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Plasmid-Associated Bacteriocin Produced by *Pediococcus pentosaceus* Isolated from Smoked Salmon: Partial Characterization and Some Aspects of his Mode of Action

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Abstract
Strain ST3Ha, isolated from commercially available smoked salmon, was identified as *Pediococcus pentosaceus* based on biochemical and physiological tests and 16S rRNA sequencing. Strain ST3Ha produces a class IIa bacteriocin active against lactic acid bacteria, *Listeria monocytogenes* and *Enterococcus faecalis*. The antimicrobial peptide was inactivated by proteolytic enzymes, confirming his proteinaceous nature, but was not affected when treated with α-amylase, SDS, Tween 20, Tween 80, urea, and EDTA. No change in activity was recorded after 2 h at pH values between 2.0 and 9.0 and after treatment at 100 °C for 120 min or 121 °C for 15 min. The mode of action against *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 was bactericidal, resulting in cell lyses and enzyme leakage. The highest level of activity (1.6×10^6 AU/mL) was recorded when cells were grown at 37 °C or 30 °C in MRS broth (pH 6.5). Antimicrobial peptide ST3Ha adsorbs at high levels to the sensitive test organisms on strain-specific manner and depending on incubation temperature, environmental pH, and presence of supplemented chemicals. Based on PCR analysis, *P. pentosaceus* ST3Ha harbor a 1044-bp plasmid-associated fragment corresponding in size to that recorded for pediocin PA-1. Sequencing of the fragment revealed a gene identical to *ped*B, reported for pediocin PA-1. The combined application of the low levels (below MIC) of ciprofloxacin and bacteriocin ST3Ha results in the synergetic effect in the inhibition of *L. ivanovii* subsp. *ivanovii* ATCC 19119. Expressed by *P. pentosaceus* ST3Ha, bacteriocin was characterized as low cytotoxic, a characteristic relevant for its application in food industry and/or in human and veterinary medical practices.

Keywords Bacteriocins · *Pediococcus pentosaceus* · *Listeria ivanovii* subsp. *ivanovii* · *Enterococcus faecalis* · Antibiotics

Introduction

In combination with the movement for more natural, beneficial, and healthier food ingredients, food safety is more than even in the focus of the consumers in their choice of nutritional products. Consumers' demand for organic, beneficial, health-promoting food ingredients was driving food industry and research centers for evaluation of different natural antimicrobial metabolites as potent biopreservatives. Antimicrobial peptides, including bacteriocins, are

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well known to the science and food industry regarding their beneficial and safe properties. Produced by different groups of microorganisms, bacteriocins were pointed as potent antimicrobials with real applications in food preservation and in last decades as potential co-factors in the human and veterinary medicine for treatment of infectious diseases [1–3]. Special focus was always given to the bacteriocins produced by lactic acid bacteria (LAB) associated with generally recognized as safe (GRAS) status for several of representatives that belong to these bacterial groups. Bacteriocins of LAB are ribosomal synthesized antimicrobial peptides [1]. Their bactericidal mechanisms may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis [4].

Bacteriocins produced by the LAB were evaluated as potent antimicrobial metabolites with application for control of foodborne and spoilage microorganisms [2], complimentary to antibiotics therapeutical agents with application in human and veterinary health care [5]; molecules involve in the intracellular communications [2]. During the last two decades, focus on scientific interests regarding applications of bacteriocins was clearly showing their potential in the control as supplementary or principal agents in medical (human and veterinary) practice in treatments of multidrug-resistant pathogen-associated infections [5, 6]. It could be underlined that food biopreservation is a movement associated with healthier lifestyle, reduction of chemically designed synthetic preservatives, and application of safe natural ingredients in the food industry [7].

Foodborne pathogen, *Listeria monocytogenes*, is considered serious health problem, associated with his specificity of the virulence and elated with mortality. Generally, contaminations with *L. monocytogenes* are health concern for the elderly, babies, pregnant women, and immunocompromised individuals [8]; however, other consumers as well can be seriously affected by this pathogen. Several countries adopted zero tolerance to *L. monocytogenes* contaminations [9]. In addition to the good manufacture practices in food industry, appropriate methods for sanitation and conservation, research progress in the selection of effective antimicrobials against *L. monocytogenes* generated large database of the different approaches for the reduction and elimination of the listeria contaminations from food and feed products. Bacteriocins were pointed as effective, selective, and safe antimicrobials with effect against *Listeria* spp. [2]. Moreover, even one group in the classifications of bacteriocins was dedicated to anti-listerial antimicrobials [10]. These anti-*Listeria* bacteriocins were shown to be carrying a conservative amino acid fragment (YGNGVXaaC), related to the specific recognition of the lipid II receptor on the cell surface of the target cells and in this way directly involved in the selectivity of antimicrobial mode of action [11].

Application of different bacteriocins was suggested for the control of *L. monocytogenes*, including application in model food systems [2, 3]. The objective of this study was to select naturally represented bacteriocinogenic LAB from commercial smoked salmon samples, to characterize expressed bacteriocin with focus on their antimicrobial properties and potential further applications as biopreservative agent in salmon processing.

Material and Methods

Isolation of LAB with Antimicrobial Properties

Six samples, representing 3 different batches from 2 different brands of the commercially available smoked salmon purchased from the local supermarkets, were evaluated for the presence of the bacteriocinogenic LAB by method previously described by dos Santos et al. [12]. Commercial samples were stored under refrigeration in the commercial stores and after purchasing were transported in isotherm bags in the presence of ice cooler. Further, microbiological manipulations were performed in time period of 1 h after purchasing. In summary, 25 g of the smoked salmon was macerated in the presence of 225 mL sterile saline (0.85% NaCl, *w/v*) for 5 min in stomacher (Seward, West Sussex, UK), and tenfold serial dilution was prepared in sterile saline. An aliquot on 100 µL from each dilution was plated on the surface of previously prepared petri plates with MRS (Difco, Franklin Lakes, NJ, USA) supplemented with 2% agar (Difco) and covered with around 10 mL 2% agar and temperate to around 50 °C. Plates were incubated in aerobic environment at 37 °C for 24–48 h until formation of well-distinguished individual colonies. Enumeration and calculations of the CFU/mL were performed. Plates with well-distinguished individual colonies were covered with additional layer of 10 mL BHI (Difco), supplemented with 1% agar and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 or *Enterococcus faecalis* ATCC 19443 at 10^5 CFU/mL final concentration and incubated for additional 24 h at 37 °C. Plates were observed for formation of inhibitory zones around the colonies, and potential producers of the antimicrobial metabolites against *L. ivanovii* subsp. *ivanovii* ATCC 19119 or *E. faecalis* ATCC 19443 were isolated. The purity of the cultures was evaluated by well-accepted microbiological practices. Morphology of the pure cultures was observed regarding colony appearance and microscopically after Gram staining. In addition, preliminary identification as LAB was based on catalase negative reactions.

Preliminary bacteriocin production by the obtained isolates was evaluated according to dos Santos et al. [12], where cell-free supernatant (CFS) of the isolates was obtained after centrifugation (10 000 × *g*, 10 min, 20 °C) of 24-h-old cultures in MRS grown at 37 °C. pH of the CFS was corrected to values between 5.5 and 6.5 with 1 M NaOH and treated for 10 min at 80 °C. Aliquots of 10 µL were spotted on the surface of previously prepared plates with BHI supplemented with 1% agar and *L. ivanovii* subsp. *ivanovii* ATCC 19119 or *E. faecalis* ATCC 19443 at 10^5 CFU/mL final concentration. Plates were incubated at 37 °C for 24 h and visually observed for formation of inhibitions zones. The presence of larger than 3-mm inhibition zone was considered evidence for potentially produced antimicrobial metabolite(s) [12].

Isolates of potential interests were cultured in MRS for 24 h at 37 °C and stored in the presence of 20% sterile glycerol in cryovial tubes at − 20 °C and − 80 °C. Other microbial cultures, applied in the determination of spectrum of activity or as controls in further performed experiments, were stored in similar conditions as described above.

Differentiation and Identification

Isolates of interest were identified as LAB based on the colony morphology, microscope observation after Gram staining, and catalase test and other test recommended by Bergey's Manual of Systematic Bacteriology of Archaea and Bacteria [13], differentiated by RAPD-PCR [12] and further identified by 16S rRNA partial sequencing [14]. DNA from the 24-h cultures grown in MRS at 37 °C were obtained by ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), following the manufacture instructions. DNA concentration and quality were evaluated on NanoDrop (Thermo Fisher, Waltham, MA, USA). Initial differentiation of the obtained isolates was based on the microscopical observations and RAPD-PCR with primers OPL5 (5′-ACG CAG GCA C-3′), OPL8 (5′-AGC AGG TGG A-3′), OPL12 (5′-GGG CGG TAC T-3′), and OPL20 (5′-TGG TGG ACC A-3′), performed on Veriti 96-well thermal cycler (Thermo Fisher). Visualization of the generated amplicons was performed on 1.5% agarose gel in the presence of ethidium bromide. For the identification, PCR targeting 16S rRNA gene was performed on Veriti 96-well thermal cycler (Thermo Fisher) with primers F8 (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and R1512 (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3′), according to recommendations from dos Santos et al. [12] and amplicons sequenced by a contracted service (Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil). Obtained sequences were analyzed on https://blast.ncbi.nlm.nih.gov/Blast.cgi and compared with existing database [12].

Safety Evaluation of the Strain

Safety evaluation of the strains selected for the further studies was performed regarding its hemolytic properties, DNase, gelatinase, and lipase activity [15], sensibility to the effect of selected antibiotics [12], and production of biogenic amines [16]. The tests were performed in at least duplicate at 30 °C and 37 °C on two different occasions.

Antibiotic discs (Oxoid Basingstoke, Hampshire, UK) were applied in the process for evaluation of the resistance/susceptibility of the selected LAB, listed in Table 1. Selected strain was cultured in MRS broth for 24 h at 37 °C and used to supplement MRS with added 1% agar in order to reach 10^6 CFU/mL final concentration. The selected antibiotic discs (Table 1) were placed on the surface of the previously prepared plate and incubated for 24 h at 37 °C. Plates were evaluated for formation of inhibition zones and measured (mm) to determine the strain sensitivity [17]. The evaluation of resistance/susceptibility test was performed in duplicate on two independent occasions.

Table 1 Antibiotic susceptibility/resistance for *P. pentosaceus* ST3Ha based on antibiotic disk diffusion method

Antibiotics	Inhibition (mm) ± SD
Amoxicillin/clavulanic acid (30 µg per disk)	34 ± 1.63
Ampicillin/sulbactam (20 µg per disk)	36 ± 0.82
Bacitracin (10 units per disk)	11 ± 1.63
Cefepime (30 µg per disk)	18 ± 1.63
Cefotaxim (30 µg per disk)	32 ± 1.83
Cefriaxon (30 µg per disk)	30 ± 1.41
Ceftiofur (30 µg per disk)	38 ± 0.82
Cefuroxim (30 µg per disk)	36 ± 0.82
Chloramphenicol (30 µg per disk)	37 ± 1.82
Ciprofloxacin (5 µg per disk)	20 ± 2.16
Clindamycin (2 µg per disk)	26 ± 1.83
Enrofloxacin (5 µg per disk)	15 ± 0.00
Erythromycin (15 µg per disk)	25 ± 1.26
Florfenicol (30 µg per disk)	17 ± 1.15
Gentamicin (10 µg per disk)	0 ± 0.00
Imipenem (10 µg per disk)	26 ± 1.63
Levofloxacine (5 µg per disk)	15 ± 0.82
Neomycin N (10 µg per disk)	18 ± 0.82
Penicillin (10 units per disk)	25 ± 1.63
Tetracycline (30 µg per disk)	28 ± 1.83
Vancomycin (30 µg per disk)	29 ± 0.81

The evaluation of resistance/susceptibility test was performed in duplicate on two independent occasions

Confirmation of Protein Nature and Stability to pH, Temperature, and Chemicals

For determination of the protein nature expressed by studied LAB antimicrobial substances, recommendations from dos Santos et al. [12] were followed. The CFS (1 mL) obtained as described above were exposed to the effect of different enzymes, including α-amylase (Sigma Diagnostics, St. Louis, MO, USA), Proteinase K (Roche, Indianapolis, IN, USA), pronase, lipase, or catalase (Roche), to a final concentration of 1.0 mg/mL. Experimental setups were incubated at 37 °C for 60 min, followed by heating treatment for 5 min at 95–97 °C, in order to deactivate added enzymes. The pH of all experimental setups was evaluated and, if needed, adjusted to 6.0–6.5. The bacteriocin activity was determined, as described above, against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443.

Moreover, the effect of some specific selected chemicals, temperature, and pH on the stability of antimicrobial activity of the evaluated bacteriocin was determined. In this experiment, 1 mL of the previously obtained CFS was incubated at 25, 30, 37, 45, and 100 °C for 30 and 120 min and at 121 °C for 15 min, representing standard autoclaving conditions. The bacteriocin activity, before and after exposure, of the studied antimicrobials was determined as described above against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443.

The effect of pH on stability of bacteriocin produced by the studied LAB was performed following suggestions from dos Santos et al. [12], and pH of CFS (10 ml) obtained as described above was adjusted to 4.0, 6.0, 7.0, 8.0, or 9.0 with sterile 1 M HCl or 1 M NaOH. The designed experimental setups were incubated for 2 h at 37 °C. The pH was readjusted to 5.5–6.5, and bacteriocin activity was evaluated, as described before, against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443.

The chemicals commonly used by the food industry or in analytical evaluations of bacteriocins were analyzed for its effect on the stability of the bacteriocin produced by selected LAB. Skim milk (Difco), NaCl, SDS, Tween 20, Tween 80, and EDTA (all from Sigma-Aldrich) were added (0.1 g) to 1 mL of CFS of studied LAB, obtained as described before, followed by incubation for 1 h at 37 °C. pH of experimental set-ups was evaluated and, in case needed, adjusted to 5.5–6.5. Bacteriocin activity was evaluated as described above against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443.

The above-described experiments were performed in duplicate on at least two independent occasions. Controls containing non-treated CFS exposed to the conditions mentioned above were included in the experimental protocols.

Evaluation of Bacterial Growth, Changes in pH, and Bacteriocin Production

For the determination of bacterial growth, acidification, and bacteriocin production dynamics, the studied bacteriocin producing LAB culture was inoculated to 300 mL MRS broth as 2% inoculum and incubated at 25 °C, 30 °C, and 37 °C. Bacterial growth, estimated based on changes in optical density (600 nm) and changes in pH of the culture, was monitored hourly for 48 h. Bacteriocin activity, expressed as AU/mL, was evaluated every 3 h, as described above, against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443. The experiments were performed in duplicate on two independent occasions.

Spectrum of Activity for Expected Bacteriocin

Aliquots (10 µL) of the previously prepared CFS were spotted on the surface of the BHI or MRS supplemented with 1% agar plates seeded with test microorganisms listed in Table 2, at a final concentration of 10^5 CFU/mL. All plates were incubated at 30 °C or 37 °C for 24 h and evaluated for inhibition zones. Inhibition zones with diameter larger than 3 mm were considered positive.

Mode of Action

Partial Purification of the Isolated Bacteriocin

CFS obtained via centrifugation (10 000 × *g*, 15 min, 4 °C) from 300 mL of the 24-h-old culture of the bacteriocinogenic LAB cultured in MRS broth at 37 °C were heated for 10 min at 80 °C. Precipitation of the proteins was carried out by 60% ammonium sulfate saturation for 4 h at 4 °C. Precipitated proteins were collected after centrifugation (20 000 × *g*, 60 min, at 4 °C), and resulting pellet was resuspended in 30 mL of 25 mM ammonium acetate buffer (pH 6.5). Then, proteins were separated via hydrophobic chromatography on a SepPak C_{18} column (Waters, Millipore, MA, USA). Proteins were eluted from the SepPak C_{18} column via a step gradient with 20, 40, 60, and 80% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). All eluted fractions were evaluated for their bacteriocin activity against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, the test microorganisms. After drying under reduced pressure (Speed-Vac, Savant, France), the bacteriocin fraction was dissolved in deionized water before being applied in subsequent experiments.

Effect of the Bacteriocin Containing CFS on Exponentially Growing Test Organisms

For evaluating the effect of studied bacteriocin on the growing of test strains (*L. ivanovii* subsp. *ivanovii* ATCC 19119

Table 2 Antagonism of bacteriocin produced by *Pediococcus pentosaceus* ST3Ha against various target strains cultured at 30 and 37 °C*

Target strains	Growth medium	Inhibition[a]
Acinetobacter baumannii	BHI	0/2
Bacteroides fragilis	BHI	0/2
Escherichia coli	BHI	0/8
Enterobacter cloacae	BHI	0/2
Ent. faecalis	MRS	12/14
Ent. faecium	MRS	8/8
Klebsiella pneumoniae	BHI	0/3
Lactobacillus acidophilus	MRS	2/2
Latilactobacillus curvatus	MRS	0/3
Lactobacillus delbrueckii	MRS	0/4
Limosilactobacillus fermentum	MRS	0/5
Lacticaseibacillus paracasei	MRS	1/7
Lactiplantibacillus plantarum	MRS	0/8
Lacticaseibacillus rhamnosus	MRS	0/3
Ligilactobacillus salivarius	MRS	0/1
Latilactobacillus sakei	MRS	0/4
Lactococcus lactis subsp. *lactis*	MRS	1/1
Leuconostoc lactis	BHI	2/2
Listeria innocua	BHI	6/6
L. ivanovii subsp. *ivanovii*	BHI	1/1
L. monocytogenes	BHI	21/21
Pediococcus acidilactici	MRS	0/1
Pediococcus pentosaceus	MRS	1/8
Staphylococcus aureus	BHI	2/14
Staph. uberis	BHI	1/1
Streptococcus agalactiae	BHI	0/3
Str. caprinus	BHI	2/2
Str. gallolyticus subsp. *macedonicus*	MRS	0/1
Str. infantarius subsp. *infantarius*	MRS	2/2
Str. pneumoniae	BHI	0/5
Bacillus subtilis	BHI	0/5

BHI - Brain Heart Infusion agar (Oxoid), *MRS* - De Man, Rogosa, Sharpe agar (Difco)

*No differences were observed when experiments were performed at both incubation temperatures. As positive were considered inhibitions zone of at least 3 mm in diameter

[a]Number of strains sensitive to bacteriocin ST3Ha from total number tested

and *E. faecalis* ATCC 19443), recommendations from Favaro et al. [18] were followed. In summary, 200 mL BHI broth was inoculated (1%) with *L. ivanovii* subsp. *ivanovii* ATCC 19119 or *E. faecalis* ATCC 19443, respectively, and initially incubated for 3 h at 37 °C, to enable bacterial cultures reaching early exponential growth phase. The previously prepared 30 mL CFS, obtained from bacteriocinogenic LAB cultured for 24 h at 37 °C, was filter-sterilized (0.22-µm Millipore sterile filters, Millipore, Burlington, MA, USA) and added to the *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, applied as test strains. Cultures were monitored by measurement of OD at 600 nm every hour during 36 h. In addition, growth of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 was determined after 9, 24, and 36 h of incubation. Determination of the CFU/mL was performed for all experimental setups, including controls (without added bacteriocin), at specified times by plating on BHI supplemented with 2% agar and further incubation at 37 °C for 48 h. The experiments were performed in duplicate on two independent occasions.

Determination of Cell Lysis by Measuring the Extracellular Levels of β-Galactosidase

Cells from the 100 mL culture of 12-h-old cultures of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, grown in BHI, were harvested by centrifugation (5000 × *g*, 15 min, 20 °C), washed twice with 0.03 M sodium phosphate buffer (pH 6.5), and resuspended in 10 mL of the same buffer. The partially purified bacteriocin, dried and resuspended in original volume deionized sterile MilliQ water (Millipore), was then sterilized (by filtration through a 0.20-µm membrane filter, Millipore). Previously prepared cell suspensions of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19433 (2 mL) were treated with equal volumes of semi-purified bacteriocin (60% isopropanol fraction) and incubated for 5 min at 25 °C. In next, 0.2 mL 0.1 M ONPG (*O*-nitrophenyl-β-D-galactopyranoside, Sigma) in 0.03 M sodium phosphate buffer (pH 6.8) was added, and after 10 min at 25 °C, 2.0 mL 0.1 M sodium carbonate was added in order to stop the reaction [19]. The cells were removed by centrifugation (8000 × *g*, 15 min, 25 °C), and absorbance of the supernatant was recorded at 420 nm. Mechanically disrupted cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19433 by 0.1 mm diameter glass beads were used as controls. The experiment was performed in duplicate on two independent assays.

Determination of Cell Lysis of Target Microorganisms in the Presence of Bacteriocin

Evaluation of the effect of studied bacteriocin on the cell lysis of test microorganisms was determined using sterile flat-bottom 96-well microtiter plates (Nunc, Thermo Fisher Scientific). Cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 were harvested by centrifugating (6000 × *g*, 4 °C, 10 min) from 10 mL of 18-h-old cultures grown in BHI, washing two times with potassium phosphate buffer (20 mM, pH 6.5), and resuspending in 5 mL of same buffer. One hundred microliters of the obtained bacterial suspensions was distributed individually in microtiter plate wells and supplemented with 50 µL of semi-purified

bacteriocin (60% iso-propanol fraction) dissolved in potassium phosphate buffer (20 mM, pH 6.5) at different serial 1/2 dilutions. These experimental sets were incubated for 24 h at 37 °C, and absorbance at 655 nm was measured on microplate reader (Thermo Fisher). Bacterial cell lysis was calculated as [100 – (At/Ao × 100)], where Ao was the absorbance measured at time 0 and At the absorbances measured at 3, 6, 9, or 24 h of incubation [20].

Determination of the Reduction of Viable Cells of Target Microorganisms in the Presence of the Bacteriocin

Cells from the early stationary phase (18-h-old) of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 target strains, grown at 37 °C in 20 mL BHI obtained by centrifugation (5000 × *g*, 5 min, 4 °C), were washed twice with sterile saline solution to eliminate traces of the growth media and resuspended in 10 mL saline solution. Equal volumes of the obtained cell suspensions and the test bacteriocin sterilized by filtration (0.22 µm, Minisart®, Sartorius) were mixed. CFU/mL, representing the viable cell numbers, was determined before and after incubation at 37 °C for 60 min by plating onto BHI with 2% agar. As controls, cell suspensions of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 without added bacteriocin were applied. The experiment was performed in duplicate on two independent occasions.

Adsorption of Bacteriocin to Test Organisms and Effect of pH, Temperature, and Chemicals

For the evaluation of the adsorption of bacteriocin, produced by studied LAB, to different target cells, recommendations from Yildirim et al. [21] were followed. The selected test organisms listed in Table 3 were grown overnight (in 10 mL MRS or BHI broth at 30 °C or 37 °C) and cells harvested by centrifugation (5000 × *g*, 15 min, 20 °C). Cells were washed twice with sterile 20 mM phosphate buffer (pH 6.5) and resuspended in 10 mL of the same buffer. When needed, the pH was adjusted to 6.5 with sterile 0.1 M NaOH. Obtained cell suspensions were individually supplemented with an equal volume of studied CFS bacteriocins prepared as described above and incubated for 1 h at 37 °C. Afterwards, the cells were removed by centrifugation (5000 × *g*, 15 min, 20 °C), and the activity of unbound bacteriocin present in remain supernatant was determined and expressed in AU/mL, as described above. The experiments were performed in duplicate on two independent occasions.

For the calculation of the percentage of adsorbed bacteriocin to studied target cells, recommendations from Yildirim et al. [21] were followed:

$$\% \text{ adsorption} = 100 - \left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}} \times 100\right)$$

For evaluating the effect of temperature and pH on the adsorption of studied bacteriocin to *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, incubation conditions, 1 h at 4, 10, 30, 37, 45, and 60 °C, respectively (pH 6.0), and at 37 °C at pH 2.0, 4.0, 6.0, 8.0, and 10.0, were evaluated. Cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, cultured in 10 mL BHI for 18 h at 37 °C, were collected by centrifugation (5000 × *g*, 15 min, 20 °C), washed twice with sterile 20 mM phosphate buffer (pH 6.5), and resuspended in 10 mL of the same buffer. The pH of the bacterial suspension

Table 3 Evaluation of the adsorption of bacteriocin produced by *Pediococcus pentosaceus* ST3Ha to sensitive and resistant test organisms

Target organism	Inhibition by bacteriocin produced by *P. pentosaceus* ST3Ha	Adsorption (%)
Bacillus subtilis ST826CD	–	40
Enterococcus faecalis ATCC 19443	+	80
Enterococcus faecium ST5Ha	+	60
Enterococcus faecium ST88Ch	+	80
Lactiplantibacillus plantarum ATCC 14917	–	40
Lactiplantibacillus plantarum ST8Sh	–	20
Latilactobacillus sakei 2a	–	20
Listeria innocua ATCC 33090	+	60
Listeria ivanovii subsp. *ivanovii* ATCC 19119	+	80
Listeria monocytogenes ATCC 15313	+	80
Pediococcus acidilactici ST3522BG	–	20
Pediococcus pentosaceus ST3633BG	–	20
Pediococcus pentosaceus ST58	–	40
Streptococcus infantarius subsp. *infantarius* K1-4	+	60

[a] – = no inhibition zone, + = inhibition zone of at least 3 mm in diameter

was adjusted to 6.0 with sterile 1 M NaOH. Bacterial suspensions were supplemented with same volume of CFS from the tested bacteriocinogenic strains, and after pH adjusted to 6.0, they were incubated for 1 h at indicated temperatures. CFS and bacteriocin activity and levels of adsorption were determined as described before.

For evaluating the effect of the pH on the adsorption of the tested bacteriocin to *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 target strains, cell suspensions of both test organisms were washed and resuspended in sterile saline solution (0.85% NaCl, *w/v*) followed by the adjustment to the previously mentioned pH values with sterile 1 M NaOH or 1 M HCl and supplemented with bacteriocin containing CFS, prepared as described above. After incubation for 1 h at 37 °C, the pH value of CFS obtained by centrifugation (5000 × *g*, 15 min, 20 °C) was corrected to 6.0. Bacteriocin activity and levels of adsorption were determined as described previously.

For evaluating the effect of inorganic salts and organic compounds (1% NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, Na_2CO_3, EDTA ($C_{10}H_{16}O_8N_2$), β-mercaptoethanol, 80% ethanol, methanol, and chloroform) on the adsorption of tested bacteriocin to *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, recommendations from Todorov et al. [22] were followed. The cells from *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, cultured in 10 mL BHI for 18 h at 37 °C, were collected by centrifugation (5 000 × *g*, 15 min, 20 °C), washed twice with 20 mM potassium phosphate buffer (pH 6.5), and resuspended in 10 mL of the same buffer. Obtained cell suspensions were supplemented with above-mentioned chemicals, and, if needed, pH values were adjusted to 6.5 with 1 M NaOH or 1 M HCl. Experimental setups were incubated for 1 h at 37 °C followed by removal of the cells by centrifugation (5000 × *g*, 15 min, 20 °C), evaluation of the bacteriocin activity in the CFS, and calculation of the levels of adsorption as described before. All experiments were performed in duplicate on two independent occasions.

Antimicrobial Action of Isolated Bacteriocin in Combination with Ciprofloxacin

In the first stage, the minimal inhibition concentration (MIC) of ciprofloxacin (Sigma) was determined for *L. ivanovii* subsp. *ivanovii* ATCC 19119. *L. ivanovii* subsp. *ivanovii* ATCC 19119 was grown in BHI at 37 °C in the presence of selected concentrations of ciprofloxacin from 0.03 to 1280 µg/mL. In the following experiment, the bacterial growth of *L. ivanovii* subsp. *ivanovii* ATCC 19119, in the presence of a combination of ciprofloxacin and the tested bacteriocin, was evaluated on sterile flat bottom microtiter plates (Nunc). Each well was filled with 170 µL of BHI and supplemented with different concentrations of 10 µL ciprofloxacin above and under MIC (final concentrations between 320 and 5 µg/mL) and 10 µL bacteriocin (final concentrations 78 125 AU/mL, 19 531 AU/mL, 4 883 AU/mL, 1 220 AU/mL, 305 AU/mL, 76 AU/mL, 19 AU/mL, 4.75 AU/mL, 1.15 AU/mL, and 0.3 AU/mL) and inoculated with 10 µL of the 18-h-old culture of *L. ivanovii* subsp. *ivanovii* ATCC 19119. Changes in OD measurements at 655 nm were recorded, each hour during 30 h, on Thermo Fisher microplate reader. *L. ivanovii* subsp. *ivanovii* ATCC 19119 grown in BHI without added bacteriocins and ciprofloxacin served as controls. The experiment was performed on two independent occasions.

Plasmid Isolation and Screening for Bacteriocin Genes on Genomic and Plasmid DNA

Plasmid DNA from studied bacteriocinogenic strain was isolated using the Zyppy^{tm} Plasmid Miniprep Kit (Zymo Research, USA) according to instructions of the manufacturer. Total DNA was isolated as described above. Plasmid and total genomic DNA obtained from the studied bacteriocinogenic strains were submitted to PCR applying primers PEDRPO (5′-CAA GAT CGT TAA CCA GTT T-3′) and PEDC1041 (5′-CCG TTG TTC CCA TAG TCT AA-3′), targeting the part of the operon encoding Pediocin PA-1/AcH [23]. PCR was performed on GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA) at following conditions: an initial denaturation step of 94 °C for 1 min followed by 35 cycles of 1 min at 94 °C, 30 s at 50 °C, and 1 min at 72 °C and final extension at 72 °C for 5 min, according to Todorov et al. [23] recommendations. The generated amplicons were visualized in a 0.8% (*w/v*) agarose gel stained with ethidium bromide.

Test for Immunity of Studied Strain to Pediocin PA-1

Evaluated bacteriocinogenic LAB was grown in MRS broth for 24 h at 37 °C and applied as test organisms for the evaluation of the sensitivity/resistance to pediocin-PA partially purified bacteriocin, according to the recommendations from Favaro et al. [24]. MRS agar was supplemented with studied bacteriocinogenic LAB culture (final concentration of 10^5 CFU/mL) and distributed to the petri plates. Semi-purified pediocin PA-1, obtained from *Pediococcus acidilactici* ST2104V, was evaluated for its inhibitory activity by depositing aliquots of 10 µL of two-fold dilution in sterile phosphate buffer (pH 6.5, 25 mM) up to 1:16 on the surface of prepared plates and incubated for 24 h at 25, 30, and 37 °C. The presence of the inhibition zones was considered evidence for inhibitory action of pediocin PA-1 again applied bacteriocinogenic strain, indication for absence of effective pediocin PA-1 immunity system [24].

Cytotoxicity of the Bacteriocin

Cytotoxicity of the semi-purified bacteriocin produced by the studied LAB was evaluated applying monkey kidney Vero cells, according to the recommendations of Wachsman et al. [25]. The monolayers of Vero cells, grown in tissue culture plates for 48 h, were supplemented to different concentrations of semi-purified bacteriocin produced by studied LAB. After incubation for 24 h, viability of Vero cells was determined based on the formation of blue formazan associated with cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) by mitochondrial succinate dehydrogenase. Results were expressed as 50% cytotoxic concentration (CC_{50}), defined as the peptide concentration (μg/mL) required to lowering the cell viability by 50%. CC_{50} values were calculated by regression analysis.

Results and Discussion

Isolation of LAB with Antimicrobial Properties

Six different samples of commercially available smoked salmon representing different lots from 2 different brands were evaluated for the presence of bacteriocinogenic LAB with activity against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443. Based on performed enumeration on MRS agar plates, average bacterial count was reasonably low, ranging from 2.43×10^3 CFU/mL to 1.57×10^4 CFU/mL for brand A and from 2.91×10^4 CFU/mL to 3.87×10^4 CFU/mL for brand B. Based on the performed, randomly isolated colonies, catalase reaction, and Gram staining, most of the presented bacteria present in the commercial samples of smoked salmon can be considered LAB, with majority presenting coccoid morphology. The presence of LAB in food commodities normally is not considered a safety hazard, since several representatives of LAB are used by food industry as beneficial cultures, namely as starter, bioprotective, probiotics, or adjunct cultures [26]. Moreover, most of the LAB are considered GRAS, since a long time being applied in food processing [27, 28]. On the other side, some LAB, especially that belonging to the genus *Streptococcus* and *Enterococcus* were clearly evaluated as serious pathogens to human and to other warm-blooded animals, based on their virulent behavior [29]. High levels of some LAB strains can also affect the shelf life of food products, associated with acidification processes and reductions of viable carbohydrates [30].

Based on the preliminary screening for bacteriocinogenic LAB, regarding the observed inhibition zones from the whole set of experiments, more than 60 isolates were selected and evaluated for potential production of antimicrobial peptides. However, according to the recommendations of dos Santos et al. [12], only 4 isolates were confirmed as potential producers of bacteriocins. The other isolates showed activity against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 only when the CFS pH was not corrected (with pH between 3.5 and 4.2), and after correction of the pH to 5.5–6.5, no inhibitory zones were observed against the applied test microorganisms. The presence of bacteriocins producers in different fermented food products can vary significantly, depending on the presence of just a specific strain or on a variety of species and strains [31]. This can be linked to the type of fermented foods, associated with different processing procedures, hygienic conditions, and different pre- and post-treatments of the food products. In the case of the smoked salmon, a non-fermented and non-pasteurized food product, application of good manufacturing practices (GMP) for manipulation of fish products, in addition to smoke exposure, vacuum packaging, and low temperature storage, can be some of the reasons for the low bacterial load of studied samples and, therefore, the low presence of bacteriocinogenic species.

Differentiation and Identification of Bacteriocinogenic Producers

Selected potential producers of bacteriocins were preidentified as LAB (Gram-positive reaction, coccoid morphology after microscope observation, and catalase negative) and differentiated by RAPD-PCR (Fig. 1) as two different strains, since 3 out of 4 tested isolates generated similar amplicon profiles. Further identification based on 16S rRNA pointed out that studied isolates belong to *P. pentosaceus* and *E. faecium* species. The isolate ST3Ha, identified as *P. pentosaceus*, was selected for future studies.

The presence of bacteriocinogenic *Pediococcus* spp. in salmon is not an unusual find. Previously, Tome et al. [32], Jang et al. [33], and Todorov et al. [34] reported on different strains belonging to genus *Pediococcus* isolated from smoked and raw salmon samples. These authors evaluated different characteristics expressed by the studied bacteriocinogenic strains, some of them suggesting their potential for application in biopreservation of food products, including in smoked salmon-based production. Moreover, Todorov et al. [20] referred to the probiotic potential for *P. acidilactici* isolated from salmon and characterized as bacteriocinogenic by Tomé et al. [35]. Additionally, the same strain was whole genome sequenced and genes of pediocin PA-1 appropriately mapped, and some additional beneficial properties of the strains were proposed by Kuniyoshi et al. [36].

Safety Evaluation of the Studied Strain

Associated with the concept "safety is a priority," one of the principal evaluations of the *P. pentosaceus* ST3Ha was

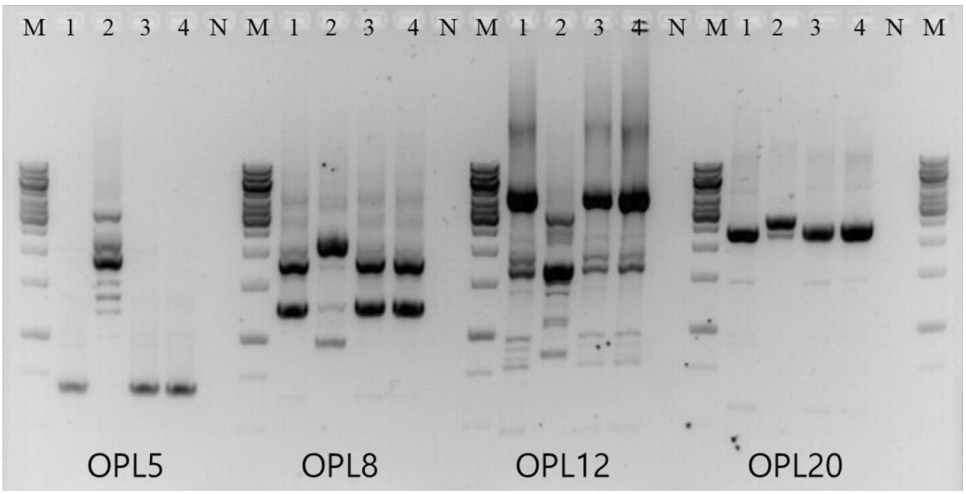

Fig. 1 RAPD-PCR (primers OPL5, OPL8, OPL12, and OPL20) profiles of 4 bacterial isolates with antimicrobial activity, obtained in the screening process for new bacteriocinogenic strains isolated from smoked salmon. M, 1-kb ladder (Thermo Fisher); N, no DNA added to the PCR reaction; 1–4, strains evaluated in present study

the confirmation of its safety profile. Based on performed experiments at 30 °C and 37 °C, *P. pentosaceus* ST3Ha studied strain can be considered safe, since it was confirmed as γ hemolytic, DNase, gelatinase, and lipase activity negative (data not shown). Evaluation of the strain sensitivity to the effect of selected antibiotics revealed that the strain was sensitive to most of the evaluated antibiotics (Table 1) and no production of biogenic amines was recorded in *P. pentosaceus* ST3Ha.

Pediococci in general are considered safe microorganisms and were applied in different fermentation processes [34, 37], as biopreservatives [35, 38], starter cultures [34, 35, 39, 40], and even suggested as probiotics with health-promoting properties [34, 41]. However, on the other side, some strains from genus *Pediococcus* were evaluated as pathogens and food spoilage microorganisms [42, 43]. Thus, it is very important to perform an accurate safety evaluation for new isolated microbial strains regarding their safety properties, and if further commercial application is an aim, a whole genome sequence and appropriate analysis of obtained sequences need to be considered an essential step.

Confirmation of Protein Nature and Stability to pH, Temperature, and Chemicals

For determination of the protein nature and classification of the antimicrobial metabolites produced by the selected LAB strain, the obtained CFS was treated for 10 min at 80 °C and exposed to different proteolytic enzymes. Complete deactivation of the antimicrobial activity after CFS exposure to the tested proteolytic enzymes was recorded (Table 4). However, after exposure to α-amylase, lipase, or catalase, no changes in the activities were observed. Thus, these results confirmed the proteinaceous nature of the expressed by *P. pentosaceus* ST3Ha antimicrobial metabolites, as well, pointing out that carbohydrates or lipids are not involved in the structure of the antimicrobial agent. Also, noninhibitory activity was associated with the presence of H_2O_2. By definition, bacteriocins are antimicrobial peptides with proteinaceous nature, produced by the cell ribosomal systems, and without post-translational modifications [1]. Testing for strain sensitivity to proteolytic enzymes is considered an easy approach for the confirmation of those characteristics [1]. Moreover, in previous studies on bacteriocins produced by different LAB, some were described as complex molecules, where in

Table 4 Stability of bacteriocin produced by *Pediococcus pentosaceus* ST3Ha after treatment with enzymes, detergents, urea, heat, and pH

	Concentration	*E. faecalis* ATCC 19443	*L. ivanovii* subsp. *ivanovii* ATCC 19119
Enzymes	1.0 mg/ml		
Lipase, α-amylase, catalase		+	+
Proteinase K, pronase		−	−
Detergents/chemicals	1% (w/v)		
Tween 20, Tween 80		+	+
SDS		+	+
EDTA		+*	+*
Skim milk, NaCl		+	+
Temperatures			
25, 30, 37, 45, 100 °C for 2 h		+	+
121 °C for 15 min		+	+
pH			
pH 4.0–9.0		+	+
Control		+	+

*Reduction of the activity with about 50% based on observed inhibition zones were observed; Activity of bacteriocin produced by *Pediococcus pentosaceus* ST3Ha was expressed in + = presence of inhibition zone (> 3 mm); − = no inhibition

addition to the protein core, a polysaccharide or lipid moiety can be involved in formation of the active structure of the antimicrobial complex, and interference with that moiety can influence the antimicrobial activity [31, 44].

Based on the evaluation of the stability of the bacteriocin produced by *P. pentosaceus* ST3Ha, it was observed that exposure to temperatures of 25, 30, 37, 45, and 100 °C for 30 and 120 min and at 121 °C for 15 min did not affect the antimicrobial activity (Table 4). A slight reduction of the inhibition zones against *L. ivanovii* subsp. *ivanovii* ATCC 19119 was observed only after treatment for 15 min at 121 °C, however not when *E. faecalis* ATCC 19443 was applied as test organism. Similar observations were reported for several other bacteriocins produced by pediococci and other LAB [37, 45–48]. This behavior is considered positive feature, since these antimicrobial proteins can be easily applied during food processing not being affected by the thermal processing stages, as well as they can be autoclaved and applied as sterile ingredients of formulations for human and veterinary medical practices.

Exposure to the different pH range did not affect antimicrobial activity. This property is also a positive feature of the studied bacteriocin since this opens possibilities for its application in different fermented food products, where pH is reduced because of the fermentation process and the presence of different LAB. Most of the bacteriocins produced by LAB showed similar behavior—stability in a large range of pH [37, 45–48]—however, some lost their activity when exposed to alkaline conditions [49].

The chemicals commonly used by the food industry or used in the analytical evaluation of the bacteriocins can be factors that may influence bacteriocin activity. In our study, skim milk, NaCl, SDS, Tween 20, and Tween 80 did not influence the antimicrobial effect of *P. pentosaceus* ST3Ha bacteriocin; however, the presence of EDTA reduced in more than 50% of the inhibition's zones against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 (Table 4). These results provide information about a potential application of the produced bacteriocin by *P. pentosaceus* ST3Ha, such as in dairy or other food products with high NaCl contents. Moreover, different detergents and chemicals normally are applied in fermentation and purification processes. Reduction of the antimicrobial activity by EDTA is a point that needs to be considered in the design of bacteriocins production and purification processes. It was already reported that some surfactants can affect bacteriocins activity by interacting with their stability or configuration of the antimicrobial molecule [22].

Evaluation of Bacterial Growth, Changes in pH, and Bacteriocin Production

Bacterial growth, acidification, and production of bacteriocin dynamics were evaluated for *P. pentosaceus* ST3Ha in MRS broth at 25 °C, 30 °C, and 37 °C, respectively. Temperature did not influence the bacterial growth; biomass concentration reached higher levels around 21 h after the start of the fermentation process (Fig. 2). The bacterial growth was following the standard bacterial behavior for representatives of genus *Pediococcus* in MRS broth, as similar observations were previously recorded in the evaluations of bacterial

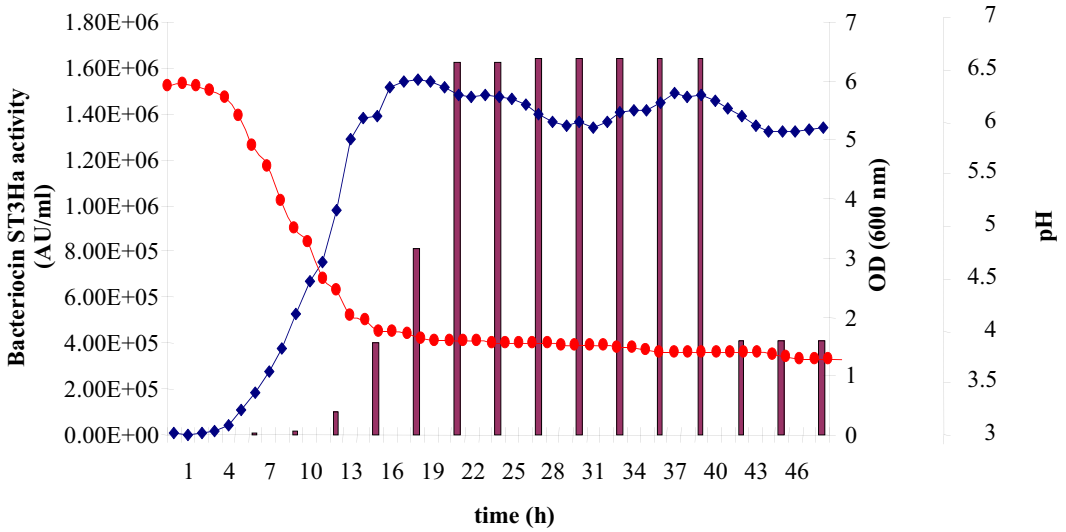

Fig. 2 Kinetics of bacterial growth (-◆-), acidification (-●-), and bacteriocin production (histogram), expressed in AU/mL for *P. pentosaceus* ST3Ha. Bacteriocin activity was evaluated against *L. ivanovii* subsp. *ivanovii* ATCC 19119. The data points represent an average of four repeats for OD and pH analyses that did not vary by more than 3%. Standard deviation bars are not shown

growth of other bacteriocinogenic pediococci cultured in MRS [37, 45–48].

In the monitored period, the pH of the culture was reduced in the first 15 h, reached pH around 4.0, and stay reasonably without changes for the following monitored period, going down to 3.72 after 48 h of fermentation (Fig. 2). Reduction of the pH occurred in line with previously reported changes on other pediococci growth in MRS broth [37, 45–48]. In the monitored period, levels of bacteriocin activity increased up to 1 622 400 AU/mL after 21 h from the beginning of fermentation process, staying stable during the next 18 h and decreasing after 42 h down to 409 600 AU/ml.

A relation between bacterial growth and increased levels of bacteriocin production was expected, since correlation between number of bacterial cells and record of the expressed bacteriocins was reported before [37, 45–49]. However, at the late stationary phase, the bacteriocin activity decreased (after 42 h from the beginning of the fermentation process). Such reduction of the bacteriocin activity is not always related to the reduction of the bacteriocin production, since in some cases, this can be associated with bacteriocin (protein) degradation resulting from the liberation of intracellular proteases, as consequence of the increased levels of dead and lysed bacterial cells in late stationary growth phase [49], or aggregation between bacteriocin molecules can occur, associated with the pH reduction [50]. However, the last hypothesis, most probably, can be regarded as the principal argument for the observed reduced levels of bacteriocin activity, since drop of the pH occurred much earlier in the monitored fermentation process.

Spectrum of Activity

Bacteriocin produced by *P. pentosaceus* ST3Ha showed inhibitory activity against the majority of the tested *Listeria* spp. and *Enterococcus* spp. strains (Table 2). In addition, some of the LAB, as well, presented sensitivity to the tested bacteriocins. It is interesting to underline that the majority of LAB applied as starter cultures or beneficial strains, including different bacteriocin producing strains, were resistant to the bacteriocin produced by *P. pentosaceus* ST3Ha. Specificity in the spectrum of activity of bacteriocins is considered beneficial characteristics, allowing to be applied for selective control of food spoilage and contaminants and specific human and other animals' pathogens [34]. Moreover, it is important to take into account that applications of bacteriocins or bacteriocin-producing cultures are not supposed to inhibit starter cultures or other beneficial microbial species/strains present in the fermentation system or playing beneficial properties in human and other animals GIT or other systems.

Specificity of bacteriocin activity against other bacteriocinogenic LAB provides interesting information regarding the nature of expressed antimicrobial proteins. The mechanisms of immunity of bacteriocin producing culture against their own bacteriocins are associated with specific immune proteins, encoded with genes normally associated to the same operon where structural bacteriocinogenic gene/s are located. By these mechanisms, the producer protects himself from the effect of his own bacteriocin [1]. Moreover, it was reported that genes associated to the immunity properties are sharing high similarity in the strains producing similar bacteriocins [51]. Based on this, it was expected that some bacteriocinogenic LAB applied as test microorganisms present a high possibility to be resistant to the effect of the bacteriocin produced by *P. pentosaceus* ST3Ha if expressed bacteriocins of sensitive strains shared a similarity to that evaluated in this study.

Mode of Action

Partial Purification of the Isolated Bacteriocin

In the preliminary experiment, different levels of ammonium sulfate (40, 60, 80% saturation) were evaluated for the selection of the appropriate approach for precipitation of produced *P. pentosaceus* ST3Ha antimicrobial peptide (data not shown). Obtained material, after 60% saturation with ammonium sulfate precipitation and further separation on SepPak C_{18} hydrophobic chromatography via step gradient with 20, 40, 60, and 80% isopropanol in 25 mM ammonium acetate buffer (pH 6.5), presented bacteriocin activity predominantly with 60% and low levels of activity in 40% iso-propanol fractions.

Purification of antimicrobial peptides is a complex process, frequently designated as "the art of biochemistry." In general, bacteriocins are described as hydrophobic cationic peptides [11]. Some standard steps were applied in the partial purification of the studied bacteriocins, including precipitation with ammonium sulfate followed by hydrophobic chromatography on SpePak C_{18}. Similar procedures were already applied in partial and complete purification processes for different bacteriocins [52–56].

Effect of the Bacteriocin Containing CFS on Exponentially Growing Test Organisms

Supplementing the exponentially growing cultures of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 with CFS containing bacteriocin from *P. pentosaceus* ST3Ha resulted in the complete growth inhibition of the tested bacteria over 36 h (Fig. 3). Moreover, when samples were withdrawn at specified time points, no viable cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 or *E. faecalis* ATCC 19443 were recorded (below detection levels of 100 CFU/

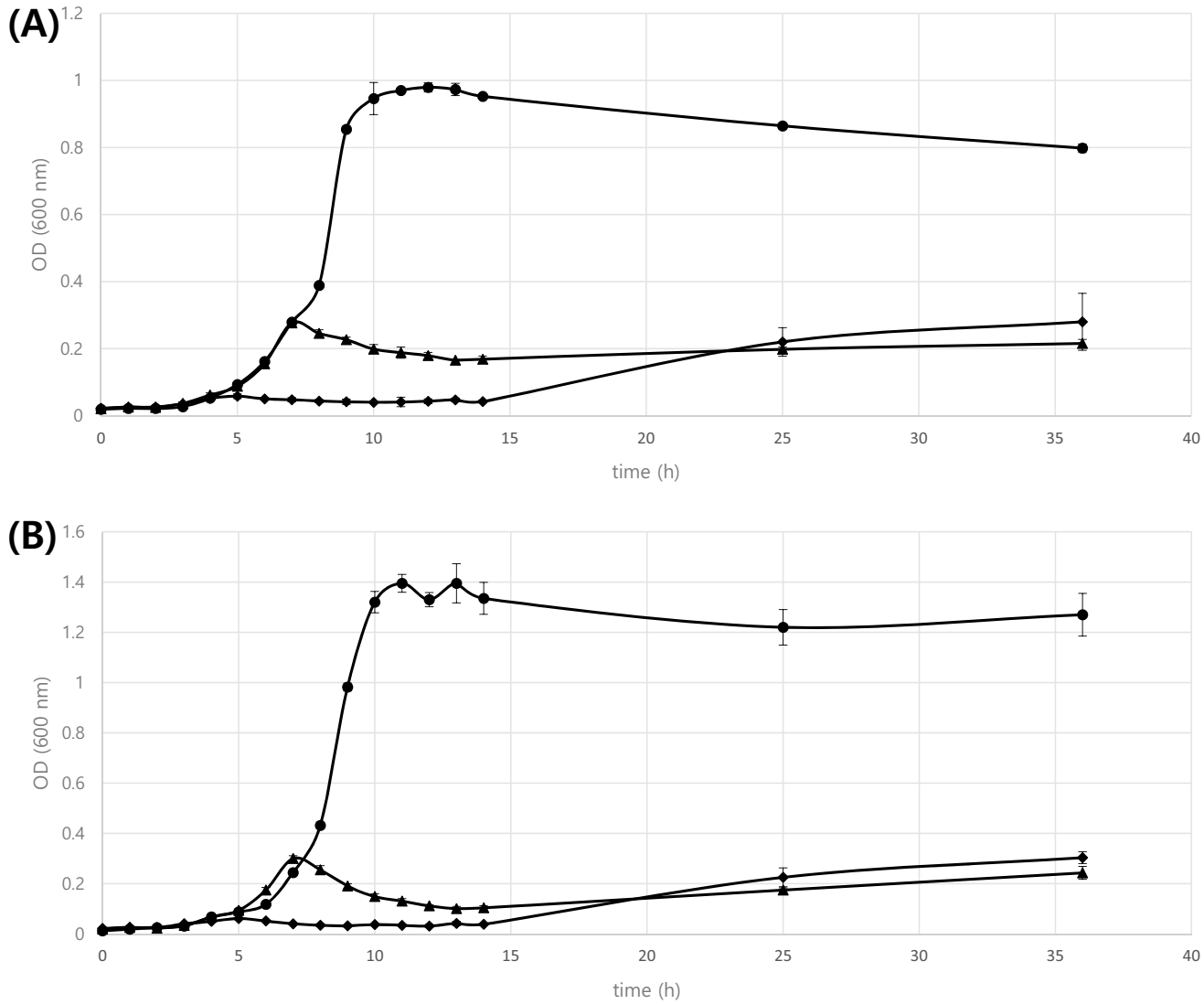

Fig. 3 Effect of bacteriocin containing CFS from *P. pentosaceus* ST3Ha *versus* (**A**) *L. ivanovii subsp. ivanovii* ATCC 19119 and (**B**) *E. faecalis* ATCC 19443. (-●-) represent growth of test microorganisms (**A**) *L. monocytogenes* ATCC 15313 and (**B**) *E. faecium* ATCC 19434 without adding bacteriocins. (-◆-) and (-▲-) represent growth of test microorganisms with added bacteriocin after 3 h and 7 h, respectively, after initial incubation. The data points represent an average of two repeats and did not vary by more than 2%. Standard deviation bars are shown

mL), evidence for the bactericidal mode of action of the studied bacteriocin. Bacteriocins from LAB were reported as bactericidal or bacteriostatic, and some authors suggested that some mechanisms of resistance can be involved [57] or simply, evidence for dose-dependent effects are crucial for the recovery of the culture after a specific time of interaction and presence of the antimicrobial peptides. A similar approach for the evaluation of the effect of bacteriocin on test organisms was previously applied in numerous research projects exploring means of inhibition of human and other animal-related pathogens [37, 45–48].

Determination of Cell Lysis by Measuring the Extracellular Levels of β-Galactosidase

Detection of the extracellular levels of β-galactosidase was done as evidence of cell disruption of test microorganisms as a consequence of bacteriocin action. The approach was previously applied in evaluation of the effect of other bacteriocins (buchnericin LB [21, 58], plantaricin 423 [59], pediocin AcH [60, 61], bacteriocin HV219 [22]). In the present study, treatments of cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 with bacteriocin

produced by *P. pentosaceus* ST3Ha resulted in increased extracellular levels of β-galactosidase based on detection of increasing transformation of ONPG to *o*-nitrophenol detected by OD readings at 420 nm (data not shown). Liberation of the intracellular material is clear evidence of the non-reversible changes of the target cells pointing to the dead of the applied test microorganisms and associated with the bactericidal mode of action of the studied antimicrobial.

Determination of Cell Lysis of Target Microorganisms in the Presence of Bacteriocin

Treatment of *L. ivanovii* subsp. *ivanovii* ATCC 19119 or *E. faecalis* ATCC 19443 in non-growing conditions with different concentrations of bacteriocin ST3Ha (from 12,800 to 200 AU/mL) led to a clear inhibitory effect, even early as after 3 h (Fig. 4). When *L. ivanovii* subsp. *ivanovii* ATCC 19119 was applied, the inhibitory effect of *P. pentosaceus* ST3Ha bacteriocin ranges from 68.99 to 87.72% and for *E. faecalis* ATCC 19443 from 78.84 to 93.98% (recorded after 18 h), depending on the specific applied concentrations of the tested bacteriocin (Fig. 4). Moreover, when applied as 200 AU/mL, at the end of the monitored period (18 h of incubation), the inhibitory effect of bacteriocin produced by *P. pentosaceus* ST3Ha on *L. ivanovii* subsp. *ivanovii* ATCC 19119 reached 79.07% and on *E. faecalis* ATCC 19443 to 81.06% (Fig. 4). Present results are additional evidence that bacteriocin produced by *P. pentosaceus* ST3Ha may be effectively applied for the control of listerial and enterococcal contaminations. Moreover, it will be reasonable to

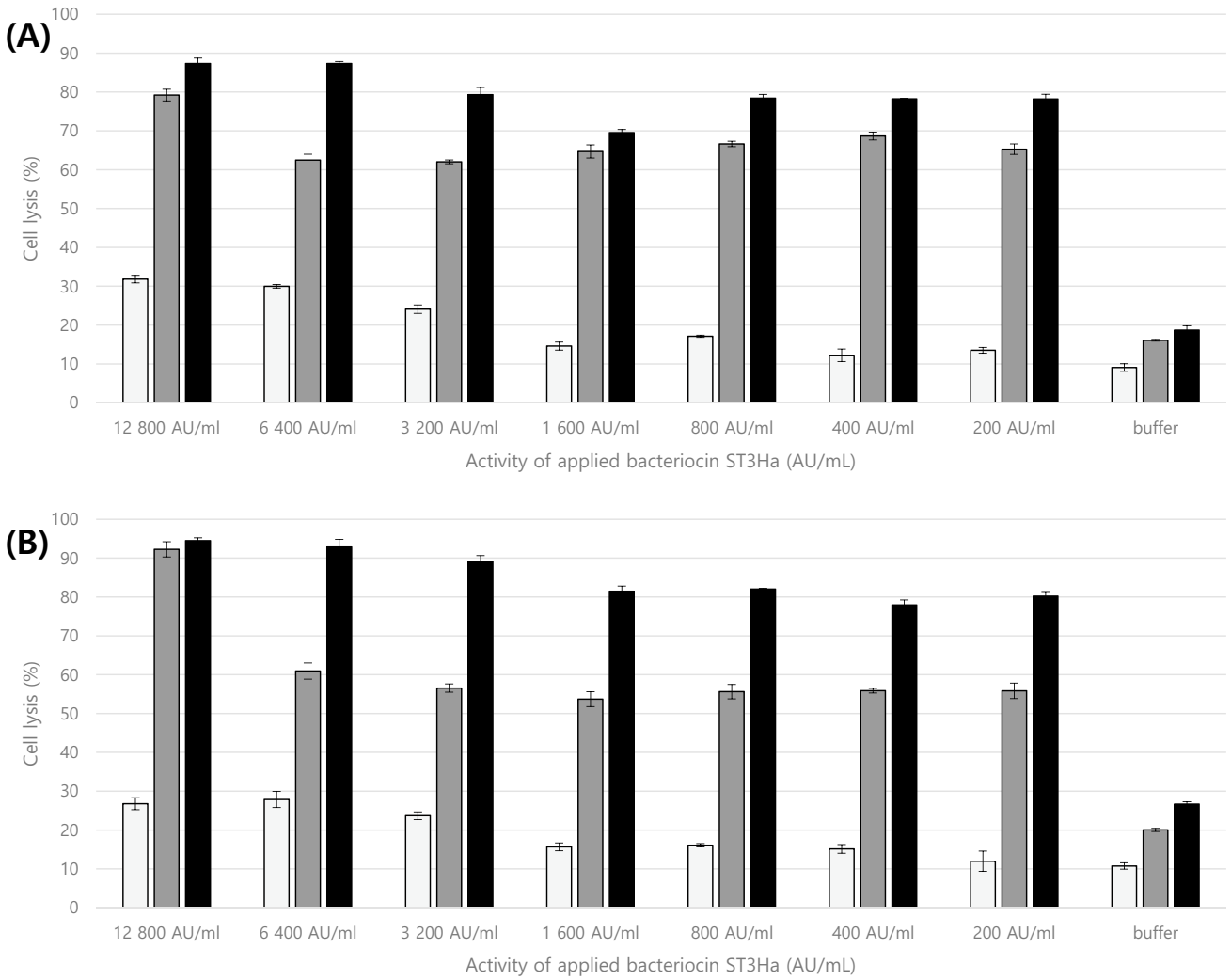

Fig. 4 Estimation of the % cell lysis of (**A**) *L. ivanovii* subsp. *ivanovii* ATCC 19119 and (**B**) *E. faecalis* ATCC 19443 in the presence of different amount of semi-purified bacteriocin produced by *P. pentosaceus* ST3Ha as detected after 3 h (light grey bars), 9 h (dark gray bars) and 18 h (black bars) of incubation at 37 °C. The data points represent an average of two repeats. Standard deviation bars are shown

clearly underline that performed experimental procedures were executed with high initial cell numbers (approximate 10^8 CFU/mL) of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443. In food processing facilities, lower cell numbers are expected even when food products are considered unfit for consumption, suggesting that this bacteriocin produced by *P. pentosaceus* ST3Ha will be a sufficient condition to control these bacterial contaminations.

Determination of the Reduction of Viable Cells of Target Microorganisms in the Presence of the Bacteriocin

When stationary phase cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 (10^8–10^9 CFU/mL) were exposed to the effect of bacteriocin produced by *P. pentosaceus* ST3Ha, complete growth inhibition was recorded. Even after 1 h of contact between test microorganisms and studied bacteriocin, no viable cells of *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443 were recorded (data not shown), taking in consideration the limit of detection of 100 CFU/mL. In parallel control experiments, for *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443, no significant changes regarding bacterial cell numbers were evidenced. Recording of the viable cells is the simplest, but in same time more evidenced, approach for the evaluation of the killing property of newly antimicrobial metabolites. Similar experimental approach was already reported in numerous studies on bacteriocins mode of action, including the one produced by different strains of pediococci [37, 45–48].

Adsorption to Test Organisms and Effect of pH, Temperature, and Chemicals

Adsorption of the bacteriocin produced by *P. pentosaceus* ST3Ha to different test organisms (sensitive and resistant to the studied bacteriocins) was a clear strain-specific feature (Table 3). However, it can be mentioned that, in general, sensitivity to the bacteriocin was associated to higher adsorption levels to the test strains. Regarding the influence of environmental factors, such as temperature, pH, and selected chemicals on adsorption processes of bacteriocins to the tests organisms, results shown that they can play modulation role and to affect bacteriocins adsorption to the test organisms (Table 5). It is a consensus that the first step in the activity of bacteriocins is their adsorption to test organisms, normally related to the recognition of specific receptors located on the cell surface, which is a lipid II in the case of nisin and some other bacteriocins [37]. Knowledge from such results can be applied in the better exploring and design appropriate conditions for enhancing the efficacy of the control of spoilage and/or pathogenic organisms in food processing operations. Moreover, applying this reasonably simple experimental procedure can predict the potential efficacy of the bacteriocins in food systems or in human and animal medical practices [62]. Furtado et al. [63] reported on similar experimental research approach in the evaluation of factors affecting adsorption of several bacteriocins to strains from genus *Listeria*; Pingitore et al. [62] also evaluated role of the selected environmental factors on the adsorption of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to *L. monocytogenes* strains revealing the potential of strain CRL35 over ST88Sh, further confirmed with in situ experiments of preparation and evaluation the role of bacteriocinogenic strains on cheese microbial safety. The scientific objective of such series of experiments is to obtain information of the effect of environmental factors (temperature, pH, and some chemical additives) on the adsorption of bacteriocins to the targeted test organisms, a characteristic associated to their efficacy and potential application for control of spoilage or pathogen microorganisms in food processing operations.

Table 5 Evaluation of the adsorption of bacteriocin produced by *Pediococcus pentosaceus* ST3Ha and effect of pH and temperature on adsorption levels to *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecalis* ATCC 19443

	Bacteriocin produced by *P. pentosaceus* ST3Ha adsorption (%) to:	
	L. ivanovii subsp. *ivanovii* ATCC 19119	*E. faecalis* ATCC 19443
Effect of pH		
2.0	60	80
4.0	80	80
6.0	80	80
8.0	60	80
10.0	60	60
Effect of temperature (°C)		
4	60	100
10	80	100
25, 30, 37	80	80
45, 60	40	60
Chemicals		
80% ethanol, 80% methanol	80	100
KCl, Tris, Na_2CO_3	60	80
EDTA (-Na), KI, NaCl	60	60
β-mercapto-ethanol	60	60
80% chloroform	80	80
KH_2PO_4, K_2HPO_4, $MgCl_2$	80	100
Control (not treated)	80	80

Antimicrobial Action of Isolated Bacteriocin in Combination with Ciprofloxacin

To evaluate the combined effect of ciprofloxacin and bacteriocin produced by *P. pentosaceus*, ST3Ha was important

to determine the MIC (minimal inhibition concentration) for the applied antibiotic. The MIC for ciprofloxacin against *L. ivanovii* subsp. *ivanovii* ATCC 19119 was estimated to be 80 µg/mL. The combined application of both antimicrobials revealed that the presence of bacteriocin produced by *P. pentosaceus* ST3Ha influenced the effect of ciprofloxacin, and strong antimicrobial mode of the action against *L. ivanovii* subsp. *ivanovii* ATCC 19119 was observed when sub-lethal levels of ciprofloxacin (below MIC) were applied (Fig. 5). Results from the performed experiment suggest that levels of ciprofloxacin below MIC (e.g., 40 µg/mL or even less) can be applied when administered in combination with the bacteriocin produced by *P. pentosaceus* ST3Ha. Growth inhibitions of *L. ivanovii* subsp. *ivanovii* ATCC 19119 were recorded during the initial 12 h of the experiment, corresponding to the general recommendations for application of antimicrobials in human and veterinary medical practices. Previously, Todorov and Dicks [40] reported on synergetic effects of the application of bacteriocin produced by *P. pentosaceus* ST44AM, a strain isolated from marula fruit. Moreover, Minahk et al. [64] showed that sub-lethal concentrations of enterocin CRL35, produced by *E. faecalis* strain isolated from Argentinian cheeses, can enhance the activity of erythromycin, chloramphenicol, and tetracycline. As previously suggested by Minahk et al. [64] and Todorov and Dicks [40], the membrane depolarization of the *L. monocytogenes* is a primary necessary step. However, it is not sufficient to produce cell death by antibiotics, and presence of the bacteriocin can enhance the effect of the antibiotic by influencing that primary step of the antimicrobial mode of action.

Plasmid Isolation and Screening for Bacteriocin Genes on Genomic and Plasmid DNA

Obtained plasmid DNA and total DNA from *P. pentosaceus* ST3Ha generated evidence for the presence of pediocin PA-1-associated gene. According to Marugg et al. [65], biosynthesis of pediocin PA-1 is associated with approximately 3.5-kb DNA fragment, including four genes *pedA*, *pedB*, *pedC*, and *pedD*. Taking in consideration that total DNA obtained represent chromosomal and plasmid DNA, obtained results pointing on the fact that genes associated with pediocin PA-1 can be considered plasmid allocated.

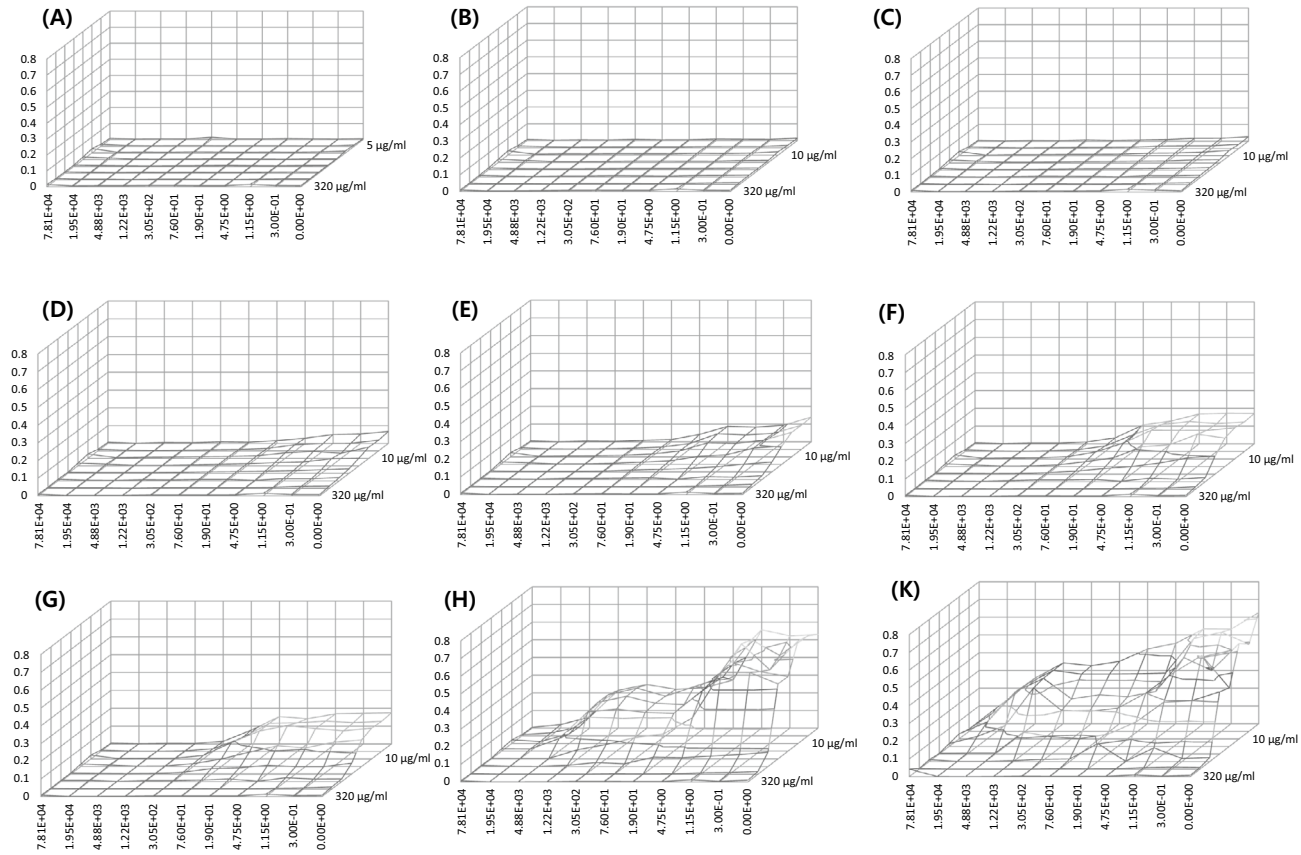

Fig. 5 Combined effect of ciprofloxacin (from 0 to 320 µg/mL) and semi-purified bacteriocin produced by *P. pentosaceus* ST3Ha (from 7.81×10^{4} to 3.0×10^{-1} AU/mL) on growth of *L. ivanovii* subsp. *ivanovii* ATCC 19119 as detected at 0 h (**A**), 2 h (**B**), 4 h (**C**), 6 h (**D**), 8 h (**E**), 10 h (**F**), 12 h (**G**), 24 h (**H**), and 30 h (**K**) of incubation at 37 °C

However, some additional experiments need to be performed with plasmid-free DNA and appropriate sequences, including whole genome sequence of the DNA from *P. pentosaceus* ST3Ha in order to confirm previous hypothesis that pediocin PA-1 is plasmid associated with the studied bacteriocinogenic strain.

Presence of pediocin PA-1 is probably one of the most frequently reported bacteriocins associated with pediococci [36, 45, 46, 61, 66–72] and even associated to other bacteriocin producing strains from different genera [73]. Pediocin PA-1 can be pointed as one of the best studied bacteriocins [65, 66, 74–77], being suggested for several biopreservation processes [60, 61, 78–80].

Pediocin-like bacteriocins are considered expressed in parallel with cognate immunity proteins and involve in the protection of the bacteriocin-producing strain to their bacteriocin. It was suggested that bacteriocins can be potential alternatives to conventional antibiotics, associated with the bacterial resistance emergency; however, immunity mechanism to own bacteriocins is still unclear [81].

In the previous experiments, it was confirmed that bacteriocin produced by *P. pentosaceus* ST3Ha can be expressed at 25, 30, and 37 °C (Fig. 2). Moreover, the reported results reveal that *P. pentosaceus* ST3Ha carry a plasmid-associated gene for production of pediocin PA-1. Regarding that bacteriocin operon carry structural and immunity gene/s as part of the same reading frame, it is presumed that expression of both will be regulated in same level. At tested temperatures (25, 30, and 37 °C), *P. pentosaceus* ST3Ha showed resistance to pediocin PA-1. Regarding the observed, it can be considered that the expression of immunity gene(s) occurred at all tested temperatures, as well as previously shown that bacteriocinogenic strain can express bacteriocins (Fig. 2). However, this is only indirect confirmation for the expression of recorded pediocin PA-1 gene on the genome of *P. pentosaceus* ST3Ha. It is a fact that immunity gene(s) can share some similarity for different bacteriocins [51] and as well is a fact that pediococci can harbor genetic determinates for the expression of more than one bacteriocin [36]. Additional proof for the identity of the expressed bacteriocin by *P. pentosaceus* ST3Ha need to be provided, with whole genome sequence and quantitative RT-PCR performed in combination with bacteriocin purification, amino-acid sequence, and mass spectrometry. This is on-going research associated to further characterization of the bacteriocin produced by *P. pentosaceus* ST3Ha.

Cytotoxicity of the Bacteriocin

Evaluation of the safety features of newly isolated strains needs to cover different aspects of physiology and biochemistry, including cytotoxicity of the expressed bacteriocins. For the studied bacteriocin produced by *P. pentosaceus ST5Ha*, it was shown that semi-purified antimicrobial peptide can be characterized with CC_{50} (50% cytotoxic concentration), evaluated on confluent non-growing Vero cells as 1200 µg/mL. The obtained as value is higher compared to the reported for bacteriocins ST4V and CRL35, produced by *Ent. mundtii*, which were of 1600 µg/mL and 2500 µg/mL, respectively [25, 82]. Bacteriocins are considered polypeptides presenting low cytotoxicity. As example, for nisin, a cytotoxicity, like that of NaCl was reported [83]. However, for some bacteriocins, including pediocin PA-1, nisin and plantaricin A was reported relatively increased cytotoxicity toward cancer cells in comparison to normal cells. Such observations in the selective toxicity open new perspectives in applications of some bacteriocins as promising candidates for further investigation and clinical trials in combat against some types of cancers [84].

Conclusions

Research on antimicrobial proteins (including bacteriocins) become a scientific trend in last few decades associated with their application as natural biopreservatives in the food industry and potential application in human and veterinary medicine as alternative or adjunct therapeutical treatment of infectious diseases and different antibiotic resistant pathogens. Driven by scientific curiosity and potential applications of antimicrobial peptides, different *Pediococcus* spp. strains were isolated from different ecological niches and their expressed bacteriocins characterized. Considered initially as simple killing molecules, nowadays bacteriocins are considered multifunctional metabolites, involve in different cellular processes, including regulation and *quorum sensing*. The fact that same bacteriocin (pediocin PA-1) can be associated with different strains of *Pediococcus* spp. and even some lactobacilli is pointing that such as prevalent protein most probably have biological role much more than a simple killing molecule, a fact that merit further evaluation and investigation. Analysis of the mode of action of bacteriocins in their inhibitory or killing properties against evaluated test organisms will contribute for a better understanding for developing processes and for predicting areas of applications, including the food and medical/veterinary sectors.

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Data Availability All data generated or analyzed during this study are included in this published article and comply with research standards.

Declarations

Ethical Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no competing interests.

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