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Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*

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ABSTRACT

The bacteriocin-producing strain *Enterococcus faecium* ST5Ha was isolated from smoked salmon and identified by biomolecular techniques. *Ent. faecium* ST5Ha produces a pediocin-like bacteriocin with activity against several lactic acid bacteria, *Listeria* spp. and some other human and food pathogens, and remarkably against HSV-1 virus. Bacteriocin ST5Ha was produced at high levels in MRS broth at 30 °C and 37 °C, reaching a maximum production of 1.0×10^9 AU/ml, checked against *Listeria ivanovii* ATCC19119 as target strain and surrogate of pathogenic strain *Listeria monocytogenes*. The molecular weight of bacteriocin ST5Ha was estimated to be 4.5 kDa according to tricine-SDS-PAGE data. *Ent. faecium* ST5Ha harbors a 1.044 kb chromosomal DNA fragment fitting in size to that of pediocin PA-1/AcH. In addition, the sequencing of bacteriocin ST5Ha gene indicated 99% of DNA homology to pediocin PA-1/AcH. The combined application of low levels (below MIC) of ciprofloxacin and bacteriocin ST5Ha resulted in a synergetic effect in the inhibition of target strain *L. ivanovii* ATCC19119. Bacteriocin ST5Ha displayed antiviral activity against HSV-1, an important human pathogen, with a selectivity index of 173. To the best of our knowledge, this is the first report on *Ent. faecium* as a potential producer of pediocin-like bacteriocin with antiviral activity.

1. Introduction

Several studies demonstrated that the microbiota of smoked salmon can be 10^6–10^8 CFU/g after 3 weeks of storage under vacuum at refrigeration temperature (Leroy et al., 1998; Stohr et al., 2001; Tomé et al., 2008). Sixty percent of this microbiota seems to be represented by lactic acid bacteria (LAB) including *Carnobacterium* spp. and *Lactobacillus* spp., while the remaining 40% are Gram-negative microorganisms like *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Aeromonas* spp., characterized by a high spoiling potential and responsible for unpleasant smell and taste (Stohr et al., 2001; Tomé et al., 2008). Members of *Staphylococcus* can be also present (Huss et al., 1995; Gonzàles-Rodrìguez et al., 2002). The absence of additives and stabilizers makes smoked salmon a product with high risk of microbial growth.

Bacteriocins are ribosomally synthesized antimicrobial peptides exhibiting antagonism mainly against related species of Gram-positive bacteria (Jack et al., 1995). Some bacteriocins are also active against certain Gram-negative bacteria, such as *Escherichia coli* and *Salmonella* Typhimurium (Gong et al., 2010) and interestingly against *Campylobacter jejuni*, the major cause of gastroenteritis worldwide (Stern et al., 2006). In the last decade, a plethora of reports were published on the production of bacteriocins by LAB isolated from different food matrices such as fermented products, vegetables, fruits, meat and fish and also from the human and animal gastrointestinal tract. Bacteriocins produced by LAB offer potential biotechnological applications because they are (i) free of

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adverse effects, (ii) stable at low pH values, (iii) easy to produce and (iv) sensitive to proteases (Todorov, 2009).

Bacteriocinogenic strains of *Enterococcus faecium* have been isolated from a variety of foods such as smoked salmon (Tomé et al., 2009), fermented meat and meat products (Callewaert et al., 2000; Strompfová and Laukova, 2007; Belgacem et al., 2010), olives (Franz et al., 1996; Todorov and Dicks, 2005a), barley beer (Todorov and Dicks, 2004a,b), cheese and milk (Ennahar et al., 2001; Sarantinopoulos et al., 2002; Leroy and De Vuyst, 2002; Cocolin et al., 2007; Ghrairi et al., 2008; Izquierdo et al., 2009), *nuka*, a Japanese rice-bran paste (Losteinkit et al., 2001) and chungkukjang, a fermented soy product (Yoon et al., 2008).

This paper reports on the isolation and identification of a strain of *Ent. faecium* from Norwegian smoked salmon, capable to produce a bacteriocin with bioactivity against Gram-positive and Gram-negative bacteria and HSV-1 virus. Further characterisation of the bacteriocin produced by this strain is also presented.

2. Materials and methods

2.1. Isolation of lactic acid bacteria and screening for bacteriocin-producing strains

Norwegian smoked salmon samples were obtained from a local market in Caracas, Venezuela. The screening for LAB bacteriocin-producing strains was performed as previously described (Todorov and Dicks, 2005b). Briefly, salmon samples were homogenized with peptone water (1:10) and serial decimal dilutions of the homogenate were plated onto MRS agar (Difco) plates supplemented with 10 mg/L actidione (Sigma) in order to prevent yeasts growth. An overlay of MRS agar containing the same concentration of actidione was added. The plates were incubated under anaerobiosis (Oxoid, Gas Generation Kit, Hampshire, England) at 37 °C for 48 h. Plates with less than 50 colonies were covered with BHI medium containing 1.0% (w/v) agar (Oxoid) and inoculated with a culture of *Listeria ivanovii* ATCC19119 to 10^6 CFU/mL (final concentration). The plates were incubated for 24 h at 37 °C. Colonies presenting zones of inhibition were selected, cultured in MRS broth (Difco) for 24 h at different temperatures (26 °C, 30 °C and 37 °C) and tested for bioactivity against *L. ivanovii* ATCC19119 by the agar spot-test (Todorov and Dicks, 2005b). The antimicrobial effect ascribed to lactic acid was eliminated by adjusting the pH of the supernatants to 6.0 with sterile 1 N NaOH. Effects of proteolytic enzymes and H_2O_2 were overcome by heat treatment (10 min at 80 °C) of cell-free supernatants. Activity was expressed as arbitrary units (AU)/mL, defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain (Todorov and Dicks, 2005b).

2.2. Identification of lactic acid bacteria strains

Strains of LAB with antagonism against *L. ivanovii* ATCC19119 were selected and identified to genus-level as proposed by Stiles and Holzapfel (1997). Carbohydrates fermentation was studied by the API50CHL and API20Strep strips (Biomérieux, Marcy-l'Etoile, France). Results were compared to carbohydrates fermentation reactions listed in Bergey's Manual of Systematic Bacteriology. Molecular identification was carried out by single PCR using specific genus primers Ent1 (5′-TAC TGA CAA ACC ATT CAT GAT G-3′) and Ent2 (5′-AAC TTC GTC ACC AAC GCG GAA C-3′) (Ke et al., 1999). A molecular DNA ladder (50 bp) from Fermentas (Canada) was used as DNA markers for agarose gels analysis.

For species identification, robust 16S rDNA sequencing was performed (Felske et al., 1997). For this purpose, the universal primers 8f (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and 1512r (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT), where Y indicates C + T and M indicates A + C, were used to amplify the 16S rDNA gene. Amplified fragments were cloned into the pGEM®-T Easy Vector system (Promega, Madison, Wisconsin) and constructs were transformed into *E. coli* DH5α. Plasmid DNAs were isolated using a QIAprep Spin miniprep kit (Qiagen®, Valencia, California, USA). DNA was sequenced using bigdye™ terminator cycle chemistry (Biosystems, Warrington, England) on an ABI Genetic Analyzer 3130XL Sequencer (Applied Biosystems, SA, Pty, Ltd.).

Further differentiation of LAB strains was performed by random amplification of polymorphic DNA (RAPD-PCR). DNA was obtained as described by Dellaglio et al. (1973) and RAPD-PCR performed with primers OPL-01 (GGC ATG ACC T), OPL-02 (TGG GCG TCA A) and OPL-20 (TGG TGG ACC A) (Kit L of the RAPD® lomer kits, Operon Biotechnologies, Cologne, Germany). Reactions of amplification were performed according to Todorov (2010). The 25 μL reaction volume contained 5 μL primer, 2.5 μL 10× *rTaq* Buffer (Takara Bio Inc, Shiga, Japan), 10 μL 5 mM $MgCl_2$ (Roche), 4 μL 2.5 mM dNTP (Takara Bio Inc, Shiga, Japan) and 0.5 μL *rTaq* DNA polymerase (Takara Bio Inc, Shiga, Japan). PCR was carried out in a DNA thermal cycler (GeneSysetm® PCR System 7900, AB Applied Biosystems) using the following conditions: 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. Extension of the amplified product was at 72 °C for 5 min. The amplified products were separated by electrophoresis in 1.4% (w/v) agarose gels in 1× TAE buffer at 100 V for 1 h. Gels were stained in TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma Diagnostics, St. Louis, Mo., USA). Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

2.3. Bacteriocin production by strain Ent. faecium ST5Ha

MRS broth was inoculated with an 18 h-old culture (2%, v/v) of *Ent. faecium* ST5Ha and incubated at 37 °C, without agitation. Antimicrobial activity (AU/mL) of the bacteriocin, and modifications in pH and optical density (OD_{600nm}) of the culture were determined at regular intervals for 48 h. *L. ivanovii* ATCC19119 was used as sensitive strain. In addition, several Gram-positive and Gram-negative strains (Table 1), from various sources, were used in the determination of spectrum of activity. Strains were grown at 30 °C or 37 °C in MRS or BHI broth as shown in Table 1.

2.4. Estimated molecular weight of bacteriocin ST5Ha

A 24 h-old culture of *Ent. faecium* ST5Ha obtained in MRS broth at 37 °C was centrifuged (15 min, 10 000 × *g*) and the pH adjusted to 6.0 with 6 N NaOH. To avoid proteolytic degradation of the bacteriocin, cell-free supernatants were treated for 10 min at 80 °C. Ammonium sulfate was slowly added to the cell-free supernatants to 60% saturation and stirred for 4 h at 4 °C and centrifuged (20 000 × *g*, 1 h, 4 °C). The precipitate was re-suspended in 10 mL of 25 mM ammonium acetate buffer (pH 6.5) and desalted by dialysis using a 1000 Da cut-off dialysis membrane (Spectrum Inc., CA, USA) against the same buffer. Tricine-SDS-PAGE was used for further separation, as described by Schägger and Von Jagow (1987). Low molecular weight markers with sizes ranging from 2.5 to 45.0 kDa (Amersham Biosciences Europe GmbH, Freiberg, Germany) were used. The gels were fixed and one half stained with Coomassie Blue R250. The position of the active bacteriocin was determined in the unstained gel, as described by Powell et al. (2007). *L. ivanovii* ATCC19119 (10^6 CFU/mL), suspended in BHI supplemented with 1% (m/v) agar, was used as sensitive strain.

2.5. Purification of bacteriocin ST5Ha

Two liters of an 24 h-old culture of *Ent. faecium* ST5Ha obtained in MRS broth at 37 °C were centrifuged (15 min, 10 000 × *g*, 4 °C)

Table 1
Antagonism of bacteriocin ST5Ha produced by *Ent. faecium* ST5Ha against various target strains.

Target strains	Growth medium	Growth temperature (°C)	Inhibition[a]
Acinetobacter baumannii	BHI	37	0/2
Bacteroides fragilis	BHI	37	0/1
Escherichia coli	BHI	37	2/6
Enterobacter cloacae	BHI	37	1/2
Ent. faecalis	MRS	30	7/7
Ent. faecium	MRS	30	2/2
Klebsiella pneumoniae	BHI	37	1/3
Lactobacillus acidophilus	MRS	30	1/1
Lb. curvatus	MRS	30	3/3
Lb. delbrueckii	MRS	30	0/2
Lb. fermentum	MRS	30	0/2
Lb. paracasei	MRS	30	1/1
Lb. plantarum	MRS	30	0/8
Lb. rhamnosus	MRS	30	1/1
Lb. salivarius	MRS	30	1/1
Lb. sakei	MRS	30	2/2
Lactococcus lactis subsp. *lactis*	MRS	30	1/1
Leuconostoc lactis	BHI	30	2/2
Listeria innocua	BHI	37	6/6
L. ivanovii subsp. *ivanovii*	BHI	37	1/1
L. monocytogenes	BHI	37	14/14
Pediococcus acidilactici	MRS	30	1/1
Pseudomonas aeroginosa	BHI	37	1/5
Staphylococcus aureus	BHI	37	2/14
Staph. uberis	BHI	37	1/1
Streptococcus agalactiae	BHI	37	0/1
Strept. caprinus	BHI	30	2/2
Strept. gallolyticus subsp. macedonicus	MRS	30	0/1
Strept. pneumoniae	BHI	37	1/5

BHI = Brain Heart Infusion agar (Oxoid), MRS = De Man, Rogosa, Sharpe agar (Difco).
[a] Number of strains sensitive to bacteriocin ST5Ha from total number tested.

and proteases in the cell supernatant inactivated by heating for 10 min at 80 °C. Ammonium sulfate was gently added to the supernatant maintained at 4 °C to obtain 60% saturation, and the mixture was stirred for 4 h at 4 °C. After centrifugation for 1 h at 20 000 × *g* at 4 °C, the resulting pellet was re-suspended in 200 mL of 25 mM ammonium acetate buffer (pH 6.5), and loaded on a SepPakC_{18} cartridge (Waters, Millipore, MA, USA). The cartridge was washed with 20% isopropanol in 25 mM ammonium acetate buffer (pH 6.5), followed by a second wash with 40% isopropanol in 25 mM ammonium acetate buffer (pH 6.5) and the bacteriocin was eluted with 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). After drying under reduced pressure (Speed-Vac, Savant, France), the 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5) bacteriocin fraction was dissolved in ultra purified water and used in the characterisation tests. Same fraction was dissolved in 0.1% trifluoroacetic acid (TFA) and applied for final purification by reverse-phase HPLC (Waters, Millford, MA, USA) on a Nucleosil C_{18} column (250 × 4.6 mm). Elution was performed using a linear gradient from 0.1% TFA (solvent A) to 90% acetonitrile in 0.1% TFA (solvent B) in 65 min. Eluted peaks were detected at 280 nm. Fractions were collected, dried under vacuum, dissolved in 1 mL deionized water, and stored at −20 °C. Antagonistic activity against *L. ivanovii* ATCC19119 was determined at each step of the purification process, using the agar spot-test (Todorov and Dicks, 2006).

2.6. Effect of enzymes, pH, NaCl, detergents, EDTA, urea and heat treatment on the activity of bacteriocin ST5Ha

Cell-free supernatant of *Ent. faecium* ST5Ha cultured in MRS broth (37 °C, 24 h), obtained by centrifugation (8000 × *g*, 10 min, 4 °C), was adjusted to pH 6.0 with 1 N NaOH. Aliquots of 2 mL were incubated for 2 h in the presence of trypsin (Sigma), pronase (Sigma), proteinase K (Sigma), pepsin (Sigma), papain (Sigma), α-chymotrypsin (Sigma), catalase (Sigma), lipase (Sigma) and α-amylase (Sigma) at final concentration of 1.0 mg/mL and then tested for their antimicrobial activity using the agar spot-test method against *L. ivanovii* ATCC19119 and *Listeria monocytogenes* ScottA (Todorov and Dicks, 2006). Plain MRS treated as described above was used as control. In a separate experiment, the effect of 1% (w/v) SDS (sodium dodecyl sulfate), Tween 20, Tween 80, Triton X-100, Triton X-114, Na-EDTA (ethylene diamine tetra acetic acid), urea and NaCl on the activity of the bacteriocin in cell-free supernatant was determined as described by Todorov and Dicks (2006). Same chemicals applied to plain MRS and incubated in similar conditions were used as controls. The experiment was repeated with the semi-purified bacteriocin. The effect of pH on the bacteriocin activity was determined by adjusting the pH of the cell-free supernatant to 2.0–12.0 with sterile 1 N HCl or 1 N NaOH. After 2 h at 30 °C, the samples were readjusted to pH 6.5 with sterile 1 N HCl or 1 N NaOH and the activity determined as described before (Todorov et al., 2006). The effect of temperature on the bacteriocin activity was tested by heating the cell-free supernatants to 25, 30, 37, 45, 60 and 100 °C for 30, 60 and 120 min (Todorov et al., 2006). In all these experiments, plain MRS was used as control.

2.7. Mode of action of bacteriocin ST5Ha

2.7.1. Growth of the test-microorganisms in presence of bacteriocin ST5Ha

A 20 mL aliquot of bacteriocin-containing filter-sterilized (0.20 μm, Millipore) supernatant (pH 6.0) was added to 100 mL culture of *L. ivanovii* ATCC19119 in early and middle exponential phases and incubated for 16 h. Optical density readings (at 600 nm) were recorded at 1 h-intervals.

2.7.2. Determination of cell lysis by measuring the extracellular levels of β-galactosidase

Eleven-hour-old cultures of *L. ivanovii* ATCC19119 and *Ent. faecalis* ATCC19433 (100 mL) were harvested and the cells washed twice with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 16 mL of the same buffer. The partially purified and dried bacteriocin obtained from 2 mL of 60% isopropanol fraction was homogenized with 10 mL sterile MilliQ water (Millipore) and sterilized by filtration through a 0.20 μm membrane filter (Millipore). Cell suspensions of *L. ivanovii* ATCC19119 and *Ent. faecalis* ATCC19433 (2 mL) were treated for 5 min at 25 °C with equal volumes of bacteriocin-containing material, followed by the addition of 0.2 mL 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside, Sigma) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37 °C, the reaction was stopped by the addition of 2.0 mL 0.1 M sodium carbonate. The cells were harvested (8000 × *g*, 15 min, 25 °C) and absorbance readings of the supernatant recorded at 420 nm. Cells of *L. ivanovii* ATCC19119 and *Ent. faecalis* ATCC19433 disrupted with 0.1 mm diameter glass beads and vortexed for 5 min served as controls. All experiments were performed in duplicate in two independent assays.

2.7.3. Determination of cell lysis of target microorganisms in the presence of bacteriocin ST5Ha

Cell lysis was assessed using sterile flat-bottom 96-well microtiter plates (TPP, Zellkultur testplatte, Switzerland). Cells of *L. ivanovii* ATCC19119, *Lactobacillus sakei* ATCC15521 and *Ent. faecalis* ATCC19433 were obtained centrifugating (8000 × g, 4 °C, 10 min) 10 mL of 18 h-old cultures, washing and resuspending in 5 mL of potassium phosphate buffer (20 mM, pH 6.5). Each bacterial suspension (100 μL) was individually placed in microtiter plate

wells and 50 μL of potassium phosphate buffer containing the semi-purified bacteriocin ST5Ha at different serial 1/2 dilutions were added. Plates were incubated at 37 °C for 24 h and absorbance at 655 nm was measured using a VERSA*max* microplate reader (Molecular Devices, Sunnyvale, CA, USA). Percentages of cell lysis were 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.

2.7.4. Determination of the reduction of viable cells of target microorganisms in the presence of bacteriocin ST5Ha

Early stationary phase (18 h-old) cultures of *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 and *Ent. faecalis* ATCC19433 were harvested (5000 × g, 5 min, 4 °C), washed twice with sterile saline and re-suspended in 10 mL saline. Equal volumes of the cell suspensions and filter-sterilized (0.20 μm, Minisart®, Sartorius) bacteriocin ST5Ha were mixed. Viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating onto MRS agar. Cell suspensions of *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 and *Ent. faecalis* ATCC19433 with no added bacteriocin served as controls.

2.7.5. Combined effect of bacteriocin ST5Ha and ciprofloxacin on the growth of L. ivanovii ATCC19119

Determination of the minimal inhibition concentration (MIC) of ciprofloxacin (Sigma) was determined by growing *L. ivanovii* ATCC19119 in BHI broth in the presence of increasing concentrations of this antibiotic from 0.03 μg/mL to 1280 μg/mL. In a separate experiment, the growth of *L. ivanovii* ATCC19119 in the presence of a combination of ciprofloxacin and bacteriocin ST5Ha was evaluated. *L. ivanovii* ATCC19119 was grown in BHI broth (Oxoid) for 18 h at 37 °C. All tests were conducted in sterile microtiter plates. Each well was filled with 190 μL of BHI broth pH 6.0 (adjusted with 1 M lactic acid) containing combinations of different concentrations of ciprofloxacin above and under MIC (final concentrations 320 μg/mL, 160 μg/mL, 80 μg/mL, 40 μg/mL, 20 μg/mL, 10 μg/mL and 5 μg/mL) and bacteriocin (final concentrations 78 125 AU/mL, 19 531 AU/mL, 4883 AU/mL, 1220 AU/mL, 305 AU/mL, 76 AU/mL, 19 AU/mL, 4.75 AU/mL, 1.15 AU/mL and 0.3 AU/mL). The wells were inoculated with 10 μL of a culture of *L. ivanovii* ATCC19119 ($OD_{600nm} = 0.3$) and OD measurements at 655 nm were recorded at intervals of 1 h for a period of 30 h using the VERSA*max* microplate reader. Cultures grown in BHI broth without added bacteriocins and ciprofloxacin served as controls.

2.8. Adsorption of the bacteriocin ST5Ha to the producer cells

The ability of the bacteriocin to adsorb to the producer cells was studied as described by Yang et al. (1992). After incubation for 18 h at 37 °C, the pH of a culture of *Ent. faecium* ST5Ha in MRS broth was adjusted to 6.0 with 1 M NaOH, and the cells harvested (10 000 × *g*, 15 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 mL 100 mM NaCl (pH 2.0), stirred for 1 h at 4 °C and then harvested (12 000 × *g*, 15 min, 4 °C). The cell-free supernatant was neutralized to pH 7.0 with sterile 1 N NaOH and tested for activity using the agar spot-test (Todorov and Dicks, 2006).

2.9. Identification of the genes involved in production of bacteriocin ST5Ha

Plasmid DNA was isolated using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, USA) following the instructions of the manufacturer. Total DNA was isolated according to the method of Dellaglio et al. (1973) and submitted to PCR using the primers PEDRPO (5′-CAA GAT CGT TAA CCA GTT T-3′) and PEDC1041 (5′-CCG TTG TTC CCA TAG TCT AA-3′), designed from the operon encoding Pediocin PA-1/AcH (Accession number M83924). PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). The following conditions were used: 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. The amplified product was visualized in a 0.8% (w/v) agarose gel stained with ethidium bromide. A band corresponding to the correct size of Pediocin PA-1/AcH was purified from the gel using the QIAquick PCR purification kit (QIAGEN GmbH). PCR purified products were ligated into pGEM-T® Easy Vector (Promega, Madison, USA) and transformed into *E. coli* DH5α according to instructions of the manufacturer. Plasmids were isolated using QIAGEN Plasmid Mini Kit and DNA fragments were sequenced in automatic sequencer (ABI Genetic Analyzer 3130XI, Applied Biosystems) using bigdye terminator chemistry (Biosystem, Wanington, England). Sequences were analysed using DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada).

2.10. Assessment of cytotoxicity

Cytotoxicity was assayed using monkey kidney Vero as described by Wachsman et al. (1999). Confluent monolayers of Vero cells were grown in tissue culture plates for 48 h and then exposed to various concentrations of bacteriocin ST5Ha. After 24 h of incubation, cell viability was determined measuring the formation of blue formazan due to cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) by mitochondrial succinate dehydrogenase. The 50% cytotoxic concentration (CC_{50}) was defined as the peptide concentration (μg/mL) required to lowering the cell viability by 50%. CC_{50} values were calculated by regression analysis.

2.11. Determination of antiviral activity

Preliminary tests were performed using the virus yield reduction assay described by Wachsman et al. (1999). Vero cells grown to confluence in 24-well culture plates for 48 h were infected with herpes simplex virus type 1 (HSV-1) strain F, obtained from the American Type Culture Collection (ATCC, Rockville, USA), at a MOI (multiplicity of infection) of 1. Virus stocks were prepared in monolayers of Vero cells grown in minimum essential medium (MEM) supplemented with 5% inactivated calf serum and 50 μg/mL gentamycin. After 1 h of adsorption at 37 °C, the cells were covered with maintenance medium MM (MEM, supplemented with 2% inactivated calf serum), containing different concentrations of semi-purified bacteriocin *Ent. faecium* ST5Ha and incubated for 24 h. Infected cells were subjected to two cycles of freeze-thawing, followed by centrifugation at low speed (1,000 × *g*). Supernatants were serially diluted (1:2) and titers determined according to the plaque formation assay (Wachsman et al., 1999). Antiviral activity was expressed as EC_{50}, i.e. bacteriocin concentration required to produce a 50% reduction in viral activity, compared to untreated (control) cultures.

3. Results and discussion

3.1. Isolation of lactic acid bacteria and screening for bacteriocin-producing strains

The average count of LAB in the smoked salmon samples was estimated to be 7.2×10^7 CFU/g. Around 4% of the colonies exhibited a zone of inhibition against *L. ivanovii* ATCC19119.

3.2. Identification of strains

Ten colonies selected among those presenting bioactivity against *L. ivanovii* ATCC19119 were successfully identified as *Enterococcus* spp. by physiological and biochemical tests. The cells presented coccoidal morphology and the carbohydrate fermentation reactions were characteristic of *Lactococcus* spp. As API 50 CHL cannot distinguish *Lactococcus* spp. from *Enterococcus* spp., the isolates were submitted to API 20 Strep strips, and the results showed that they belonged to genus *Enterococcus*. Amplification with genus-specific primers Ent1 and Ent2 has led to a DNA fragment of 112 bp (Fig. 1), characteristic of genus *Enterococcus*. The RAPD-PCR banding patterns of the ten strains were identical (not shown) pointing that these isolates are copies of the same strain. Identification of one of the isolates (ST5Ha) by 16S rDNA sequencing indicated 98% homology to *Ent. faecium*.

3.3. Bacteriocin production

Similar growth and bacteriocin production were observed when the strain *Ent. faecium* ST5Ha was cultured for 24 h in MRS broth at 26 °C, 30 °C or 37 °C. In all tested temperatures, the activity against *L. ivanovii* ATCC19119 was 1.0×10^8 AU/mL. This is in good agreement with results obtained for pediocin PA-1/AcH (Ray, 1992) and mundticin ST4SA (Todorov and Dicks, 2009b). Based on these results, all further experiments were conducted at 37 °C.

The maximal bacteriocin ST5Ha production (1.0×10^9 AU/mL) was recorded after 42–45 h of growth in MRS broth (Fig. 2), when a decrease in activity to 1.0×10^8 AU/ml was observed after 45 h of growth. During the same period of growth, the pH of the medium decreased from 6.38 to 4.15. The cell density increased from 0.01 to 3.29 in 24 h, but decreased to 2.56 in the next 24 h (Fig. 2). Low levels of bacteriocin ST5Ha activity (approximately 5.1×10^4 AU/mL) were recorded after 3 h of growth in MRS broth at 37 °C.

Several studies have shown that bacteriocin production is dependent on the biomass. Todorov and Dicks (2009b) reported that optimal levels of mundticin ST4SA, produced by *Ent. mundtii* ST4SA, were obtained in growth media that supported high biomass production, such as MRS. Similar bacteriocin production profile was reported for bacteriocin ST311LD, produced by *Ent. faecium* ST311LD isolated from fermented olives, in which maximal bacteriocin production was reported at 20 h in MRS broth, followed by a decrease in activity in the next 5 h (Todorov and Dicks, 2005a). The decrease in activity of bacteriocin ST5Ha at the end of the monitored period might be explained by the degradation of the bacteriocin by extracellular proteolytic enzymes. Similar decrease has been observed for bacteriocins produced by *Ent. faecium* ST311LD (Todorov and Dicks, 2005a), *Ent. mundtii* ST4SA (Todorov and Dicks, 2009b) and *Pediococcus acidilactici* NRRL B5627 (Anastasiadou et al., 2008). From a metabolic point of view, this trend is characteristic of a primary metabolite production.

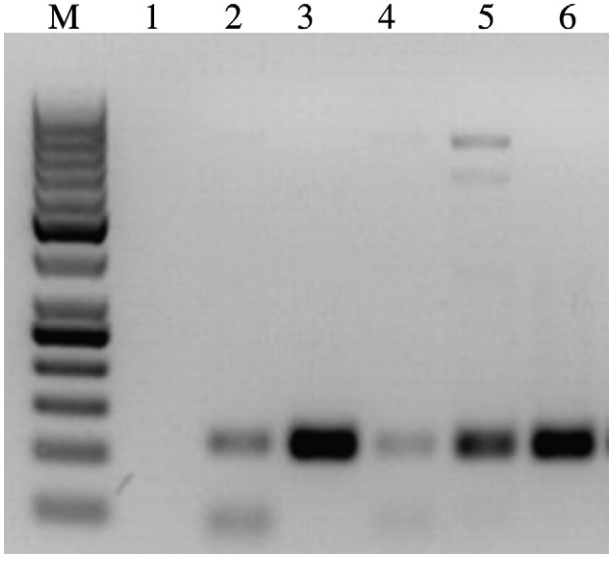

Fig. 1. DNA banding patterns obtained after PCR with genus-specific primers. Lane M: 100 bp-DNA markers (Fermentas), lane 1: negative control, lanes 2–5 different strains of *Enterococcus* spp., lane 6: Strain ST5Ha.

3.4. Spectrum of activity

Bacteriocin ST5Ha presented a broad spectrum of activity, being inhibitory against many food spoilage bacteria and foodborne pathogens (Table 1). Similar results were recorded for the cell-free supernatant and for the semi-purified bacteriocin. It is important to outline the bioactivity against *L. monocytogenes*, a foodborne pathogen of increasing importance. Activity against Gram-negative bacteria is also a relevant characteristic, detected by several authors in other enterococci. Mundticin ST4SA, produced by *Ent. mundtii* ST4SA isolated from soy beans, and bacteriocin ST311LD, produced by *Ent. faecium* ST311LD isolated from olives were also active against Gram-negative bacteria (Knoetze et al., 2008; Todorov and Dicks, 2009b) and presented antilisterial activity as well. However, it should be highlighted that the antilisterial activity of bacteriocin ST5Ha was higher than that of mundticin ST4SA (Todorov and Dicks, 2009b).

3.5. Molecular weight of bacteriocin ST5Ha

The molecular weight of the bacteriocin ST5Ha is around 5.0 kDa, as determined by Tricine-SDS-PAGE (Fig. 3), and it is within the range of most bacteriocins reported for the genus *Enterococcus*.

3.6. Purification of bacteriocin ST5Ha

As the activity of the bacteriocin in the culture medium (1.0×10^8 AU/mL) was much higher than that detected in the surface of the bacteriocin-producing *Ent. faecium* ST5Ha (6.4×10^3 AU/mL), cell-free supernatants from 24-h cultures in MRS broth at 37 °C were used for bacteriocin purification. The activity against *L. ivanovii* ATCC19119 presented by the proteins precipitated with ammonium sulfate and reconstituted in ammonium acetate buffer was similar to that presented by the fractions after chromatography on SepPakC_{18} column and elution with 40, 60 or 80% isopropanol (1.0×10^8 AU/mL). As shown in Fig. 4, the active fraction was eluted at 50 min and, when re-injected in the column, a purified active fraction was obtained at 44 min. Similar purification protocol was used by other researchers for purification of bacteriocins produced by *Lactobacillus plantarum* and *Ent. mundtii* (Todorov et al., 1999, 2004, 2005; Granger et al., 2005).

3.7. Effect of enzymes, pH, NaCl, detergents, EDTA, urea and heat treatment on activity of bacteriocin ST5Ha

Treatment of the cell-free supernatant with proteinase K, pronase or α-chymotrypsin resulted in complete inactivation of antimicrobial activity (Table 2). Same results were observed when cell-free supernatant and semi-purified bacteriocin ST5HA were tested. Slight activity (200–400 AU/ml) was observed against *L. ivanovii* ATCC19119 upon treatment with papain and trypsin,

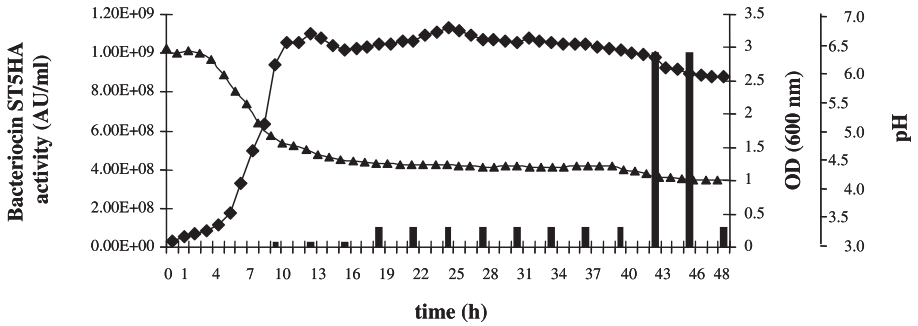

Fig. 2. Production of bacteriocin ST5Ha in MRS broth (pH 6.5 at 37 °C). Antimicrobial activity is presented as AU/mL (bars) against *L. ivanovii* ATCC19119. Modifications in OD (◆) and pH (▲) are indicated.

which may be attributed to the high level of activity of bacteriocin ST5HA against *L. ivanovii* ATCC19119. However, no bioactivity was detected when the experiment was repeated with the cell-free supernatant previously treated with papain and trypsin.. Treatment with catalase did not affect the activity against the target strains (Table 2), discarding clearly the involvement of H_2O_2 in the antagonism process. Moreover, treatment with α-amylase and lipase did not affect the antimicrobial activity (Table 2), suggesting that bacteriocin ST5Ha does not belong to the controversial group IV of the bacteriocins, which contain carbohydrates or lipids in the active molecule. According to De Vuyst and Vandamme (1994), most bacteriocins are polypeptides but others, like leuconocin S produced by *Leuconostoc paramesenteroides* (Lewus et al., 1992), and carnocin 54 produced by *Leuconostoc carnosum* (Keppler et al., 1994) are typical examples of amylase-sensitive bacteriocins.

Bacteriocin ST5Ha activity was not affected by SDS, Tween 20, Tween 80, Triton X-100 and X-114, urea, EDTA or NaCl (Table 2). The sensitivity to detergents, NaCl and urea seems to be bacteriocin-dependent and affected by the experimental conditions. Bioactivity of plantaricin C19 was not affected by the presence of SDS or Triton X-100 (Atrih et al., 2001) contrarily to bacteriocins produced by *Ent. faecium* ST311LD (Todorov and Dicks, 2005a), plantaricin 423 (Verellen et al., 1998), pediocin PA-1/AcH (Biswas et al., 1991), lactacin B (Barefoot and Klaenhammer, 1984) and lactocin 705 (Vignolo et al., 1995).

Bacteriocin ST5Ha was not affected by the pH as remained active after incubation for 2 h at pH ranging from 2.0 to 12.0 (Table 2). However, when the experiment was conducted at pH 12 with the bacteriocin diluted to 1.0×10^4 AU/mL, a substantially lower activity was recorded (data not shown). This loss of activity might be ascribed to proteolytic degradation, protein aggregation or instability of proteins at this extreme pH (Parente et al., 1994; Parente and Riccardi, 1994; De Vuyst et al., 1996; Aasen et al., 2000). Activity was still present after exposure for 24 h to pH 2.0–10.0. Similar observation has been previously reported for pediocin PA-1/AcH (Gonzales and Kunka, 1987; Bhunia et al., 1988).

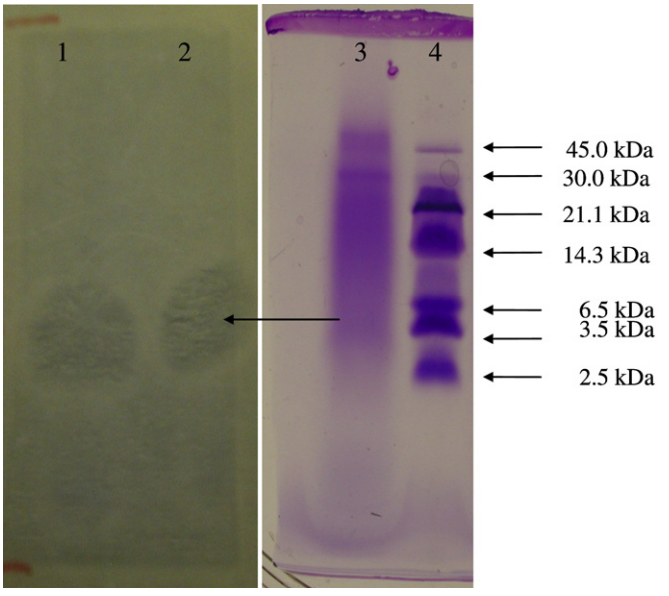

Fig. 3. Tricine-SDS-PAGE of bacteriocin ST5Ha. Lanes 1 and 2: zones of growth inhibition, corresponding to the position of the peptide band representing bacteriocin ST5Ha. Lane 3: peptide bands stained with Coomassie Blue R250 (60% ammonium sulfate saturated), lane 4: molecular mass marker. The gel was covered with viable cells of *L. ivanovii* ATCC19119 (approx. 10^6 CFU/mL), imbedded in BHI supplemented with 1% agar. Incubation was at 37 °C for 24 h.

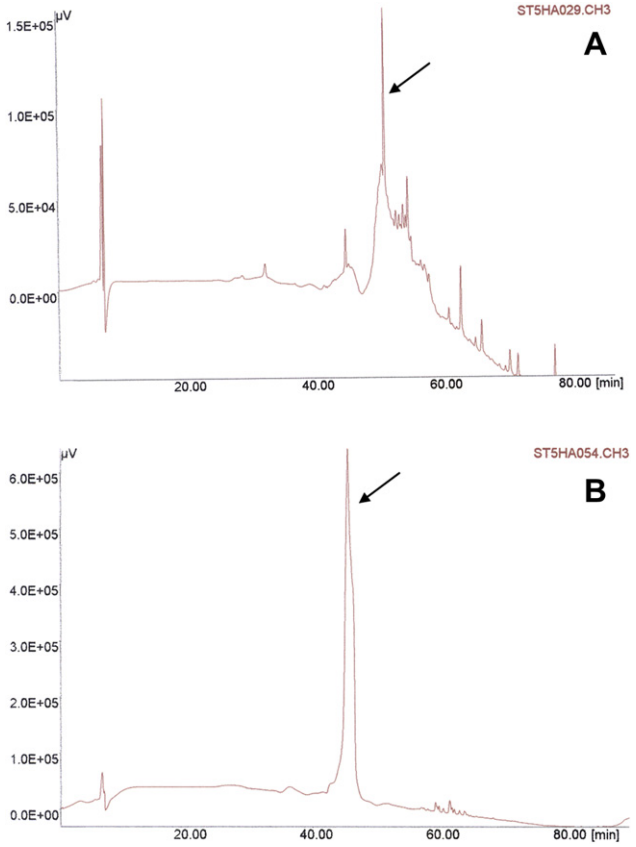

Fig. 4. HPLC Chromatogram of the primary separation (A) and reinjection (B) of the cell-free supernatant of bacteriocin ST5Ha.

Table 2
Stability of bacteriocin ST5Ha after treatment with enzymes, detergents, Urea, heat and pH.

	Concentration	*L. monocytogenes* ScottA	*L. ivanovii* ATCC19119
Enzymes	0.1 and 1.0 mg/ml		
Lipase, α-amylase, catalase		+	+
Proteinase K, Pronase		−	−
α-chymotrypsin		−	−
Papain, Trypsin		−	+[a]
Detergents/chemicals	1% (w/v)		
Tween 20, Tween 80		+	+
Triton X-100, Triton X-114, SDS		+	+
Urea		+	+
EDTA		+	+
NaCl		+	+
Temperatures			
25, 30, 37, 45, 60, 100 °C for 2 h		+	+
121 °C for 20 min		+	+
pH			
pH 2–10		+	+
pH 12		+	+
Control		+	+

[a] Low inhibition; activity of bacteriocin ST5Ha was expressed in + = presence of inhibition zone (>2 mm); − = no inhibition.

Bacteriocin ST5Ha remained stable after 2 h at 25, 30, 45, 60 or 100 °C (Table 2). Decrease in activity from 1.0×10^8 AU/mL to 1.0×10^4 AU/mL was observed upon heat treatment at 121 °C for 20 min at pH 6.0. Similar results were reported for pediocin PA-1/AcH, which was resistant to heat treatment at 80 °C for 60 min and 100 °C for 10 min, but not to 121 °C (Ray et al., 1992). Heat resistance of pediocin PA-1/AcH was pH dependent, as at pH 6.0, 84% of the activity was lost after heating at 121 °C for 15 min and no activity was registered after the same heat treatment at pH 7.0 and 8.0. At pH 4.0, only 11% of the activity was lost. Similar results were recorded for other pediocins and enterocin (Bhunia et al., 1988; Moreno et al., 2003).

3.8. Mode of action

Addition of cell-free supernatant of a 24 h-old culture of *Ent. faecium* ST5Ha to a 3 h-old culture of *L. ivanovii* ATCC19119 (early exponential phase) repressed cell growth over 14 h (Fig. 5). When the supernatant was added to a 7 h-old culture, a similar inhibition was observed (Fig. 5). After treatment with bacteriocin ST5Ha, no viable cells of *L. ivanovii* ATCC19119 were detected after 14 h, 25 h or 36 h, underlining the bactericidal mode of action of this bacteriocin.

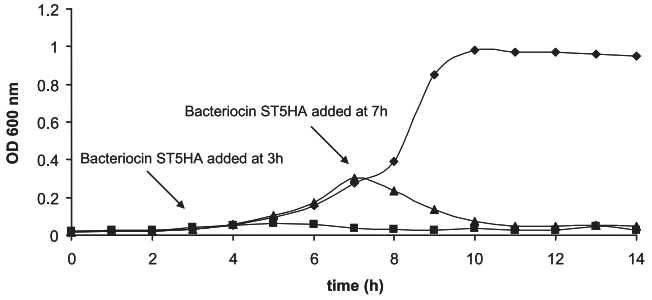

Fig. 5. Effect of bacteriocin ST5Ha on growth of *L. ivanovii* ATCC19119. (◆) represent the growth of *L. ivanovii* ATCC19119 without added bacteriocin (control). The arrow indicates the point at which the bacteriocin was added [3 h (■) and 7 h (▲)].

The treatment of *L. ivanovii* ATCC19119 and *Ent. faecalis* ATCC19433 with bacteriocin ST5Ha resulted in leakage of β-galactosidase from the cells (Table 3) indicating destabilization of the cell membrane permeability. Similar results were reported for other bacteriocins like buchnericin LB (Yildirim et al., 1999, 2002), plantaricin 423 (Todorov and Dicks, 2006), pediocin PA-1/AcH (Bhunia et al., 1991), mundticin ST4SA (Knoetze et al., 2008) and bacteriocin HV219 (Todorov et al., 2006).

When *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 or *Ent. faecalis* ATCC19433 were cultured in MRS broth in the presence of concentrations of bacteriocin ST5Ha ranging from 1.3×10^4 AU/mL to 2.0×10^2 AU/mL, the antagonistic effect could be observed as early as 3 h of incubation (Fig. 6). For *L. ivanovii* ATCC19119, the percentage of the cell lysis varied from 9.5 to 34.8%, for *Lb. sakei* ATCC15521 from 7.8 to 22.13% and for *Ent. faecalis* ATCC19433 from 11.1 to 33.1%, depending on the concentration of bacteriocin. A more drastic inhibitory effect of bacteriocin ST5Ha was observed at 9 h and 18 h of incubation (Fig. 6). After 18 h, the percentage of cell lysis varied from 82.9 to 96.7% for *L. ivanovii* ATCC19119, from 82.1 to 99.9% for *Lb. sakei* ATCC15521, and from 71.0 to 87.7% for *Ent. faecalis* ATCC19433. These results indicate a potential application for bacteriocin ST5Ha in the control of listerial or enterococcal food contamination. Noteworthy, in these experiments the effect of the bacteriocin was tested against high numbers of the indicator microorganisms (approximately 10^8 CFU/mL). In real food systems, where the level of contamination is much lower, a better effect of bacteriocin ST5Ha can be expected.

Treatment of cells of *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 and *Ent. faecalis* ATCC19433 (10^8–10^9 CFU/mL) at stationary phase with bacteriocin ST5Ha resulted in complete death. After 1 h contact time, no viable cells of *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 and *Ent. faecalis* ATCC19433 were detected (data not shown), while the counts remained the same in the untreated control samples. Similar results were obtained when lower levels of contamination were tested (data not shown). Other bacteriocins, such as those produced by *Ped. acidilactici* HA-6111-2 and *Ent. faecium* HKLHS, presented a similar behaviour (Albano et al., 2007).

3.9. Combined effect of bacteriocin ST5Ha and ciprofloxacin on the growth of L. ivanovii ATCC19119

The minimal inhibitory concentration (MIC) of ciprofloxacin for *L. ivanovii* ATCC19119 was 80 μg/mL. When sub-lethal levels of ciprofloxacin were combined with bacteriocin ST5Ha, a strong enhancement of the bioactivity was observed (Fig. 7). The bacteriocin increased the effectiveness of the antibiotic through dissipation of the proton gradient responsible for the extrusion of these

Table 3
Effect of bacteriocin ST5Ha on *L. ivanovii* ATCC19119 and *Ent. faecalis* ATCC19443 as determined by detection of the extracellular levels of β-galactosidase.

	Detection of evel of β-galactosidase (OD 420 nm)
***L. ivanovii* ATCC19119 in presence of bacteriocin ST5HA**	**0.062 ± 0.02**
L. ivanovii ATCC19119 non treated cells	0.026 ± 0.01
L. ivanovii ATCC19119 mechanically broken cells by glass beads	0.059 ± 0.01
***Ent. faecalis* ATCC19433 in presence of bacteriocin ST5Ha**	**0.071 ± 0.02**
Ent. faecalis ATCC19433 non-treated cells	0.016 ± 0.01
Ent. faecalis ATCC19433 mechanically broken cells by glass beads	0.062 ± 0.02
Bacteriocin ST5Ha (fraction from SepPakC_{18} purification)	0.008 ± 0.01

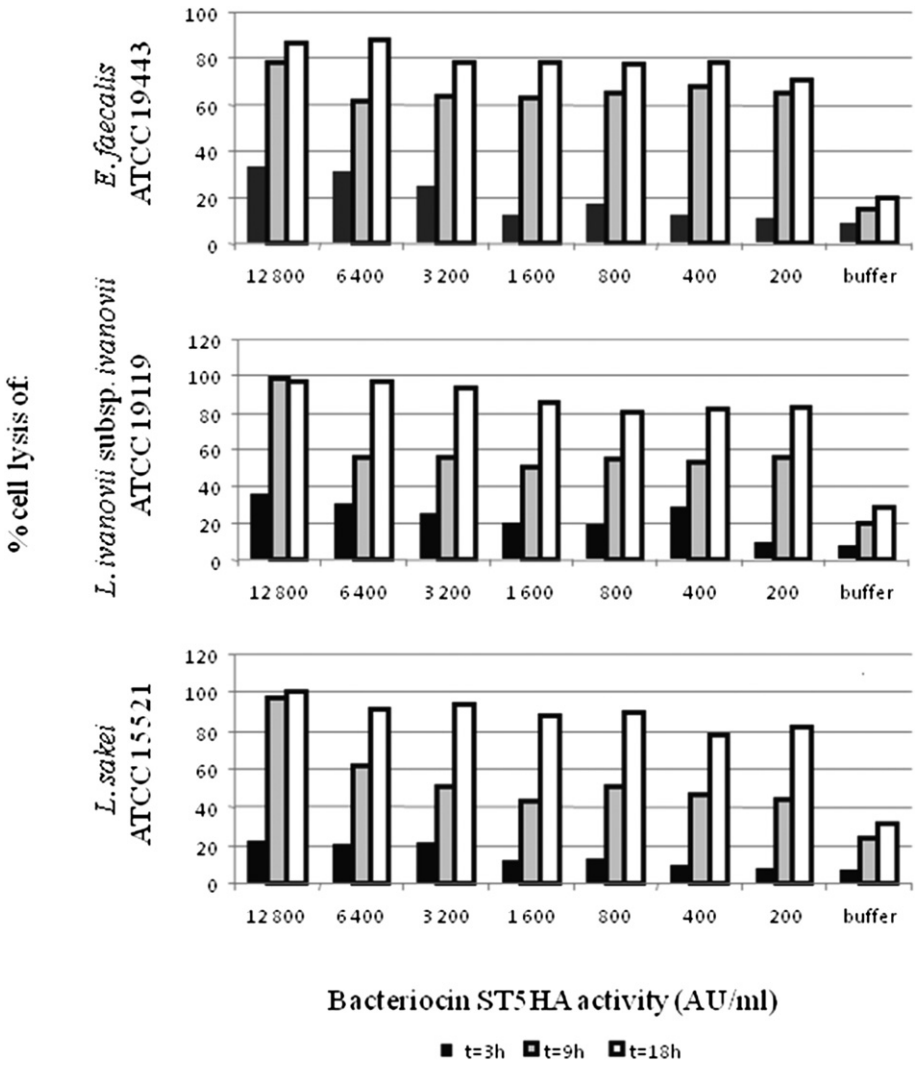

Fig. 6. Determination of the % cell lysis of *L. ivanovii* ATCC19119, *Lb. sakei* ATCC15521 and *Ent. faecalis* ATCC19433 in presence of different amount of bacteriocin ST5Ha as detected after 3 h, 9 h and 18 h of incubation at 37 °C.

antibacterial compounds, resulting in a synergistic effect. The combined use of antibiotics, particularly ciprofloxacin, and bacteriocins is a promising approach to reduce the amounts of antibiotics required for the treatment of infectious diseases in human and veterinary medicine, overcoming the development of resistant strains.

As illustrated in Fig. 7, when ciprofloxacin and bacteriocin ST5Ha were added simultaneously to the culture of *L. ivanovii* ATCC19119, a concentration of ciprofloxacin as low as 40 μg/mL was enough to inhibit growth. Remarkably, the inhibition was more evident in the first 12 h of growth of *L. ivanovii* ATCC19119 (Fig. 7g), whilst the effect of bacteriocin ST5Ha diminished after 24 h (Fig. 7h) and 30 h (Fig. 7k). For a given time, combination of ciprofloxacin and bacteriocin resulted in a stronger inhibition of *L. ivanovii* ATCC19119 than when ciprofloxacin or bacteriocin ST5Ha was used alone. The reduction of the inhibitory effect of bacteriocin ST5Ha after 24 h might be a result of the proteolytic degradation of the bacteriocin, aggregation of the bacteriocin molecules or development of resistance in *L. ivanovii* ATCC19119. This reduction could be also ascribed to exhaustion of the antimicrobials in the system.

Minahk et al. (2004) have also studied the effect of sub-lethal concentrations of enterocin CRL35, a cationic peptide produced by *Ent. mundtii* CRL35, combined to erythromycin, chloramphenicol and tetracycline, observing that the peptide induced a significant membrane gradient dissipation without appreciable cell death. A plausible explanation is that membrane depolarization is necessary, but not sufficient to provoke cell death, and another concentration dependent step may be required. It has been described that pleurocidin and derivatives which are antimicrobial peptides from eukaryotic organisms lost their ability to damage cell membranes at sub-lethal concentrations, whilst maintaining their capacities to inhibit macromolecular synthesis (Patrzykat et al., 2002). Todorov and Dicks (2009a) also reported on synergism between bacteriocin produced by *Pediococcus pentosaceus* and ciprofloxacin. To our knowledge, this is the first report on the synergistic effect between ciprofloxacin and bacteriocin produced by *Ent. faecium*.

3.10. Adsorption of bacteriocin ST5Ha to the producer cells

Treatment of the *Ent. faecium* ST5Ha cell suspension with 100 mM NaCl (pH 2.0) for 1 h caused adsorption of the bacteriocin to the producer cells at 6400 AU/mL activity level. In contrast, no bacteriocin adsorption was reported for plantaricin ST31 (Todorov et al., 1999), pediocin ST18 (Todorov and Dicks, 2005c),

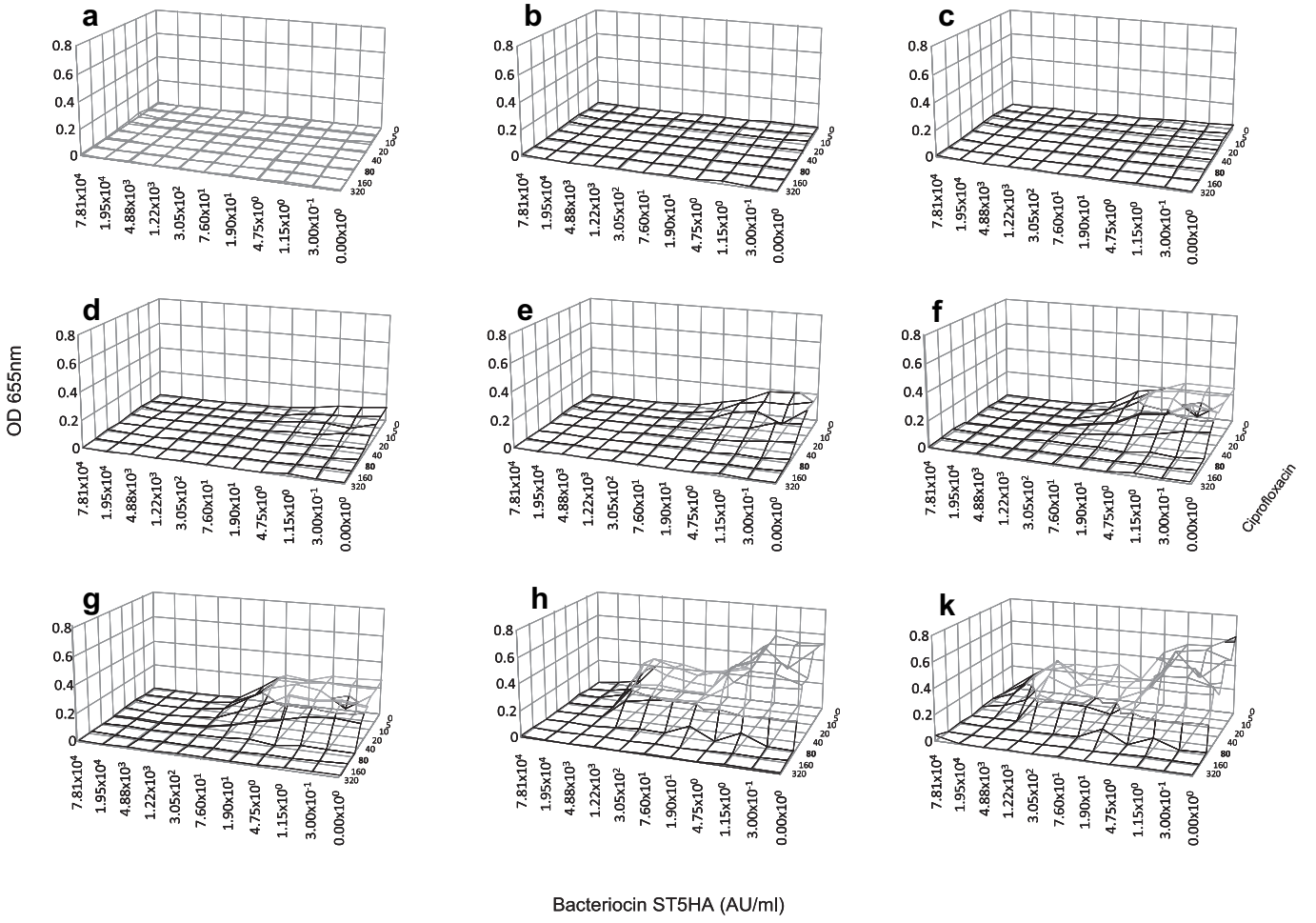

Fig. 7. Combined effect of ciprofloxacin (from 0 to 320 μg/mL) and bacteriocin ST5Ha (from 7.8×10^4 to 3.0×10^{-1} AU/mL) on growth of *L. ivanovii* ATCC19119 as detected at 0 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), 10 h (f), 12 h (g), 24 h (h) and 30 h (k) of incubation at 37 °C.

bacteriocins HA-6111-2 and HA-5692-3 (Albano et al., 2007) or bozacin B14 (Ivanova et al., 2000).

3.11. Identification of genes coding for bacteriocins

Ent. faecium ST5Ha did not harbor any plasmid DNA (data not shown). PCR, performed with primers targeting pediocin PA-1/AcH gene (PEDRPO and PEDC1041) in the chromosomal DNA isolated from *Ent. faecium* ST5Ha, indicated that this strain harboured a 1044 kb fragment, corresponding in size to that of pediocin PA-1/AcH (not shown). The sequences of *ped*A, *ped*B and part of *ped*C were identical to those reported for pediocin PA-1/AcH (Marugg et al., 1992). Presence of pediocin genes in a strain of *Ent. faecium* is unexpected as most pediocins and pediocin-like bacteriocins are produced by *Pediococcus* spp. Several pediocins were already described and all present antilisterial activity, are thermostable and present molecular weight between 2867 and 4685 Da (Henderson et al., 1992; Daba et al., 1991; Fimland et al., 2002; Bauer et al., 2005; Diep et al., 1996; Gonzales and Kunka, 1987; Bhunia et al., 1988; Motlang et al., 1992). Pediocin PA-1/AcH is the best known pediocin (Henderson et al., 1992; Marugg et al., 1992; Nieto-Lozano et al., 2002; Bukhtiyarova et al., 1994; Motlagh et al., 1994; Venema et al., 1995) and is considered therefore a potential biopreservative for application in foods (Bhunia et al., 1988, 1991; Pucci et al., 1988; Yousef et al., 1991; Foegeding et al., 1992). This is the first report on potential production of pediocin by a *Ent. faecium* strain isolated from smoked salmon. Interestingly, the results indicate that genes involved in the production of pediocin PA-1/AcH are highly conserved and distributed in lactic acid bacteria other than *Pediococcus* spp.

3.12. Cytotoxicity of bacteriocin ST5Ha

The CC_{50} (50% cytotoxic concentration) for bacteriocin ST5Ha on confluent non-growing Vero cells was 8645 μg/mL. This value is higher than those obtained for bacteriocins ST4V and CRL35, produced by *Ent. mundtii*, which were of 1600 μg/mL and 2500 μg/mL, respectively (Wachsman et al., 1999; Todorov et al., 2005).

3.13. Antiviral activity of bacteriocin ST5Ha

The EC_{50} (50% effective concentration) value of bacteriocin ST5Ha against HSV-1 was 50 μg/mL, with an SI (Selectivity Index, ratio CC_{50}/CE_{50}) of 173. This high effective concentration is promising as HSV-1 is an important viral pathogen that causes primary and recurrent infections of mucosal membranes, orofacial and genital lesions, and encephalitis (Whitley and Roizman, 2001). The mode of action of bacteriocins against viruses is not known. Possible explanations could be the aggregation of virus particles, blockage of receptor sites on the host cell, or inhibition of key reactions in the multiplication cycle (Wachsman et al., 2003).

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