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Original Article

INFLUENCE OF PEPTIDE P34 ON GENE EXPRESSION OF *LISTERIA MONOCYTOGENES* AND *LISTERIA SEELEGERI*

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ABSTRACT

Objective: Investigate the influence of the antimicrobial peptides P34 and nisin on the expression of genes associated with components of the cell surface of *Listeria monocytogenes* and *Listeria seeligeri*.

Methods: Antimicrobial activity was determined by addition of peptide P34 and nisin (12.5 µg/ml) onto Brain Heart Infusion agar (BHI) plates previously inoculated with indicator strains (*L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4) after incubation for 24 h at 37 °C or 240 h at 4 °C. Ribonucleic acid (RNA) was directly extracted from bacterial colonies at the border of the inhibition zones, and the expression levels of genes D-alanine-D-alanyl carrier protein ligase *(dltA),* putative phospholipid lysinylation (*Imo 1695*) and EIIAB^{Man} of mannose-specific PTS (*mptA*) were determined using real-time PCR.

Results: A non-significant increase in the levels of transcription of genes *dltA, Imo1695* and *mptA* was observed for *L. monocytogenes* treated with peptide P34 or nisin. Both peptides caused a similar decrease in *dltA* gene expression in *L. seeligeri*. The expression of gene *Imo1695* significantly decreased (about 2000-fold) after treatment with the peptide P34 at 37 °C, while at 4 °C a reduction of 12-fold and 5-fold was detected for P34 and nisin, respectively. A significant decrease in *mptA* gene expression was observed by exposition to peptide P34 (31.872-fold) and nisin (16.047-fold) for 24 h at 37 °C.

Conclusion: The results suggest that both peptide P34 and nisin influence the expression of genes related with the cell-surface/cell-membrane structure of *L. seeligeri* and in lesser extent *L. monocytogenes*.

Keywords: Bacteriocin, *L. monocytogenes, L. seeligeri*, Gene expression

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INTRODUCTION

The genus *Bacillus* encompasses a number of industrially important species that have been recognised for years and are considered safe for biotechnological purposes. *Bacillus subtilis, Bacillus thuringiensis*, *Bacillus stearothermophilus, Bacillus licheniformis, Bacillus megaterium* and *Bacillus cereus* are examples of typical producers [1-12].

Bacteriocins and bacteriocin-like substance (BLS) are antimicrobial peptides produced by diverse bacteria and are often effective against closely related species [4, 13]. Currently, BLS have gaining increased attention due to their potential use as natural preservatives in the food industry, due to activity against many pathogenic bacteria and the highest activity against *Listeria* species [14, 15]. An example is a nisin, a non-toxic bacteriocin hydrolyzed by digestive enzymes that have been used for over 50 y as a food preservative in different countries without the substantial development of bacterial resistance [16, 17]. Until recently, it was believed that bacteria could not acquire resistance to antimicrobial peptides, so these substances would be strong candidates for new preservative in foods [18-20]. The development of resistance to antimicrobial peptides from previously sensitive strains has been viewed as difficult not impossible, but some studies have revealed that certain genes can provide increased resistance to antimicrobial peptides [21].

Studies on the structure, activity and gene expression of *Listeria* species indicate that the use of bacteriocins can induce the development of resistance in some target bacterial strains [22-24]. These studies have suggested that the interaction of class II bacteriocins is highly dependent on EII^{Man} and that the mechanism of resistance to class II bacteriocins can be observed in strains of *L. monocytogenes*, in which resistance seems to be associated to reduced expression of a mannose-specific phosphotransferase system (EIIAB^{Man} PTS, encoded by *mptA*) [25]. The expression of genes like *dltA* and *Imo 1695*, which can potentially influence the cell-surface charge by D-alanyl-esterification of teichoic acid and lysinylation of membrane phospholipids, has been investigated as well. In addition, it has been described cross-resistance to class I and class II bacteriocins [26, 27], and the combined use of different bacteriocins can be successful to reduce the appearance of resistant strains.

A novel antimicrobial peptide was produced by a *Bacillus* sp. isolated from aquatic environments of Brazilian Amazon basin [28], with the potential to be used as food preservative. This peptide, named as P34, was purified and characterised as described elsewhere [29]. The peptide P34 was active against Gram-positive and Gram-negative bacteria, including pathogenic and spoilage microorganisms, with remarkable inhibitory activity on *L. monocytogenes* [30]. As the peptide P34 targets the cell envelope of *L. monocytogenes*, it is conceivable that P34 may also influence the expression of some genes after direct incubation with *Listeria* species. Thus, the aim of this study was to investigate the influence of antimicrobial peptide P34 on the gene expression of *L. monocytogenes* and *L. seeligeri* after direct plate inoculation.

MATERIALS AND METHODS

Bacterial strains and media

L. monocytogenes ATCC 7644 and *L. seeligeri* AC 82/4 and *Bacillus* sp. strain P34, belonging to our own culture collection (UFRGS, Porto Alegre, Brazil), were grown in Brain Heart Infusion (BHI; Oxoid, Basingstoke, UK) broth or agar. Bacterial strains were maintained as stock cultures were frozen at-21 °C in BHI broth supplemented with 20% glycerol. For the production of the peptide, *Bacillus* sp. strain P34 was grown in BHI broth and detection of antimicrobial activity was performed in BHI agar plates.

Peptide P34

The peptide P34 was purified as described elsewhere [29]. Briefly, *Bacillus* sp. was cultivated in 500 ml Erlenmeyer flasks containing 200 ml of BHI broth for 24 h at 30 °C in a rotary shaker at 180 rpm (LAC-INA-800, Láctea). Cells were harvested by centrifugation at 10.000 x *g* for 15 min at 12 °C, and the resulting supernatant was filtered through 0.22 µm membranes (Millipore, Bedford, MA, USA). The peptide was purified from the supernatant by ammonium sulfate precipitation and sequential liquid chromatography on Sephadex G-100 and DEAE-Sepharose (Pharmacia Biotech, Uppsala, Sweden). The purification factor was 175-fold and the concentration used was 12.5 µg ml^{-1}. Nisin (Nisaplin®; Danisco, Copenhagen, Denmark) was suspended in 0.02 mol l^{-1} HCl and then diluted in phosphate buffer saline (PBS; 35 mmol l^{-1} phosphate buffer, 150 mmol l^{-1} NaCl, pH 7.4) to obtain a solution of 12.5 µg ml^{-1}. The concentrations of peptide P34 and nisin were determined by the Folin-phenol reagent method [31], using a calibration curve developed with bovine serum albumin as the protein standard.

Detection of antimicrobial activity

Antimicrobial activity was determined essentially as described elsewhere [32]. Briefly, aliquots of 20 µl of purified peptide P34 and nisin were applied on cellulose disks (6 mm) on BHI agar plates previously inoculated with indicator strain suspension (*L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4), which corresponded to 0.5 McFarland turbidity standard solution. The plates were incubated for 24 h at 37 °C for bacteriological incubator (MA 032/3, Marconi, Piracicaba, SP, Brazil) or 240 h at 4 °C. After incubation, inhibition zones around the disks were measured. PBS was used as a negative control. The experiments were performed in triplicate (n=3) and the expressed values as mean±SD of three independent experiments.

Relative levels transcript of genes for real-time PCR quantification

Bacterial cells of *L. monocytogenes* ATCC 7644 and *L. seeligeri* AC 82/4 were removed directly at the border around the inhibition zones after 24 h and 240 h of incubation of negative control, peptide P34, and nisin, respectively. The cell concentration was adjusted spectro-photometrically by O. D. 600 nm for a final concentration of 2 x 10^4cells/ml. The TRIZOL® (Invitrogen, Carlsbad, CA) reagent was utilised for total RNA extraction, according to the manufacturer's instructions. Then, the extracted RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA), its quality was assessed by running samples on a 1% formaldehyde-agarose gel, and quantified spectrophotometrically. The primers used in this study were previously described by [12, 47] were named: *dltA* F 5'CACAAGATCAGCTAATGGACGC3', *dltA* R 5'CTGGA-ACTTCTCCGAAATGTTT3', *Imo1695* F 5'GGGATTGACTA-TCCGTCGCTA3', *Imo1695* R 5' TTCCGGGCTTTGAGAAGTTAA3', *mptA* F 5'CAGGAC-TTAATTTGCCAATGTTG3', *mptA* R 5'CGCGAACACCTTCTT-GAGCT3', *rpoD* F 5'ACTGAAAAAGTTCGGGAAATCCT3' and *rpoD* R 5' TCGCCT-AGATGTGAATCGTCTTC3'. The real-time PCR amplification reaction was carried using SYBR® Green One-Step qRT-PCR with Rox (Invitrogen, Carlsbad,CA), according to the manufacturer`s instructions. cDNA was synthesised from 0.5 µg of total RNA of *L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4, using the forward and reverse primers (100 µM) specific for each target gene. Amplification conditions were: 48 °C for 30 min, 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All PCR reactions were run on the 7500 Real-Time PCR System (Applied Biosystems®, USA). The assays were accomplished in duplicated (n=2) for each gene, and include cDNA of the samples and control without template on three independent experiments.

The data were analysed using Sequence Detection System (SDS) software version 1.6.3. Results were obtained as C_T (threshold cycle) values. The mean (C_T) value was calculated for each triplicate reaction in two independent experiments. The (ΔC_T) values were calculated and normalised by subtracting the mean (C_T) value of *L. monocytogenes* ATCC 7644; *L. see ligers* AC 82/4 and control by mean (C_T) values endogenous control *(the rpo*D gene encoding the σ70 subunit of the RNA polymerase) to give the ($\Delta\Delta C_T$) value. The ($\Delta\Delta C_T$) value was calculated subtracting (ΔC_T) value of *L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4 by (ΔC_T) value control. The relative expression of each gene can be quantitatively calculated as ($\mathbf{2^{-\Delta\Delta C_T}}$), as described by [12].

Statistical analysis

Treatment comparisons were performed using Tukey's *t*-test and the values were considered significantly different each other at $P<0.05$.

RESULTS AND DISCUSSION

Increased resistance of microbial strains has been associated with the use of antimicrobials applied to the food preservation, including strains of *L. monocytogenes* [34]. Some strains isolated from foods have become resistant to conventional bacteriocins such as nisin, pediocin PA-1 and bavaricin [35, 36]. Under the experimental conditions of this work, *L. monocytogenes* was more sensitive than *L. seeligeri* when exposed to nisin and peptide P34. The inhibition zones of peptide P34 and nisin against *L. monocytogenes* and *L. seeligeri* after the incubation time of 24 h at 37 °C and 240 h at 4 °C are shown in table 1. This study showed that nisin produced larger inhibitory halos than the peptide P34 against the strain of *L. seeligeri*, although both substances were inhibitory to the *Listeria* strains tested. Our results for the antimicrobial activity were similar to a previously published study [37].

Table 1: Antimicrobial activity of purified peptide P34, nisin, and control

Indicator strain	Conditions	Inhibition zone (mm)*
	24 h incubation at 37 °C	
Listeria monocytogenes ATCC7644	Control	0
	P34	9.0±0.2
	Nisin	10.0±0.3
	240 h incubation at 4 °C	
	Control	0
	P34	10.0±0.1
	Nisin	12.0±0.2
	24 h incubation at 37 °C	
Listeria seeligeri AC82/4	Control	0
	P34	10.0±0.3
	Nisin	12.0±0.3
	240 h incubation at 4 °C	
	Control	0
	P34	9.0±0.2
	Nisin	10.0±0.1

*Diameter of the inhibition zone in mm around the disk. The experiments were performed in triplicate (n=3) and the expressed values as mean±SD of three independent experiments.

Based on the inhibition of *L. monocytogenes* and *L. seeligeri* by peptide P34 and nisin, the expression of three different genes of *Listeria* genus, namely *dltA, imo1695*, and *mptA,* were evaluated by real-time PCR. It has been reported that changes in cell surface may occur after the interaction of class IIa bacteriocins with specific receptors of bacterial cell wall, which could be associated with an increase in the transcription level of certain genes in some strains of *L. monocytogenes* [38]. Another gene investigated was the *mptA*, which has been reported as a mannose permease, named EII_t^{Man}, belonging to the phosphotransferase system (PTS). The PTS is responsible for the transport and concomitant phosphorylation of sugars inside both Gram-negative and Gram-positive bacteria [39].

The values of ΔC_T and $\Delta\Delta C_T$ to calculate the relative expression levels ($2^{-\Delta\Delta C_T}$) of strains of *L. monocytogenes* and *L. seelegeri*, are shown in table 2.

Table 2: Calculated and analysed relative transcript levels of genes by real-time PCR quantification after incubation of *L. monocytogenes* ATCC 7644 (A) and *L. seeligeri* AC 82/4 (B) with antimicrobial peptides

A

Gene	Time and temperature of incubation	Group	ΔC_T	$\Delta\Delta C_T$*	$2^{-\Delta\Delta C_T}$
dltA	24 h at 37 °C	Control	0.12±0.045	0	1.0
		P34	1.27±0.042	1.16±0.003	0.44
		Nisin	1.99±0.142	1.87±0.097	0.27
	240 h at 4 °C	Control	0.54±0.065	0	1.0
		P34	0.80±0.082	0.25±0.017	0.84
		Nisin	1.84±0.097	1.29±0.032	0.40
Imo 1695	24 h at 37 °C	Control	1.18±0.052	0	1.0
		P34	3.39±0.187	2.21±0.135	0.21
		Nisin	4.05±0.121	2.87±0.069	0.13
	240 h at 4 °C	Control	1.28±0.057	0	1.0
		P34	1.52±0.071	0.25±0.014	0.84
		Nisin	2.92±0.139	1.65±0.082	0.31
mptA	24 h at 37 °C	Control	-0.23±0.076	0	1.0
		P34	0.26±0.057	0.49±0.019	0.71
		Nisin	1.69±0.154	1.92±0.078	0.26
	240 h at 4 °C	Control	-0.45±0.057	0	1.0
		P34	0.27±0.086	0.72±0.029	0.60
		Nisin	1.65±0.128	2.10±0.071	0.23

B

Gene	Time and temperature of incubation	Group	ΔC_T	$\Delta\Delta C_T$*	$2^{-\Delta\Delta C_T}$
dltA	24 h at 37 °C	Control	4.90±0.302	0	1.0
		P34	1.88±0.183	-3.02±0.119	8.11*
		Nisin	1.83±0.190	-3.07±0.112	8.39*
	240 h at 4 °C	Control	4.99±0.197	0	1.0
		P34	1.72±0.111	-3.27±0.086	9.64*
		Nisin	1.19±0.148	-3.80±0.049	13.92*
Imo 1695	24 h at 37 °C	Control	3.62±0.342	0	1.0
		P34	-7.57±0.201	-11.19±0.141	2336.2*
		Nisin	2.61±0.175	-1.00±0.119	2.0
	240 h at 4 °C	Control	5.52±0.223	0	1.0
		P34	1.83±0.118	-3.69±0.105	12.9*
		Nisin	3,07±0.165	-2.44±0.058	5.42*
mptA	24 h at 37 °C	Control	-7.38±0.277	0	1.0
		P34	-7.58±0.154	-14.96±0.123	31872.0*
		Nisin	-6.59±0.132	-13.97±0.145	16047.0*
	240 h at 4 °C	Control	-2.70±0.228	0	1.0
		P34	0.07±0.147	2.78±0.081	0.14
		Nisin	-0.17±0.165	2.52±0.063	0.17

*Relative gene expression considered significant for values ≤-3 or ≥ 3. The experiments were performed in duplicated (n=2) for each gene on three independent experiments. *dltA* (D-Alanine-D-alanyl carrier protein ligase), *Imo 1695* (Putative phospholipid lysinylation) and *mptA* ($EIIAB^{Man}$ of mannose-specific PTS).

A significant change in the gene expression level was considered when a three-fold decreased or increased expression was obtained in comparison to control. Our results showed a non-significant increase in the transcription levels of genes *dltA, Imo1695* and *mptA* after incubation of *L. monocytogenes* with peptide P34 and nisin for 24 h at 37 °C or 240 h at 4 °C.

For the expression levels of *dltA* and *Imo1965*, our results are consistent with those proposed by [40], who also found no significant changes in expression of these genes in wild-type strains of *L. monocytogenes*. The *L. monocytogenes* used in this study is a collection strain (ATCC) and the decrease of *mptA* gene expression has been reported only for resistant or mutant strains of *L. monocytogenes* [38, 40, 41]. Therefore the result of a non-significant increase in *mptA* gene expression in *L. monocytogenes* ATCC 7644 is consistent with the literature.

In the strain of *L. seeligeri*, a significant decrease of *dltA* gene expression was observed, with similar values to the peptide P34 and nisin after incubation for 24 h at 37 °C. The expression of this gene was highly suppressed after incubation for 240 h at 4 °C, and the treatment with nisin resulted in lower *dltA* expression as compared with the peptide P34. The gene *Imo1695* showed a significant decrease in its expression (2336-fold) when *L. seeligeri* was treated with the peptide P34 for 24 h at 37 °C. A significant decrease of *Imo1695* gene expression was observed after incubation with peptide P34 and nisin for 24 h at 37 °C. Under this condition, the

gene *Imo1695* was two times less expressed by nisin treatment as compared with the peptide P34.

A significant decrease in *mptA* gene expression was also observed for *L. seeligeri* after incubation with peptide P34 and nisin for 24 h at 37 °C. This gene was 31.872-fold less expressed after treatment with the peptide P34 as compared with the control. In addition, the expression of this gene was significantly lower after treatment with nisin. Interestingly, the inoculation of the plate with the peptide P34 and nisin and further incubated for 240 h at 4 °C showed a non-significant increase of gene expression. *Listeria* thrives at refrigeration temperatures and therefore is feasible that the exposition to low temperature would influence the expression of genes related with energetic metabolisms, such as *mptA*. Recently, the influence of P34 on specific gene expression of *L. monocytogenes* after the inoculation in Minas Frescal cheese was investigated. A significant increase in the expression of the genes *dltA, Imo 1695* and *mptA* was observed after 96 h in the presence of peptide P34 at 5 °C [12].

Previous studies have shown that a 1000-fold increased resistance to class IIa bacteriocins in *L. monocytogenes* and *Enterococcus faecalis* resulted from the loss of *mptA* expression [38, 42, 43]. The cell wall of *L. monocytogenes* is composed by a thick peptidoglycan layer containing two types of anionic polymers: teichoic acids (TA), which are covalently linked to the peptidoglycan, and lipoteichoic acids (LTAs), which are poly phosphoglycerols substituted with a D-alanyl ester or a glycosyl residue and anchored in the membrane by their glycolipid moiety [44]. Some authors suggest that bacteriocin-induced lysis could be due to the release of autolytic enzymes that are usually electrostatically bound to anionic polymers (TA and LTAs) of the cell wall, which are displaced by cationic bacteriocins from their binding sites [45-47].

CONCLUSION

In this study, we observed a non-significant influence of peptide P34 and nisin on the expression of *L. monocytogenes* genes after the plate activity assay. However, in *L. seeligeri* the peptide P34 and nisin significantly influenced the expression of genes (*dltA, Imo1685*, and *mptA*) after incubation for 24 h at 37 °C. After an incubation period of 240 h at 4 °C, it was observed the more significant changes in gene expression (*dltA* and *Imo1685*) in *L. seeligeri*, but no significant change was observed for *mptA* gene. These results indicate the influence of nisin and peptide P34 on the expression of structural cell-surface/cell-membrane-associated genes.

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CONFLICT OF INTERESTS

Declared none

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