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Inhibition of multidrug resistant *Listeria monocytogenes* by peptides isolated from combinatorial phage display libraries

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ABSTRACT

The aim of the study was to isolate and characterize novel antimicrobial peptides from peptide phage library with antimicrobial activity against multidrug resistant *Listeria monocytogenes*. Combinatorial phage-display library was used to affinity select peptides binding to the cell surface of multidrug resistant *L. monocytogenes*. After several rounds of affinity selection followed by sequencing, three peptides were revealed as the most promising candidates. Peptide L2 exhibited features common to antimicrobial peptides (AMPs), and was rich in Asp, His and Lys residues. Peptide L3 (NSWIQAPDTKSI), like peptide L2, inhibited bacterial growth *in vitro*, without any hemolytic or cytotoxic effects on eukaryotic cells. L1 peptide showed no inhibitory effect on *Listeria*. Structurally, peptides L2 and L3 formed random coils composed of α-helix and β-sheet units. Peptides L2 and L3 exhibited antimicrobial activity against multidrug resistant isolates of *L. monocytogenes* with no haemolytic or toxic effects. Both peptides identified in this study have the potential to be beneficial in human and veterinary medicine.

1. Introduction

Listeria monocytogenes is a widely distributed pathogen causing arthritis and severe central nervous system infection collectively referred as listeriosis. Listeriosis represents a public health problem as it is fatal in up to 30% of all reported cases, and it has been widely associated with food-borne epidemics during the last decade (Farber and Peterkin, 1991; Ramaswamy et al., 2007). *Listeria* is a facultative intracellular pathogen that replicates in the cytosol of eukaryotic non-phagocytic cells (Cossart and Kocks, 1994; Ramaswamy et al., 2007). *Listeria* are tolerant to extreme conditions such as low pH, low temperature and high salt concentrations, and can be found in a variety of environments (Van Renterghem et al., 1991; MacGowan et al., 1994; Sleator et al., 2003). In veterinary medicine, *L. monocytogenes* is an unwelcome pathogen responsible for significant economic losses at the farm level due to the high morbidity and mortality of animals (Oevermann et al., 2010). In ruminants (cattle, sheep and goats) *L. monocytogenes* is mostly acquired through the consumption of contaminated feed and food; and manifests itself as central nervous system (CNS) disease and uterine infections (Low and Donachie, 1997; Kathariou, 2002). Conversely, contaminated animal-derived food products which are not processed before consumption (*e.g.*, raw food) represent a direct link between human infections, and *L. monocytogenes* in farm animals and the environment (Nightingale et al., 2004). In humans, *Listeria* affects preferentially individuals with impaired cell-mediated immunity, pregnant women and the elderly (Wilson and Drevets, 1998). Experimental data show that *L. monocytogenes* is able to invade and infect the CNS through several different mechanisms and can result in meningitis, meningoencephalitis, rhombencephalitis, cerebritis and brain abscess (Wilson and Drevets, 1998).

In general, listeriosis is treated with ampicillin and Bactrim (trimethoprim-sulfamethoxazole), which are considered as first-line drugs for invasive diseases, while trimethoprim-sulfamethoxazole is recommended in the case of penicillin allergy (Gyurko et al., 2000). Shortly after the initial detection of the first multidrug resistant clinical strain of *L. monocytogenes*, other strains of *Listeria* spp. resistant to one or more antibiotics were reported (Poyart-Salmeron et al., 1990; Charpentier et al.,

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1995; Aureli et al., 2003; Li et al., 2007). Currently, food-borne and clinical isolates of *Listeria* have shown resistance to multiple antibiotics including trimethoprim, gentamicin, streptomycin, erythromycin, kanamycin, sulfamethoxazole and rifampin (Roberts et al., 1996; Charpentier and Courvalin, 1997; Purwati et al., 2001; Christensen et al., 2011). Multidrug resistance of *L. monocytogenes* has been linked to the presence of a self-transferable plasmid or by conjugative transposons both originating in *Enterococcus* and *Streptococcus* (Poyart-Salmeron et al., 1990; Doucet-Populaire et al., 1991).

Short cationic peptides with antimicrobial and/or immunomodulatory activity represent a promising alternative to traditional antibiotics. Antimicrobial peptides have been found in multicellular organisms where they constitute part of the innate immunity (Boman, 1991; Gudmundsson et al., 1996; Zasloff, 2002; Radek and Gallo, 2007; Schauber and Gallo, 2008). The mode of antimicrobial action of these defence peptides is related to the disruption of cell membrane or interaction with intracellular components (Hale and Hancock, 2007; Mihajlovic and Lazaridis, 2010), resulting in inhibition of cell-wall, nucleic-acid and protein synthesis or inhibition of enzymatic activity (Cudic and Otvos, 2002; Nicolas, 2009). The advantage of antimicrobial peptides as defence weapons consists in their non-specific mechanism of action, which makes it difficult for microbial pathogens to develop resistant mutants to overcome peptide intervention.

Previously, it was shown that *L. monocytogenes* was strongly inhibited by human defensins, protamine and magainin, as well as plant-derived peptides such as thionin and snaking (Lopez-Solanilla et al., 2003). Listeriocidal activity has also demonstrated for the novel antibiotic Khal. Khal was derived from the AMP halocidin, and is capable penetrating the host cell without causing membrane damage, and attack intracellular resident *Listeria*. *In vivo* experiments clearly demonstrated that intravenous administration of Khal has significant therapeutic effect on the survival of infected animals (Jang et al., 2007). The high susceptibility of *Listeria* to antimicrobial peptides opens the possibility of using such molecules in therapeutic strategies, and in food preservation (Lopez-Solanilla et al., 2003).

Combinatorial phage-display is a powerful tool for the selection of peptides binding to a cell surface, and has been used to generate therapeutic peptides (Kay and Castagnoli, 2003; Bishop-Hurley et al., 2005; Fang et al., 2006; Bishop-Hurley et al., 2010). In this contribution, combinatorial phage display was used to isolate peptides binding to the outer surface of *L. monocytogenes*. Bound phage clones were sequenced and based on their percent occurrence three peptides were selected and tested for their antimicrobial activities *in vitro*. In further analyses, the listeriocidal properties of the L2 and L3 peptides were confirmed. The L2 and L3 peptides share a number of features common to AMPs, and have the potential to play a significant role in the development of therapeutic strategies against multidrug resistant *Listeria*.

2. Material and methods

2.1. *Cultivation of* L. monocytogenes

Multidrug resistant *L. monocytogenes* isolated from the cerebrospinal fluid was obtained from Palacky University in Olomouc, Czech Republic. *Listeria* was grown on blood agar overnight at 37 °C and then in the enrichment broth (Oxoid, Slovakia). The culture centrifuged and bacterial cells were counted using flow cytometer (FACS single-laser flow cytometer Accuri™ C6, Becton-Dickinson, Heidelberg, Germany). The suspension was then diluted according to experimental procedure.

2.2. *Selection of phages with affinity to* L. monocytogenes

Phage display library Ph.D-12 (New England Biolabs, Frankfurt am Main, Germany) (2×10^{10} PFU) was incubated with *L. monocytogenes* (4×10^5 cells) in 100 μl LB medium at 37 °C for 1 h with gentle shaking. After incubation, the culture was centrifuged at 2500*g* for 15 min at room temperature, and unbound phages were washed with PBS-T (PBS containing 0.1% Tween 20) for at least three times. The bound phages were eluted with 0.2 M Glycin-HCl (pH 2.2). Eluate containing phages with affinity to the bacteria was neutralised with 1 M Tris–HCl (pH 9.0) and the phages were separated from bacterial cells by centrifugation at 2500*g* for 15 min at room temperature. Supernatant containing phages was recovered, and phages were amplified in *Escherichia coli* ER 2738 (New England Biolabs, Frankfurt am Main, Germany) for 4.5 h at 37 °C by shaking. Amplified phages were precipitated from cleared *E. coli* culture with polyethylene-glycol precipitation (20% PEG8000 in 2.5 M NaCl) as per manufacturer's instructions (New England Biolabs, Frankfurt am Main, Germany). Four rounds of the biopanning were performed. In each round of biopanning the titer of the phages was determined, and the efficiency was evaluated.

2.3. *Isolation of phage DNA and sequencing*

Phages from the fourth round of bioapanning were used to prepare phage stocks to isolate genomic DNA and subsequent nucleotide sequencing. The procedure is described in details in manufacturer's instructions (Ph.D-12, New England Biolabs, Frankfurt am Main, Germany). DNA sequencing of isolated phage clones was performed using an Applied BiosystemsAvant 3010 automated sequencing system with the ABI BigDye™ Terminator sequencing kit (Life Technologies, Bratislava, Slovakia). The DNA sequences were translated into amino acid sequences (Biomatters, San Francisco, USA).

2.4. *Peptide sequence analysis using bioinformatic tools*

Physicochemical properties of the peptides (Molecular weight, pI) were predicted using Geneious software. Hydrophobicity, net charge and sequence similarity with existing peptides deposited in the repository was performed using Antimicrobial Peptide Database server (http://aps.unmc.edu/AP/main.php). Homology modelling was done using the package MODELLER (https://salilab.org/modeller/) (Modeller, San Francisco, USA). Biotinylated form of peptides was synthesized commercially (Shafer-N, Copenhagen. Denmark).

2.5. *Conformation analysis of peptides by CD spectrometry*

The secondary structure of peptides was assessed by CD spectrometry using JASCO J-810 (JASCO, Easont, USA). Peptides were diluted in 50 mM phosphate buffer (50 mM Na_2HPO_4 + 50 mM NaH_2PO_4) pH 7.4 to a final concentration of 300 μM. The measurements were recorder using JASCO J-810CD spectrometer (JASCO, Easont, USA) under nitrogen gas flow of 8 l/h at a temperature of 25 °C. Spectra were recorded between 190 and 270 nm, using a 1 mm cuvette at a scan speed of 50 nm/min. We performed an average of five measurements for each sample. The average absorption was corrected by buffer and then baseline to zero using the average of readings between 190 and 270 nm.

2.6. *ATR (attenuated total reflectance) Fourier transform infrared spectroscopy of peptides*

Synthesized peptides were purchased from Schafer-N (Denmark). Peptides were dissolved in ultra-pure water to

a final concentration of 6 mM. The spectra of liquid samples with 6 mM peptides were recorded with a Nicolet 8700 Fourier transform infrared (FTIR) spectrometer equipped with Smart OMNI-Sampler (diamond crystal) (Thermo Scientific, Waltham, USA). Every spectrum was measured as an average of 128 interferograms in the amide I region (1720–1600 cm^{-1}) with a resolution of 2 cm^{-1}. The ultra-pure water background was subtracted from the peptide spectrum. For the deconvolution of the recorded FTIR spectra we used the curve-fitting program Fityk 0.9.7. (Tian et al., 2015).

2.7. Evaluation of bacteriostatic/cidal effect of peptides

Minimum inhibitory concentration (MIC) of selected peptides was determined by microdilution method (in 96 well microtiter plate) as recommended by Clinical and Laboratory Standard Institutes (Wayne, 2002; Azad et al., 2011). M100-S12.Overnight culture was diluted in LB medium and grown to mid-log-phase at 37 °C with shaking and diluted to 0.5 McFarland standard to a final volume 100 μl. Bacteria were incubated with decreasing concentrations (500 μM, 200 μM, 100 μM, 75 μM, 30 μM and 15 μM) of peptides at 37 °C with shaking overnight. Bacteria untreated with peptides served as growth control. Results were recorded by visual inspection (inhibition of growth assessed by cloudiness of culture) and cell count was assessed by flow cytometry.

Listeriocidal effect of the peptides was also examined. A cell suspension (10 μl) was drawn from each well and plated on blood agar. After overnight incubation, the CFU was determined. The assay was repeated three separate times to ensure reproducibility.

2.8. Assessment of metabolic activity

Metabolic activity was assessed by ATP inhibition using BacTiter-Glo assay kit™ (Promega, Madison, USA) according to the manufacturer's instructions. In short, *L. monocytogenes* was incubated with peptides (1 × MIC in case of L2 and L3, and 500 μM in case of L1) for 24 h at 37 °C by gentle shaking. Control cells were incubated in medium without peptides (negative control). As positive control peptides treated with 0.1% Triton X-100 were used. The level of ATP was measured as a luminescent signal using FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany). The luminescent signal correlates with the number of viable microbial cells based on the amount of ATP present after the addition of peptide. Assay was repeated three separate times to ensure reproducibility.

2.9. Toxicity on mammalian cells

The toxicity of peptides to endothelial cells (ECs) was determined by XTT test (AppliChem, St. Loius, USA) per manufacturer's instructions. The ECs cultured in Dulbecco's modified Eagleís medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 1 ng/ml bFGF (basic fibroblast grow factor, Roche, Basel, Switzerland) to obtain a confluent monolayer. The peptides in concentrations of 0.5× and 1 × MIC were added and incubated for 24 h at 37 °C, 90% humidity and 5% CO_2 atmosphere. Untreated cells served as negative control. Cells treated with 0.1% Triton X-100 were used as positive control. After incubation, the mixture of XTT and activation reagent (5:0.1) was added to the cell cultures and incubated for additional 4 h. The fluorescence was read using MULTISKAN FC (Thermo Scientific, Waltham, USA) at 450 nm. Assay was repeated three separate times to ensure reproducibility.

2.10. Assessment of haemolytic activity

Red blood cells (RBCs) were harvested from fresh sheep blood, washed three times and diluted in phosphate buffer saline (1.7×10^7/ml). Cell suspension was transferred into wells of 96-well plate (1.7×10^6cells/well) and peptides diluted at 0.5× and 1 × MIC were added. RBCs were incubated for 1 h at 37 °C by gentle shaking. Untreated RBCs served as negative control. As positive control Triton-X 100 (final concentration of 0.1%) was added. After incubation cells were centrifuged at 2000*g* for 10 min at room temperature and supernatant was transferred into new wells. The release of haemoglobin was measured with microplate reader (Dynatech, Laboratories, El Paso, USA) at absorbance of 450 nm. Assay was repeated three separate times to ensure reproducibility.

2.11. Statistical analysis

Mean values ± SD (Standard Deviation) of three independent repeats were calculated using Graph Pad prism 6 (GraphPad software La Jolla, USA). A one-way ANOVA was used to determine significance. Values were considered significant when $P < 0.05$.

3. Results

3.1. Phage display selection of peptides binding to L. monocytogenes

Selection of peptides exhibiting binding affinity to *L. monocytogenes* was performed using combinatorial phage library displaying random 12-mer peptides. After four rounds of biopanning, DNA was isolated from 20 phage clones (20 plaques picked from 10E-8dilution plate), sequenced and translated into amino acid sequence. Ten clones encoded NHLSTPVWSITG (referred as L1), four clones encoded DQFVHDVKGTKH (L2) and six clones encoded NSWIQAPDTKSI (L3).

3.2. Assessment of antimicrobial peptide features

Features of the peptides are summarized in Table 1. The amino acid sequences of L1, L2 and L3 indicated hydrophilic nature of the peptides. Peptide L1 had a positive net charge (+1), showed a tendency to form a β-sheet structure (Fig. 1A) and sequence similarity between L1 and other antimicrobial peptides were: VmCT2—37.5%, Leurocin II—33.33% and CPF-St7-33.33%. Peptide L2 had a net positive charge (+2), showed a tendency to form α-helical structures (Fig. 1 B) and had sequence similarity with other antimicrobial peptides such as Myxinidin (33.33%) and Fallaxidin 3.2 (33.33%). Peptide L3 had a neutral net charge, showed a tendency to fold into a β-sheet structure (Fig. 1C). The sequence similarities of L3 with other antimicrobial peptides, exhibiting anti-Gram positive bacterial properties and tendency to structurally fold form into β-sheet structures, were as follows: VmCT2 (37.5%), Mastoparan-VT4 (33.3%), Mastoparan-X (33.3%) and Leucrocin I (33.3%).

3.3. Analysis of peptide secondary structure

CD spectra of the short peptides revealed the tendency to form a random coil structure (Fig. 2A). Using FTIR analysis we focused on the differences in the secondary structure between the isolated peptides. Component analysis of the peptide's secondary structure was performed using deconvolution of obtained FTIR spectra (Fig. 2B, Table 2). Deconvolution confirmed data obtained from CD measurements. All peptides showed a high content of turns and a highly disordered structure. The FTIR spectra of L1 showed the high absorption of the peptide at 1652 cm^{-1}, which is correlated with the presence of an α-helix. The peak 1621 cm^{-1} indicated the

Table 1
In silico analysis of peptides.

Clone	Sequence	Frequency	Net Charge	pI	Hydrophobicity	Lys/Arg	MW[a] (kDa)
L1	NHLSTPVWSITG	10/20	+1	7.55	33%	0	1.311
L2	DQFVHDVKGTKH	4/20	+2	7.71	25%	2	1.411
L3	NSWIQAPDTKSI	6/20	0	6.34	33%	1	1.360

[a] Molecular weight.

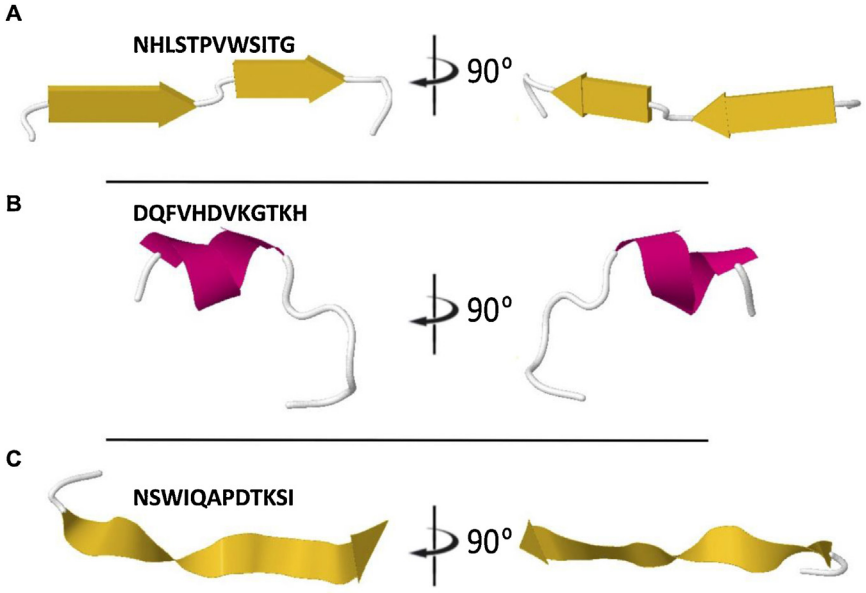

Fig. 1. Structural prediction of peptides. (A) peptide L1, (B) peptide L2 and (C) peptide L3. Yellow arrows represent β-sheets. Magenta arrows represent α-helix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analysis of peptide secondary structure.

Structure	L1		L2		L3	
	peak (cm^{-1})	%	peak (cm^{-1})	%	peak (cm^{-1})	%
α-helix	1651	13.0	1654	14.7	1652	13.1
β-sheet	1621	23.0	1622	21.8	1620	24.0
Turns β-turns	167016841699	15.39.711.0	168416701698	10.617.015.6	168416701700	10.215.014.2
Unordered	1637	19.3	1637	11.2	1637	10.6
Side-chain contributions	1604	4.1	1619	24.0	1606	7.8

presence of β-sheets, peaks 1670 cm^{-1} and 1684 cm^{-1} turns, while 1699 cm^{-1} correlates to β-turns (Fig. 2A). The L2 peptide showed high absorption at 1654 cm^{-1} corresponding to the presence of an α-helix. The peak at 1622 cm^{-1} indicated β-sheet structures. The peaks at 1684 cm^{-1} and 1670 cm^{-1} represent turns, whilst the peak at 1698 cm^{-1} correlates to β-turns (Fig. 2 B). Peptide L3 on the other hand, displayed a higher prevalence of β-sheet structures (peak at 1620 cm^{-1}). The peak 16512 cm^{-1} was correlated with the presence of an α-helix. Peaks 1684 cm^{-1} and 1670 cm^{-1} represent turns, while peak 1700 cm^{-1} correlates to β-turns (Fig. 3).

3.4. *Peptides L2 and L3 showed antimicrobial activity*

In microdilution performed to calculate MIC, peptides L2 and L3 stopped bacterial expansion at 30 μM (no cloudiness was present in microdiluted cultures), while peptide L1 did not inhibit bacterial growth even at the highest tested peptide concentration (Table 3). The inhibitory effects of the peptides were also confirmed by flow cytometry, where the L2 and L3 peptides reduced cell count significantly at concentrations of 30 μM or greater (L2 $p < 0.01$, L3 $p < 0.001$) compared to cultures free of peptide. Peptide L1 showed no inhibitory even at 500 μM (Fig. 4).

Listeria incubated with peptides was also plated on blood agar and CFU was determined to assess cidal effect. Both L2 and L3 peptides were listericidal at concentrations of 200 μM as determined by the absence of any single colony on the plate. CFU count for L1 remained unchanged (even at the 500 μM concentration) compared to negative control (no peptides added to the *Listeria* culture), thus indicating no cidal effect.

3.5. *Peptides L2 and L3 decrease metabolic activity of* L. monocytogenes

The effect of peptides on the metabolic activity of the pathogen was assessed by ATP assay. A significant decrease in ATP level was detected when *Listeria* were incubated with 1 × MIC of L2 ($p < 0.01$) and L3 ($p < 0.05$). Peptide L1 did not reduce ATP levels even at 500 μM (Fig. 5).

Table 3
Antimicrobial effect of peptides measured by microdilution method.

Concentration/Peptides	15 μM	30 μM	75 μM	100 μM	200 μM	500 μM
L1	+++	+++	+++	++	++	++
L2	+	–	–	–	–	–
L3	+	–	–	–	–	–

+++ high turbidity (cloudiness) indicating growth of bacteria.
++ moderate turbidity.
+ very low turbidity.
– none turbidity.

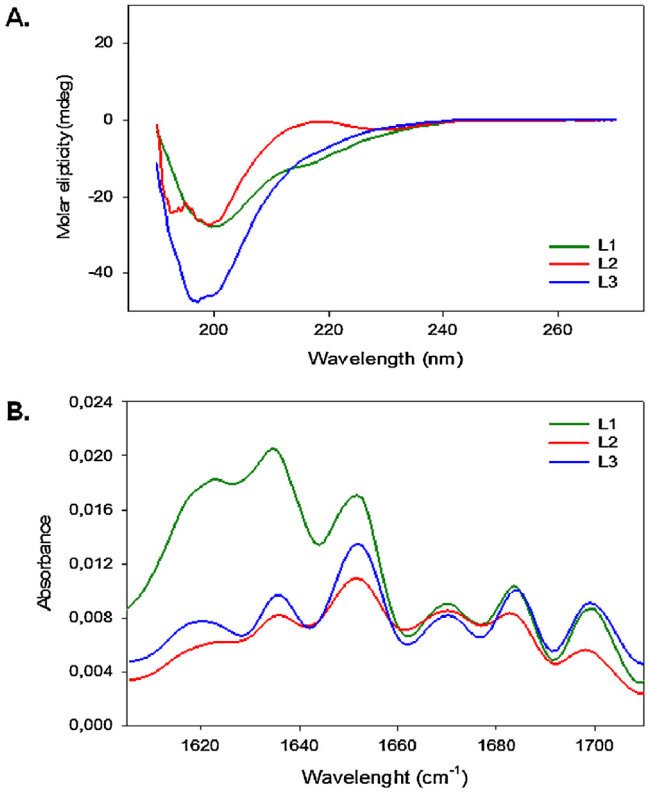

Fig. 2. Panel A: CD spectra of peptides. CD spectra were measured in the UV region for peptides. All peptides are at 300 μM concentration diluted in 50 mM PBS. L1 showed in green, L2 in red and L3 in blue. Panel B Infrared spectra of peptides. FTIR spectra in the amide I region (1600–1720 cm^{-1}) of peptides L1 (green), L2 (red) and L3 (blue). All peptides have a concentration of 6 mM and are diluted in water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Cytotoxic and haemolytic activity of peptides

The toxicity of peptides (L2 and L3) to eukaryotic cells was assessed by XTT assay. Study of the toxicity and haemolytic activity of L1 was irrelevant owing to its inability to inhibit *Listeria*. None of the peptides were toxic for ECs (Fig. 6A). The haemolytic activity of peptides against sheep RBCs was determined by measuring haemoglobin release. After 1 h incubation, peptides showed no haemolytic activity (Fig. 6B).

4. Discussion

Most of the clinical and environmental *Listeria* spp. isolates are susceptible to antibiotics active against gram-positive bacteria. However, there is growing evidence of multi-resistant strains of *Listeria* spp. (Poyart-Salmeron et al., 1990; Charpentier et al., 1995; Qu and Lehrer, 1998; Aureli et al., 2003; Li et al., 2007). Emergence of drug resistance bacteria has focussed attention towards the development of antimicrobial peptides as novel anti-infective leads. Human defensins, cathelicidins, and AMPs from bacteria (bacteriocins), viruses, plants, vertebrates and invertebrates have the potential to replace and/or supplement traditional antibiotics.

Most antimicrobial peptides synthesized to date are either variants of natural antimicrobial peptides, or isolated from synthetic combinatorial libraries (Sainath Rao et al., 2013). Combinatorial phage library and whole-cell phage display technologies were used to select peptides able to bind to the cell surface of multi-resistant *L. monocytogenes*. Ten phage clones encoded the peptide sequence NHLSTPVWSITG (L1), four clones encoded DQFVHDVKGTKH (L2), whereas six encoded NSWIQAPDTKSI (L3) (Table 1). A conventional amino acid alignment revealed that there was no clear consensus pattern among the peptides. All peptides were then subjected for assessment of their physicochemical properties.

Numerous studies have dealt with understanding of the mode of action of AMPs, with antimicrobial activity having been shown to depend on the amino acid composition as well as on its physical and chemical properties (interfacial activity). This phenomenon can be described as the ability of a peptide to bind a bacterial membrane and partition itself at the membrane-water interface, which results in the alteration of packaging and organization of lipids within the membrane (Wimley, 2010). Determination of the physicochemical properties of the active peptides showed a positive net charge (+2) for peptide L2 and net charge (0) for peptide L3, and a total hydrophobic ratio of 25% and 33%, respectively. A positive net charge for AMPs is important for the binding to the negatively charged surface of bacterial membranes (Dzwolak et al., 2004; Mukherjee et al., 2007).

Peptides with higher hydrophobicity have been shown to undergo a structural transition in contact with bacterial-type membranes, and have a tendency to form α −helical or β-sheet structures, whereby triggering the mode of action of the AMPs (Huang et al., 2010). Peptides L2 and L3 are rich in hydrophilic residues, which helps explain their mode of action. Furthermore, *in silico* analysis based on the antimicrobial peptide database predicted the potential of peptides L2 and L3 as antibacterial peptides, due to their highly hydrophilic nature.

The CD spectra analysis proposed a random coil structure for both peptides. Differences in the peptide secondary structure revealed the presence of α-helix and β-sheet, turns and β-turns in the L2 and L3 peptides. The amide I band between 1600 and 1700 cm^{-1} was the most intense absorbance band being associated with the C═O stretching vibration and directly related to the backbone conformation. After deconvolution of spectra, a predicted value of 14.7% and 25.8% for α-helix and β–sheet, respectively was proposed for L2, while 13.1% and 24% for α-helix and β–sheet, respectively for L3. The high content of unstructured conformers in L2 can accounted for by the presence of glycine, a non-chiral amino acid, which gives the peptide flexibility. The L3 peptide on the other hand contains proline (also known as the "helix breaker") and can explain the high number of turns.

Peptide L2 shares some degree of sequence similarity with AMPs in literature such as Myxinidin (33.33%) and Fallaxidin (33.33%), which exhibit antimicrobial activity against Gram-positive bacte-

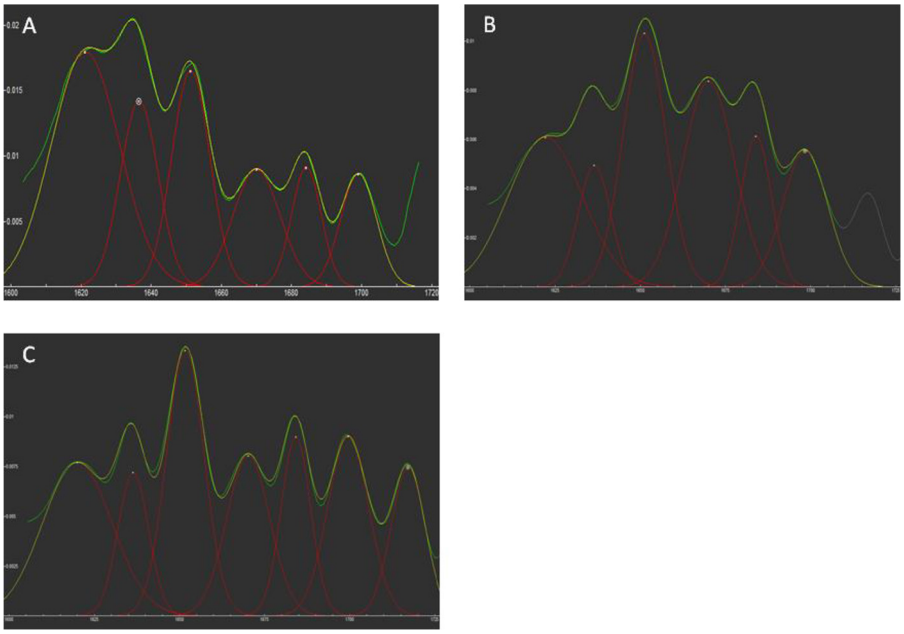

Fig. 3. Deconvolution of peptide FTIR spectra. Deconvolution of FTIR spectra in the amide I region using Fityk 0.9.7. Panel A. peptide L1, panel B. peptide L2 and panel C. peptide L3.

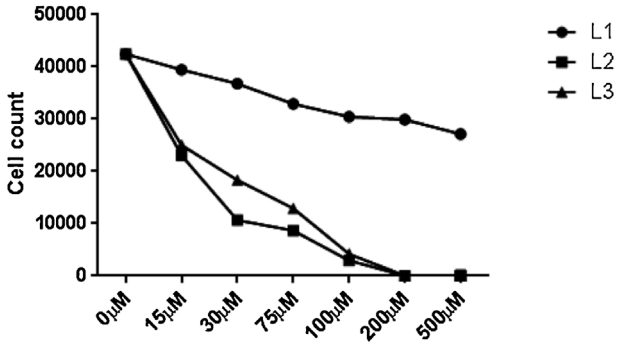

Fig. 4. Assessment of inhibitory effect of peptides. Detection of the peptide inhibitory effect against *L. monocytogenes* assessed by flow cytometry. Peptides L2 and L3 inhibited the bacterial growth at a concentration of 30 μM (L2 $p < 0.01$, L3 $p < 0.001$). L1 did not show any bactericidal effect.

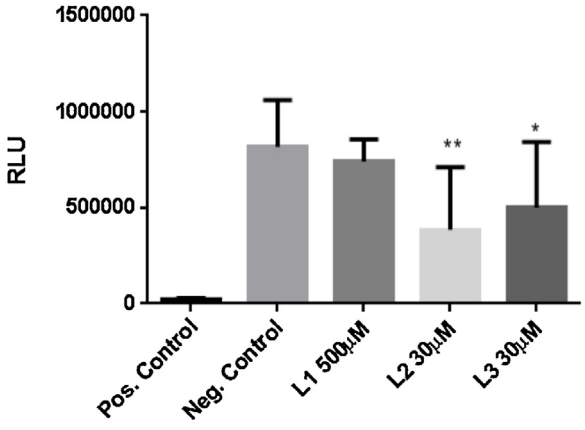

Fig. 5. Assessment of ATP level. The level of ATP was assessed by luciferase assay. Decrease in ATP level was observed when *Listeria* were incubated with peptide L2 ($p < 0.01$) and L3 ($p < 0.05$). RLU—relative luminescence unit.

ria. The mode of action of Myxinidin is cytoplasmic membrane disruption, pore/channel formation (Syvitski et al., 2005), and inhibition of cell wall and nucleic acid synthesis (Patrzykat et al., 2002). Fallaxidin, a peptide secreted by *Litoria fallax* has been shown to induce transmembrane pore formation in DMPC/DMPG membranes with antimicrobial activity against bacteria (Sherman et al., 2009).

Peptide L3 showed a degree of similarity (sequence similarity) with anti-Gram positive peptides such as VmCT2 (37.5%) and Mastoparan-X (33.33%). VmCT2 is isolated from scorpion's family group Vaejovidae, and exhibits antimicrobial activity against Gram-positive bacteria at a concentration of 10–20 μM (Ramirez-Carreto et al., 2012; Quintero-Hernandez et al., 2015). Mastoparan-X was shown to stimulate the activity of phospholipase A2 (PLA2) which is able to induce changes in membrane composition, and activate G proteins (Wakamatsu et al., 1992). Secretory PLA2 was shown to kill *L. monocytogenes* at a concentration of 0.1 nM/ml. (Qu and Lehrer, 1998). Peptides L2 and L3 inhibited bacterial growth at a concentration of 30 μM and showed bactericidal effect at a concentration of 200 μM after 24 h incubation with the *L. monocytogenes*. L2 and L3 also had a considerable impact on energy metabolism, and caused significant decrease in ATP levels. Affected ATP synthesis can be attributable to inhibition of the respiratory chain. The precise mode of action of these peptides on *L. monocytogenes* is currently unknown and will be subject to further study. Many studies support the notion that AMPs mediated membrane damage is bactericidal, however other studies point to a multi-hit mechanism in which the peptide binds to intracellular targets (Shai, 2002). In either case, peptide–membrane interactions are essential for ensuring antimicrobial activity (membrane damage or self-promoted uptake).

Safety analysis of both L2 and L3 peptides towards mammalian cells revealed no cytotoxicity. When tested with sheep red blood cells in hemolytic assay, both peptides showed no lytic activity to mammalian red blood cells at peptide concentrations 1 × MIC.

Using combinatorial phage display library and whole-cell approach, we have isolated and identified novel cationic anti-*Listeria* peptides. The listeriocidal potency, absence of toxicity towards mammalian cells and no-hemolytic activity suggests that they might have potential uses in human and animal health-related applications.

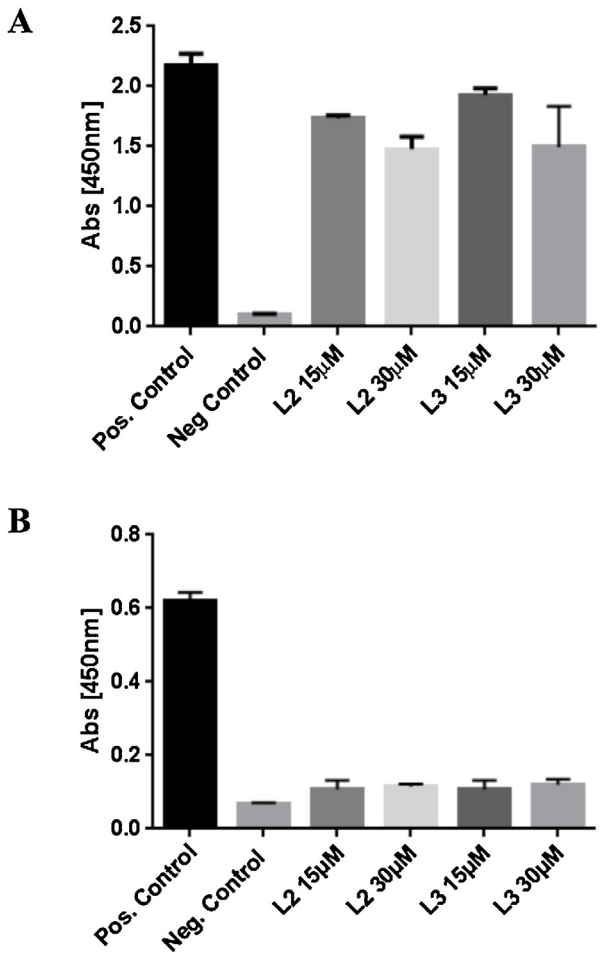

Fig. 6. Panel A- Assessment of cell proliferation. Cell proliferation was detected by XTT test on ECs after 24 h incubation with peptides. None of the peptides had an impact on cell proliferation. Panel B. Assessment of haemolytic activity. Suspension of sheep erythrocytes 1.7×10^7 was incubated with peptide (0.5× and 1 × MIC) in 96-well microtiter plates. No haemolytic effect (no release of haemoglobin) of peptides L2 and L3 was observed. Positive control—treatment of red blood cells with Triton-X 100. No statistical significance was observed between negative control and tests.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

Author contributions

M.B., Z.F. L.P. and E.B conceived and designed the experiments. Z.F., L.P., E.B., L.P., L.C and Z.B. performed experiments. Z.F. and M.B. analyzed data. Z.F. and M.B. wrote the paper and prepared the figures. All authors discussed the results and commented on the manuscript.

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