Food Control 60 (2016) 103–112

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

# Characterization of a two-peptide plantaricin produced by *Lactobacillus plantarum* MBSa4 isolated from Brazilian salami

M.S. Barbosa [a, c], S.D. Todorov [a, *], I.V. Ivanova [b, c], Y. Belguesmia [c], Y. Choiset [c], H. Rabesona [c], J.-M. Chobert [c], T. Haertlé [c], B.D.G.M. Franco [a]

[a] Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil
[b] University of Sofia, Faculty of Biological Sciences, Department of Microbiology, Sofia, Bulgaria
[c] Institut National de la Recherche Agronomique, UR 1268 Biopolymères Interactions Assemblages, Equipe Fonctions et Interactions des Protéines, BP 71627, 44316 Nantes cedex 3, France

ARTICLE INFO

*Article history:*
Received 15 March 2015
Received in revised form
16 July 2015
Accepted 21 July 2015
Available online 23 July 2015

*Keywords:*
*Lactobacillus plantarum*
Two-peptides bacteriocin
Plantaricin
Bacteriocins

ABSTRACT

The aim of this study was to explore the biochemical and genetic features of the two-peptide bacteriocin produced by a *Lactobacillus plantarum* strain isolated from Italian type salami produced in Brazil (*Lb. plantarum* MBSa4). Identification of bacteriocinogenic *Lb. plantarum* MBSa4 was performed by 16S rRNA sequencing. Expressed bacteriocin was evaluated for spectrum of activity, heat and pH stability, mechanism of action, and molecular mass. Partial purification was achieved by cation-exchange, and reversed phase - HPLC. Total DNA of *Lb. plantarum* MBSa4 was extracted and tested for presence of previously described bacteriocin genes. Bacteriocin MBSa4 was heat-stable, unaffected by pH 2.0 to 6.0 and active against all tested *Listeria monocytogenes* strains and most of tested fungi. Maximal production (1600 AU/ml) in MRS broth occurred after 22 h at 25 °C, presenting bacteriostatic activity as result of combined action of two components. The molecular mass determined by SDS-PAGE was 2.3 kDa. PCR-amplified DNA indicated the same nucleotide sequence of plantaricin W. Results indicate that *Lb. plantarum* MBSa4 produces plantaricin W, a two-peptide lantibiotic with remarkable anti-*Listeria* activity.



## 1. Introduction

The term bacteriocin is applied to a wide range of genetically-encoded antibacterial peptides, known to be active against closely related bacteria (Dobson, Cotter, Ross, & Hill, 2012). Some studies have also reported on activity against unrelated strains, especially those that are pathogenic and responsible for food spoilage (Corsetti, Settanni, & Van Sinderen, 2004). Although a variety of Gram-positive bacteria have been reported to produce bacteriocins, those produced by lactic acid bacteria (LAB) have been more widely investigated because of their potential application as bio-preservatives in food products (Cotter, Hill, & Ross, 2005; Dobson et al., 2012).

Bacteriocins produced by LAB are small and thermo-stable proteins, classified initially in four classes (Klaenhammer, 1993) and latter in three classes (Cotter et al., 2005; Deegan, Cotter, Hill, & Ross, 2006). Lantibiotics (Class I bacteriocins) and non-lantibiotics (Class II bacteriocins) are common in all classifications, and are the best studied. Class I bacteriocins are small (<5 kDa) and thermostable peptides with residues of lanthionine and methyl lanthionine (thioether amino acids), and class II are small (≤10 kDa), thermo-stable non-lanthionine containing peptides. Classes III/IV are thermo-labile sometimes hydrophilic proteins or protein complexes consisting of phospholipids and/or carbohydrates. Deegan et al. (2006) divided Class I bacteriocins into subclasses Ia, composed of long, flexible and positively charged peptides, which act on the cytoplasmic membrane by pore formation, and subclass Ib, composed of spherical, rigid, and neutral or negatively charged peptides. The class II was also divided into two subclasses: IIa, which contains bacteriocins presenting the N-terminal consensus sequence YGNGVXCXXXXCXV and IIb, which is composed by bacteriocins that require two peptides for antimicrobial activity. Research has focused mostly on bacteriocins belonging to classes I and IIa, since they are the most abundant and have potential for industrial application.

Besides *Lactobacillus sakei*, *Lactobacillus plantarum* is the most

* Corresponding author. Present address: Universidade Federal de Viçosa, Veterinary Department, Campus UFV, 36570-900, Viçosa, Minas Gerais, Brazil.
*E-mail address:* slavi310570@abv.bg (S.D. Todorov).

http://dx.doi.org/10.1016/j.foodcont.2015.07.029
0956-7135/

common species of lactobacilli in meat and meat products (Belfiore, Fadda, Raya, & Vignolo, 2013; Freiding, Gutsche, Ehrmann, & Vogel, 2011; Gao, Li, & Liu, 2014; Rivas, Castro, Vallejo, Marguet, & Campos, 2014; Todorov, Ho, Vaz-Velho, & Dicks, 2010). Some *Lb. plantarum* strains of meat origin have been characterized as bacteriocin producers (Enan, El-Essawy, Uyttendaele, & Debevere, 1996; Hugas, Garriga, Aymerich, & Monfort, 1995; Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Müller, Carrasco, Tonarelli, & Simonetta, 2009; Rattanachaikunsopon, & Phumkhachornt, 2006; Todorov, Ho, et al., 2010), evidencing their potential application as bio-preservatives in meat products and possibly other food products.

Some studies done in the past have suggested that certain strains of *Lb. plantarum* are capable to produce more than one peptide with synergistic antibacterial effect, possibly class IIb bacteriocins (Diep, Håvarstein, & Nes, 1996; Jiménez-Díaz et al., 1995; Maldonado, Ruiz-Barba, & Jiménez-Díaz, 2003). Since then, there was a great advance on the knowledge on properties of bacteriocins and this study contributes with the description of isolation and characterization of a two-peptide bacteriocin produced by a *Lb. plantarum* strain isolated from Italian type salami produced in Brazil.

## 2. Material and methods

### 2.1. Isolation of LAB with anti-Listeria activity and assay of bacteriocin production

Samples of 50 g of Italian type salami, obtained from a local supermarket, Sao Paulo, SP, Brazil, were homogenized in a stomacher (Seward 400, London, UK) with equal volume of 0.1% sterile peptone water (Difco, USA) and decimal dilutions of the homogenates were plated on De Man, Rogosa, Sharpe (MRS) agar (Difco) in duplicates. After 48 h at 30 °C, plates presenting well diferenciated colonies were overlaid with Brain-Heart Infusion (BHI) (Oxoid, UK) containing 0.75% bacteriological agar (Difco) and a suspension of *Listeria monocytogenes* Scott A ($10^5$–$10^6$ CFU/ml). After incubation for 24 h at 37 °C, plates presenting colonies surrounded by an inhibition zone were selected and colonies transferred to MRS broth (Difco) and incubated at 30 °C for 24 h (Todorov & Dicks, 2005), followed by streaking on MRS agar and incubation for 24 h at 30 °C. Individual colonies were submitted to Gram-staining and tested for catalase production using 3% hydrogen peroxide (v/v). Gram positive and catalase negative strains presenting anti-*Listeria* activity were freeze-dried in 10% sterile milk and stored at −20 °C until future tests.

Production of bacteriocin was assayed by the spot-on-the-lawn method described by Todorov, Wachsman, et al. (2010) with modifications. Isolates were grown in MRS broth for 24 h at 30 °C. Cell free supernatants (CFS) were obtained by centrifugation at 4000 × *g* for 15 min at 4 °C (Hettich Zentrifugen, model Mikro 22R, Germany) and pH adjusted to 6.0–6.5 with 1 M NaOH (Synth, Brazil) followed by heat treatment at 70 °C for 30 min and filter sterilization (Millex GV 0.22 μm, Millipore, USA). *L. monocytogenes* Scott A ($10^5$–$10^6$ CFU/ml), selected as antimicrobial activity indicator, was mixed with 5 ml of BHI supplemented with 0.85% (w/v) bacteriological agar. The mixtures were transferred to plates containing 10–12 ml of 1.5% bacteriological agar, and let allowed to solidify at room temperature. An aliquot of 10 μl of each CFS was spotted on the surface of plates and after complete absorption of the CFS, they were incubated at 37 °C for 12 h and examined for the formation of a clear zone of inhibition around of the spotted CFS. The proteinaceous nature of the CFS was checked as described by Todorov, Wachsman, et al. (2010), observing the disappearance of the anti-*Listeria* activity after treatment with proteolytic enzymes protease K, trypsin, pepsin, α-chymotrypsin and protease Type XIV (0.1 mg/ml). Control tests, with non-treated CFS, were also performed.

The critical dilution approach was used to determine the amount of bacteriocin produced by the strains. The CFS of each strain was submitted to two-fold serial dilutions in 100 mM phosphate buffer pH 6.5 and tested by the spot-on-the-lawn method as described by Todorov, Wachsman, et al. (2010). One arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest dilution that resulted in a clear zone of inhibition of at least 2 mm in diameter. Results were expressed in AU/ml, taking in consideration the volume of the spotted material and the dilution factor.

### 2.2. Identification of bacteriocin-producing strains

Bacteriocin-producing strains were submitted to 16S rRNA sequence analysis by amplification of genomic DNA with primers 8f (5′-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3′) and 1512r (5′-GTG AAG CTT ACG G(C/T)T AGC TTG TTA CGA CTT-3′), according to Felske, Rheims, Wokerink, Stackebrandt, and Akkermans (1997). DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), following the manufacturer protocol. The amplification was carried out in a DNA thermocycler MasterCycler® PCR (Eppendorf Scientific, Germany), in 20 μl reaction volume, which contained 100 pmol of each primer, 1× PCR buffer (Fermentas, MD, USA), 25 μM dNTP, 2 mM $MgCl_2$ (Fermentas, MD, USA) and 0.0125 U *Taq* DNA polymerase (Fermentas, MD, USA). PCR conditions included denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 10 s, primer annealing at 61 °C for 20 s, polymerization at 68 °C for 2 min and then at 72 °C for 7 min. PCR-amplified DNA were analyzed by 0.8% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide (0.1 mg/ml) fluorescence using an UVP Bioimaging System (DIGIDOC-IT System, USA). The amplified products were extracted from the gel and purified with QIAquick® PCR Purification kit (Qiagen, Germany) according to the manufacturer's instructions and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

### 2.3. Spectrum of activity

The spectrum of activity of bacteriocin producing strains was determined against a variety of Gram-negative and Gram-positive bacteria (Table 1) and moulds and yeasts (Table 2). *Lb. plantarum* MBSa4 presented special antimicrobial features when compared to the other strains, and results are shown for only this strain. For determination of the activity against bacteria, lactobacilli and enterococci were grown in MRS broth at 30 °C for 24 h, while the other strains were grown in BHI at 37 °C for 24 h. The spot-on-the-lawn method was used in this determination (Todorov, Wachsman, et al., 2010).

Antagonism of *Lb. plantarum* MBSa4 against moulds and yeasts was tested using the dual culture overlay methodology described by Magnusson, Ström, Roos, Sjögren, and Schnürer (2003), with some modifications. Yeast cells and mould spores, grown on Potato Dextrose Agar (AES, Bruz, France) at 30 °C for 48–96 h, were collected and transferred to saline (0.8% NaCl) and enumerated using a counting chamber. The suspensions were standardized to a final concentration of $10^4$–$10^5$ cells or spores per ml. For testing, an overnight culture of *Lb. plantarum* MBSa4 in MRS broth (Biokar, France) at 30 °C was used to inoculate tubes containing modified

MRS soft agar (MRS broth without sodium acetate, supplemented with 0.85% [w/v] of bacteriological agar [Biokar, France]). The inoculated soft agar was transferred to the wells of 12-well plates and incubated at 30 °C for 48 h, when 100 μl of the yeast cells and mould spores suspensions ($10^4$–$10^5$ cells or spores per ml) were added. Plates were incubated at 30 °C for 72 h and observed for growth of the inoculated yeasts and moulds.

Testing for the antifungal activity of compounds produced by *Lb. plantarum* MBSa4 was performed adapting the agar well diffusion method used for testing activity against bacteria. Those yeasts and moulds that were inhibited in the dual culture overlay test were spread plated ($10^4$–$10^5$ cells or spores per ml) on the surface of plates containing MRS soft-agar (MRS broth supplemented with 0.85% [w/v] of bacteriological agar) and the CFS of *Lb. plantarum* MBSa4 was added to 8 mm diameter wells cut in the soft agar. After incubation for 72 h at 30 °C, the plates were examined for clear zones of inhibition around the wells.

### 2.4. Characterization of the bacteriocins produced by Lactobacillus plantarum MBSa4

#### 2.4.1. Effect of pH and temperature on activity/stability

The pH of the CFS obtained from *Lb. plantarum* MBSa4 was adjusted from 2.0 to 10.0 with 1 M NaOH (Synth, Brazil) or concentrated phosphoric acid (Synth, Brazil) and incubated for 1 h at 25 °C. Before testing for antibacterial activity, the pH of the CFS was corrected back to 6.0–6.5 with 1 M NaOH or phosphoric acid. The effect of temperature on the activity of the bacteriocin was evaluated after incubating the CFS at 4 °C, 25 °C, 30 °C, 37 °C, 45 °C, 60 °C, 80 °C and 100 °C for 60 min, and at 121 °C for 15 min. All samples were tested for anti-*Listeria* activity using the spot-on-the-lawn method described by Todorov, Wachsman, et al. (2010).

#### 2.4.2. Bacteriocin production during growth

Bacteriocin production by *Lb. plantarum* MBSa4 in MRS broth was evaluated at 25 °C, 30 °C and 37 °C. Growth was monitored every 2 h, up to 24 h, by spectrophotometric measurements (Ultrospec 2000 spectrophotometer, Pharmacia Biotech, UK) at 600 nm. The amount of bacteriocin in the CFS was determined using the critical dilution approach described above.

#### 2.4.3. Determination of Minimal Inhibitory Concentration (MIC)

CFS of *Lb. plantarum* MBSa4 was obtained as described before, and bacteriocin in the CFS was precipitated by saturation with 60% of ammonium sulfate. After stirring at 4 °C for 4 h, the mixture was centrifuged at 10 000 × *g* at 4 °C for 1 h and the obtained pellet was re-suspended in 25 mM ammonium acetate buffer (pH 6.5). Proteins were separated by hydrophobic chromatography on a Sep-Pak C18 column (Waters, USA) and eluted with 25 mM ammonium acetate buffer (pH 6.5) containing increasing concentrations of isopropanol (20%, 40%, 60% and 80%). Anti-*Listeria* activity of the bacteriocins in each fraction was tested using the spot-on-the-lawn method. Fractions of interests were pooled and dehydrated under reduced pressure (Speed-Vac, France) and stored at −20 °C.

The MIC (Minimal Inhibitory Concentration) was determined as described by Nielsen, Dickson, and Crouse (1990) with modifications. The pooled fractions with anti-*Listeria* activity were submitted to serial two-fold dilutions until a final concentration of 0.1 AU/ml in 96-well microtiter-plates (TPP, Switzerland) containing BHI. An overnight culture of *L. monocytogenes* Scott A in BHI at 37 °C was added to each well at a final concentration of $10^5$–$10^6$ CFU/ml, followed by incubation at 37 °C for 24 h. The MIC was determined as the lowest concentration that caused absence of visible bacterial growth after 24 h.

#### 2.4.4. Mode of action

The bacteriostatic or bactericidal mode of action of the bacteriocin produced by *Lb. plantarum* MBSa4 was determined according to Todorov, Wachsman, et al. (2010). A 24 h culture of *L. monocytogenes* Scott A ($10^5$–$10^6$ CFU/ml) grown on BHI was transferred to fresh BHI and bacteriocin MBSa4 (160 AU/ml final concentration) was added to the culture at times 0 h and 6 h of incubation at 37 °C. *L. monocytogenes* growth was monitored by spectrophotometric measurements (Thermo Fisher Scientific Multiskan® FC, Germany) at 595 nm for 24 h, every 2 h. A culture of *L. monocytogenes* Scott A without addition of bacteriocin was used as control (Kruger et al., 2013).

#### 2.4.5. Adsorption to L. monocytogenes cells

Adsorption of bacteriocin MBSa4 to *L. monocytogenes* Scott A was tested as described by Yildirim, Avşar, and Yildirim (2002) with modifications. An overnight culture of *L. monocytogenes* Scott A grown in BHI at 37 °C was centrifuged at 4000 × *g* (4 °C) for 15 min and washed twice with sterile 5 mM phosphate buffer (pH 6.5). Cells were suspended using the same buffer in order to obtain a suspension with optical density at 600 nm equal to 1.0. Cell suspension was added to the same volume of CFS containing the bacteriocin, incubated at 37 °C for 1 h and centrifuged at 4000 × *g* (4 °C) for 15 min. The supernatant was tested for anti-*Listeria* activity by the spot-on-the-lawn test. This activity corresponded to unbound bacteriocin. The adsorption percentage was calculated using the following equation:

$$\text{Adsorption}\,(\%) = 100 - [(\text{AU/ml after treatment}/\text{AU/ml original}) \times 100]$$

#### 2.4.6. Estimation of the molecular weight

The molecular weight of the bacteriocin MBSa4 was estimated using Tricine-SDS-PAGE performed in continuous gradient gel designed for low molecular weight proteins (Schägger & Von Jagow, 1987), using low molecular weight markers ranging from 26,600 Da to 1060 Da (Sigma). The position of the bacteriocin was determined by the antagonistic test with *L. monocytogenes* Scott A ($10^5$–$10^6$ CFU/ml).

#### 2.4.7. Partial purification

Bacteriocin produced by *Lb. plantarum* MBSa4 was partially purified according to Batdorj et al. (2006), with modifications. *Lb. plantarum* MBSa4 was grown on MRS broth for 18 h at 25 °C and CFS was obtained as described before. After neutralization of pH to 6.8 with 10 M NaOH (Euromedex, France) CFS was loaded into an SP-Sepharose Fast Flow cation exchange column (GE Healthcare, Amersham, Sweden) equilibrated with 20 mM phosphate buffer pH 6.8 (buffer A). The column was washed with buffer A and subsequently the absorbed substances were eluted with a linear gradient from 0 to 100 % buffer B (20 mM sodium phosphate [Sigma–Aldrich, USA] and 1 M NaCl [Euromedex, France] pH 6.8). The fractions were collected and activity was tested against *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 using the spot-on-the lawn test.

Active fractions were pooled (Fraction 1) and applied into a reversed phase (RP) column (SOURCE™15RPC 10 ml; GE Healthcare, Amersham, Sweden) equilibrated with solvent A (0.05% trifluoroacetic acid [TFA] (Sigma–Aldrich), 95% $H_2O$ and 5% solvent B [80% acetonitrile (Biosolve, Netherlands), 10% isopropanol (Sigma–Aldrich), 10% $H_2O$, 0.03% TFA (Sigma–Aldrich)]). Elution was performed with solvent B with a linear gradient from 0 to 100 % in 25 min, at a flow rate of 5 ml/min. After drying under reduced

**Table 1**
Spectrum of activity of the bacteriocin produced by *Lactobacillus plantarum* MBSa4.

| Target microorganism | Origin | Diameter of inhibition zone (mm) |
|---|---|---|
| *Bacillus cereus* ATCC 1178 | ATCC | 0 |
| *Staphylococcus aureus* ATCC 29213 | ATCC | 0 |
| *Staphylococcus aureus* ATCC 25923 | ATCC | 7 |
| *Staphylococcus aureus* ATCC 6538 | ATCC | 0 |
| *Listeria innocua* ATCC 33090 | ATCC | 7 |
| *Listeria welshimeri* USP | USP | 7 |
| *Listeria seeligeri* USP | USP | 0 |
| *Listeria monocytogenes* Scott A | ATCC | 9 |
| *Listeria monocytogenes* 103 serotype 1/2a | USP | 6 |
| *Listeria monocytogenes* 106 serotype 1/2a | USP | 6 |
| *Listeria monocytogenes* 104 serotype 1/2a | USP | 10 |
| *Listeria monocytogenes* 409 serotype 1/2a | USP | 9 |
| *Listeria monocytogenes* 506 serotype 1/2a | USP | 7 |
| *Listeria monocytogenes* 709 serotype 1/2a | USP | 9 |
| *Listeria monocytogenes* 607 serotype 1/2b | USP | 8 |
| *Listeria monocytogenes* 603 serotype 1/2b | USP | 8 |
| *Listeria monocytogenes* 426 serotype 1/2b | USP | 6 |
| *Listeria monocytogenes* 637 serotype 1/2c | USP | 6 |
| *Listeria monocytogenes* 422 serotype 1/2c | USP | 5 |
| *Listeria monocytogenes* 712 serotype 1/2c | USP | 9 |
| *Listeria monocytogenes* 408 serotype 1/2c | USP | 7 |
| *Listeria monocytogenes* 211 serotype 4b | USP | 9 |
| *Listeria monocytogenes* 724 serotype 4b | USP | 8 |
| *Listeria monocytogenes* 101 serotype 4b | USP | 9 |
| *Listeria monocytogenes* 703 serotype 4b | USP | 8 |
| *Listeria monocytogenes* 620 serotype 4b | USP | 8 |
| *Listeria monocytogenes* 302 serotype 4b | USP | 5 |
| *Escherichia coli* ATCC 8739 | ATCC | 0 |
| *Escherichia coli* O157:H7 ATCC 35150 | ATCC | 0 |
| *Enterobacter aerogenes* ATCC 13048 | ATCC | 0 |
| *Salmonella* Typhimurium ATCC 14028 | ATCC | 0 |
| *Salmonella* Enteritidis ATCC 13076 | ATCC | 0 |
| *Enterococcus faecalis* USP | USP | 0 |
| *Enterococcus hirae* D105 | USP | 11 |
| *Enterococcus faecium* ST5HA | USP | 12 |
| *Enterococcus faecium* S154 | USI | 0 |
| *Enterococcus faecium* S100 | USI | 0 |
| *Enterococcus faecium* ST62 | USP | 8 |
| *Enterococcus faecium* ST211Ch | USP | 0 |
| *Enterococcus faecium* ET12 | UCV | 0 |
| *Enterococcus faecium* ET88 | UCV | 0 |
| *Enterococcus faecium* ET05 | UCV | 0 |
| *Lactobacillus* sp. V94 | USP | 0 |
| *Lactobacillus fermentum* ET35 | UCV | 9 |
| *Pediococcus pentosaceus* ET34 | UCV | 0 |
| *Lactobacillus curvatus* ET06 | UCV | 0 |
| *Lactobacillus curvatus* ET31 | UCV | 0 |
| *Lactobacillus curvatus* ET30 | UCV | 0 |
| *Lactobacillus sakei* subsp. *sakei* 2a | USP | 0 |
| *Lactobacillus sakei* ATCC 15521 | ATCC | 8 |
| *Lactobacillus delbrueckii* B5 | USP | 0 |
| *Lactobacillus delbrueckii* ET32 | UCV | 0 |
| *Lactobacillus acidophilus* La14 | DuPont | 0 |
| *Lactobacillus acidophilus* Lac4 | DuPont | 0 |
| *Lactobacillus acidophilus* La5 | Chr. Hansen | 0 |
| *Lactococcus lactis* V69 | USP | 0 |
| *Lactococcus lactis* B16 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* MK02R | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* D2 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* B1 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* D4 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* B2 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* B15 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* D3 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* D5 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* B17 | USP | 0 |
| *Lactococcus lactis* subsp. *lactis* 7-4 | USP | 0 |

USP = Food Microbiology Laboratory, Faculty Pharmaceutical Science, University of Sao Paulo (USP), Sao Paulo, Brazil.
UCV = Science and Food Technology Institute, School of Biology, Central University of Venezuela (UCV), Caracas, Venezuela.
USI = Department for Research in Animal Production, AGRIS, Sardegna, Olmedo, Italy.

**Table 2**
Activity of *Lactobacillus plantarum* MBSa4 against fungi.

| Target | Antifungal activity[a] |
|---|---|
| *Penicillium roqueforti* | + |
| *Penicillium expansum* | + |
| *Fusarium* sp. | + |
| *Geotrichum candidum* | − |
| *Mucor plumbeus* | + |
| *Cladosporium* sp. | + |
| *Debaromyces hansenii* | + |

All fungal cultures were from INRA, Nantes, France.
[a] + inhibited the strain; − not inhibited the strain.

pressure (Speed-Vac), each fraction was tested for anti-*Listeria* activity.

Fractions with anti-*Listeria* activity were pooled (Fraction 2) and submitted to another purification step by reversed phase high performance liquid chromatography (RP-HPLC) using Unicorn 3.21 software (Amersham Pharmacia Biotech, Sweden). Fraction 2 was loaded into a preparative $C_{18}$ column (Symmetry 300™ $C_{18}$, 5 μm 4.6 × 50 mm, Waters, UK) equilibrated with solvent C (0.05% TFA), 5% solvent D (80% acetonitrile, 20% $H_2O$, 0.03% TFA). Elution was performed with solvent D using a linear gradient from 25 % to 60 % in 35 min, at a flow rate of 6 ml/min. Fractions were detected by monitoring absorbance at 220 nm. Fractions were collected, dried under vacuum, dissolved in sterile ultra-pure water (Milli-Q, Millipore, USA) and tested for anti-*Listeria* activity. Then, active fraction was combined with non-active fraction (1/1) and tested again for anti-*Listeria* activity. Fractions 1 and 2, separated and combined, were stored at −20 °C.

### 2.4.8. Identification of genes encoding bacteriocin production

*Lb. plantarum* MBSa4 was investigated for the presence of known bacteriocin genes, using PCR and appropriate primers (Table 3). Total DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and submitted to amplification in a reaction mixture (20 μl) containing approximately 25 ng/μl of extracted DNA, PCR buffer (Fermentas, MD, USA), 100 μM $MgCl_2$ (Fermentas), 200 μM dNTPs (Fermentas), 0.025 U *Taq* polymerase (Fermentas) and 1 pmol each primer. Amplification was achieved in 35 cycles using a DNA thermocycler MasterCycler®PCR (Eppendorf Scientific, Germany). PCR conditions are show on Table 4. PCR-amplified DNA were separated by 0.8 %–2.0 % (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide (0.1 mg/mL) fluorescence using a UVP Bioimaging System (DIGIDOC-IT System, USA). For each bacteriocin gene set of primers, the band corresponding to the correct size (Table 3) was purified from the gel using QIAquick® PCR Purification kit (Qiagen, Germany) following the manufacturer instructions and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

## 3. Results and discussion

The preliminary screening for bacteriocin-producing LAB in Italian type salami indicated several isolates with good potential for further study, but one of them (named MBSa4) presented special antibacterial features when compared to the others. The 16S rRNA sequencing indicated that this strain is *Lb. plantarum*. The loss of antimicrobial activity when the CFS was treated with proteases confirmed that *Lb. plantarum* MBSa4 is a bacteriocin-producing strain (Table 5). The bacteriocin was active against all tested *L. monocytogenes* strains (all serotypes) and *Listeria innocua* ATCC 33090 and *Listeria welshimeri* USP (Table 1). When tested against *Staphylococcus aureus*, antimicrobial activity was observed only towards *St. aureus* ATCC 25923. Bacteriocin MBSa4 was active against three out of ten strains of *Enterococcus* spp. and two (*Lb. sakei* ATCC 15521 and *Lactobacillus fermentum* ET35) out of 25 strains of LAB. Bacteriocin MBSa4 did not inhibit the tested *Salmonella*, *Escherichia coli* and *Enterobacter*, nor *Bacillus cereus*. Notably, *Lb. plantarum* MBSa4 was active against all tested fungi, except *Geotrichum candidum* (Table 2). However, the CFS did not inhibit the tested fungi, indicating that most probably a different compound produced by *Lb. plantarum* MBSa4 was involved in this inhibitory feature.

Activity of the bacteriocin was not affected by the temperature from 4 °C to 100 °C, remaining active even after treatment at 121 °C for 15 min (Table 5). Full residual activity was observed at acid pH (2.0–6.0), but part of activity was lost at pH 8.0 (20.8% residual activity) and complete inactivation was observed at pH 10.0 (Table 5).

The stability to heat and low pH of bacteriocin produced by *Lb. plantarum* MBSa4 confers to this strain promising technological properties, required for application in acidified products with long shelf-life. It is important to highlight the antagonistic effect of *Lb. plantarum* MBSa4 against fungi, which are common spoilage organisms and can produce health-damaging mycotoxins (Dalié, Deschamps, & Richard-Forget, 2010). However, it should be noted that the antifungal activity was a consequence of interaction between the tested fungi and *Lb. plantarum* MBSa4 and not caused by an antimicrobial compound in the CFS of *Lb. plantarum* MBSa4. In the food industry, fungal spoilage of fermented food products can be minimized by addition of propionic acid and its salts, use of modified atmosphere packaging, irradiation or pasteurization, but modern biopreservation techniques based on interactions between microorganisms are also promising, especially involving LAB that have well-accepted GRAS status. LAB produce an array of antimicrobial compounds, such as organic acids, peptides, proteins and many other low molecular weight substances (Cotter et al., 2005), but the mechanism of antifungal action is difficult to elucidate due to complex and commonly synergistic interactions between these compounds (Schnürer & Magnusson, 2005).

*Lb. plantarum* MBSa4 growth, pH reduction and bacteriocin-production (AU/ml) in MRS broth incubated at 25 °C, 30 °C and 37 °C are presented in Fig. 1. Good growth was achieved at the three temperatures, but decrease of pH was faster at 30 °C and 37 °C than at 25 °C, a feature that may be important if this strain is applied in fermentation processes. The highest level of bacteriocin production was observed at 25 °C after 22 h of incubation (1600 AU/ml). Bacteriocin production was first detected only after 14 h at this temperature (100 AU/ml), but was detected as early as 12 h at 30 °C, at level of 200 AU/ml.

Bacteriocin production by *Lb. plantarum* MBSa4 started at late exponential phase and reached its maximum at the stationary phase (22 h at 25 °C, Fig 1), suggesting that it is a primary metabolite. Similar trends have been reported for nisin (Hurst, 1981). However, high biomass values occurred at 30 °C and 37 °C (Fig 1). Decrease of temperature below the optimum for growth improved bacteriocin production. Many reports have shown that unfavorable growth conditions such as low temperature, nutrient limitation, osmotic stress, etc. stimulate bacteriocin production (Delgado et al., 2007; Mataragas, Metaxopoulos, Galiotou, & Drosinos, 2003), probably due to an increased availability of amino acids and energy at low growth rates and enzymatic reactions.

The determined value of MIC for the extracted bacteriocin MBSa4 against *L. monocytogenes* Scott A was 160 AU/ml. The bactericidal or bacteriostatic effect of the bacteriocin at MIC value

**Table 3**
Primers used for detection of bacteriocin genes.

| Bacteriocin | Primers | Sequence (5′-3′) | Expected size (bp) | Reference |
|---|---|---|---|---|
| Plantaricin S | PlanS-F | GCC TTA CCA GCG TAA TGC CC | 450 | Stephens et al., 1998 |
| | PlanS-R | CTG GTG ATG CAA TCG TTA GTT T | | |
| Plantaricin NC8 | PlanNC8-F | GGT CTG CGT ATA AGC ATC GC | 207 | Maldonado et al., 2003 |
| | PlanNC8-R | AAA TTG AAC ATA TGG GTG CTT TAA ATT CC | | |
| Plantaricin W | PlanW-F | TCA CAC GAA ATA TTC CA | 165 | Holo et al., 2001 |
| | PlaW-R | GGC AAG CGT AAG AAA TAA ATG AG | | |
| Sakacin T-α | SakT-α −F | TCGGTGGCTATACTGTCTAAACA | 160 | Macwana & Muriana, 2012 |
| | SakT-α −R | TGTCCTAAAAATCCACCAATGC | | |
| Sakacin T-β | SakT-β −F | AAGAAATGATAGAAATTTTTGGAGG | 151 | Macwana & Muriana, 2012 |
| | SakT-β −R | TGTGAAATCCAATCTTGTCCTG | | |
| Sakacin Q | SakQ-F | GAA (T/A)T(A/G) (C/A) (A/C)A NCA ATT A(C/T) (A/C) GGT GG | | Dortu, Huch, Holzapfel, Franz, & Thonart, 2008 |
| | SakQ-R | TAC CAC CAG CAG CCA TTC CC | | |
| Sakacin X | SakX-F | AGCTATGAAAGGTATTGTCGGG | 156 | Macwana & Muriana, 2012 |
| | SakX-R | TAAGATTTCCAGCCAGCAGC | | |
| Sakacin P | SakP-F | ATG GAA AAG TTT ATT GAA TTA | 186 | Reminger, Ehrmann, & Vogel, 1996 |
| | SakP-R | TTA T TT ATT CCA GCC AGC GTT | | |
| Sakacin G1 | SakGA1-F | TTA GAA CTA CAC TGA TCG TG | | Todorov et al., 2011 |
| | SakGA1-R | TGG AAG AAT GAG TAC TTG TT | | |
| Sakacin G2 | SakGA2-F | CGT TAC AAC AGA ACT TCA AG | | Todorov et al., 2011 |
| | SakGA2-R | TGG AAG AAT GAG TAC TTG TT | | |
| Curvacin A | CurA-F | GTA AAA GAA TTA AGT ATG ACA | 171 | Reminger et al., 1996 |
| | CurA-R | TTA CAT TCC AGC TAA ACC ACT | | |
| Enterocin A | EntA-F | GAG ATT TAT CTC CAT AAT CT | | Aymerich et al., 1996 |
| | EntA-R | GTA CCA CTC ATA GIG GAA | | |
| Enterocin B | EntB-F | GAA AAT GAT CAC AGA ATG CCT A | 159 | Du Toit, Franz, Dicks, & Holzapfel, 2000 |
| | EntB-R | GTT GCA TTT AGA GTA TAC ATT TG | | |
| Enterocin P | EntP-F | ATG AGA AAA AAA TTA TTT AGT TT | 216 | Gutiérrez et al., 2005 |
| | EntP-R | TTA ATG TCC CAT ACC TGC CAA ACC | | |
| Enterocin L50B | EntL50B−F | ATG GGA GCA ATC GCA AAA TTA | | Cintas, Casaus, Fernandez, & Hernandez, 1998 |
| | EntL50B-R | TAG CCA TTT AAT TTG TTG ATC | | |
| Nisin | Nis-F | ATG AGT ACA AAA GAT TTC AAC TT | 203 | Kruger et al., 2013 |
| | Nis-R | TTA TTT GCT TAC GTG AAC GC | | |
| Pediocin PA1 | PedPA1-F | CAA GAT CGT TAA CCA GTT T | 1238 | Todorov, Ho, et al., 2010; Todorov, Wachsman, et al., 2010 |
| | PedPA1-R | CCG TTG TTC CCA TAG TCT AA | | |

against *L. monocytogenes* Scott A is shown in Fig. 2. When added at 0 h or at early exponential phase (6 h), the bacteriocin MBSa4 presented a clear bacteriostatic effect. When tested for adsorption to the *L. monocytogenes* Scott A cells, the bacteriocin was 100% adsorbed after 1 h at 37 °C.

The mode of action of bacteriocin produced by *Lb. plantarum* MBSa4 against *L. monocytogenes*, when added in the MIC amount (160 AU/ml), was bacteriostatic (Kruger et al., 2013). It is known that the electrostatic interactions with cytoplasmic membranes of bacteria are responsible for the initial binding of class II bacteriocins (Drider, Fimland, Héchard, McMullen, & Prévost, 2006). The bacteriocin MBSa4 presented a strong interaction with the *L. monocytogenes* cells (100% adsorption), differently from bacteriocin AMA-K (Todorov, 2008) and pentocin 31-1 (Liu, Lv, Li, Zhou, & Zhang, 2008) that showed adsorption of 75% and 50%, respectively. Considering the high level of adsorption and the bacteriostatic mode of action, bacteriocin MBSa4 most probably binds to the cell surface, but does not reach the specific receptors needed to perform the killing effect. Such a high level of adsorption of

**Table 4**
Optimized cycling conditions used for the amplification of bacteriocin genes.

| Primers | Initial denaturation | Denaturation | Annealing | Elongation |
|---|---|---|---|---|
| PlanS-F | 94 °C, 3 min | 94 °C, 30 s | 45 °C, 1 min | 72 °C, 1 min |
| PlanNC8 | 94 °C, 3 min | 94 °C, 1 min | 51 °C, 1 min | 72 °C, 30 s |
| PlanW | 94 °C, 3 min | 94 °C, 1 min | 41 °C, 1 min | 72 °C, 30 s |
| SakT-α | 95 °C, 15 min | 95 °C, 30 s | 58 °C, 1 min | 72 °C, 1 min |
| SakT-β | 95 °C, 15 min | 95 °C, 30 s | 56 °C, 1 min | 72 °C, 1 min |
| SakQ | 95 °C, 15 min | 95 °C, 30 s | 53 °C, 1 min | 72 °C, 1 min |
| SakX | 95 °C, 15 min | 95 °C, 30 s | 58 °C, 1 min | 72 °C, 1 min |
| SakP | 94 °C, 3 min | 94 °C, 30 s | 40 °C, 1 min | 72 °C, 1 min |
| SakG | 94 °C, 4 min | 94 °C, 30 s | 38 °C, 30 s | 72 °C, 30 s |
| CurA | 94 °C, 3 min | 94 °C, 30 s | 40 °C, 1 min | 72 °C, 1 min |
| EntA | 94 °C, 2 min | 94 °C, 1 min | 34 °C, 30 s | 72 °C, 2 min |
| EntB | 94 °C, 2 min | 94 °C, 1 min | 42 °C, 1 min | 72 °C, 2 min |
| EntP | 94 °C, 4 min | 94 °C, 1 min | 41 °C, 1 min | 72 °C, 1 min |
| EntL50B | 95 °C, 5 min | 95 °C, 1 min | 45 °C, 1 min | 72 °C, 1 min |
| Nis | 94 °C, 4 min | 94 °C, 45 s | 48 °C, 30 s | 72 °C, 45 s |
| PedPa1 | 94 °C, 4 min | 94 °C, 30 s | 44 °C, 30 s | 72 °C, 30 s |

**Table 5**
Effect of different treatments on the activity of bacteriocin produced by *Lactobacillus plantarum* MBSa4.

| Treatment | Residual bacteriocin activity (%) |
|---|---|
| *Enzymes* | |
| Protease K | 0 |
| Trypsin | 0 |
| Pepsin | 0 |
| α-Chymotrypsin | 0 |
| Protease Type XIV | 0 |
| *Temperature (time)* | |
| 4 °C (60 min) | 100 |
| 25 °C (60 min) | 100 |
| 30 °C (60 min) | 100 |
| 37 °C (60 min) | 100 |
| 45 °C (60 min) | 100 |
| 60 °C (60 min) | 100 |
| 80 °C (60 min) | 100 |
| 100 °C (60 min) | 100 |
| 121 °C (15 min) | 100 |
| *pH* | |
| 2 | 100 |
| 4 | 100 |
| 6 | 100 |
| 8 | 20.8 |
| 10 | 0 |

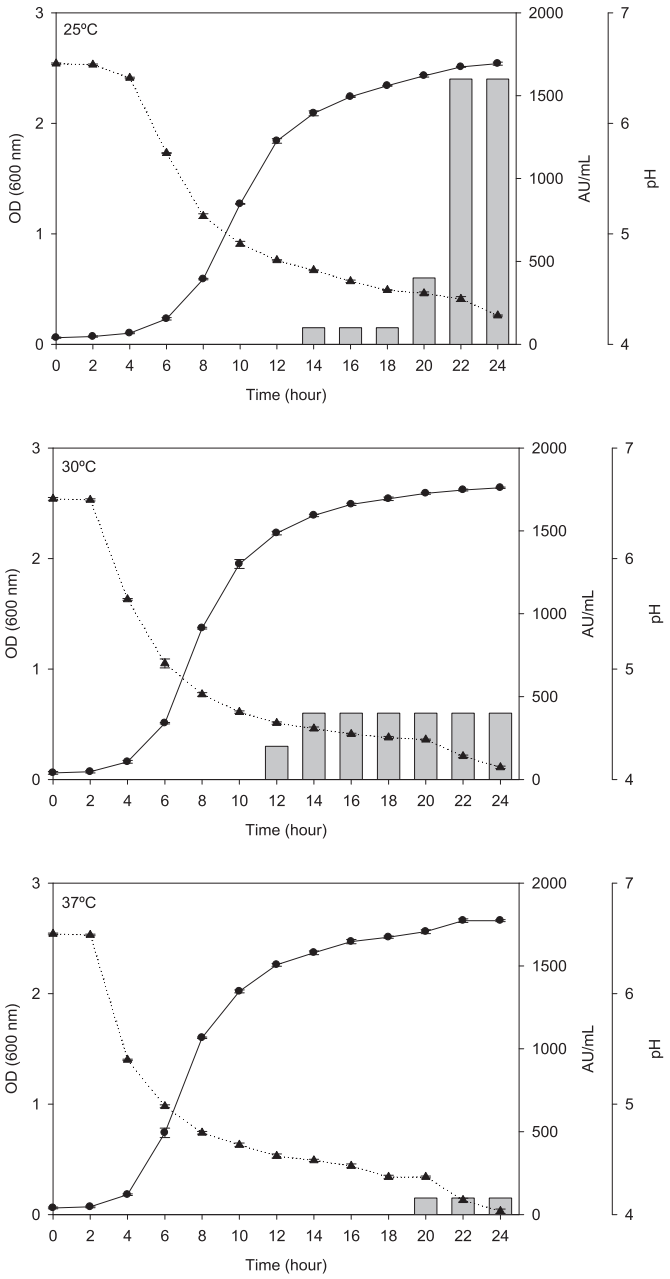


**Fig. 1.** Growth (solid lines, ●), pH (dotted lines, ▲) and bacteriocin production (bars) by *Lactobacillus plantarum* MBSa4 in MRS broth at 25 °C, 30 °C and 37 °C.

antimicrobial peptides to the cell surface of *Listeria* may be interpreted as a defense mechanism against bacteriocins.

The molecular weight of the bacteriocin produced by *Lb. plantarum* was determined by SDS-PAGE to be around 2.5 kDa (Fig. 3). Results of the last step of the partial purification of the bacteriocin by $C_{18}$ RP-HPLC are shown in Fig. 4, where several peaks can be seen. The anti-*Listeria* activity (*L. ivanovii* subsp. *ivanovii* ATCC 19119) of the fractions correspondent to each peak is shown in Fig. 5a, indicating that only fraction 9 was active. However, when fraction 9 was combined with the other fractions (ratio 1:1) (Fig. 5b), fractions 1 to 8 still presented low or absence of activity, while strong inhibition zone was observed for fractions 10 to 12, pointing a synergistic effect between this fractions.

Only primers PlanW-F/PlanW-R targeting plantaricin W generated an amplicon of approximately 165 bp. Nucleotides sequencing

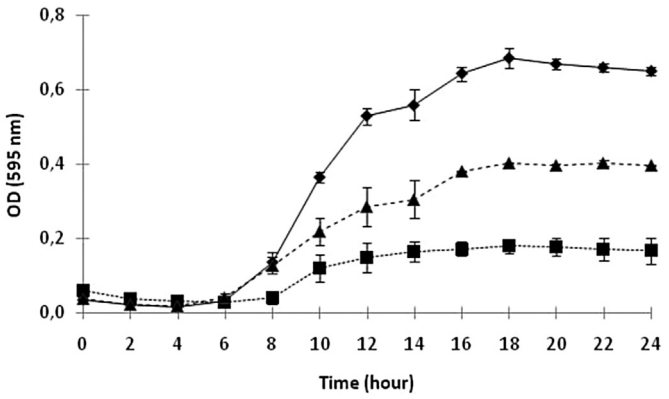


**Fig. 2.** Growth of *Listeria monocytogenes* Scott A in BHI at 37 °C after addition of the bacteriocin produced by *Lactobacillus plantarum* MBSa4 at time 0 h (■) and 6 h (▲). Control without bacteriocin (◆).

of this amplicon corresponded to plantaricin W and the translated genetic sequence resulted in MKISKIEAQA RKDFFKKIDT NSNLLNVNGA KCKWWNISCE peptide.

According to the criteria of Deegan et al. (2006) for classification of bacteriocins, the antimicrobial peptide produced by *Lb. plantarum* MBSa4 belongs to class IIb (two-peptides nonlantibiotic bacteriocins), as the antimicrobial activity of some fractions obtained in the purification procedure was dependent on the complementation with the active fraction. The size of the active fraction of this bacteriocin in SDS-PAGE was approximately 2.5 kDa, similar to the plantaricin S described by Jiménez-Díaz, Rios-Sánchez, Desmazeaud, Ruiz-Barba, and Piard (1993), which is also a two-peptide nonlantibiotic bacteriocin (Table 6). In addition, the DNA of *Lb. plantarum* MBS4 contained a fragment homologous to plantaricin W gene, as determined by the specific primers for PlanW-F/PlanW-R. Holo, Jeknic, Daeschel, Stevanovic, and Nes (2001) reported that plantaricin W from *Lb. plantarum* LMG 2379 is a two-

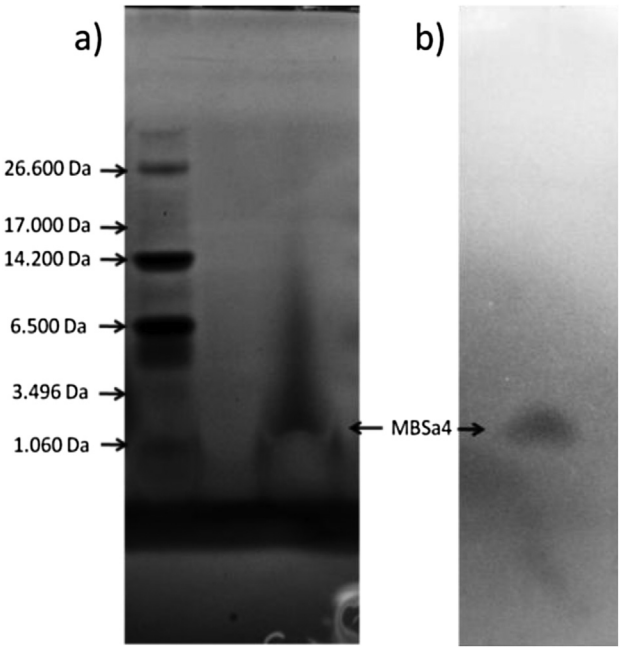


**Fig. 3.** SDS-PAGE gel containing the bacteriocin produced by *Lactobacillus plantarum* MBSa4. Gel stained with Coomassie Brilliant Blue R250 and gel overlaid with BHI soft-agar inoculated with *Listeria monocytogenes* Scott A and incubated at 37 °C for 12 h (b).

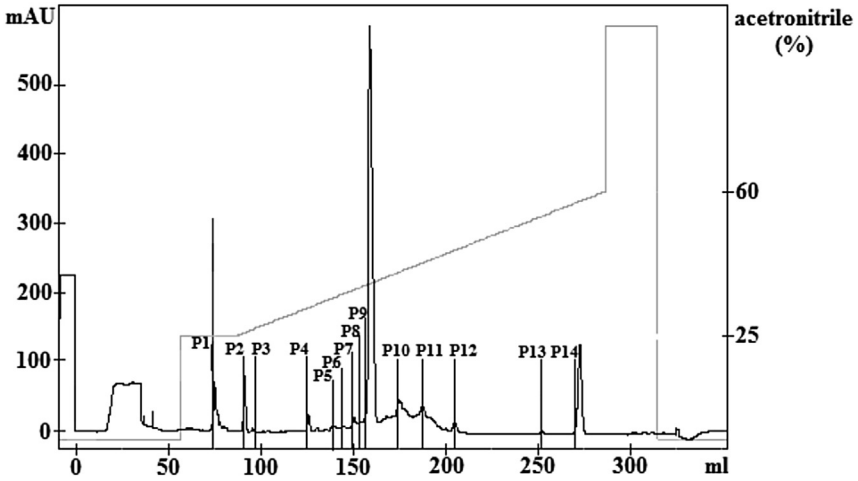


**Fig. 4.** Chromatogram of the bacteriocin produced by *Lactobacillus plantarum* MBSa4 ($C_{18}$ reverse-phase HPLC).

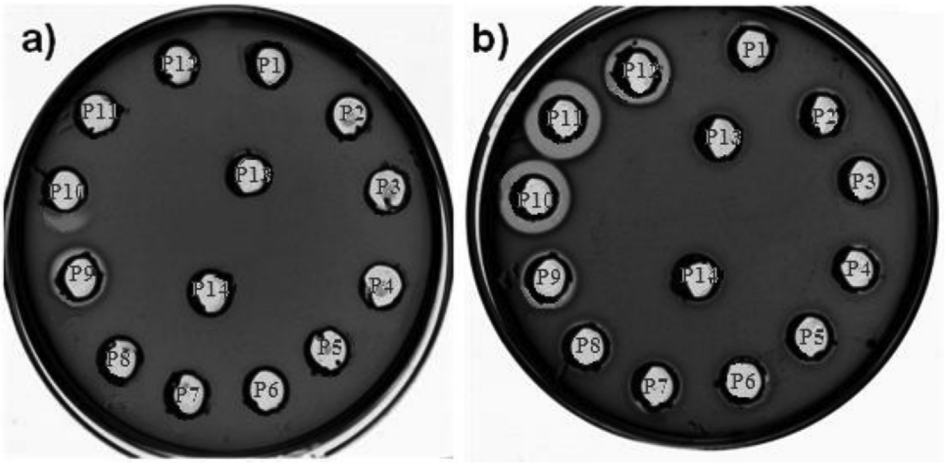


**Fig. 5.** Anti-*Listeria* activity of the purified fractions of the bacteriocin produced by *Lactobacillus plantarum* MBSa4 ($C_{18}$ reverse-phase HPLC). Single fractions (a) and single fractions combined with fraction 9 (b).

**Table 6**
Summary of known two-peptide lantibiotic and nonlantibiotic bacteriocins.

| Strain | Source | Bacteriocin | Classification | Reference |
|---|---|---|---|---|
| *Bacillus thuringiensis* DPC 6431 | Human feces | Thuricin CD | nonlantibiotic | Rea et al., 2010 |
| *Brochothrix campestris* ATCC 43754 | Soil | Brochocin-C | nonlantibiotic | McCormick et al., 1998 |
| *Enterococcus faecalis* C901 | Human colostrum | Enterocin C | nonlantibiotic | Maldonado-Barragán et al., 2009 |
| *Enterococcus faecalis* NKR-4-1 | Thai fermented fish | Enterocin W | lantibiotic | Sawa et al., 2012 |
| *Enterococcus faecalis* | Clinical isolates | Cytolysin | lantibiotic | Booth et al., 1996 |
| *Enterococcus faecalis* BFE 1071 | Feces of minipigs | Enterocin 1071 | nonlantibiotic | Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000 |
| *Enterococcus faecalis* FAIR-E 309 | Argentinian cheese | Enterocin 1071 | nonlantibiotic | Franz et al., 2002 |
| *Enterococcus faecium* L50 | Dry-fermented sausage | Enterocin L50 | nonlantibiotic | Cintas et al., 1998 |
| *Enterococcus faecium* KU-B5 | Sugar apples | Enterocin X | nonlantibiotic | Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010 |
| *Lactobacillus casei* CRL 705 | Fermented sausage | Lactocin 705 | nonlantibiotic | Cuozzo, Sesma, Palacios, De Ruíz Holgado, & Raya, 2000 |
| *Lactobacillus johnsonii* VPI11088 | Human intestine | Lactacin F | nonlantibiotic | Allison, Fremaux, & Klaenhammer, 1994 |
| *Lactococcus lactis* LMG 2081 | | Lactococcin G | nonlantibiotic | Nissen-Meyer, Holo, Havarstein, Sletten, & Nes, 1992 |
| *Lactococcus lactis* QU 4 | Corn | Lactococcin Q | nonlantibiotic | Zendo, Koga, Shigeri, Nakayama, & Sonomoto, 2006 |
| *Lactococcus lactis* subsp. *lactis* DPC3147 | Irish kefir grains | Lacticin 3147 | lantibiotic | Ryan et al., 1999 |
| *Lactobacillus salivarius* DPC6005 | Porcine intestine | Salivaricin P | nonlantibiotic | Barret et al., 2007 |
| *Lactobacillus salivarius* UCC118 | Human gastrointestinal tract | ABP-118 | nonlantibiotic | Flynn et al., 2002 |
| *Lactobacillus plantarum* C11 | Fermented cucumbers | Plantaricin A<br>Plantaricin EF<br>Plantaricin JK | nonlantibiotic | Andersen et al., 1998 |
| *Lactobacillus plantarum* LPCO10 | Fermented green olives | Plantaricin S | nonlantibiotic | Jiménez-Díaz et al., 1995 |
| *Lactobacillus plantarum* NC8 | Grass silage | plantaricin NC8 | nonlantibiotic | Maldonado et al., 2003 |
| *Lactobacillus plantarum* LMG 2379 | Pinot Noir wine | Plantaricin W | lantibiotic | Holo et al., 2001 |
| *Leuconostoc* MF215B | | Leucocin H | nonlantibiotic | Blom et al., 1999 |
| *Staphylococcus aureus* C55 | Human skin | Staphylococcin C55 | lantibiotic | Navaratna, Sahl, & Tagg, 1998 |
| *Streptococcus bovis* HJ50 | Raw milk | Bovicin HJ50 | lantibiotic | Xiao et al., 2004 |
| *Streptococcus mutans* UA140 | Caries-active dental patient | Mutacin IV | nonlantibiotic | Qi, Chen, & Caufield, 2001 |
| *Streptococcus thermophilus* SFi13 | Nestle strain collection | Thermophilin 13 | nonlantibiotic | Marciset, Jeronimus-Stratingh, Mollet, & Poolman, 1997 |

peptide lantibiotic bacteriocin. Many other two-peptide lantibiotic and nonlantibiotic bacteriocins produced by bacteria other than *Lb. plantarum* have been more recently reported (Table 6), indicating that a new bacteriocin classification is needed.

Kaur et al. (2011) described development of resistance of some food-borne pathogens to Class IIa bacteriocins and reviewed possible mechanisms involved in this process. Macwana and Muriana (2012) reported that use of mixtures of bacteriocins with different modes of action could exert greater inhibition than mixtures of bacteriocins with the same mode of action. Therefore, more studies of characterization and application of these bacteriocins are important for more effective use as biopreservatives in foods.

## Acknowledgments

The authors acknowledge Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Project 08/58841-2), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, COFECUB, project 3592-11-1), CAPES-COFECUB (project #730/11) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for fellowships and financial support.

## References

Allison, G. E., Fremaux, C., & Klaenhammer, T. R. (1994). Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within the lactacin F operon. *Journal of Bacteriology, 176*, 2235–2241.

Anderssen, E. L., Diep, D. B., Nes, I. F., Eijsink, V. G. H., & Nissen-Meyer, J. (1998). Antagonistic activity of *Lactobacillus plantarum* C11: two new two- peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. *Applied and Environmental Microbiology, 64*, 2269–2272.

Aymerich, T., Holo, H., Havarstein, L. S., Hugas, M., Garriga, M., & Nes, I. F. (1996). Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology, 62*, 1676–1682.

Balla, E., Dicks, L. M. T., Du Toit, M., Van Der Merwe, M. J., & Holzapfel, W. H. (2000). Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. *Applied and Environmental Microbiology, 66*, 1298–1304.

Barrett, E., Hayes, M., O'Connor, P., Gardiner, G., Fitzgerald, G. F., Stanton, C., et al. (2007). Salivaricin p, one of a family of two-component antilisterial bacteriocins produced by intestinal isolates of *Lactobacillus salivarius*. *Applied and Environmental Microbiology, 73*, 3719–3723.

Batdorj, B., Dalgalarrondo, M., Choiset, Y., Pedroche, J., Métro, F., Prévost, H., et al. (2006). Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *Journal of Applied Microbiology, 101*, 1364–5072.

Belfiore, C., Fadda, S., Raya, R., & Vignolo, G. (2013). Molecular basis of the adaption of the anchovy isolate *Lactobacillus sakei* CRL1756 to salted environments through a proteomic approach. *Food Research International, 54*, 1334–1341.

Blom, H., Katla, T., Holck, A., Sletten, K., Axelsson, L., & Holo, H. (1999). Characterization, production, and purification of leucocin H, a two- peptide bacteriocin from Leuconostoc MF215B. *Current Microbiology, 39*, 43–48.

Booth, M. C., Bogie, C. P., Sahl, H.-G., Siezen, R. J., Hatter, K. L., & Gilmore, M. S. (1996). Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. *Molecular Microbiology, 21*, 1175–1184.

Cintas, L. M., Casaus, P., Fernandez, M. F., & Hernandez, P. E. (1998). Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and foodborne pathogenic bacteria. *Food Microbiology, 15*, 289–298.

Corsetti, A., Settanni, L., & Van Sinderen, D. (2004). Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. *Journal of Applied Microbiology, 96*, 521–534.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews in Microbiology, 3*, 777–788.

Cuozzo, S. A., Sesma, F., Palacios, J. M., De Ruíz Holgado, A. P., & Raya, R. R. (2000). Identification and nucleotide sequence of genes involved in the synthesis of lactocin 705, a two-peptide bacteriocin from *Lactobacillus casei* CRL 705. *FEMS Microbiology Letters, 185*, 157–161.

Dalié, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria – potential for control of mould growth and mycotoxins: a review. *Food Control, 21*, 370–380.

Deegan, L. H., Cotter, P. D., Hill, C., & Ross, P. (2006). Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal, 16*, 1058–1071.

Delgado, A., López, F. N. A., Brito, D., Peres, C., Fevereiro, P., & Garrido-Fernández, A. (2007). Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. *Journal of Biotechnology, 130*, 193–201.

Diep, D. B., Håvarstein, L. S., & Nes, I. F. (1996). Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *Journal of Bacteriology, 178*, 4472–4483.

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin production: a probiotic trait? *Applied and Environmental Microbiology, 78*, 1–6.

Dortu, C., Huch, M., Holzapfel, W. H., Franz, C. M. A. P., & Thonart, P. (2008). Antilisterial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Letters in Applied Microbiology, 47*, 581–586.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews, 70*, 564–582.

Du Toit, M., Franz, C., Dicks, L. M. T., & Holzapfel, W. H. (2000). Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces. *Journal of Applied Microbiology, 88*, 482–494.

Enan, G., El-Essawy, A. A., Uyttendaele, M., & Debevere, J. (1996). Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization production and bactericidal action of plantaricin UG1. *International Journal of Food Microbiology, 30*, 189–215.

Felske, A., Rheims, H., Wokerink, A., Stackebrandt, E., & Akkermans, A. D. L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology, 143*, 2983–2989.

Flynn, S., van Sinderen, D., Thornton, G. M., Holo, H., Nes, I. F., & Collins, J. K. (2002). Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. *Microbiology, 148*, 973–984.

Franz, C. M. A. P., Grube, A., Herrmann, A., Abriouel, H., Stärke, J., Lombardi, A., et al. (2002). Biochemical and genetic characterization of the two-peptide bacteriocin enterocin 1071 produced by *Enterococcus faecalis* FAIR-E 309. *Applied and Environmental Microbiology, 68*, 2550–2554.

Freiding, S., Gutsche, K. A., Ehrmann, M. A., & Vogel, R. F. (2011). Genetic screening of *Lactobacillus sakei* and *Lactobacillus curvatus* strains for their peptidolytic system and amino acid metabolism, and comparison of their volatiles in a model system. *Systematic and Applied Microbiology, 34*, 311–320.

Gao, Y., Li, D., & Liu, X. (2014). Bacteriocin-producing *Lactobacillus sakei* C2 as starter culture in fermented sausages. *Food Control, 35*, 1–6.

Gutiérrez, J., Bourque, D., Criado, R., Choi, Y. J., Cintas, L. M., Hernández, P. E., et al. (2005). Heterologous extracellular production of enterocin P from *Enterococcus faecium* P13 in the methylotrophic bacterium *Methylobacterium extorquens*. *FEMS Microbiology Letters, 248*, 125–131.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S., & Nes, I. F. (2001). Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology, 147*, 643–651.

Hugas, M., Garriga, M., Aymerich, M. T., & Monfort, J. M. (1995). Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sake* CTC494. *Journal of Applied Bacteriology, 79*, 322–330.

Hu, C.-B., Malaphan, W., Zendo, T., Nakayama, J., & Sonomoto, K. (2010). Enterocin X, a novel two-peptide bacteriocin from *Enterococcus faecium* KU-B5, has an antibacterial spectrum entirely different from those of its component peptides. *Applied and Environmental Microbiology, 76*, 4542–4545.

Hurst, A. (1981). Nisin. *Advances in Applied Microbiology, 27*, 85–122.

Jiménez-Díaz, R., Rios-Sánchez, R. M., Desmazeaud, M., Ruiz-Barba, J. L., & Piard, J.-C. (1993). Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Applied and Environmental Microbiology, 59*, 1416–1424.

Jiménez-Díaz, R., Ruiz-Barba, J. L., Cathcart, D. P., Holo, H., Nes, I. F., Sletten, K. H., et al. (1995). Purification and partial amino acid sequence of plantaricin S, a bacteriocin produced by *Lactobacillus plantarum* LPCO10, the activity of which depends on the complementary action of two peptides. *Applied and Environmental Microbiology, 61*, 4459–4463.

Kaur, G., Malik, R. K., Mishra, S. K., Singh, T. P., Bhardwaj, A., Singroha, G., et al. (2011). Nisin and class IIa bacteriocin resistance among *Listeria* and other foodborne pathogens and spoilage bacteria. *Microbial Drug Resistance, 17*, 197–205.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews, 12*, 39–85.

Kruger, M. F., Barbosa, M. D. S., Miranda, A., Landgraf, M., Destro, M. T., Todorov, S. D., et al. (2013). Isolation of bacteriocinogenic strain of *Lactococcus lactis* subsp. *lactis* from rocket salad (*Eruca sativa* Mill.) and evidences of production of a variant of nisin with modification in the leader-peptide. *Food Control, 33*, 467–476.

Liu, G., Lv, Y., Li, P., Zhou, K., & Zhang, J. (2008). Pentocin 31-1, an anti-*Listeria* bacteriocin produced by *Lactobacillus pentosus* 31-1 isolated from Xuan-Wei Ham, a traditional China fermented meat product. *Food Control, 19*, 353–359.

Macwana, S., & Muriana, P. M. (2012). Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *Journal of Microbiological Methods, 88*, 7–13.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., & Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters, 219*, 129–135.

Maldonado-Barragán, A., Caballero-Guerrero, B., Jiménez, E., Jiménez-Díaz, R., Ruiz-

Barba, J. L., & Rodríguez, J. M. (2009). Enterocin C, a class IIb bacteriocin produced by *E. faecalis* C901, a strain isolated from human colostrum. *International Journal of Food Microbiology, 133*, 105–112.
Maldonado, A., Ruiz-Barba, J. L., & Jiménez-Díaz, R. (2003). Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. *Applied and Environmental Microbiology, 69*, 383–389.
Marciset, O., Jeronimus-Stratingh, M. C., Mollet, B., & Poolman, B. (1997). Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. *Journal of Biological Chemistry, 272*, 14277–14284.
Mataragas, M., Metaxopoulos, J., Galiotou, M., & Drosinos, E. H. (2003). Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Meat Science, 64*, 265–271.
McCormick, J. K., Poon, A., Sailer, M., Gao, Y., Roy, K. L., Mcmullen, L. M., et al. (1998). Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by Brochothrix campestris ATCC 43754. *Applied and Environmental Microbiology, 64*, 4757–4766.
Messi, P., Bondi, M., Sabia, C., Battini, R., & Manicardi, G. (2001). Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *International Journal of Food Microbiology, 64*, 193–198.
Müller, D. M., Carrasco, M. S., Tonarelli, G. G., & Simonetta, A. C. (2009). Characterization and purification of a new bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus plantarum* lp 31 strain isolated from dry-fermented sausage. *Journal of Applied Microbiology, 106*, 2031–2040.
Navaratna, M. A. D. B., Sahl, H.-G., & Tagg, J. R. (1998). Two-component anti-*Staphylococcus aureus* lantibiotic activity produced by *Staphylococcus aureus* C55. *Applied and Environmental Microbiology, 64*, 4803–4808.
Nielsen, J. W., Dickson, J. S., & Crouse, J. D. (1990). Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology, 56*, 2142–2145.
Nissen-Meyer, J., Holo, H., Havarstein, L. S., Sletten, K., & Nes, I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *Journal of Bacteriology, 174*, 5686–5692.
Qi, F., Chen, P., & Caufield, P. W. (2001). The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. *Applied and Environmental Microbiology, 67*, 15–21.
Rattanachaikunsopon, P., & Phumhachorn, P. (2006). Isolation and preliminary characterization of a bacteriocin produced by *Lactobacillus plantarum* N014 isolated from Nham, a traditional Thai fermented pork. *Journal of Food Protection, 69*, 1937–1943.
Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., et al. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against *Clostridium difficile*. *Proceedings of National Acadademy of Science of USA, 107*, 9352–9357.
Reminger, A., Ehrmann, M. A., & Vogel, R. F. (1996). Identification of bacteriocin-encoding genes in Lactobacilli by polymerase chain reaction (PCR). *Systematic and Applied Microbiology, 19*, 28–34.
Rivas, F. P., Castro, M. P., Vallejo, M., Marguet, E., & Campos, C. A. (2014). Sakacin Q produced by *Lactobacillus curvatus* ACU-1: functionality characterization and antilisterial activity on cooked meat surface. *Meat Science, 97*, 475–479.
Ryan, M. P., Jack, R. W., Josten, M., Sahl, H.-G., Jung, G., Ross, R. P., et al. (1999). Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. *Journal of Biological Chemistry, 274*, 37544–37550.
Sawa, N., Wilaipun, P., Kinoshita, S., Zendo, T., Leelawatcharamas, V., Nakayama, J., et al. (2012). Isolation and characterization of enterocin W, a novel two-peptide lantibiotic produced by *Enterococcus faecalis* NKR-4-1. *Applied and Environmental Microbiology, 78*, 900–903.
Schägger, H., & Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Annals of Biochemistry, 166*, 368–379.
Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science and Technology, 16*, 70–78.
Stephens, S. K., Floriano, B., Cathcart, D. P., Bayley, S. A., Witt, V. F., Jiménez-Díaz, R., et al. (1998). Molecular analysis of the locus responsible for production of plantaricin S, a two-peptide bacteriocin produced by *Lactobacillus plantarum* LPCO10. *Applied and Environmental Microbiology, 64*, 1871–1877.
Todorov, S. D. (2008). Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of adsorption of bacteriocin AMA-K to *Listeria* spp. *Brazilian Journal of Microbiology, 38*, 178–187.
Todorov, S. D., & Dicks, L. M. T. (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against gram-negative bacteria. *Enzymes and Microbiological Technology, 36*, 318–326.
Todorov, S. D., Ho, P., Vaz-Velho, M., & Dicks, L. M. T. (2010). Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science, 84*, 334–343.
Todorov, S. D., Rachman, C., Fourrier, A., Dicks, L. M. T., van Reenen, C. A., Prévost, H., et al. (2011). Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe, 17*, 23–31.
Todorov, S. D., Wachsman, M., Tomé, E., Dousset, X., Destro, M. T., Dicks, L. M. T., et al. (2010). Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. *Food Microbiology, 27*, 869–879.
Xiao, H., Chen, X., Chen, M., Tang, S., Zhao, X., & Huan, L. (2004). Bovicin HJ50, a novel lantibiotic produced by *Streptococcus bovis* HJ50. *Microbiology, 150*, 103–108.
Yildirim, Z., Avşar, Y. K., & Yildirim, M. (2002). Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactocobacillus buchneri*. *Microbiology Research, 157*, 103–107.
Zendo, T., Koga, S., Shigeri, Y., Nakayama, J., & Sonomoto, K. (2006). Lactococcin Q, a novel two-peptide bacteriocin produced by *Lactococcus lactis* QU 4. *Applied and Environmental Microbiology, 72*, 3383–3389.