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Isolation and characterization of a nisin-like bacteriocin produced by a *Lactococcus lactis* strain isolated from charqui, a Brazilian fermented, salted and dried meat product

V. Biscola [a,*], S.D. Todorov [a], V.S.C. Capuano [a], H. Abriouel [b], A. Gálvez [b], B.D.G.M. Franco [a]

[a] Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, 580, Professor Lineu Prestes, 13 B, Sao Paulo, SP, 05508-000, Brazil
[b] Department of Health Sciences, Faculty of Experimental Sciences, University of Jaén, Campus las Lagunillas, s/n, 23071 Jaén, Spain

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ABSTRACT

A *Lactococcus lactis* subsp. *lactis* strain (*L. lactis* 69) capable to produce a heat-stable bacteriocin was isolated from charqui, a Brazilian fermented, salted and sun-dried meat product. The bacteriocin inhibited, *in vitro*, *Listeria monocytogenes*, *Staphylococcus aureus*, several lactic acid bacteria isolated from foods and spoilage halotolerant bacteria isolated from charqui. The activity of the bacteriocin was not affected by pH (2.0–10.0), heating (100 °C), and chemical agents (1% w/v). Treatment of growing cells of *L. monocytogenes* ScottA with the cell-free supernatant of *L. lactis* 69 resulted in complete cell inactivation. *L. lactis* 69 harbored the gene for the production of a nisin-like bacteriocin, and the amino acid sequence of the active peptide was identical to sequences previously described for nisin Z. However, differences were observed regarding the leader peptide. Besides, the isolate was able to survive and produce bacteriocins in culture medium with NaCl content up to 20%, evidencing a potential application as an additional hurdle in the preservation of charqui.

1. Introduction

Charqui is a traditional fermented, salted and sun-dried meat product largely consumed in Brazil. Its preservation is based on the application of the hurdle technology, where salt, dehydration and fermentation are hurdles sequentially applied to prevent the growth of undesired microorganisms. The manufacture of charqui is an artisanal process. Large meat pieces are injected with a marinating solution, salted with coarse salt, piled up for juices draining, and then dried on racks exposed to the sun. The piles are inverted every 24 h, and the whole process takes several days. The Aw of the final product is around 0.7–0.8. During piling, a fermentation step, carried out by lactic acid bacteria (LAB) naturally present in the raw meat, is responsible for the unique flavor characteristics of charqui (Youssef, Garcia, Yamashita, & Shimokomaki, 2007).

The artisanal manufacture process hinders the standardization of the product. Another important issue concerning charqui quality is the growth of halophilic and halotolerant spoilage bacteria. Halophilic bacteria such as *Halobacterium cutirubrum* may cause the red spoilage "*vermelhão*", when the product becomes slimy and presents red spots and off-odors (Pinto, Ponsano, Franco, & Shimokomaki, 2002; Shimokomaki et al., 1998).

One technological option for the control of the charqui fermentation process and for the prevention of growth of halophilic and halotolerant spoilage bacteria in the product is the application of LAB capable to produce antimicrobial compounds, especially bacteriocins. The interest in the application of bacteriocinogenic LAB and/or their bacteriocins in food preservation has increased in the last years and several bacteriocins have been described, however, only nisin and pediocin are commercially available and permitted for applications in foods, such as cheeses and meat products (Castellano, Belfiore, Fadda, & Vignolo, 2008; Fadda, López, & Vignolo, 2010; Gálvez, López, Abriouel, Valdivia, & Ben Omar, 2008).

It is well known that the success of food preservation by the use of bacteriocin-producing LAB is dependent on the ability of the strain to grow and produce the antimicrobial compound in the food matrix (Cotter, Hill, & Ross, 2005; Gálvez et al., 2008; Hartmann, Wilke, & Erdmann, 2011). Sometimes the *in vitro* activity of bacteriocins produced by LAB is not confirmed *in situ*, thus the effectiveness of the application of a bacteriocinogenic strain or its bacteriocin in a food system for the control of a target bacteria requires careful testing and confirmation (Ammor & Mayo, 2007; Gálvez et al., 2007; Gálvez et al., 2008; Hartmann et al., 2011; Urso, Rantsiou, Cantoni, Comi, & Cocolin, 2006).

As LAB are normally found in raw and fermented meat products, they are well adapted to these environments, increasing the chances of success of application of bacteriocinogenic strains isolated from these products for the control of spoilage microorganisms in similar

* Corresponding author. Tel.: +55 11 3091 2191; fax: +55 11 3815 4410.
E-mail address: vbiscola@usp.br (V. Biscola).

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products (Ammor & Mayo, 2007; Deegan, Cotter, Hill, & Ross, 2006; Fadda et al., 2010; Signorini, Ponce-Alquicira, & Guerrero-Legarreta, 2006).

The aim of this study was to isolate and characterize bacteriocin-producing LAB from charqui, testing the activity against different bacteria isolated from the same product, foreseeing a potential technological application for the improvement of the quality of this peculiar meat product.

2. Material and methods

2.1. Isolation and identification of halophilic and halotolerant bacteria from charqui

Charqui samples presenting red spoilage (25 g) were homogenized with 225 mL of peptone water (0.1% w/v), submitted to serial ten-fold dilutions in peptone water (0.1% w/v), and spread on the surface of plates containing Tryptone Soya Agar (TSA) (Oxoid, Basingstoke, UK) supplemented with 3% (w/v) of NaCl (Synth, Diadema, Brazil), for the isolation of medium halotolerant bacteria or 10% (w/v) of NaCl (for the isolation of highly halotolerant bacteria). After aerobic incubation at 37 °C for 72 h, the colonies were randomly selected, transferred to Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) and streaked on TSA to obtain pure cultures. The cultures were stored at −80 °C in the presence of 20% w/v glycerol as crioprotector.

For identification of halophilic and halotolerant bacteria, total DNA of the strains was extracted using the commercial Kit Ilustra bacteria genomicPrep Mini Spin (GE Healthcare, UK), according to the manufacturer's recommendations. The DNA concentration was determined by NanoDrop 2000 (Thermo Scientific, USA) and the total DNA was used as template for amplification of the almost complete 16S rDNA gene by PCR, according to Cibik, Lepage, and Tailliez (2000). The reaction mixture (100 μL) contained 100 ng of template DNA, 1.5 mM of $MgCl_2$, 10 μL of 1× *Taq* polymerase buffer, 2.5 U of *Taq* DNA polymerase, 200 μM of deoxynucleoside triphosphates and 50 pM of each primer described in Table 1. The amplification program comprised a first denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1.5 min (extension), and a final extension step at 72 °C for 5 min. The amplified products were separated by electrophoresis on 1.0% agarose gels in 1× TBE (Tris-borate EDTA, pH 8.0) buffer (Sigma, New York, USA). Gels were stained in TBE buffer containing 0.5 μg/mL ethidium bromide (Sigma, New York, USA).

The amplification products were purified using a Quantum Prep PCR Kleen Spin column (BioRad, Madrid, Spain), and then sequenced using primers Sp3, Sp4 and Sp5 (Table 1), according to Weisburg, Barns, Pelletier, and Lane (1991), in a CEQ2000 XL DNA Analysis System (Beckman Coulter, CA, USA). The obtained sequences were compared with those available in GenBank database, using the Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

2.2. Isolation of LAB with antimicrobial activity from charqui

Potential bacteriocinogenic LAB were isolated from charqui using the triple-agar layer method (Todorov & Dicks, 2004). Briefly, 25 g of non-spoiled charqui, donated by a Brazilian meat industry, was homogenized with 225 mL peptone water (0.1% w/v) in a stomacher (Seward Medical, Worthing, UK). Homogenates were submitted to serial ten-fold dilutions in peptone water and surface-plated on *de Man Rogosa and Sharpe* (MRS) agar (Oxoid, Basingstoke, UK). The plates were overlaid with a thin layer of 1.5% (w/v) bacteriological agar (Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h. Plates containing less than 50 colonies were overlaid with another layer of semi-solid MRS agar (0.75% w/v agar) containing *Lactobacillus sakei* ATCC 15521 (10^6 CFU/mL). The plates were incubated for additional 24 h at 30 °C and colonies presenting inhibitory zones were transferred to MRS broth and streaked on MRS agar for obtention of pure cultures for confirmation of the antimicrobial activity.

Table 1
Primers used in this study.

Target	PCR Primers (5′–3′)	Reference
16S–23S rDNA	F: AGAGTTTGATCMTGGCTC R: CNCGTCCTTCATCGCCT	Brosius, Palmer, Kennedy, and Noller (1978); Brosius, Dull, and Noller (1980)
16S sequencing	SP3: TACGCATTTCACCKCTACA SP4: CTCGTTGCGGGACTTAAC SP5: GNTACCTTGTTACGACTT	Ben Omar et al. (2006)
Lactococcus lactis	F: CGTTATGGATTTGATGGATAT AAAGC R: ACTCTTCTTAAGAAC AAGTTTAACAGC	Petrov et al. (2008)
Nisin	nisA F: GGATAGTATCCATGTCTG nisA R: CAATGATTTCGTTCGAAG	Li and O'Sullivan (2002)
Lacticin 3147	Lac3147 F: GTCTTTGTGTTGTTTGGAGATG Lac3147 R: CAACTCCCGAAATAAATCATCG	Alegria et al. (2010)
Lacticin 481	Lact481 F: CCAATGTCATTGCATCTGCAC Lact481 R: GTCCTTATGTTGCTATTCATC	Alegria et al. (2010)
Lactococcin A	LactABM F: GAAGAGGCAATCAGTAGAG LactA R: GTGTTCTATTTATAGCTAATG	Alegria et al. (2010)
Lactococcin B	LactABM F: GAAGAGGCAATCAGTAGAG LactB R: CCAGGATTTTCTTTGATTTACTTC	Alegria et al. (2010)
Lactococcin M	LactABM F: GAAGAGGCAATCAGTAGAG LactM R: GTGTATGGTCTAGC ATAAG	Alegria et al. (2010)
Lactococcin G	LactGQ F: GAAAGAATTATCAGAAAAAG LactGQ R: CCACTTATCTTTATTTCCCTCT	Alegria et al. (2010)
Lactococcin Q	LactGQ F: GAAAGAATTATCAGAAAAAG LactGQ R: CCACTTATCTTTATTTCCCTCT	Alegria et al. (2010)
Lactococcin 972	Lcn972 F: TTGTAGCTCCTGCAGAAGGAACATGG Lcn972 R: GCCTTAGCTTTGAATTCTTACCAAAAG	Alegria et al. (2010)

2.3. Confirmation of the antimicrobial activity of LAB isolates

The antimicrobial activity of LAB isolates was confirmed by the spot-on-the-lawn test (Lewus & Montville, 1991), using the cell-free supernatant (CFS). LAB isolates were grown in MRS broth at 30 °C for 24 h, the cells were harvested by centrifugation (8000×*g*, 4 °C, 10 min) and the pH of the CFS was adjusted to 6.0 with 1 M NaOH (Synth, Diadema, Brazil) and heat treated for 10 min at 80 °C to destroy extracellular proteases and H_2O_2. For the spot-on-the-lawn test, 10 μL of CFS was spotted on the surface of plates containing MRS agar, previously inoculated with 10^6 CFU/mL of the indicator strain *L. sakei* ATCC 15521. Plates were incubated at 30 °C for 24 h and the presence of an inhibition zone of at least 2 mm around the spotted CFS indicated a positive result. The bacteriocin activity was quantified by the critical dilution method, according to Mayr-Harting, Hedges, and Berkeley (1972) and expressed as arbitrary units (AU) per mL, with 1 AU defined as the highest dilution showing a clear zone of growth inhibition.

2.4. Sensitivity of the inhibitory substance to proteolytic enzymes

The effect of proteolytic enzymes on the antimicrobial activity of the CFS, prepared as described above in Section 2.3, was tested according to Van Reenen, Dicks, and Chikindas (1998). One milliliter of the CFS was mixed with pepsin (1 mg/mL) or protease (1 mg/mL) and incubated at 37 °C for 30 min. The enzymes were purchased from Sigma, New York, USA. The enzymatic reactions were stopped by heating at 100 °C for 3 min and the antimicrobial activity was assessed by the spot-on-the-lawn test.

2.5. Effect of pH, temperature and chemical agents on the antimicrobial activity

The effect of pH on the antimicrobial activity of the CFS was tested adjusting the pH of aliquots (5 mL) of the CFS from 2.0 up to 12.0 (with increments of one pH unit) with 1 M NaOH or 1 M HCl (Synth, Diadema, Brazil). After incubation at 25 °C for 30 min, the pH was readjusted to 7.0. The effect of temperature on the antimicrobial activity of the CFS was tested heating aliquots of 1 mL of CFS at 60 °C, 80 °C and 100 °C for 30 min, 60 min and 120 min. The effect of sodium dodecyl sulfate (SDS) (BioAgency, Sao Paulo, Brazil), ethylenediamine tetraacetic acid (EDTA) (Invitrogen, Carlsbad, USA), tween 80 (Synth, Diadema, Brazil) and urea (Synth, Diadema, Brazil) on the antimicrobial activity was tested by adding 1% (w/v) of these substances to the CFS. For all tests the antimicrobial activity was determined by the spot-on-the-lawn test, using *L. sakei* ATCC 15521 as indicator microorganism. In all experiments, the bacteriocin-producing strain *L. sakei* subsp. *sakei* 2a was used as positive control (De Martinis & Franco, 1998).

2.6. Spectrum of antibacterial activity

Two isolates (69 and 94) that produced antimicrobial substances sensitive to the proteolytic enzymes and not affected by pH, heat and chemical agents, were considered bacteriocin producers and thus tested for antimicrobial activity against the target strains listed in Table 2. In addition to the halophilic and halotolerant bacteria isolated from charqui, these strains included reference strains (ATCC and others) and food isolates belonging to the culture collection at Food Microbiology Laboratory of University of Sao Paulo, Sao Paulo, Brazil. The activity was evaluated using the spot-on-the-lawn test. A target microorganism was considered sensitive to the bacteriocin producer strain when the diameter of the growth inhibition zone was at least 5 mm. The growth conditions of each tested microorganism are shown in Table 2.

2.7. Mode of action

For the determination of the mode of action of the antimicrobial compounds, 20 mL of filter sterilized (0.22 μm, Millipore, USA) CFS was added to a culture (100 mL) of *L. monocytogenes* ScottA in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) in early (3 h) exponential phase and the optical density (600 nm) and number of viable cells were measured every hour up to 10 h (Todorov & Dicks, 2005). The numbers of CFU/mL of *L. monocytogenes* ScottA in the mixture were determined by plating on TSA supplemented with 0.6% (w/v) yeast extract (Oxoid, Basingstoke, UK).

The adsorption of the bacteriocin produced by isolate 69 to *L. monocytogenes* ScottA, *L. monocytogenes* 620 4b (University of Sao Paulo culture collection), *L. monocytogenes* 637 1/2c (University of Sao Paulo culture collection), *L. sakei* ATCC 15521 and *Enterococcus faecium* ATCC 19443 was measured according to Todorov (2008). The target microorganisms were grown overnight in BHI broth at 37 °C and then centrifuged (8000 ×*g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended in the same buffer to OD at 600 nm equal to 1.0. The pH was adjusted to 6.5 with sterile 0.1 M NaOH. One milliliter of each cell suspension was mixed with 1 mL of CFS and incubated at 37 °C for 1 h. After removal of cells (8000 ×*g*, 15 min, 25 °C), the activity of unbound bacteriocin in the supernatant was determined by the critical dilution method. The adsorbed bacteriocins were determined as:

$$\%\ \text{adsorption} = \left(100 - \frac{AU/mL_1}{AU/mL_0}\right) \times 100$$

AU/mL_0 and AU/mL_1 refer to the bacteriocin activity before and after treatment, respectively.

In addition, the influence of temperature (4 °C, 25 °C, 30 °C and 37 °C), pH (4.0, 6.0, 8.0 and 10.0) and presence of 1% (w/v) of NaCl, tween 20, tween 80, glycerol and SDS on the adsorption of the bacteriocin produced by isolate 69 to *L. monocytogenes* ScottA was determined according to Todorov (2008).

2.8. Growth and survival in media containing different concentrations of NaCl

To investigate the capability of isolate 69 to grow in the presence of NaCl, the microorganism was inoculated (10^5 CFU/mL) in 100 mL of MRS broth containing increasing concentrations of NaCl (0%, 3%, 5%, 10%, 15% and 20% w/v) and incubated at 30 °C up to 72 h. The growth in MRS broth containing 0%, 3% and 5% NaCl was monitored by enumeration of viable cells performed every 2 h up to 12 h, and

Table 2
Spectrum of activity of isolates 69 and 94, obtained from charqui.

Test microorganism	Origin	Growth médium	Incubation time/ temperature	Activity[a]	
				Isolate 69	Isolate 94
B. cereus	ATCC 11778	BHI	37 °C/48 h	0/1	0/1
S. aureus (3 strains)	ATCC 29213/ 25923/ 6538 (29213/ 259236538 nofaltaono(29213/ 25923/6538)	BHI	37 °C/48 h	1/3	0/3
L. monocytogenes (18 strains)	Poultry meat	TSAYE	37 °C/48 h	18/18	1/18
L. monocytogenes	ATCC 7644	TSAYE	37 °C/48 h	1/1	0/1
L. monocytogenes	ScottA	TSAYE	37 °C/48 h	1/1	0/1
E. aerogenes	ATCC 13048	BHI	37 °C/48 h	0/1	0/1
L. sakei subsp. *sakei* 2a	Meat product	MRS	30 °C/48 h	1/1	0/1
L. acidophilus (3 strains)	Rhodia LA5/ LAC4/LA14	MRS	30 °C/48 h	0/3	0/3
L. paracasei	Rhodia LBC82	MRS	30 °C/48 h	0/1	0/1
L. cremoris	CH R704	MRS	30 °C/48 h	1/1	0/1
E. hirae	Goat milk	BHI	37 °C/48 h	0/1	0/1
L. bulgaricus (5strains)	Goat milk	MRS	30 °C/48 h	4/5	0/5
L. lactis (6 strains)	Goat milk	MRS	30 °C/48 h	3/6	4/6
E. faecium (2 strains)	Goat milk	BHI	37 °C/48 h	2/2	1/2
Isolate 69	Charqui	MRS	30 °C/48 h	0/1	0/1
Isolate 94	Charqui	MRS	30 °C/48 h	1/1	0/1
S. xylosus (5 strains)	Charqui	TSA 3% NaCl	37 °C/72 h	5/5	5/5
S. pasteuri/ *S. warneri* (3 strains)	Charqui	TSA 3% NaCl	37 °C/72 h	2/3	2/3
Staphylococcus sp.	Charqui	TSA 3% NaCl	37 °C/72 h	1/1	1/1
S. xylosus (4 strains)	Charqui	TSA 10% NaCl	37 °C/72 h	2/4	0/4
Staphylococcus sp. (4 strains)	Charqui	TSA 10%	37 °C/72 h	1/4	0/4
S. saprophyticus	Charqui	TSA 10% NaCl	37 °C/72 h	1/1	0/1

[a] Number of tested strains sensitive to the bacteriocin/total number of tested strains.

then every 12 h up 72 h. In MRS broth containing 10%, 15% and 20% NaCl, the growth was monitored at 24 h, 30 h, 36 h, 48 h, 60 h and 72 h of incubation. MRS agar was used for the determination of number of CFU/mL in each culture.

2.9. Bacteriocin production in media containing 20% NaCl

The capability of isolate 69 to produce bacteriocin in media containing high concentration of NaCl was tested by cultivating it in MRS broth containing 20% (w/v) NaCl (pH 6.0). After 24 h, 48 h and 72 h, the antimicrobial activity of CFS was tested against *L. sakei* ATCC 15521, using the critical dilution method and the results were compared to those obtained in MRS without NaCl.

2.10. Identification of isolate 69

The identification of isolate 69 was based on 16S rDNA sequencing followed by sub-species specific PCR. The DNA extraction was performed as described by Abriouel et al. (2006), using a combination of lysozyme, mutanolysin, proneinase K, isopropanol and phenol/chloroform, all from Sigma, Madrid, Spain. The 16S rDNA amplification was carried as described above. The sub-species-specific PCR was carried out according to Petrov, Urshev, and Petrova (2008), in a 50 μL (final volume) reaction mixture composed by 50 ng of DNA template, 5 μL of 1 × *Taq* polymerase buffer, 2.5 U of *Taq* DNA polymerase, 200 μM of deoxynucleoside triphosphates and 50 pM of each primer (Table 1). The amplification program was composed by a first denaturation step at 95 °C for 2 min, followed by 40 cycles of: 95 °C for 30 s (denaturation), 45 °C for 30 s (annealing) and 72 °C for 30 s (extension) with a final extension step at 72 °C for 7 min.

2.11. Screening for genes encoding known bacteriocins produced by L. lactis

The screening for genes encoding known bacteriocins previously described for *L. lactis* was done using PCR of the total genomic DNA of isolate 69, identified as *L. lactis* subsp. *lactis*. Screening for nisin gene (*nisA*) was performed as described by Li and O'Sullivan (2002) and screening for genes encoding lacticin 3147, lacticin 481, lactococcin A, lactococcin B, lactococcin M, lactococcin G, lactococcin Q and lactococcin 972 was carried out according to Alegria, Delgado, Roces, López, and Mayo (2010). The primers used in the tests are described in Table 1. The generated PCR products were purified in a Quantum Prep PCR Kleen Spin column and sequenced using a CEQ 2000 XL DNA Analysis System with the specific primers and then compared with the sequences for known bacteriocins produced by *L. lactis* strains, using the *Lipman–Pearson Protein Alignment* program.

3. Results and discussion

Among the halotolerant bacteria successfully isolated from the charqui samples, nine were medium halotolerant, *i.e.* were isolated using TSA supplemented with 3% of NaCl, and nine were highly halotolerant, *i.e.* were isolated using TSA supplemented with 10% of NaCl. The amplification of the 16S rDNA indicated that five medium halotolerant isolates were *Staphylococcus xylosus*, three were *Staphylococcus pasteuri*/*Staphylococcus warneri* and one could not be identified at species level. Among highly tolerant isolates, four were *S. xylosus*, one was *Staphylococcus saprophyticus* and four could not be identified at species level. This prevalence of staphylococci among halotolerant bacteria in fermented meat products has been described in several other studies (Cagno et al., 2008; Chevallier et al., 2006). A number of studies have reported that *S. xylosus* and *S. saprophyticus* are the main Gram-positive, coagulase-negative cocci species found in fermented meat products (Cocolin, Manzano, Cantoni, & Comi, 2001; Fontana, Cocconcelli, & Vignolo, 2005; Lu et al., 2010; Samelis, Metaxopoulos, Vlassi, & Pappa, 1998; Tu, Wu, Lock, & Chen, 2010; Van Reenen et al., 1998), and are responsible for the sensorial characteristics like color, texture and flavor (Aymerich, Martín, Garriga, & Hugas, 2003; Casquete et al., 2011; Comi et al., 2005; Fontán, Lorenzo, Prada, Franco, & Carballo, 2007; Pinto et al., 2002; Rantsiou et al., 2005; Shimokomaki et al., 1998; Talon, Leroy, & Lebert, 2007).

Using the triple-layer agar technique, one hundred isolates of LAB obtained from charqui presented activity against *L. sakei* ATCC 15521. The antilisterial activity may have been caused by many known antimicrobial compounds produced by LAB (Castellano et al., 2008), but results of the spot-on-the-lawn test indicated that five isolates were potential bacteriocin producers. The effect of enzymes, pH and chemical agents on the antimicrobial activity of the CFS of these five LAB isolates is shown in Table 3. The antimicrobial activity of two isolates (69 and 94) was sensitive to proteolytic enzymes, revealing the proteinaceous nature of the inhibitory substances, supporting the hypothesis of bacteriocin production. The antibacterial activity of CFS of isolates 69 and 94 was not affected by pH and most of the tested chemical agents.

Regarding the effect of temperature on the antimicrobial activity, the antimicrobial substances produced by isolates 69 and 94 were heat-stable (Table 4). The bacteriocin produced by isolate 94 was not affected by heating at 80 °C up to 120 min, while that produced by isolate 69 was resistant to heating at 100 °C up to 120 min. Similar resistance was previously reported for other bacteriocins produced by *Lactococcus* spp. such as for the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* MA23 (Akkoç, Ghamat, & Akçelik, 2011), stable at pH values ranging from 2.0 to 7.0 and heating up to 100 °C for 15 min. The Lactococcin BZ, produced by *L. lactis* subsp. *lactis* BZ isolated from boza, maintained its activity after treatment at 90 °C up to 30 min, pH 2.0–7.0 and in the presence of SDS, urea, tween 80, and triton X-100 (Sahingil, Isleroglu, Yildirim, Alçelik, & Yildrim, 2011). The bacteriocins produced by *Lactobacillus pentosus* SM7, isolated from "lukanka", a dry Bulgarian sausage, maintained the activity after treatment at 80 °C up to 10 min (Stovanovski et al., 2009). Bacteriocin J46, produced by *L. lactis* subsp. *cremoris*, was stable in the presence of several chemical agents, various temperatures and pH levels (Hout, Maghrous, & Barena-Gonzales, 1996). Treatment of bozacin B14, produced by *L. lactis* subsp. *lactis* B14, with SDS did not result in any activity loss, however, heating at 90 °C for 10 min caused the complete inactivation of the bacteriocin (Ivanova, Kabadjova, Pantev, Danova, & Dousset, 2000). Moreover, lactocin NK24, produced by *L. lactis* NK24, lost 87.5% of its activity after 30 min at 100 °C and was completely inactivated after 15 min at 121 °C (Lee & Paik, 2001). In the case of lactocin MMFII, produced by *L. lactis* MMFII, only 8.3% activity was recorded after 30 min at 110 °C and 25% after 30 min at 80 °C and 90 °C (Ferchichi, Frere, Mabrouk, & Manai, 2001). Nisin, produced by *L. lactis* subsp. *lactis* WNC20, was inactivated after 15 min at 121 °C when incubated at pH 7.0, but not when incubated at pH 3.0 (Noonpakdee, Santivarangkna, Jumriangrit, Sonomoto, & Panyim, 2003).

Isolate 69 presented broader spectrum of antimicrobial activity than isolate 94 (Table 2). Isolate 69 was able inhibit, *in vitro*, all tested *L. monocytogenes* strains, one out of three *Staphylococcus aureus*

Table 3
Antimicrobial activity of the CFS* of the five lactic acid bacteria isolates after treatment with proteolytic enzymes, pH and chemical agents.

Isolates	Enzymes		pH					Chemical agents			
	Pepsin	Protease	2	4	8	10	12	Urea	SDS	EDTA	Tween 80
10	+	+	−	−	−	−	−	−	−	−	−
69	−	−	+	+	+	+	−	+	+	+	+
71	−	+	−	−	−	−	−	+	−	−	−
93	−	+	−	+	−	−	−	−	−	−	−
94	−	−	+	+	+	+	−	+	+	−	+
L. sakei 2a	−	−	+	+	+	+	+	+	+	+	+

* Cell free supernatant; (+) not affected by the treatment, (−) no activity after treatment.

Table 4
Effect of heating at 60 °C, 80 °C and 100 °C on the activity of the antimicrobial substances present in the CFS* of the LAB isolates.

Isolates nº	Temperature								
	60 °C			80 °C			100 °C		
	30 min	60 min	120 min	30 min	60 min	120 min	30 min	60 min	120 min
10	−	−	−	−	−	−	−	−	−
69	+	+	+	+	+	+	+	+	+
71	−	−	−	−	−	−	−	−	−
93	−	−	−	−	−	−	−	−	−
94	+	+	+	+	+	+	−	−	−
L. sakei 2a	+	+	+	+	+	+	+	+	+

* Cell free supernatant; (+) not affected by the treatment, (−) no activity after treatment.

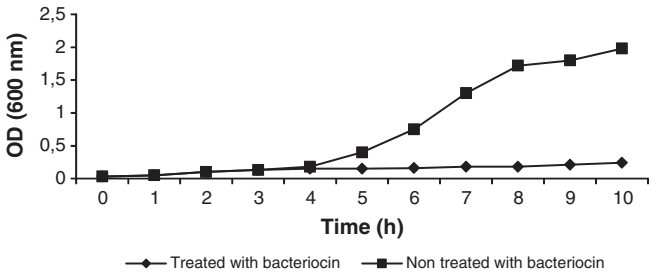

Fig. 1. Optical density of *L. monocytogenes* ScottA cells, in BHI broth, treated and non-treated with cell free supernatant of *L. lactis* subsp. *lactis* 69.

strains, most food isolates of LAB and twelve out of eighteen spoilage halotolerant bacteria isolated from charqui, indicating its potential application for preservation of salted meat products.

Treatment of early exponential phase cells of *L. monocytogenes* ScottA with the bacteriocin produced by isolate 69 (CFS containing active bacteriocin) resulted in complete cell inactivation of the test microorganism, as no viable cells were detected after 10 h incubation, suggesting a bactericidal mode of action. Besides, no significant increase in the optical density was detected in the samples treated with CFS of isolate 69, in comparison with the results observed with non-treated samples (Fig. 1).

Different levels of adsorption of CFS of isolate 69 to *L. monocytogenes* ScottA, *L. monocytogenes* 620 4b, *L. monocytogenes* 637 1/2c, *L. sakei* ATCC 15521 and *E. faecium* ATCC 19443 were recorded (Table 5). The highest adsorption was obtained for *L. sakei* ATCC 15521 (100%), followed by *L. monocytogenes* (66.66%) and *E. faecium* (33.33%). The absorption to *L. monocytogenes* ScottA was influenced by temperature, pH and presence of chemicals (Table 6). The highest adsorption rates were recorded at 4 °C, 25 °C and 30 °C; pH 4.0 and pH 6.0 and in the presence of NaCl, glycerol and SDS. Adsorption of the bacteriocin to the target cells is important because it intermediates the insertion of the bacteriocin in the cell membrane and pore formation, leading to the cell death (Todorov, 2009). Therefore, the knowledge of the influence of pH, temperature and chemicals on the adsorption levels is of relevance when the potential application of bacteriocin in food preservation is under evaluation. Other studies have also demonstrated that pH, temperature and presence of chemical agents may influence the adsorption level of bacteriocins to the target cells (Todorov, 2008). Results for isolate 69 evidence its potential application at neutral or moderately acid pH, temperatures ranging from 4 °C to 30 °C and in the presence of NaCl, which is of great interest considering salted meat products like charqui.

Isolate 69 was able to grow in the presence of NaCl in concentrations up to 5% (Fig. 2). In medium with 5% salt, the counts after 72 h were the same as in the medium with no added NaCl. However, when the concentration of salt was 10%, the counts remained stable up to 72 h, indicating that this strain may be tolerant to the salt content in charqui. Increase of the concentration of salt to 15% or 20% caused only a slight reduction in the counts of viable cells.

When grown in MRS with 20% (w/v) NaCl for 24 h, 48 h and 72 h, isolate 69 was capable to produce bacteriocins active against *L. sakei* ATCC 15521. However, the activity of the bacteriocin varied according to the incubation time: after 24 h, the activity in MRS containing 20% NaCl was half of that in MRS without added salt (6400 AU/mL and 12,800 AU/mL, respectively). After 48 h and 72 h, the activity in the two media decreased similarly regardless of the presence of NaCl (6400 AU/mL and 400 AU/mL, respectively). The capability to produce antimicrobial substances in media with 20% of salt reinforces that isolate 69 presents an important potential as biopreservative in salted meat products.

Table 5
Adsorption of the bacteriocin produced by *L. lactis* subsp. *lactis* 69 to *L. monocytogenes*, *E. faecium* and *L. sakei*.

Microorganisms	Adsorption rate (%)
L. monocytogenes ScottA	66.66
L. monocytogenes 620, 4b	66.66
L. monocytogenes 637, 1/2 c	66.66
E. faecium ATCC 19443	33.33
L. sakei ATCC 15521	100.00

Table 6
Influence of pH, temperature and chemical agents on the adsorption of the bacteriocin produced by isolate 69 to *L. monocytogenes* ScottA.

Treatments		Adsorption rate (%)
Temperature	4 °C	100.00
	25 °C	100.00
	30 °C	100.00
	37 °C	66.66
pH	4.0	100.00
	6.0	100.00
	8.0	66.66
	10.0	66.66
Chemical agents	Tween 80	33.33
	Tween 20	33.33
	SDS	66.66
	NaCl	66.66
	Glycerol	66.66

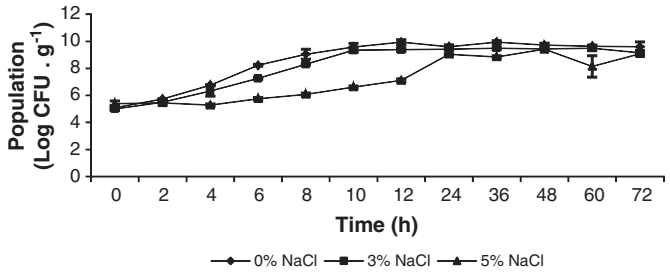

Fig. 2. Growth of *L. lactis* subsp. *lactis* 69 in MRS supplemented with 0%, 3% and 5% of NaCl (w/v).

Table 7
Amino acid sequences of nisin Z produced by *L. lactis* subsp. *lactis* 69, *L. lactis* MS27 and *L. lactis* K213.

Microorganism	Sequence	Reference
L. lactis subsp. *lactis* 69	-20 -10 1 10 20 30 MSTK**I LTWIW YLFR**K KDSGA SPRIT SISLC TPGCK TGALM GCNMK TATCN CSIHV SK Leader peptide	Strain isolated in this study
L. lactis subsp. *lactis* MS27	-20 -10 1 10 20 30 MSTK**D FNLDL VSVS**K KDSGA SPRIT SISLC TPGCK TGALM GCNMK TATCN CSIHV SK Leader peptide	Aso *et al.*, 2008 (GenBank accession nº: AB375441.1)
L. lactis subsp. *lactis* K213	-20 -10 1 10 20 30 MSTK**D FNLDL VSVS**K KDSGA SPRIT SISLC TPGCK TGALM GCNMK TATCN CSIHV SK Leader peptide	Park *et al.*, 2003 (GenBank accession nº: AB083093)

Letters in bold represent the differences between the amino acid sequence of nisin Z produced by *L. lactis* 69 and the amino acid sequences of nisin Z produced by *L. lactis* MS27.

The 16S rDNA amplification indicated that isolate 69 presented 99% identity with the 16S rDNA sequences reported for *L. lactis* subsp. *lactis* in the GenBank database, such as strain IL1403 (accession number: AE005176; Bolotin et al., 2001). The amplification of genomic DNA with sub-species-specific primers yielded a 500 bp fragment, which corresponded in size to that expected for *L. lactis* subsp. *lactis* strains (Petrov et al., 2008). According to these results, the isolate 69 was determined to be an *L. lactis* subsp. *lactis* strain.

The amplification of genomic DNA extracted from *L. lactis* 69 with primers targeting known bacteriocins generated positive results for nisin and the sequence of this amplicon presented 98% homology to the nisin Z gene. However, no amplification was recorded for the genes enconding lacticin 3147, lacticin 481, lactococcin A, lactococcin B, lactococcin M, lactococcin G, lactococcin Q and lactococcin 972.

Based on the genetic sequence of the amplified PCR product, the amino acid sequences of the bacteriocin (including part of the leader peptide) produced by *L. lactis* 69 was reconstructed and compared with the sequence reported for nisin Z (Table 7). The amino acid sequence of the active peptide (amino acids 1 to 34) is identical to other sequences previously deposited for nisin Z (Aso et al., 2008; Park, Itoh, Kikuchi, Niwa, & Fujisawa, 2003). However, differences were observed regarding the leader peptide (amino acids −10 to −19).

Leader peptides have no antimicrobial activity. They are cleaved off from the mature bacteriocins before their secretion out of the cell. However, leader peptides play an important role in the bacteriocin synthesis, as they are responsible for important enzymatic reactions, such as those mediated by dehydratase and cyclase. In addition, it has been speculated that leader peptides prevent the mature peptides from displaying antimicrobial activity intracellularly (Kuipers, Rollema, de Voz, & Siezen, 1993; Moll, Kuipers, & Rink, 2010). The exact mechanism of enzymatic induction by leader peptides remains unknown. Further studies are needed to clarify the role of the differences observed in the leader peptide of the nisin Z produced by isolate *L. lactis* 69 on the synthesis and secretion of this bacteriocin. Moreover, results of studies currently in development in salted meat models will clarify the role played by bacteriocinogenic *L. lactis* 69 in the fermentation step during charqui processing.

4. Conclusions

Results reported in the presented work indicate that the *L. lactis* 69, isolated from charqui, is a nisin Z-producing strain, evidencing a potential for application as an additional hurdle in the preservation of salted meat products. Further studies in salted meat models will indicate how this strain or its bacteriocin will behave in the control of food spoilage bacteria in these products.

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