a medically important foodborne infection with severe health consequences caused by the ingestion of contaminated food products with *Listeria monocytogenes* [1,2]. This Gram-positive pathogen possesses biofilm-forming activity that contributes to prolonging its survival and persistence in different environments, such as dairy products, deli meats, and ready-to-eat foods, utensils, and equipment [3,4]. Additionally, *Listeria* exhibits an intrinsic ability to grow under a broad range of environmental conditions such as pH (4.3–9.8), temperatures (1–45 °C), and high salt concentrations (up to 10 % NaCl) [4]. Overall, the impact is translated into a high mortality rate in vulnerable groups and a demand for the introduction of novel agents in food safety programs [5–7].

After success in the pharmaceutical market, antimicrobial peptides (AMPs) have recently been proposed as sustainable antimicrobial preservatives for the food industry [8–10]. Novel studies are finding additional applications and emergent niches that can benefit from the multiple biological properties of AMPs. Specifically, frog-derived AMPs are natural antibacterial agents with a wide spectrum of bioactivity [11–14]. Moreover, the screening of Ecuadorian biodiversity has provided a library of new AMPs belonging to the cruzioseptin family. Recent efforts have

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characterised their effects in a diverse group of microorganisms, including Gram-negative bacteria (*Escherichia coli, Salmonella* Typhimurium), Gram-positive bacteria (*Staphylococcus aureus*), yeast (*Candida albicans*), and parasites (*Leishmania* spp.) [15–19]. Imaging modalities and spectrofluorimetric assays suggested the mechanism of action (MOA) of the cruzioseptins is the cell membrane [20].

The membrane-active mechanism is the most well-described pathway employed for AMPs to kill medically relevant pathogens [21], with different reductionist models being proposed. The combinational approach, including microscopy and omics strategies has been reshaping the current understanding of the underlying processes, creating more detailed and coherent pictures of the bactericidal activity of AMPs, which consider intracellular events and changes in transcriptomics, metabolomics and proteomics levels [22,23]. Despite, the evident beneficial insights, few studies have explored this framework in food-borne pathogens. Hence, this study aims to broaden the screening of cruzioseptin members and elucidate their impact on bacteria cells in a more holistic view.

2. Material and methods

2.1. Chemical synthesis and in silico analysis of cruzioseptin sequences

An automated microwave peptide synthesizer (Liberty Blue, CEM Corporation) was used to produce three members of the cruzioseptin family using the Fmoc (9-fluorenylmethyloxy carbonyl) strategy. The reagents used for the solid resin support, deprotection, coupling, and cleavage steps were previously described by Ref. [18]. Synthesised peptides were washed and precipitated with cold diethyl ether and then lyophilized in vacuum conditions at −80 °C and stored at −20 °C.

Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed with a binary pump chromatograph (Agilent Technologies) coupled to a C18 analytical column (250 × 4.6 mm, 5 μm) and with an ultraviolet–visible (UV–Vis) light detector at 220 nm to evaluate the purity of synthetic peptides. One mg of peptide was dissolved in 500 μL of water/trifluoroacetic acid (TFA) solution (99.9/0.1 v/v) and then injected into the equipment. Peptides were eluted using a linear gradient system from 99 % mobile phase A (99.9 % water, 0.1 % TFA) to 99 % mobile phase B (99.9 % acetonitrile, 0.1 % TFA) at a flow rate of 1 mL/min for 55 min. Finally, the chemical identity of peptides was determined by matrix-assisted laser ionization/desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik).

Physicochemical properties of peptides were predicted by the HELIQUEST server [24] and the Bachem Peptide Calculator (Retrieved from https://www.bachem.com/knowledge-center/peptide-calculator/, 10 September 2023).

2.2. Anti-Listeria activities of synthetic cruzioseptins

The minimum inhibitory concentration (MIC) and maximum bactericidal concentration (MBC) of three cruzioseptins were determined against *Listeria monocytogenes* CECT 934 through a broth microdilution assay. The procedure was based on the protocol routinely employed with minor modifications [18]. Serial dilutions of peptide concentrations (1–512 μg/mL) were prepared with dimethyl sulfoxide (DMSO). *L. monocytogenes* was grown in Brain Heart Infusion (BHI) at 37 °C until obtaining a bacterial suspension of 1×10^6 CFU/mL. The bacterial suspension was transferred to a 96-well sterile microplate, which contained peptide dilutions. As controls, we included DMSO instead of peptide and BHI sterile medium. The microplates were incubated at 37 °C for 18 h. Growth was monitored at 600 nm in a microplate reader (Synergy HTX, Biotek). The MIC value was determined as the lowest peptide concentration that did not show visible growth. Additionally, the MBC was performed by subculturing 10 μL of each concentration without bacterial growth on BHI agar plates and incubating at 37 °C overnight. The MBC value was defined as the minimal concentration without any bacterial growth.

2.3. Effect of high temperatures and autoclaving on antibacterial activity

The stability of [K4K15]CZS-1 (512 μg/mL) in higher temperatures was determined. The peptide (512 mg/L) was subjected to heat treatments (60, and 80 °C for 1 h) or autoclaved (121 °C for 15 min) and left to cool at room temperature for 30 min. After this period, the bioactivity of [K4K15]CZS-1 under these conditions was verified using the diffusion agar technique against *L. monocytogenes*. All experiments were repeated three times.

2.4. Protease stability studies

[K4K15]CZS-1, in a 512 μg/mL concentration, was incubated with trypsin (1 mg/mL). This sample was incubated for 2 or 24 h at 37 °C. Then, the reaction was stopped using TFA 0.1 %. After this incubation, the effect of trypsin treatment on the antibacterial properties was assessed using the diffusion agar method, as stated before. In addition, the peptide fragments produced under this enzymatic treatment were analysed by MALDI TOF- MS. All experiments were repeated three times.

2.5. Measurement of release nucleic acids induced by engineered cruzioseptin

A nucleic acid release assay was performed to evaluate the bacterial membrane integrity after incubation with [K4K15]CZS-1. In summary, *L. monocytogenes* CECT 934 was grown on a BHI medium at 37 °C. The bacterial suspension (optical density of 0.3) was washed and resuspended with phosphate buffer solution (PBS) two times. Then, the bacterium was exposed to different concentrations of [K4K15]CZS-1 (1 xMIC, 2 xMIC, 4 xMIC) and samples were incubated at 37 °C for 2 h. PBS instead of AMP was used as a negative control. Samples were filtered using 0.22 μm syringe filters to remove the bacterial cells and the supernatant was measured at 260 nm using a microplate reader (Synergy HTX, Biotek).

2.6. Scanning electron microscopy (SEM)

L. monocytogenes of the mid-exponential growth phase (1×10^8 CFU/mL) was harvested by centrifugation at 1000*g* for 10 min. The bacterial pellet was washed and resuspended with PBS. The mixture of bacterial cells and the peptide (4 xMIC of [K4K15]CZS-1) was incubated at 37 °C for 2 h. Later, the bacterial cells were fixed with 2.5 % (v/v) glutaraldehyde at 4 °C overnight. Subsequently, bacterial cells were washed with PBS and dehydrated through a graded ethanol series (50 %, 70 %, 90 %, and 100 % ethanol) for 10 min each. No peptide treatment was used as a negative control. Finally, all the samples were transferred to a coverslip, dried, and coated with gold. The samples were examined using a scanning electron microscope with 15 kV resolution (LEO 435VP).

2.7. Transmission electron microscopy (TEM)

Bacteria cells for TEM samples were grown and incubated with [K4K15]CZS-1, as described above for SEM sample preparation.

After pre-fixation with 2.5 % (v/v) glutaraldehyde, the bacterial pellet was washed twice with PBS. Subsequently, a post-fixation was performed with 2 % osmium tetroxide (w/v) in PBS for 2 h and cells were washed with PBS. Samples were dehydrated in a graded ethanol series (50 %, 70 %, and 90 %, for 8 min in each step) and incubated in 100 % (v/v) ethanol (10 min), a 1:1 mixture of 100 % ethanol and acetone (10 min), and pure acetone (10 min). The samples were immersed in a mixture of absolute acetone and epoxy resin (1:1) for 30 min and pure epoxy resin overnight. Finally, an ultramicrotome was used to cut ultrathin slices, which were then stained with uranyl acetate and lead citrate. Samples were examined using a transmission electron microscope (Morgagni 268D).

2.8. Metabolomic analysis: GC/MS sample preparation, derivatization and spectral acquisition

L. monocytogenes metabolite profiling was analysed through gas chromatography coupled with mass spectroscopy (GC-MS). *L. monocytogenes* was incubated under the same conditions as described in the MIC evaluation step. Bacterial cells were treated with [K4K15]CZS-1 (1x MIC) for 18 h at 37 °C, then centrifuged, washed with PBS, and dried in a vacuum concentrator. To extract intracellular metabolites, 1 mL of methanol was added to each sample, followed by a shaking step for 30 s, sonication for 15 min, and drying again in a vacuum concentrator. Three biological replicates were performed for each treatment.

For derivatization, a solution containing 100 μl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (50 % v/v) and a solvent mixture of acetonitrile/dichloromethane/cyclohexane (5:4:1) and 5 % (v/v) trimethylamine (50 %) was prepared and added to each sample. The samples were then vortexed for 30 s and incubated at 60 °C in a thermobath for 1 h, followed by centrifugation at 12000*g* for 2 min. For GC-MS (Shimadzu TQ-8050) analysis, 2 μL of the supernatant was injected at a split ratio of 1:10. Helium gas was used as a carrier at a constant flow rate of 1 mL/min. The method used was GCMSsolution Smart MRM version 4.2 and the run time for each sample was 67 min. The injection, interface and ionization source temperatures were 280 °C, 280 °C and 250 °C respectively. The initial temperature was set at 100 °C, then increased to 320 °C in a linear ramp of 4 °C/min and finally maintained at 320 °C for 8 min.

The GC-MS/MS data were analysed using the software MetaboAnalyst 5.0. To visualise the distribution and grouping of each sample, Principal Component Analysis (PCA) was used. Furthermore, the most significant metabolites of L. monocytogenes incubated with or without [K4–K15]CZS-1 were displayed. The determination of stress

indicators (biomarker meta-analysis) was performed using the meta-analysis approach based on the combination of P-values. Identified metabolites with mean local confidence scores ≥80 % were compared in the KEGG PATHWAY database.

2.9. Statistical analysis

GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. The acquired data were reported as mean ± standard deviation and evaluated using analysis of variance (ANOVA), followed by post-hoc Tukey multiple comparisons. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Peptide characterisation, purity analysis, and physicochemical properties of cruzioseptins

[K4K15]CZS-1 is a peptide inspired by the primary sequence of frog AMP cruzioseptin-1. In our recent work, the functional characterisation of that peptide was evaluated and demonstrated that this peptide inhibited the growth of *Staphylococcus aureus, Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Escherichia coli,* and *Candida albicans* [18,19]. [K4K15]CZS-1 is a 21-mer peptide with a hydrophobicity value of 0.523 and a net charge of +6 (Table 1). In addition, two AMPs, isolated from the amphibian skin secretion of *Cruziohyla calcarifer* [15], were chosen for this work. CZS-9 and CZS-12 share high similarity in the primary structure. Both peptides have a length of 27 amino acids with only four different residues (Table 1). Another difference is that CZS-12 is more cationic than CZS-9, while CZS-9 is more hydrophobic than CZS-12.

The three synthetic AMPs were chemically synthesised and presented a purity of 82.37 % for CZS-9, 89.92 % for CZS-12, and 94.48 % for [K4K15]CZS-1 (Fig. 1). The chemical identity of cruzioseptins was corroborated through mass spectra and the theoretical mass (Fig. 1 and Table 1).

3.2. Antimicrobial activity of cruzioseptins

The antibacterial activity of the peptides was tested against *L. monocytogenes* CECT 934 (Table 2). The three cruzioseptins displayed antibacterial activity against the foodborne pathogen *L. monocytogenes*. These results are in line with other studies where AMPs isolated from bacteria, animals, and plants also showed antilisterial properties [5,25–27]. Overall, [K4K15]CZS-1 was the most active peptide, followed by CZS-12, with CZS-9 the least potent. Indeed, [K4K15]CZS-1 inhibited bacterial growth in the same concentration (2 μg/mL) as defensin-1, human defensin-2, and thionin [27]. On the contrary, other previously reported AMPs displayed antibacterial activity at concentrations >1 mg/mL [5,25,26]. This fact reaffirms the strong inhibition displayed by our engineered cruzioseptin, [K4K15]CZS-1, which could be considered a good candidate for development as a biopreservative agent. Furthermore, [K4K15]CZS-1 induced low toxicity against red blood cells and macrophage cells at the anti-listerial concentration [18,19].

Considering the amino acid sequences and physicochemical properties of these studied peptides, the increase in the charge net may contribute to anti-listerial bioactivity, as previously reported [28,29]. The positively charged residues in the primary sequence are required for interaction with the anionic membrane surface of bacteria, which is the initial step in the membranolytic effect of AMPs [30].

Table 1
Primary sequences and physicochemical characteristics of cruzioseptins.

AMPs	Sequence	Length (aa)	Net Charge	Hydrophobicity <H>	Theoretical mass (Da)
CZS-9	GFLDVITHVGKAVGKAALNAVNEMVNQ-NH_2	27	+1	0.393	2795.26
CZS-12	GFLDVVKHVGKAVGKAALNAVNDLVNQ-NH_2	27	+2	0.335	2776.22
[K4K15]CZS-1	GFLKIVKGVGKVALKAVSKLF-NH_2	21	+6	0.523	2201.8

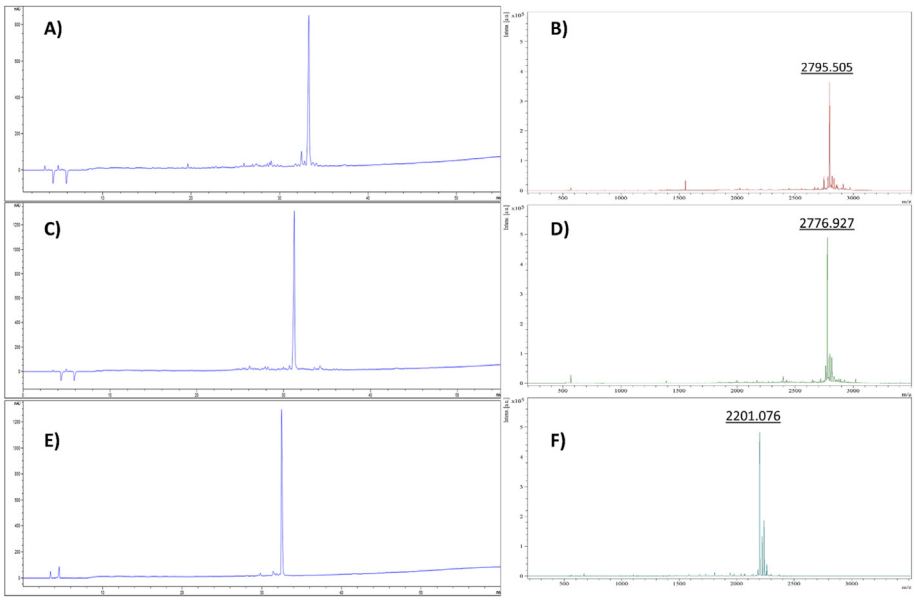

Fig. 1. Chromatogram report and mass spectra of synthetic cruzioseptins: A-B) CZS-9 with a molecular mass of 2795.505 Da, (C–D) CZS-12 with a molecular mass of 2776.927 Da, and (E–F) [K4K15]CZS-1 with a molecular mass of 2201.076 Da.

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cruzioseptins against *L. monocytogenes*.

Listeria monocytogenes CECT 934	*Listeria monocytogenes* CECT 934	
AMPs	MIC μg/mL (μM)	MBC μg/mL (μM)
CZS-9	512 (183.17)	>512 (>183.17)
CZS-12	128 (46.11)	256 (92.21)
[K4K15]CZS-1	2 (0.91)	16 (7.26)

3.3. [K4K15]CZS-1 is thermostable but degraded in proteolytic environments

Some food manufacturing processes involve thermal treatments [31]. Therefore, heat stability is a major criterion for the application of AMPs as biopreservatives. Due to the antimicrobial potency of [K4K15]CZS-1, this peptide was chosen to evaluate its stability after thermal treatment. For this purpose, an agar diffusion method was used to determine if the peptide retained its antibacterial potency under several temperature conditions (Table 3). Indeed, the anti-listerial activity of [K4K15]CZS-1 was not altered by heat treatments. Similar findings have been reported for AMPs from different sources, such as bacteria [29] and *de novo* design [32].

Proteolytic degradation of AMPs into their constitutive amino acids could be considered an additional safety aspect of the peptide for biopreservative use [33]. In this sense, trypsin was used as the proteolytic enzyme due to its presence in the human gut [34]. Trypsin cleaves peptide bonds at the C-terminal side of arginine and lysine residues, but this protease is not cut at this site before proline residues (-Arg-Pro- or -Lys-Pro). As shown in Table 3, [K4K15]CZS-1 loses its antibacterial activity after trypsin incubation for 2 and 24 h. Subsequently, these samples were examined by MALDI-ToF, and the results indicated that the peptide was digested (Fig. 2b). These findings suggest that [K4K15]CZS-1 will not survive the digestion process in the gut, supporting its safety in the potential application in the food industry.

3.4. Membranolytic effect of [K4K15]CZS-1 on L. monocytogenes

The cell membrane is a common target for AMPs active against Gram-positive and Gram-negative bacteria [35]. The mechanism of action of [K4K15]CZS-1 towards *L. monocytogenes* was further investigated using a nucleic acid leakage assay. The release of nucleic acids can be detected by measuring absorbance at 260 nm, as an initial assessment of compromised cell membranes. As can be seen from Fig. 3a, [K4K15]CZS-1 released a significantly higher amount of nucleic acids compared to the control ($P < 0.05$). Therefore, the membrane integrity was altered after exposure to peptide treatment, resulting in a leakage of genetic material in a concentration-dependent manner. These results were consistent with other AMPs with antimicrobial properties against Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* [36,37].

A closer understanding of the effect of [K4K15]CZS-1 (at 4 xMIC for 2 h) on the cell morphology and ultrastructure of *L. monocytogenes* was performed using SEM and TEM1. The SEM micrographs of untreated cells showed a smooth and intact surface (Fig. 3B). On the contrary, bacterial cells co-cultured with [K4K15]

Table 3
Anti-listerial activity of [K4K15]CZS-1 after heat treatment and trypsin treatments. Results are expressed as a plus sign (+) to indicate antibacterial activity or a minus sign (−) to demonstrate the lack of bioactivity after treatment.

	Treatments					
		Temperature			Trypsin	
	Control	60 °C for 1 h	80 °C for 1 h	121 °C for 15 min	1 mg/mL for 2 h	1 mg/mL for 24 h
[K4K15]CZS-1	+	+	+	+	−	−

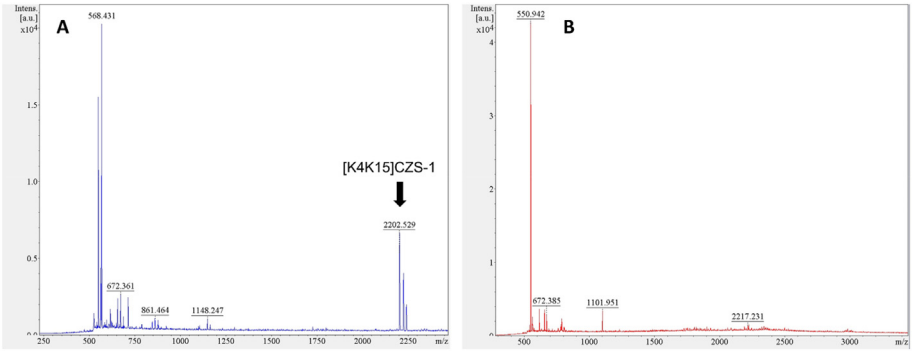

Fig. 2. MALDI-ToF MS spectra of [K4K15]CZS-1: **(A)** control without protease treatment and **(B)** trypsin-incubated peptide for 2 h.

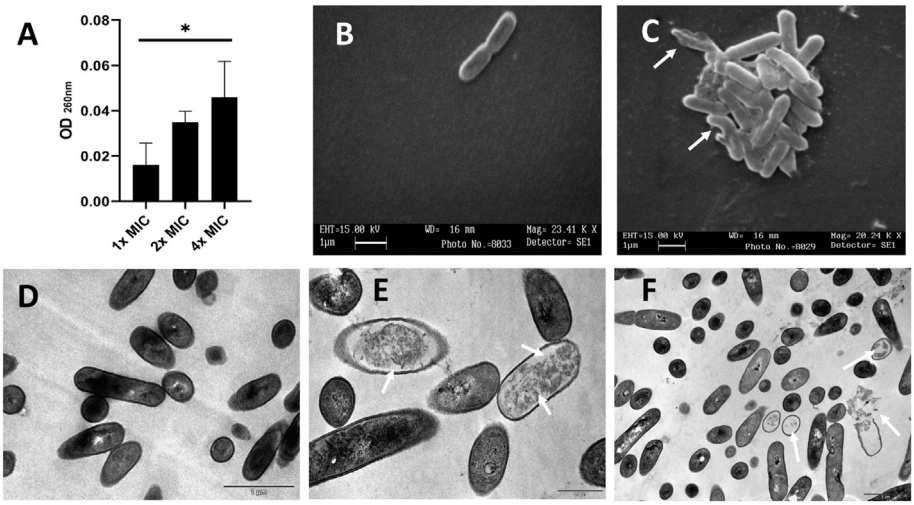

Fig. 3. Alterations in the cell membrane and intracellular content of *L. monocytogenes* after peptide treatment. **(A)** Significant release of DNA/RNA after incubation with peptide (*P < 0.05 compared to the control group). SEM micrographs of *L. monocytogenes*: **(B)** no peptide-treatment (control) and **(C)** bacterial cells treated with 4 xMIC of [K4K15]CZS-1. TEM images of *L. monocytogenes*: **(D)** control sample without peptide and **(E–F)** bacterial cells incubated with 4 xMIC of [K4K15]CZS-1. The scale bar value for 3B, 3C, 3D, and 3F is 1 μm, while for 3E is 0.5 μm.

CZS-1 displayed an abnormal morphology, including membrane roughening and corrugation (Fig. 3C). TEM observations of the control group exhibited intact cell membranes and a dense cytoplasm (Fig. 3D). Conversely, [K4K15]CZS-1 induced several alterations in the cell membrane, such as deformation, cell membrane lysis, and intracellular content leakage. In addition, the cytoplasm is condensed with empty areas inside the cells (Fig. 3E and F). The closely packed cytoplasm after peptide treatment has been observed in other studies [38,39]. Overall, we propose [K4K15]CZS-1 inhibits bacterial growth through a membrane-lytic mechanism, consistent with other reported anti-listerial AMPs [40–42].

3.5. Effect of [K4K15]CZS-1 treatment on intracellular metabolite profiles of L. monocytogenes

Identifying the MOA is a critical barrier to the discovery and development of innovative therapeutic candidates [43]. Understanding the MOA is essential for informing the design of new peptide antimicrobial therapies. Aside from their membrane-disrupting properties, AMPs can penetrate the bacterial cell and interfere with intracellular processes [44,45]. Indeed, biochemical and gel electrophoresis assays demonstrated that jelleine-1 and LL-1 interact with the bacterial membrane, bacterial DNA genomic, and energy metabolism [46,47].

The metabolomic analysis could reveal intracellular changes in

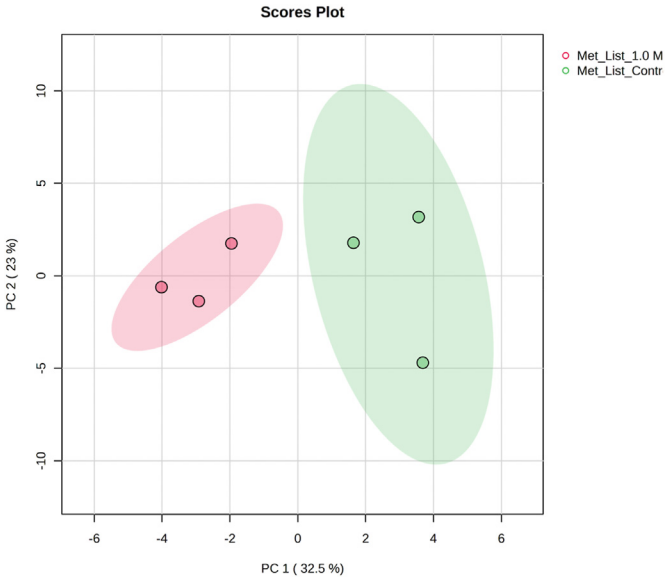

Fig. 4. Principal component analysis of intracellular metabolites of *Listeria monocytogenes* between the peptide treatment group (1 xMIC) and the control group.

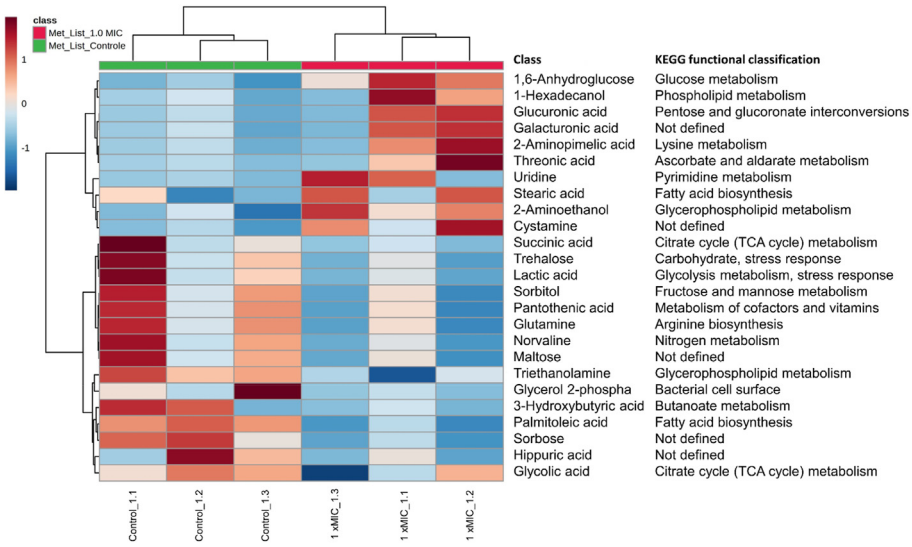

Fig. 5. Hierarchical cluster analysis of the 25 most significant metabolites with their KEGG functional classification. Intracellular metabolites identified in *L. monocytogenes* treated without or with [K4K15] CZS-1. Blue or red indicate lower or higher relative metabolite abundance, respectively.

the metabolic pathways of bacteria after peptide treatments. Characterising metabolic alterations induced by AMPs and understanding how these modulations in bacterial metabolism may deepen our knowledge about MOA. Our TEM results revealed the condensed cytoplasm, which may indicate other molecular targets different from the bacterial membrane. In light of this, the alterations in the metabolite profile of bacteria treated with the lethal concentration (1 xMIC) were investigated using an untargeted metabolomic approach by GC-MS combined with multivariate statistical analysis. This investigation was performed under the same conditions as MIC assays. The principal component analysis (PCA) was performed, and comparisons were made between the metabolic profiles of the peptide treatment group and the control group (Supplementary Table 1). We saw group separation between treatments and changes induced by the peptide in the metabolite profile of bacteria (Fig. 4).

The hierarchical cluster analysis (HCA) is in line with the PCA analysis, showing group pattern clustering. The 25 most significant metabolites with their respective KEGG functional classifications (Fig. 5). 10 metabolites were up-regulated in the peptide treatment such as 1,6-anhydroglucose, 1-hexadecanol, glucuronic acid, galacturonic acid, 2-aminopimelic acid, threonic acid, uridine, stearic acid, 2-aminoethanol, cystamine. The other 15 metabolites down-regulated are involved in different metabolic pathways such as the tricarboxylic acid cycle, glycerophospholipid metabolism, fatty acid biosynthesis, and the bacterial cell surface, among others.

[K4K15]CZS-1 negatively affected the production of succinic and glycolic acids in bacteria. These metabolites are involved in the TCA pathway. The TCA cycle is one of the main metabolic pathways, generating energy in the form of adenosine triphosphate (ATP) and providing precursor molecules for several biosynthetic routes [23]. This finding shows the disruption of the TCA cycle caused by the peptide treatment and agrees with other studies [19,23,48]. In addition, the results suggested that [K4K15]CZS-1 may interfere with glycerophospholipid metabolism, phospholipid metabolism, and fatty acid biosynthesis. For instance, palmitoleic acid and stearic acid are precursors involved in the fatty acid biosynthesis and these metabolites are dysregulated in the peptide treatment. Likewise, 1-hexadecanol is up-regulated, which is a precursor molecule in the phospholipid metabolism. In contrast, triethanolamine is down-regulated after peptide treatment and is involved in glycerophospholipid metabolism. Fatty acids, phospholipids and glycerophospholipids are the main components for the biogenesis of the membrane bilayer, essential for bacterial survival and adaptability under environmental stress [49]. Therefore, the potential response to membrane disruption and metabolism alterations induced by [K4K15]CZS-1 and the need to make a new membrane might reinforce the low development of resistance for AMPs compared to conventional antibiotics. This interference in several metabolic pathways related to maintaining the bacterial membrane structure is also observed in other studies [19,49–51].

4. Conclusions

[K4K15]CZS-1 showed strong antibacterial activity against *L. monocytogenes* with a MIC value of 0.91 μM. The anti-listerial activity of this peptide was retained at high temperatures, which is a crucial characteristic in developing antimicrobials for the food industry. Further insights on the mechanism of action using electron microscopy and metabolomics demonstrated that the effect of [K4K15]CZS-1 involved a membranolytic mechanism and modulation of metabolic pathways. Taken together, [K4K15]CZS-1 is a promising molecule for the development of food biopreservatives.

CRediT authorship contribution statement

Sebastián Bermúdez-Puga: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Meriellen Dias:** Methodology, Investigation. **Iara Lima Reis:** Writing – original draft, Investigation. **Taciana Freire de Oliveira:** Methodology, Investigation. **Sonia Regina Yokomizo de Almeida:** Investigation. **Maria Anita Mendes:** Methodology, Investigation. **Simon J. Moore:** Writing – review & editing, Formal analysis. **José R. Almeida:** Writing – review & editing, Formal analysis. **Carolina Proaño-Bolaños:** Writing – review & editing, Investigation, Formal analysis. **Ricardo Pinheiro de Souza Oliveira:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.05.022.

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