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Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate

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ABSTRACT

The aims of this study were to isolate LAB with anti-*Listeria* activity from salami samples, characterize the bacteriocin/s produced by selected isolates, semi-purify them and evaluate their effectiveness for the control of *Listeria monocytogenes* during manufacturing of salami in a pilot scale. Two isolates (differentiated by RAPD-PCR) presented activity against 22 out of 23 *L. monocytogenes* strains for bacteriocin MBSa2, while the bacteriocin MBSa3 inhibited all 23 strains in addition to several other Gram-positive bacteria for both antimicrobials and were identified as *Lactobacillus curvatus* based on 16S rRNA sequencing. A three-step purification procedure indicated that both strains produced the same two active peptides (4457.9 Da and 4360.1 Da), homologous to sakacins P and X, respectively. Addition of the semi-purified bacteriocins produced by *Lb. curvatus* MBSa2 to the batter for production of salami, experimentally contaminated with *L. monocytogenes* (10^4–10^5 CFU/g), caused 2 log and 1.5 log reductions in the counts of the pathogen in the product after 10 and 20 days respectively, highlighting the interest for application of these bacteriocins to improve safety of salami during its manufacture.

1. Introduction

Lactic acid bacteria (LAB), especially *Lactobacillus sakei* and *Lactobacillus curvatus*, are part of the microbiota of many types of fermented meat products. These two species of LAB are well adapted to the meat environment, conveying the improved flavor and accelerated maturation of fermented meat products (Chaillou et al., 2005; Lahtinen et al., 2011). LAB are also essential in fermented food products, preventing growth of spoilage and pathogenic microorganisms by acidification and production of antimicrobial compounds, contributing to improved safety and quality (Fadda et al., 2010; Balciunas et al., 2013; Mangia et al., 2013).

Bacteriocins produced by LAB are antimicrobial proteinaceous compounds synthesized via the ribosomes, presenting variable spectrum of activities. Most bacteriocins are small molecules with amphipathic characteristics and high isoelectric points. The producer cells are resistant to the bacteriocins they produce due to synthesis of specific immunity proteins (Deegan et al., 2006; Mills et al., 2011; Dobson et al., 2012; Nishie et al., 2012). Numerous bacteriocins produced by different LAB species have been already described (Balciunas et al., 2013). According to Cotter et al. (2005), between 30 and 99% of the prokaryotes (Bacteria and Archaea) produce at least one bacteriocin.

Bacteriocins produced by LAB are well known for their activity against *Listeria monocytogenes*, a ubiquitous Gram-positive pathogen that has caused several food related outbreaks in the last decades (Kumar, 2011; Scallan et al., 2011). In the most recent classification of bacteriocins (Heng et al., 2007), there is a special class dedicated to bacteriocins with anti-*Listeria* activity. Control of *L. monocytogenes* in processed foods is a serious problem, due to the capability to survive the hurdles usually encountered during manufacture of dry fermented products, such as low pH, salt and presence of nitrites (Vogel et al., 2010). Due to this anti-*Listeria* activity, bacteriocinogenic LAB and their bacteriocins are beneficial as preservation agents in fermented products, and can be used as technological alternatives to chemical preservatives, fitting the increased demand for foods with less or no additives (Dickson-Spillmann et al., 2011).

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Many surveys in Brazil indicate that *L. monocytogenes* is a frequent contaminant in fermented meat products consumed in the country, such as sausages and salamis (Borges et al., 1999; Sakate et al., 2003; Martins and Germano, 2011). In our study, we describe the isolation of LAB with anti-*Listeria* activity from Italian type salami produced in Brazil, characterization and purification of the bacteriocins produced by selected LAB and evaluation of their effectiveness in the control of *L. monocytogenes* during the fermentation step of salami manufacture.

2. Material and methods

2.1. Isolation and identification of bacteriocinogenic LAB from salami

Italian type salami samples were purchased in retail markets in the city of Sao Paulo (Brazil), and 50 g of each sample were submitted to isolation of bacteriocinogenic LAB as described by Todorov et al. (2010). Identification of the strains was done using recommended morphological and biochemical tests and 16S rRNA sequence analysis, using 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′) as described by Felske et al. (1997). Purified amplified PCR products were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil and sequences were compared to known sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The genetic similarity of the bacteriocinogenic isolates was tested by Random Amplification of Polymorphic DNA (RAPD) with primers OPL-01 (5′- GGC ATG ACC T-3′), OPL-02 (5′- TGG GCG TCA A-3′), OPL-04 (5′- GAC TGC ACA C-3′), OPL-14 (5′- GTG ACA GGC T-3′) and OPL-20 (5′- TGG TGG ACC A-3′) as described by Todorov et al. (2010).

2.2. Determination of bacteriocin activity

The amount of bacteriocin produced by two selected bacteriocinogenic isolates (*Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3) was determined testing two-fold dilutions of cell free supernatants (CFS) for antimicrobial activity according to Todorov et al. (2010), using *L. monocytogenes* Scott A as indicator strain. For preparation of the CFS, bacteriocin producer strains were grown in MRS broth (Difco, Detroit, MI, USA) for 24 h at 30 °C and cells were removed by centrifugation at 4000 × *g* for 15 min at 4 °C (Hettich Zentrifugen, model Mikro 22R, Tuttlingen, Germany). The pH of CFS was adjusted to 6.0–6.5 with 1 M NaOH (Synth, Sao Paulo, Brazil), heated for 30 min at 70 °C and filter-sterilized (Millex GV 0.22 μm, Millipore, Billerica, MA, USA). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition of *L. monocytogenes*. Results were expressed in AU/mL (Todorov et al., 2010).

2.3. Characterization of the bacteriocinogenic strains

2.3.1. Growth and bacteriocin production in MRS broth

The bacteriocinogenic strains *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 were tested for growth and bacteriocin production in MRS broth at 25 °C, 30 °C and 37 °C. Growth was monitored measuring absorbance at 600 nm (Ultrospec 2000; Pharmacia Biotech, Little Chalfont, UK) every 2 h up to 24 h. Changes in pH of the cultures were recorded. Presence of bacteriocins in the CFS was monitored every 2 h up to 24 h, using the spot-on-the-lawn method and *L. monocytogenes* Scott A as indicator of activity (Todorov et al. (2010)).

2.3.2. Influence of NaCl content and pH of MRS broth on bacterial growth

Bacteriocinogenic strains *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 were tested for growth in MRS broth containing increasing NaCl contents and at acid pH, simulating conditions occuring during the manufacture of salami. Strains were grown in MRS broth for 24 h at 30 °C and then aliquots (10^6–10^7 CFU/mL) were transfered to MRS broth containing 1%–10% NaCl, with pH adjusted to 4 or to 6 with 1 M lactic acid, and incubated at 30 °C. Growth was monitored every 2 h up to 24 h, measuring changes in absorbance as described in 2.3.1.

Table 1
Spectrum of activity of the bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

Target microorganism	Source	Diameter of the inhibition zone (mm)	
		MBSa2	MBSa3
Listeria ivanovii subsp. *ivanovii*	ATCC 19119	15	16
Listeria innocua	ATCC 33090	18	21
Listeria innocua 225/07 serovar 6a	FIOCRUZ[a]	15	16
Listeria innocua 224/07 serovar 6a	FIOCRUZ	11	15
Listeria innocua 047/07 serovar 6a	FIOCRUZ	15	14
Listeria innocua 588/08 serovar 6a	FIOCRUZ	14	11
Listeria monocytogenes Scott A	USP[b]	13	13
Listeria monocytogenes 602/08 serovar 1/2a	FIOCRUZ	13	13
Listeria monocytogenes 046/07 serovar 1/2c	FIOCRUZ	11	14
Listeria monocytogenes 103 serovar 1/2a	USP	0	15
Listeria monocytogenes 106 serovar 1/2a	USP	13	14
Listeria monocytogenes 104 serovar 1/2a	USP	14	15
Listeria monocytogenes 409 serovar 1/2a	USP	12	14
Listeria monocytogenes 506 serovar 1/2a	USP	14	14
Listeria monocytogenes 709 serovar 1/2a	USP	11	12
Listeria monocytogenes 607 serovar 1/2b	USP	18	17
Listeria monocytogenes 603 serovar 1/2b	USP	10	20
Listeria monocytogenes 426 serovar 1/2b	USP	10	14
Listeria monocytogenes 637 serovar 1/2c	USP	10	14
Listeria monocytogenes 422 serovar 1/2c	USP	12	15
Listeria monocytogenes 712 serovar 1/2c	USP	13	15
Listeria monocytogenes 408 serovar 1/2c	USP	14	15
Listeria monocytogenes 211 serovar 4b	USP	15	16
Listeria monocytogenes 724 serovar 4b	USP	19	16
Listeria monocytogenes 101 serovar 4b	USP	18	18
Listeria monocytogenes 703 serovar 4b	USP	18	20
Listeria monocytogenes 620 serovar 4b	USP	20	20
Listeria monocytogenes 302 serovar 4b	USP	15	14
Enterococcus hirae D105	USP	10	13
Enterococcus faecium S105	AGRIS[c]	10	15
Enterococcus faecium S154	AGRIS	0	11
Enterococcus faecium ST62BZ	USP	10	10
Lactobacillus fermentum ET35	UCV[d]	10	10
Lactobacillus curvatus ET31	UCV	0	9
Lactobacillus sakei	ATCC 15521	10	11

No activity have been recorded against: *Bacillus cereus* ATCC 1178, *Staphylococcus aureus* ATCC 29213, ATCC 25923 and ATCC 6538, *Listeria welshimeri* USP, *Listeria seeligeri* USP, *Escherichia coli* ATCC 8739, *Escherichia coli* O157:H7 ATCC 35150, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 12755, *Enterococcus faecium* S100 AGRIS, ST211Ch USP, ET12 UCV[d], ET88 UCV and ET05 UCV, *Lactococcus lactis* V94 USP, V69 USP and B16 USP, *Pediococcus pentosaceus* ET34 UCV, *Lactobacillus curvatus* ET06 UCV and ET30 UCV, *Lactobacillus sakei* subsp. *sakei* 2a USP, *Lactobacillus delbrueckii* B5 USP and ET31 UCV, *Lactobacillus acidophilus* La14 Rhodia, Lac4 Rhodia and La5 Rhodia, *Lactococcus lactis* subsp. *lactis* MK02R USP, D2 USP, D3 USP, D4 USP, D5 USP, B1 USP, B2 USP, B15 USP, B17 USP and R704 Chr. Hansen.

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2.4. Characterization of the bacteriocins

2.4.1. Effect of temperature, pH and salt content on activity

CFS of culture of *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3, prepared as described in 2.2 were tested for antimicrobial activity after exposing them to 4 °C, 25 °C, 30 °C, 37 °C, 45 °C, 60 °C, 80 °C and 100 °C for 60 min, and to 121 °C for 15 min. The influence of pH on their activity was tested after adjustment of the pH of the CFS to values ranging from 2 to 10, using 1 M NaOH or 10 M phosphoric acid, and incubation for 1 h at 25 °C. Before testing the antimicrobial activity, the pH of each preparation was adjusted to pH 6.0–6.5. The effect of salt on bacteriocin activity was tested by the addition of 1%–10% NaCl to the CFS of the cultures and incubation at 7 °C, 30 °C and 37 °C for 2 h. Sterile MRS broths containing the same amounts of NaCl were used as negative controls. For all tests, the residual antimicrobial activity of the treated CFS was measured using the spot-on-the-lawn method and *L. monocytogenes* Scott A as indicator of activity, as described in 2.3.1.

2.4.2. Spectrum of activity

The CFS of the cultures of *Lb. curvatus* MBSa2 and MBSa3, prepared as described in 2.2, were tested for antimicrobial activity against the Gram-negative and Gram-positive bacteria listed in Table 1. The activity is presented as diameter of inhibition zone by the spot-on-the-lawn method, as described in 2.3.1.

2.4.3. Search for presence of bacteriocin genes

Lb. curvatus MBSa2 and MBSa3 were investigated for the presence of known bacteriocin genes using PCR and the primers listed in Table 2. Total DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and submitted to the amplification in a reaction mixture (20 μL) containing 25 ng/μL of extracted DNA, 1x PCR buffer (New England BioLabs), 100 mM $MgCl_2$ (Fermentas), 200 mM dNTPs (Fermentas), 0.025 U *Taq* polymerase (New England BioLabs) and 1 pM each primer. Amplification was achieved in 35 cycles using a DNA thermocycler MasterCycler® PCR (Eppendorf Scientific). PCR conditions are show in Table 2. PCR-amplified DNA fragments were separated by 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide (0.1 mg/mL) and visualized using the UVP BioImaging System (DIGIDOC-IT System). For each primer, the corresponding bands (Table 2) were purified with QIAquick® PCR Purification kit (Qiagen) 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).

2.5. Purification of expressed bacteriocins

Bacteriocins produced by *Lb. curvatus* MBSa2 and MBSa3 were purified as described by Batdorj et al. (2006), with some modifications. MRS broth was inoculated with a 1% (v/v) overnight culture of the bacteriocinogenic strain and incubated for 18 h at 25 °C, then the cells were removed by centrifugation at 6000 × *g* for 15 min at 4 °C (Centrifuge GR 2022, Jouan, France). The pH of the CFS was adjusted to 6.8 with 10 M NaOH (Euromedex, Souffelweyersheim, France) and loaded into an SP-Sepharose Fast Flow cation-exchange column (GE Healthcare, Amersham, Uppsala, Sweden) equilibrated with 20 mM phosphate (Sigma–Aldrich) buffer pH 6.8 (buffer A). The column was washed with buffer A and the absorbed substances were eluted with a linear gradient from 0 to 100% buffer B (20 mM sodium phosphate + 1 M NaCl [Euromedex] pH 6.8). The fractions were collected and tested for antimicrobial activity using the spot-on-the-lawn method and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a less pathogen, model strain for *L. monocytogenes* as sensitive microorganism (Todorov et al., 2010). Fractions presenting activity were pooled and submitted to Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) using Unicorn 3.21 software (Amersham Pharmacia Biotech). The pools were loaded on the preparative C_{18} column (Symmetry 300™ C_{18}, 5 μm, 4.6 × 50 mm, Waters, Hertfordshire, UK) equilibrated with solvent A (0.05% trifluoroacetic acid (TFA), 5% solvent B [80% acetonitrile, 20% H_2O, 0.03% TFA], 95% H_2O). Elution was performed with solvent B using a linear gradient from 0 to 100% in 25 min, at a flow rate of 5 mL/min. Peaks were detected by monitoring absorbance at 220 nm. Fractions were collected, dried under vacuum, dissolved in sterile ultra-pure water (Milli-Q, Millipore, Billerica, MA, USA) and tested for anti-*Listeria* activity. The protein concentration in this material, corresponding to purified bacteriocins, was measured in microtiter plates using Pierce® BCA protein assay kit (Thermo Fisher Scientific, Schwerte, Germany) with albumin (Sigma–Aldrich) as standard. Molecular mass measurement was performed on a quadrupole-time-of-flight hybrid mass spectrometer (Q-TOF Global, Waters, Manchester UK), equipped with an electrospray ionization (ESI) source and operated in the positive ion

Table 2
Targeted bacteriocin genes and primers used in the study.

Target bacteriocin	Primer[a]	Sequence (5′-3′)[b]	Size (bp)	Reference	Initial denaturation	Denaturation	Annealing	Elongation
Sakacin A	SakA-F	GAAWTRMMANCAATTAYMGGTGG	150	Dortu et al., 2008	95 °C, 15 min	95 °C, 30 s	55 °C, 1 min	72 °C, 1 min
	SakA-R	CAGCCGCTAATCATACCACC						
Sakacin T-α	SakT-α –F	TCGGTGGCTATACTGTCTAAACA	160	Macwana and Muriana, 2012	95 °C, 15 min	95 °C, 30 s	58 °C, 1 min	72 °C, 1 min
	SakT-α –R	TGTCCTAAAAATCCACCAATGC						
Sakacin T-β	SakT-β –F	AAGAAATGATAGAAATTTTTGGAGG	151	Macwana and Muriana, 2012	95 °C, 15 min	95 °C, 30 s	56 °C, 1 min	72 °C, 1 min
	SakT-β -R	TGTGAAATCCAATCTTGTCCTG						
Sakacin Q	SakQ-F	GAA RTW SYA NCA ATT ADN GGT GG	130	Dortu et al., 2008	95 °C, 15 min	95 °C, 30 s	53 °C, 1 min	72 °C, 1 min
	SakQ-R	TAC CAC CAG CAG CCA TTC CC						
Sakacin P	SakP-F	ATG GAA AAG TTT ATT GAA TTA	186	Reminger et al., 1996	94 °C, 3 min	94 °C, 30 s	40 °C, 1 min	72 °C, 1 min
	SakP-R	TTA T TT ATT CCA GCC AGC GTT						
Sakacin G	SakGA1-F	TTA GAA CTA CAC TGA TCG TG		Todorov et al., 2011	94 °C, 4 min	94 °C, 30 s	38 °C, 30 s	72 °C, 30 s
	SakGA1-R	TGG AAG AAT GAG TAC TTG TT						
Sakacin G	SakGA2-F	CGT TAC AAC AGA ACT TCA AG		Todorov et al., 2011	94 °C, 4 min	94 °C, 30 s	38 °C, 30 s	72 °C, 30 s
	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	94 °C, 3 min	94 °C, 30 s	40 °C, 1 min	72 °C, 1 min
	CurA-R	TTA CAT TCC AGC TAA ACC ACT						

[a] F- Forward; R- Reverse.
[b] Deoxyinosine; R, T/A; W, A/G; S, C/A; Y, A/C; D, C/T; N, A/C.

mode. Fractions collected from the HPLC were diluted in a mixture of water and acetonitrile (1/1, v/v) acidified with 0.1% formic acid and infused into the mass spectrometer at a continuous flow rate of 5 mL/min. Following parent mass determination, ions were fragmented in the collision cell of the mass spectrometer using an appropriate energy. The obtained MS/MS spectra were interpreted to reconstruct a sequence tag of the peptide. Results were searched against NCBI databank using the BLAST program.

2.6. Control of *L. monocytogenes* in salami by bacteriocin produced by *Lb. curvatus* MBSa2

2.6.1. Preparation of the bacteriocin for application in salami

The bacteriocinogenic strain *Lb. curvatus* MBSa2 was selected for the tests of control of *L. monocytogenes* in salami. The CFS were obtained by centrifugation (4000 × *g* for 15 min at 4 °C) of cultures obtained in MRS broth for 24 h at 30 °C, and then subjected to ammonium sulfate precipitation (80%) at 4 °C for 4 h, followed by centrifugation at 10,000 × *g* at 4 °C for 1 h. The pellets were re-suspended with 25 mM ammonium acetate buffer pH 6.5 (1/10 from original volume) and 5 ml loaded to SepPak C_{18} columns (Waters) for separtion of proteins, using 5 ml increasing concentrations of iso-propanol (20%, 40%, 60% and 80%) in 25 mM ammonium acetate buffer, pH 6.5. The collected fractions were tested for antimicrobial activity using the spot-on-the-lawn method and *L. ivanovii* subsp. *ivanovii* ATCC 19119 as sensitive microorganism. Fractions presenting activity were pooled, dehydrated under reduced pressure (Speed-Vac) and stored at −20 °C.

2.6.2. Determination of minimal inhibitory concentration (MIC)

The Minimal Inhibitory Concentration (MIC) of the semi-purified bacteriocin MBSa2 was determined by the micro dilution method described by Nielsen et al. (1990) using 96-well microplates containing 100 μL of BHI. A culture of *L. monocytogenes* Scott A (10^4–10^5 CFU/mL) was used as indicator of the antimicrobial activity.

2.6.3. Manufacture of salami and experimental contamination with *L. monocytogenes Scott A*

Salami (arrox. 250 g each) was manufactured in the pilot plant of a meat industry located in Sao Paulo, SP, Brazil following the routine procedure used in this industry. Salami batter was formulated with 10% ground bovine meat (3 mm disc), 75% ground pork shoulder (8 mm disc) and 15% chopped lard (cubes of approximately 125 mm^3). The batter was added of 1.3% NaCl, 1% of a pre formulated mixture of maltodextrin, sugar, garlic and onion powders, ground red and white pepper, sodium nitrate, sodium erythorbate, and garlic and nutmeg essential oils (Compact Salami 160, Kraki and Kienast Ltda, Brazil), and 0.02% Bactoferm™ T-SPX starter culture (*Pediococcus pentosaceus* and *Staphylococcus xylosus*, CHR Hansen, Denmark). The ingredients were mixed in a stainless steel meat homogenizer (CAF HG 120/114S, Brazil) for 3–5 min and the resulting batter was kept under refrigeration until used. The salami batter was divided into four parts: one was added of *L. monocytogenes* Scott A, the second was added of *L. monocytogenes* Scott A and the semi-purified bacteriocin MBSa2 at the concentration determined in the MIC test, the third was added of *L. monocytogenes* Scott A and sterile water (same volume as the semi-purified bacteriocin) and the forth was added of nothing (control). The level of contamination with *L. monocytogenes* was 10^4–10^5 CFU/g, The batters were transferred into caliber 60 collagen casings (Fibran S.A., Brazil), pre-hydrated in 15% saline solution for 30 min using a small-scale stainless steel filling machine (Filizola, Brazil). Prior each use, the cylinder and the piston of the filling machine were autoclaved at 121 °C for 15 min. The casings containing the batters (approximately 20 cm long) were transferred to maturation hambers (EL111, Electrolab, Brazil) where the temperature and relative humidity (RH) were controlled as follows: 4 days at 20 °C and 97% RH (fermentation step), 5 days at 18 °C and RH from 95% to 87% and then for 20 days at 15 °C and RH from 87% to 75% (maturation step). These experiments were performed in triplicates.

2.6.4. Counts of *L. monocytogenes Scott A* in the experimentally inoculated salami

Counts of *L. monocytogenes* were performed in the batters (time 0) and at 4, 10, 20 and 30 days of manufacture of salami. For the tests, 25 g of the product were removed and homogenized with 225 mL sterile 0.1% peptone water in a stomacher. The mixtures were submitted to decimal serial dilutions in sterile 0.1% peptone water and surface plated on Oxford agar (Difco) in duplicates. Plates were incubated at 37 °C for 24 h, when colonies were counted. Results were expressed in log CFU/g.

2.6.5. pH and water activity (a_w) measurements

The pH and the a_w of the samples were measured at times 0, 4, 10, 20 and 30 days of manufacture of salami, using a HI1090B6 pH electrode (Hannah Instruments, USA) and Novasina AWC500 (Novasina AG, Switzerland), respectively. Both measurements were made in duplicates.

2.6.6. Statistic analyses

Counts of *L. monocytogenes* were submitted to analysis of variance (ANOVA) and to Tukey's Test when applicable. The Statistica software version 7.0 was used in these tests and the adopted level of significance was 5% ($p < 0.05$).

3. Results and discussion

3.1. Isolation of the bacteriocinogenic strains and characterization of the produced bacteriocins

Several LAB strains isolated from the salami samples presented capability to produce inhibitory substances against the tested microorganisms. However, when submitted to the appropriate tests for bacteriocin production (Todorov et al., 2010), only two of them proved to be bacteriocinogenic, as demonstrated by the sensitivity to the proteolytic enzymes α-chymotrypsin, *Streptomyces griseus* protease type XIV, trypsin, pepsin and proteinase K. The 16S rRNA sequencing indicated that the two strains were *Lb. curvatus* (MBSa2 and MBSa3). The RAPD-PCR performed with primers OPL-01, OPL-02, OPL-04, OPL-14 and OPL-20 indicated that they were two distinct strains (data not shown).

Despite the common presence of LAB in meat and meat products there are not too many reports on strains with antimicrobial activity isolated from these products. Sudirman et al. (1993) isolated *Lactobacillus* spp. strains obtained from semi-dry sausages; Cintas et al. (1995) reported LAB isolates from Spanish dry-fermented sausages; Aymerich et al. (2000) failed in the isolation of LAB from fuet, chorizo and salchichon; Belgacem et al. (2008) reported on LAB isolated from gueddid, a Tunisian fermented meat; Vermeiren et al. (2004) obtained LAB from meat products; Todorov et al. (2013) reported on *Lb. sakei* isolated from Portuguese fermented meat products.

As shown in Fig. 1, production of bacteriocins MBSa2 and MBSa3 in MRS broth started in the early exponential growth phase (4 h of incubation). When the incubation was performed at 37 °C, after 12 h the amount of produced bacteriocins started to decrease, for both strains. The maximum production of bacteriocin MBSa2 (12,800 AU/mL) occurred at 8 h at 25 °C and 37 °C, and 6 h at 30 °C.

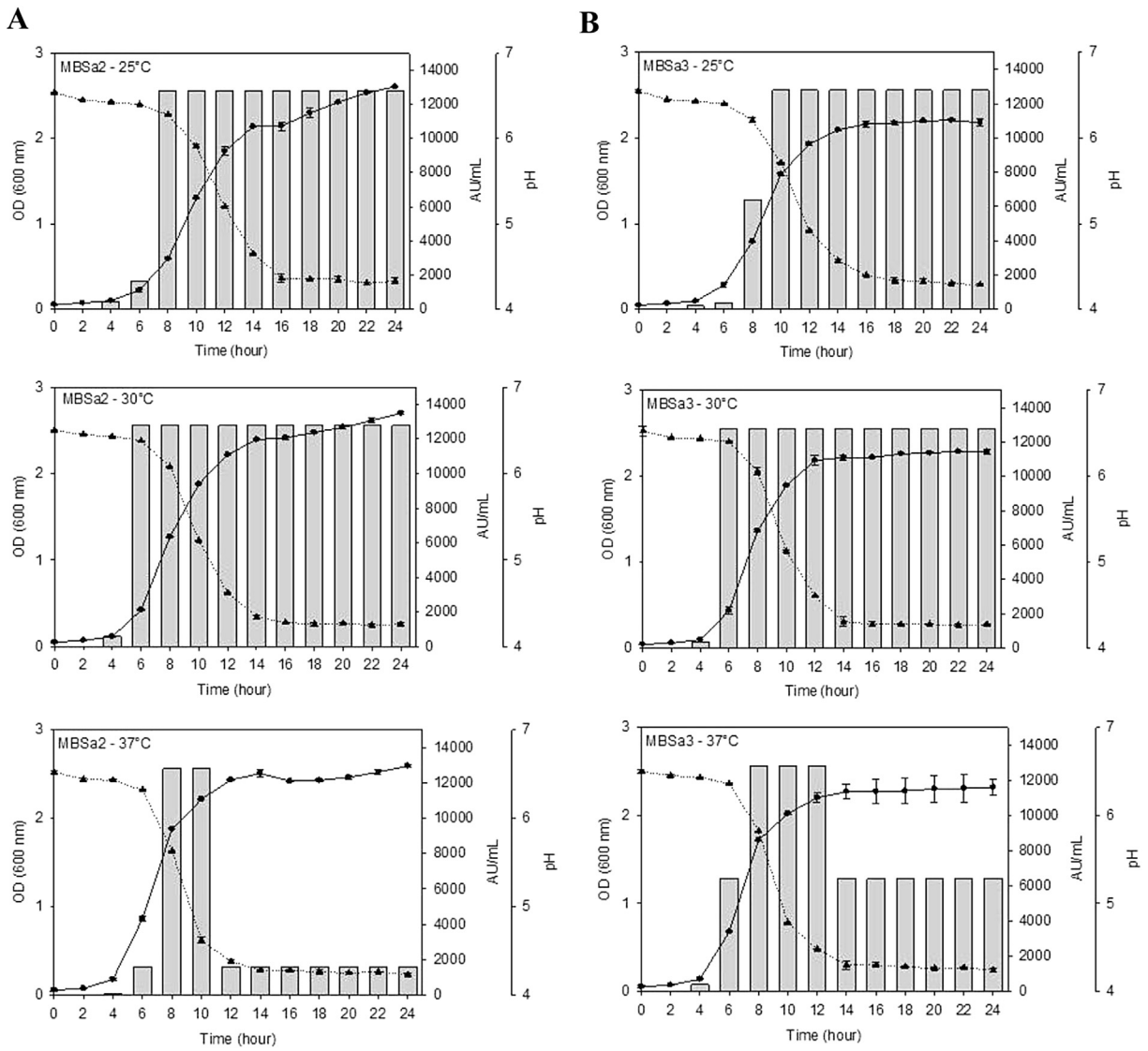

Fig. 1. Growth (-●-) and bacteriocin-production (bars) expressed in AU/mL by *Lactobacillus curvatus* MBSa2 (A) and *Lactobacillus curvatus* MBSa3 (B) in MRS broth at 25 °C, 30 °C and 37 °C. (-▲-) indicates the pH of the MRS broth. Experiments were performed in 3 independent occasions. Standard deviations (SD) were indicated.

Despite presenting a similar profile, the maximum production of bacteriocin MBSa2 at 25 °C occurred at 10 h. These features indicate primary metabolite kinetics, also observed for other bacteriocins, such as sakacin K produced by *Lb. sakei* CTC 494 (Leroy and De Vuyst, 1999), sakacin P produced by *Lb. sakei* CCUG 42687 (Moretro et al., 2000), curvacin A produced by *Lb. curvatus* LTH 1174 (Messens and de Vuyst, 2002), curvaticin L442 produced by *Lb. curvatus* L422 (Xiraphi et al., 2006) and the bacteriocin produced by *Lb. sakei* R1333 (Todorov et al., 2011).

The spectra of activity of bacteriocins MBSa2 and MBSa3 is summarized in Table 1. The bacteriocin MBSa2 inhibited 22 out of 23 *L. monocytogenes* strains, while the bacteriocin MBSa3 inhibited all 23 strains. The other Gram-positive bacteria were inhibited in a similar pattern and the tested Gram-negative bacteria were not inhibited. These results are similar to that reported for several other bacteriocins isolated from meat products (Sudirman et al., 1993; Vermeiren et al., 2004; Belgacem et al., 2008; Todorov et al., 2010, 2013).

When the concentration of NaCl in MRS broth was equal to or above 6%, a negative effect on the growth of both bacteriocinogenic strains was observed (Fig. 2). Such as results are not surprising, since is well known that species part of genera *Lactobacillus* are less tolerant to presence of NaCl compared to these from genera *Enterococcus*. However, at higher tested concentrations of NaCl both strains (MBSa2 and MBSa3) can survive and recover when effect of NaCl has been removed (data not shown). It should be noted that both strains grew and produced bacteriocins at 4–6% NaCl, which is an important technological feature as they can be applied in salted meat products, even if the production is lower than in the absence of salt. It is well known that lactobacilli do not grow well in presence of high levels of NaCl, but the growth capability is strain-dependent. Coppola et al. (1997) reported that all 183 strains of *Lactobacillus* spp. isolated from fermented sausage during maturation were able to grow in MRS broth containing 8% NaCl and most of them in 10% NaCl. Papamanoli et al. (2003) observed that among 49 strains of *Lb. sakei*, 24 strains of *Lb. curvatus* and 7 strains of *Lb. plantarum*, 24%, 17% and 100%, respectively, grew in the presence of 10% NaCl. Other studies have shown that salt may affect the activity of bacteriocins in different intensity. Garcia et al. (2004) observed that 2, 4 and 6% NaCl did not affect the activity of enterocin EJ97

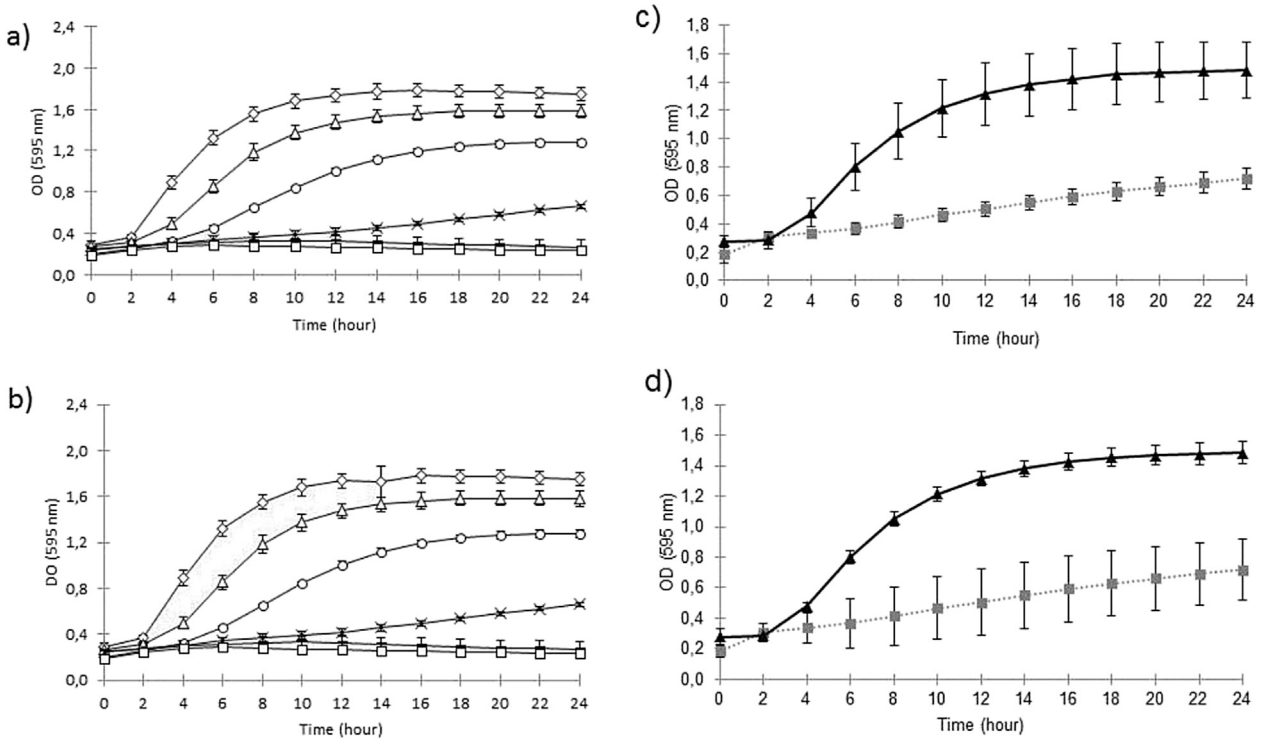

Fig. 2. Growth of *Lactobacillus curvatus* MBSa2 (a) and *Lactobacillus curvatus* MBSa3 (b) in MRS broth supplemented with 0% (◊), 2% (Δ), 4% (○), 6% (×), 8% (−) and 10% (□) NaCl, at 30 °C and growth of *Lactobacillus curvatus* MBSa2 (c) and *Lactobacillus curvatus* MBSa3 (d) in MRS broth at pH 4.0 (-■-) and pH 6.0 (-▲-), at 30 °C. Experiments were performed in 3 independent occasions. Standard deviations (SD) were indicated.

against *L. monocytogenes* CECT 4032 while Bouttefroy et al. (2000) reported that 1%–6% NaCl reduced the antimicrobial activity of curvacin 13.

As shown in Table 3, the activity of the two bacteriocins was the same, regardless the pH (2, 4, 6 or 8). However, the residual antimicrobial activity of bacteriocin produced by MBSa3 at pH 10 was reduced to 26%, while that of bacteriocin produced by MBSa2 remained unchanged. As for stability at acidic pH, detected for the bacteriocins MBSa2 and MBSa3, several studies have shown that most bacteriocins are stable over a wide pH range, such as pediocin L50 (Cintas et al., 1995), piscicocin CS526 (Yamazaki et al., 2005), acidocin D20079 (Deraz et al., 2005) and pediocin NV5 (Mandal et al., 2011). Lower pH stability was described for plantaricin LP31 (Müller et al., 2009). This tolerance to pH is a convenient characteristic of these strains because they may be used in acidic as well as non-acidic foods for biopreservation.

For both strains, a better growth was detected at pH 6.0 than at pH 4 (Fig. 2). Effect of pH on growth of LAB in general and of bacteriocinogenic strains in particular is another strain-dependent feature. Papamanoli et al. (2003) reported that none of the 49 *Lb. sakei* strains isolated from salami was able to grow in MRS at pH 4, but 10 out of 24 *Lb. curvatus* strains and all 7 *Lb. plantarum* strains grew well in these conditions.

Results shown in Table 3 indicate that the bacteriocins MBSa2 and MBSa3 were heat stable molecules, as both maintained the antimicrobial activity after autoclaving at 121 °C for 15 min. This property indicates that both of them can be used in foods that are submitted to heat treatment, without affecting their biopreservative characteristics. Usually, low molecular weight bacteriocins are heat-stable because they are small polypeptides. Same properties have been already described for sakacin M (Sobrino et al., 1992), pediocin L50 (Cintas et al., 1995), piscicocin CS526 (Yamazaki et al., 2005), acidocin D20079 (Deraz et al., 2005), plantaricin LP31 (Müller et al., 2009), sakacin P (de Carvalho et al., 2010) and pediocin NV 5 (Mandal et al., 2011).

The purification of bacteriocins MBSa2 and MBsa3 achieved by the three-step procedure (cation-exchange, followed by sequential hydrophobic-interaction and reversed-phase chromatography) resulted in two peaks (P1 and P2) in the final chromatogram of each bacteriocin (Table 4) with a purification yields of 20% and 10%, respectively. This three-step procedure resulted in successful purification of both bacteriocins MBSa2 and MBsa3. Other studies have used other purification methods with different degrees of success. The direct injection of bacterial culture supernatants on cation-exchange chromatography columns was used for purification of pediocin PA-1 (Uteng et al., 2002), divergicin M35 (Tahiri et al., 2004) and enterocin A5-11 Batdorj et al. (2006). Todorov et al. (2004) observed that purification with and without a previous precipitation with ammonium sulfate achieved similar results.

Mass spectrometry analysis performed on purified materials indicated that peak 1 (P1) contained two peptides with molecular masses of 4457.9 Da and 2228.16 Da and partial amino acid sequences AAANWATGGNAG and AGNSSNFLHKLQQLFT, respectively.

Table 3
Effect of temperature, pH and NaCl (%) on residual antimicrobial activity of bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3 in MRS broth.

Condition		Residual activity (%)	
		MBSa2	MBSa3
Temperature/time	4, 25, 30, 37, 45, 60, 80, 100° C/60 min	100	100
	121° C/15 min	100	100
pH	2, 4, 6, 8	100	100
	10	100	26
NaCl (%)	2, 4, 6, 8, 10	100	100

Table 4
Purification of bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

Purification stages		Volume (mL)	Activity (AU/mL)	Protein (mg/mL)	Specific activity (AU/mg protein)	Purification factor	Yield (%)
MBSa2							
Supernatant		200	800	3.10	257.65	1.00	100
Cation-exchange		700	200	2.46	81.20	0.31	87.5
Reversed phase		20	6400	2.54	2519.56	9.78	80
C_{18} RP-HPLC	P1	2	16,000	2.18	7353.19	28.54	20
	P2	2	8000	1.89	4242.23	16.46	10
MBSa3							
Supernatant		200	800	4.41	181.26	1.00	100
Cation-exchange		700	200	1.93	103.85	0.57	87.5
Reversed phase		20	6400	2.32	2753.78	15.19	80
C_{18} RP-HPLC	P1	2	16,000	2.14	7491.33	41.33	20
	P2	2	8000	1.88	4263.16	23.52	10

Database screening indicated that the first peptide is sakacin P and the second corresponds to a bacteriocin-type signal sequence domain protein found in *Lb. curvatus* CRL 705. The peak 2 (P2) contained one peptide of 4360.1 Da and partial amino acid sequence AVANLTTGGAGG, also present in sakacin X. These results suggest that both *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 produce two different bacteriocins.

When the DNA extracted from *Lactobacillus curvatus* MBSa2 and *L. curvatus* MBSa3 was tested for the bacteriocin genes listed in Table 2, only primers SakP-F/SakP-R that target sakacin P structural gene (*sak*A) resulted in positive amplicons. The sequencing of the amplified 186 bp fragment presented homology to sakacin P structural gene and was detected in both strains (Fig. 3).

The literature describes several LAB capable to produce two or more bacteriocins. *Carnobacterium piscicola* V1 produced piscicocin V1a with molecular mass 4416 Da and piscicocin V1b with molecular mass 4526 Da (Bhugaloo-Vial et al., 1996). *Leuconostoc mesenteroides* TA33a produces three bacteriocins: leucocin A-TA33a (3933 Da), leucocin B-TA33a (3466 Da) and leucocin C-TA33a (4598 Da) (Papathanasopoulos et al., 1997). *Lb. sakei* 5 produced sakacin 5T, 5X and 5P, and *L. mesenteroides* 6 produced leucocin 6A and leucocin 6C (Vaughan et al., 2001). *Enterococcus durans* A5-11 is a strain that produces two different bacteriocins with molecular mass 5206 Da (enterocin A5-11A) and 5218 Da (enterocin A5-11B) (Batdorj et al., 2006). *Lb. sakei* subsp. *sakei* 2a produces at least three compounds with antimicrobial activity: sakacin P (4.4 kDa), a ribosomal protein S21 (6.8 kDa) and a histone-like DNA-binding protein (9.5 kDa) produced as well by *Lb. sakei* subsp. *sakei* 23 K (de Carvalho et al., 2010). *Enterococcus faecium* L50 produces four enterocins: L50A, L50B, Q and P (Criado et al., 2006) and *E. faecium* NKR-5-3 also produces four enterocins: NKR-5-3A (5242.3 Da), NKR-5-3B (6316.4 Da), NKR-5-3C (4512.8 Da) and NKR-5-3D (2843.5 Da) (Ishibashi et al., 2012). This ability to produce multiple bacteriocins may be advantageous for a strain, enhancing its ability to compete with other bacteria in the same environment (Vaughan et al., 2001).

3.2. Control of *L. monocytogenes* by bacteriocins produced by *Lb. curvatus MBSa2* in salami

The MIC value of the semi-purified bacteriocin(s) produced by *Lb. curvatus* MBSa2 against *L. monocytogenes* was 200 AU/mL, which corresponded to the concentration used in the salami batter (200 AU/g) for evaluation of the capability to control the growth of this pathogen.

Measurements of pH and a_w (data not shown) and counts of *L. monocytogenes* in the batter and salami during the manufacturing process are presented in Fig. 4. The pH dropped from an average of 5.81 in the batter to 4.81 in the product at the 4th day of manufacturing (fermentation step), increasing again to 5.36 and 5.43 at the 20th and 30th day of manufacturing (maturation step). At the end of the fermentation period (4th day), the average pH in the four types of salami was similar, in the range 4.6–4.9, and the same occurred in the maturation step. The aw dropped gradually from 0.99 in the batter to 0.88 in the product at the 30th day of production.

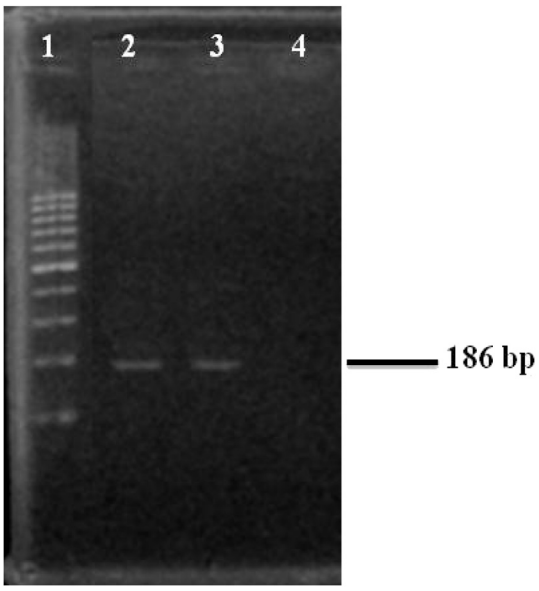

Fig. 3. DNA fragments obtained after PCR with genomic DNA from *Lactobacillus curvatus* MBSa2 and *Lactobacillus curvatus* MBSa3 using sakacin P specific primers (SakP-F/SakP-R). Lane 1, molecular weight marker (100 bp); lane 2, genomic DNA of *Lactobacillus curvatus* MBSa2; lane 3, genomic DNA of *Lactobacillus curvatus* MBSa3; lane 4, control, no DNA.

The semi-purified bacteriocin produced by *Lb. curvatus* MBSa2 caused a small reduction (0.5 log) in the counts of *L. monocytogenes* (Fig. 4) after its addition to the batter, but the counts remained the same up to the 4th day of fermentation ($p > 0.05$) and started to decrease afterward. The decrease was more evident in the samples

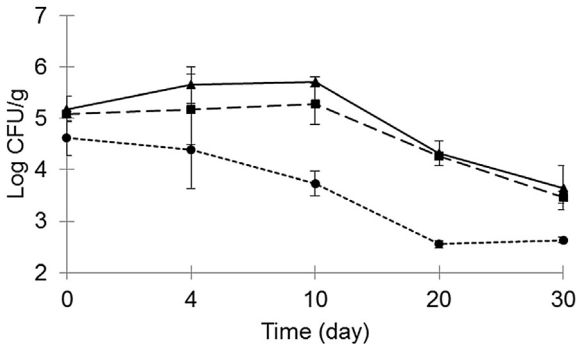

Fig. 4. Counts of *Listeria monocytogenes* Scott A in salami containing the semi-purified bacteriocin produced by *Lactobacillus curvatus* MBSa2 (-●-), in salami containing sterile water instead of the bacteriocin (-■-) and in salami containing only *Listeria monocytogenes* Scott A (-▲-). Counts were performed in the salami batter (time 0) and in the product up to the end of manufacturing (time 30 days). Counts of *Listeria monocytogenes* Scott A was performed from each experimental salami (3 for each experimental conditions) in at least 2 repetitions and were submitted to analysis of variance (ANOVA) and to Tukey's Test when applicable. The Statistica software version 7.0 was used in these tests and the adopted level of significance was 5% ($p < 0.05$).

containing the bacteriocin. On the 10th day the counts of *L. monocytogenes* were almost 2 log lower than in samples without added bacteriocin. At the end of the maturation step (30th day) the detected difference in the CFU/g counts was 1.77 log. Future experiments of combined application of semi-purified bacteriocin/s and/or bacteriocinogenic producer strain/s and antimicrobial plant extracts need to be explored in order to obtain better inhibition of *L. monocytogenes* in order to face "no tolerance" rules for this food-borne pathogen.

The manufacturing process of Italian type salamis, such as the one used in this study, is expected to reduce the counts of pathogens present in these products. However, the reduction may be insufficient for effective control of pathogens frequent in such products, which may cause disease, like *L. monocytogenes*. Nightingale et al. (2006) have shown that counts of *Salmonella* spp. in experimentally contaminated Italian-style salami batter dropped from 7.4 log CFU/g to 4.5 log CFU/g when the moisture/protein ratio in the product was 1.4/1. However, *L. monocytogenes* populations in these products reduced less than 1 log CFU/g, indicating that additional measures are necessary to achieve the expected 5 log reduction determined by the Unites States regulatory agencies. In Brazil, *L. monocytogenes* is a frequent contaminant detected in salami (Sakate et al., 2003; Petruzzelli et al., 2010; Di Pinto et al., 2010; Okada et al., 2012), so the application of bacteriocins produced by LAB can be a technological alternative to be considered to improve of the sanitary status of these products.

A number of studies have tested the effect of adding purified or semi-purified bacteriocins to foods for the control of pathogenic bacteria, with controversial results. The application of enterocin CCM 4231 (12,800 AU/g) in dry fermented Hornad salami reduced the counts of *L. monocytogenes* immediately after addition of the bacteriocin and maintained these counts until the end of trial period when compared with control samples (Lauková and Czikková, 1999). The effect of pediocin AcH produced by *Lb. plantarum* WHE 92 applied to sliced cooked sausage was inefficient to kill all *L. monocytogenes* (Mattila et al., 2003). The inhibitory effect of nisin towards *L. monocytogenes* in experimentally contaminated Turkish fermented sausages (suçuk) depended on the concentrations of the bacteriocin (Hampikyan and Ugur, 2007). The enterocin AS-48 (148 AU/g) caused a drastic decrease in *L. monocytogenes* population (5.5 log CFU/g) in fuet (a low acid fermented sausage) during its maturation (Ananou et al., 2010). The inhibitory effects of pediocin PA-1 (5000 AU/mL) produced by *P. acidilactici* MCH14 was studied in frankfurters, decreasing by 2 and 0.6 log cycles of the counts of *L. monocytogenes* after storage at 4 °C for 60 days and at 15 °C for 30 days, respectively, when compared to the control (Nieto-Lozano et al., 2010).

In conclusion, *Lb. curvatus* MBSa2 and MBSa3 isolated from Italian type salami samples produce two bacteriocins (sakacin P and sakacin X) with high stability (heat, pH and NaCl) and remarkable activities against *L. monocytogenes*. The semi-purified bacteriocins extracted from cultures of *Lb. curvatus* MBSa2 and applied to the batter for salami production caused about 2 log CFU/g count reduction in the final product when compared to salami without addition of bacteriocins, suggesting that application of these bacteriocins can be an additional measure to improving the safety of these ready-to-eat products with regards to *L. monocytogenes*.

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