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ORIGINAL PAPER

Characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CRL 1584 isolated from a *Lithobates catesbeianus* hatchery

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Abstract *Lactococcus lactis* CRL 1584 isolated from a *Lithobates catesbeianus* hatchery inhibits the growth of *Citrobacter freundii* (a bullfrog pathogen) and *Listeria monocytogenes* by a synergistic effect between lactic acid, hydrogen peroxide and a bacteriocin-like molecule. The chemical characterization of the bacteriocin in cell-free supernatants indicates that it has a proteinaceous nature. Hexadecane and ethyl acetate did not modify the bacteriocin activity, while 10 and 20 % (v/v) chloroform decreased the activity by 29 and 43 %, respectively. The antimicrobial peptide was heat stable since 85 % of residual activity was detected when neutralized supernatants were heated at 80 °C for 30 min. Moreover, no bacteriocin inactivation was observed when supernatants were kept at −20 °C for 3 months. The synthesis of the bacteriocin was associated with bacterial growth, highest production (2,100 AU/ml) being detected at the end of the exponential growth phase. At pH ranges of 5–6.5 and 5.0–5.5 the inhibitory molecule was stable when stored for 2 days at 4 and 25 °C, respectively. Moreover, it had a bactericidal effect on *L. monocytogenes* and the ultrastructural studies of pathogenic cells revealed clumping of the cytoplasmic material, increased periplasmic space and cell wall modifications. The deduced amino acid sequence of the bacteriocin was identical to nisin Z and the genetic determinants for its production are harbored in the chromosome. These results, described for the first time in *L. lactis* from a bullfrog hatchery, will increase knowledge of the bacteriocin under study with a view to its potential inclusion in probiotics for raniculture or biopreservatives.

Keywords *Lactococcus lactis* · Raniculture · Bacteriocin · Infectious diseases · Probiotic

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Introduction

The ability of lactic acid bacteria (LAB) to produce inhibitory metabolites against pathogenic microorganisms has been demonstrated for many strains isolated from different aquaculture-related environments and activities (Irianto and Austin 2002; Balcázar et al. 2006; Gatesoupe 2008; Muñoz-Atienza et al. 2013) and represents one of the most frequently used criteria in the selection of beneficial microorganisms to be included in beneficial or probiotic products (Verschuere et al. 2000). The bactericidal effect can be exerted by individual metabolites or by a combination of organic acids, hydrogen peroxide, diacetyl and bacteriocins as well as by the production of siderophores or enzymes such as lysozymes and proteases (Martínez Cruz et al. 2012).

Bacteriocins, ribosomally synthesized proteinaceous compounds lethal to cells closely related to the producing bacteria, could act as anti-competitor molecules that prevent the invasion of pathogenic species in a microbial community or as communication molecules in some bacterial consortia such as biofilms (Desriac et al. 2010). Moreover, bacteriocins have acquired relevance in the food

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and pharmaceutical industries as food preservatives and drugs, respectively (Kumari et al. 2012). One of these antimicrobial compounds, nisin, is produced by *Lactococcus lactis* subsp. *lactis* (a food-grade bacterium) and has been approved for use in foods. It inhibits Gram-positive bacteria and spores and is generally ineffective against Gram-negative ones, since they possess an outer cell membrane that blocks the active site, a situation that can be overcome by the combination of antimicrobial compounds with food-grade chelators (Delves-Broughton 2005). Nisin, which became available as *Nisaplin*® in 1953, is now used in 50 countries and in different ecosystems. In the aquaculture industry, nisin is applied to control botulism in vacuum-packed fish and *Listeria monocytogenes* in smoked salmon and crabmeat (Delves-Broughton 2005). However, it is interesting to point out that probiotics, which are used to prevent some infectious disease outbreaks, can be formulated with specific strains with beneficial characteristics or combined with prebiotics and/or bacteriocins.

Raniculture is an aquaculture activity that implies the captive breeding of different amphibian species, *Lithobates catesbeianus* (American bullfrog) being the selected species to obtain meat and by-products. This activity represents an intensive growth process where epizootics occur regularly, the red-leg syndrome (RLS) or bacterial dermatosepticemia being the most representative infection, causing both high mortality and economic losses (Densmore and Earl Green, 2007).

Lactococcus lactis subsp. *lactis* CRL 1584 is an autochthonous LAB strain isolated from a bullfrog hatchery that inhibits RLS-related pathogens (*Proteus vulgaris* and *Citrobacter freundii*) and food-borne bacteria (*L. monocytogenes* Scott A) (Pasteris et al. 2006, 2009b, 2011). These characteristics support the use of this LAB strain as a potential candidate to be included in the design of probiotic products for raniculture, the resulting bacteriocin being an excellent option to be used as a biopreservative of bullfrog carcasses according to its inhibitory effect on *L. monocytogenes* growth (Pasteris et al. 2009b). The purpose of this work was to characterize the bacteriocin produced by *L. lactis* subsp. *lactis* CRL 1584 in cell-free supernatants to increase knowledge of this antimicrobial compound with a view to its potential application in different biotechnological areas.

Materials and methods

Microorganisms and culture conditions

Lactococcus lactis subsp. *lactis* CRL 1584 was isolated from an Argentinean bullfrog hatchery and identified by phenotypic and genotypic approaches (Pasteris et al. 2008, 2009a). The strain was grown in LAPTg broth (in g/l: peptone, 15; yeast extract, 10; tryptone, 10; glucose, 10; tween 80, 1 ml) (Raibaud et al. 1963), pH 6.8, for 8 h at 37 °C in a 5 % CO_2-enriched chamber (microaerophilia). In these conditions, the LAB strain supernatants inhibited the growth of *L. monocytogenes* Scott A by a combined effect of organic acids, H_2O_2 and a bacteriocin-like metabolite (Pasteris et al. 2009a).

Listeria monocytogenes Scott A was grown in Brain Heart Infusion (BHI) broth, pH 6.9, for 6 h at 37 °C in microaerophilia.

The microorganisms were stored at −20 °C in their specific growth media supplemented with 20 % glycerol.

Chemicals and enzymes used were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO (USA). LAPTg components were supplied by Britania laboratories (Buenos Aires, Argentina) while BHI medium was obtained from Merck Darmstadt, Germany.

Preliminary characterization of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CRL 1584

Five-hundred milliliters of a 10 h culture of *L. lactis* CRL 1584 were centrifuged at 3,000*g* at 4 °C for 20 min. Then, 2 ml fractions of crude (untreated), neutralized (pH = 7.0) and neutralized + catalase (treated) supernatants were used to determine the chemical nature and the stability of the bacteriocin-like molecule. Its activity was evaluated by the agar-well diffusion assay according to Pasteris et al. (2009a). Briefly, soft BHI agar (0.7 % w/v) plates were inoculated with 1×10^5 c.f.u./ml of *L. monocytogenes*. Then, 100 µl of LAB supernatants were added to the plates into which 10 mm holes had been punched. The supernatants were previously adjusted to pH 6.5 with 1 N NaOH and treated with 0.5 mg/ml catalase (1 h at 25 °C) to abolish the inhibitory effect of organic acids and H_2O_2, respectively.

The antimicrobial titer was defined as the reciprocal of the highest two fold dilution producing a clear zone of inhibition and was expressed as arbitrary units per milliliter of culture medium (AU/ml).

Physico-chemical characterization

Two-milliliter fractions of treated supernatants were supplemented with 1 mg/ml of hydrolytic enzymes: pepsin, α-chymotrypsin and trypsin. Enzymes were suspended in the specific buffer solutions indicated by the suppliers. Positive and negative controls included enzyme solutions and treated fractions diluted with sterile water, respectively. For α-chymotrypsin, samples were incubated for 1 h at 25 °C and for 1 h at 37 °C for the other enzymes.

Moreover, 5 ml fractions of untreated and treated supernatant were supplemented with 10 and 20 % (v/v) hexadecane, ethyl acetate, chloroform and ethanol according to Pasteris et al. (2009a, b).

Temperature stability

Five-milliliter fractions of untreated and treated supernatants were exposed to different temperatures for different time periods: 80 and 100 °C for 5, 10, 15 and 30 min and 115 °C (autoclave) for 5 min. Samples without any treatment were used as control.

Lactococcus lactis subsp. *lactis* CRL 1584 growth and kinetics of bacteriocin production

Growth of the LAB strain was monitored in LAPTg broth for 10 h in the conditions stated above. Samples were taken at different time intervals for growth determinations (optical density $\lambda = 540$ nm and number of c.f.u./ml). Fluid supernatants were obtained by centrifugation at 3,000*g* at 4 °C for 20 min and used for both pH determinations and quantification of the bacteriocin activity.

Effect of pH and storage on bacteriocin stability

The combined effect of pH and temperature on bacteriocin stability during storage was studied. Thus, 20 ml fractions of *L. lactis* supernatant containing bacteriocin were heated at 80 °C for 30 min, adjusted to different pH values (2–9) with sterilized 1 N HCl and NaOH solutions and kept at 4 and 25 °C. Samples were removed every 24 h for 3 days, neutralized and treated with catalase before bacteriocin activity determinations.

The effect of low temperature storage on the bacteriocin stability was also evaluated. Thus, treated and untreated supernatants were heated at 80 °C for 30 min and stored later at −20 °C for 3 months. For these assays, samples were removed every week and processed for AU/ml determinations.

Mode of action of the bacteriocin on *Listeria monocytogenes* cells

The effect of the bacteriocin on *L. monocytogenes* Scott A was evaluated following the guidelines of the Clinical Laboratory Standard Institute (CLSI Document M26-A). Thus, pathogenic cells were harvested from a 6 h culture at 37 °C, washed twice with sterile phosphate-buffered saline (PBS) (pH 7.0 and suspended to 1×10^7 c.f.u./ml). The bacteriocin-containing medium was prepared by concentration (10×) of the LAB strain supernatant (60 °C, 10 min in a Büchi R110 rotavap). Then, 4.75 ml of a-treated supernatant and b-treated supernatant + chymotrypsin were supplemented with 0.2 ml BHI broth to reach the nutritional conditions suitable for bacterial growth and inoculated with the cell suspension to obtain 5×10^5 c.f.u./ml. The bacteriocin concentration in each treatment was 4,200 AU/ml.

Treated supernatant + chymotrypsin were heated at 115 °C for 5 min to abolish the effect of chymotrypsin on *L. monocytogenes* cell wall components.

All the samples were incubated at 37 °C in microaerophilic conditions. The number of viable cells was determined every 30 min for 300 min, while samples to evaluate the ultrastructural cell damages were collected at 1 and 2 h of incubation.

Transmission electron microscopy (TEM)

Cells obtained under each of the above experimental conditions were harvested, suspended in LAPTg medium supplemented with 3 % glutaraldehyde (1:1 v/v) and incubated for 30 min at room temperature; then they were centrifuged at 3,000*g* at 4 °C for 5 min and suspended in 3 % glutaraldehyde (Karnovsky1965). Finally, cells were processed according to Venable and Coggeshall (1965) and observed by TEM.

Molecular characterization of the bacteriocin produced by *L. lactis* subsp. *lactis* CRL 1584

Genomic DNA and plasmid isolation

Genomic DNA of *L. lactis* CRL 1584 was isolated as described by Pospiech and Neumann (1995), while plasmid DNA was isolated by the procedure of Muriana and Klaenhammer (1991). DNA extraction samples were resolved on agarose gel electrophoresis according to Sambrook and Russell (2001). Agarose gel electrophoresis was performed in a Tris–acetate buffer containing 40 mM Tris–acetate and 1 mM EDTA (pH 8.0). Gels contained 0.8 % (w/v) agarose, and electrophoresis was performed at 100 V for 1.5 h. Gels were stained with GelRed™(Biotium, California, USA), imaged using a 254-nm transilluminator and photographed.

DNA amplification, sequencing and analysis

To identify the bacteriocin genes in *L. lactis* CRL 1584, degenerate primers were designed based on the genetic sequence of antimicrobial peptides from LAB (Table 1). Primers were synthesized by Invitrogen (Buenos Aires, Argentina). PCRs were carried out in a MyCycler thermal cycler (BioRad, California, USA). PCR mixtures (50 µl) contained DNA (15 ng), $MgCl_2$ (2.5 mM), the four dNTPs

Table 1 Primers used to identify the genes coding for bacteriocin produced by *L. lactis* subsp. *lactis* CRL 1584

Primer set	Sequence (5′ → 3′)	Product size (bp)	Bacteriocin target	References
NisAf1-Fw	AAAATGAGTACAAAAGATTTYAAC	456	Nisin	Espeche et al. (2009)
NisBr3-Rev	TGCATAACATCATAGAGTTTAGG			
ABP118-Fw	GGNAAACGNGGNCCNAAC	343	ABP-118	Flynn et al. (2002)
ABP118-Rev	CCACCAGAAATTCCACCCGCT			
B2-Fw	ATGAATAGCGTAAAAGAATTA	197	Carnobacteriocin B2	Quadri et al. (1994)
B2-Rev	CGGTCTCCTACCAATGGATCC			
BM1-Fw f	ATGAAAAGCGTTAAAGAACTAAAT	184	Carnobacteriocin BM1	Quadri et al. (1994)
BM1-Rev	ATGTCCCATTCCTGCTAAACT			
Cur-Fw	ATGAATAATGTAAAAGAATTAAGT	180	Curvacin A	Tichaczek et al. (1993)
Cur-Rev	TTACATTCCAGCTAAACCACTAGC			
Ent A-Fw	GGTACCACTCATAGTGGAAA	137	Enterocin A	Aymerich et al. (1996)
Ent A-Rev	CCCTGGAATTGCTCCACCTAA			
Ent B-Fw	GAAAATGATCACAGAATGCCTA	151	Enterocin B	Casaus et al. (1997)
Ent B-Rev	AGAGTATACATTTGCTAACCC			
Ent P	GCTACGCGTTCATATGGTAAT	86	Enterocin P	Cintas et al. (1997)
Ent Pr	TCCTGCAATATTCTCTTTAGC			

N = A/T/G/C; Y = C/T

(100 μM), each primer (1 μM) and 2.5 U of *Taq* polymerase in *Taq* buffer (Invitrogen, Buenos Aires, Argentina). PCR conditions included 3 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 or 50 °C (depending on the average melting temperature of the primer set) for 30 s, and extension at 72 °C for 3 min. The reactions were stopped by incubating for 5 min at 72 °C and then chilled at 4 °C. PCR products were purified by agarose gel electrophoresis in the above conditions with the commercial GFX^{TM} PCR DNA gel band purification kit (Amersham Biosciences, New York, USA).

Biosciences, New York, USA). The fragments were cloned in *Escherichia coli* DH10B into pCR2.1-TOPO vector by using the Invitrogen TOPO TA cloning kit. *E. coli* transformants were selected by their growth in Luria–Bertani agar supplemented with ampicillin (50 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 μg/ml) and IPTG (Isopropyl β-D-1-thiogalactopyranoside) (0.1 mM). The cloned PCR products were sequenced by Ruralex SRL (Buenos Aires, Argentina). Database searches were performed using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequence data is available at the DDBJ/EMBL/GenBank databases under the accession number JN398478.

Plasmid curing

Plasmid-curing experiments were performed in order to determine whether there was a relationship between the presence of plasmid in *L. lactis* CRL 1584 and bacteriocin production. Therefore, LAPTg broth (4 ml) containing 10 μg/ml novobiocin was inoculated (1 % w/v) with an overnight *L. lactis* CRL 1584 culture and incubated at 37 °C for 24 h. Then, appropriate dilutions were plated onto LAPTg agar and incubated at 37 °C for 48 h. Colonies were selected, plasmid DNA was isolated and the profiles were analysed. The plasmid-cured strains were evaluated for their ability to produce bacteriocin, which was confirmed by four subsequent cultures in LAPTg broth at 37 °C without the curing agent, and then stored as stated above.

Statistical analysis

The values shown correspond to the media of three separate assays. One-way analysis of variance (ANOVA) was applied to the experimental data by using Student's *t* test for multiple mean comparisons (95 % confidence interval). Statistical treatments were performed using INFOSTAT software (2008 student version).

Results

Identification of the inhibitory metabolites produced by *Lactococcus lactis* subsp. *lactis* CRL 1584

The cell-free supernatant obtained from *L. lactis* CRL 1584 cultures inhibited the growth of *L. monocytogenes* Scott A

Table 2 Effect of physico-chemical treatments on bacteriocin activity

Treatment	Bacteriocin activity (AU/ml)
Control[a]	2,100
Enzymes[a]	
Pepsin	1,500
Trypsin	1,200
α-Chymotrypsin	0.0
Organic solvents (%)[a]	
Hexadecane, 10	2,100
Hexadecane, 20	2,100
Ethyl acetate, 10	2,100
Ethyl acetate, 20	2,100
Chloroform, 10	1,500
Chloroform, 20	1,200
Ethanol, 10	0.0
Temperature (°C)[a]	
80/5 min	1,800
80/10 min	1,800
80/15 min	1,800
80/30 min	1,800
100/5 min	1,800
100/10 min	1,800
100/15 min	1,800
100/30 min	1,200
115/5 min	600
−20/3 months[b]	2,100

Data represent the bacteriocin activity of [a]treated and [b]untreated supernatants

The antimicrobial activity was determined by the agar-well diffusion assay against *L. monocytogenes* Scott A. All tests were performed in three separate assays, and the variation in the bacteriocin activity was ≤0.05

by organic acids and H_2O_2, since the inhibition halos decreased to 2 mm when the cell-free supernatants were neutralized and to 3 mm when the neutralized supernatants were treated with catalase. This antimicrobial activity decreased when fractions of the latter supernatant were then treated with different proteolytic enzymes, and a complete inhibition of the antibacterial effect was detected only on chymotrypsin-treated samples (Table 2). These results indicate that the inhibitory metabolite has a proteinaceous nature. Therefore, growth of *L. monocytogenes* was inhibited by a synergistic effect among organic acids, H_2O_2 and a bacteriocin-like molecule.

Chemical nature and temperature stability of the bacteriocin

The effect of some physico-chemical factors on bacteriocin stability is shown in Table 2. The addition of 10 and 20 % (v/v) hexadecane and ethyl acetate to untreated or treated supernatants did not modify the bacteriocin activity, while the addition of 10 and 20 % (v/v) chloroform decreased it by 29 and 43 %, respectively. 10 % (v/v) ethanol caused the complete inhibition of the antimicrobial peptide.

When the treated supernatants were heated at 80 °C, the bacteriocin activity remained stable (residual activity = 85 %). 43 % inactivation was observed when the supernatants were heated at 100 °C for 30 min, while the antimicrobial peptide was sensitive when heated at 115 °C for 5 min (residual activity = 29 %). When untreated supernatants were heated at the above temperatures and times, the bacteriocin activity was preserved.

No inactivation of the bacteriocin was observed when untreated and treated supernatants of *L. lactis* were kept at −20 °C for 3 months.

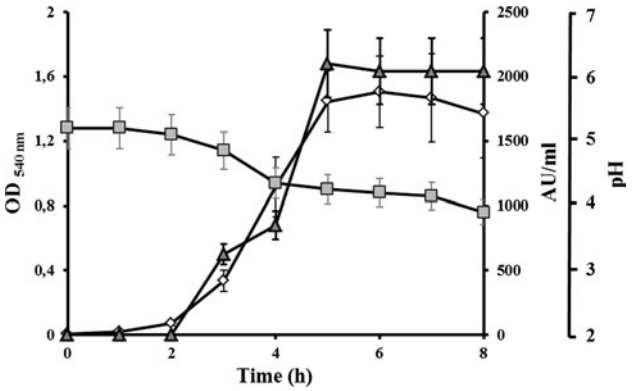


Fig. 1 Growth and kinetics of bacteriocin production by *L. lactis* subsp. *lactis* CRL 1584. *Diamond* O.D. (540 nm), *square* pH, *triangle* AU/ml. All the tests were performed in three separate assays ($P \leq 0.05$)

Lactococcus lactis subsp. *lactis* CRL 1584 growth and kinetics of the bacteriocin production

L. lactis CRL 1584 was grown in LAPTg broth and reached the stationary phase at 5 h of incubation under microaerophilic conditions, while the number of viable cells increased by more than 3 $\log_{10}$ units and the pH decreased 2.5 units at 8 h of incubation ($P \leq 0.05$). Bacteriocin production was associated with cell growth, the highest production level (2,100 AU/ml) being observed at the end of the exponential growth phase (5 h) (Fig. 1).

Effect of pH and temperature of storage on bacteriocin stability

The combined effect of pH and temperature on bacteriocin stability during storage at 4 and 25 °C was evaluated. Optimal bacteriocin activities were determined at pH values lower than 7.0. At a pH range of 5–6.5 the bacteriocin

activity was not affected when stored at 4 °C for 2 days, while at 3 days of storage, a slight decrease in activity (20 %) was detected. The same behavior was observed when bacteriocin-containing supernatants were adjusted to pH 5.0 and 5.5 and stored at 25 °C. At alkaline pH values, bacteriocin inactivation was higher, mainly at 25 °C. At both storage temperatures a normal data distribution was observed around pH 5.5 (Fig. 2).

Mode of action of the bacteriocin on *Listeria monocytogenes* and evaluation of cell damage

The addition of the bacteriocin to *L. monocytogenes* Scott A cells harvested at the end of the exponential growth phase resulted in a slow decrease in the number of viable cells during the first 30 min of incubation (5.58 $\log_{10}$ units). From this time on, a rapid decline was observed and no viable cells were detected at 150 min ($P \leq 0.05$) (Fig 3). The results indicate that the bacteriocin has a bactericidal effect on *L. monocytogenes* cells. An increase of growth ($\sim$1 $\log_{10}$ unit) was observed at 150 min in the control samples: (a) *Listeria* growth, (b) neutralized + catalase + chymotrypsin supernatants, (c) chymotrypsin + heating.

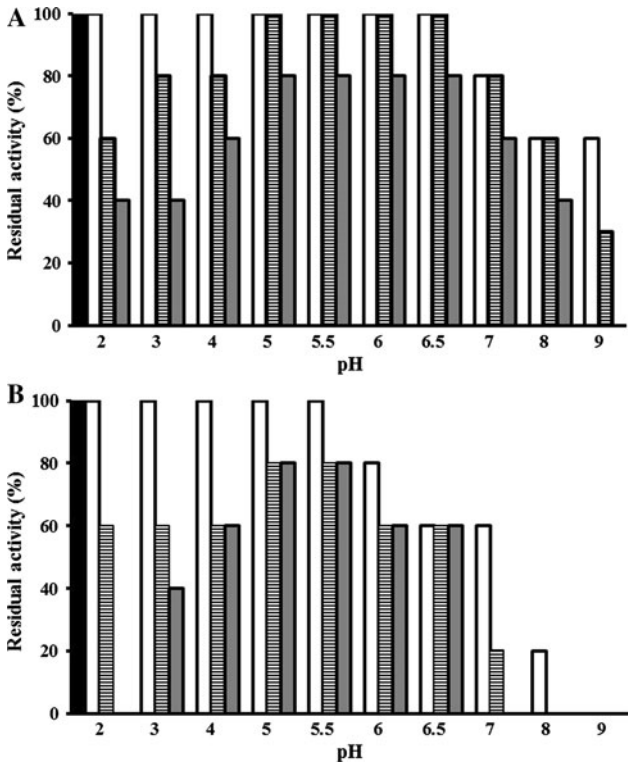


Fig. 2 Effect of pH and temperature of storage on bacteriocin activity. **a** 4 °C; **b** 25 °C. *Black bar* 0, *white bar* 1, *striped bar* 2 and *grey bar* 3 days. Data represent the average of three separate assays. The % of the residual activity during storage was calculated by considering 100 % of initial activity (*black bar*)

Fig. 4 Transmission electronic microscopy of *L. monocytogenes* ▶ cells incubated with a bacteriocin-containing medium. 1 h treatment: **a** (X34300), **b** (X96300), **c** (X160500): *arrows* indicate the microvesicles formation, **d** (X220000): *asterisk* shows the area of the cell wall with increased electron density. 2 h treatment: **e** (X96300), **f** (X34300); **g** (X220000) and **h** (X220000): (*CM*) cellular membrane. Control culture: 1 h incubation, **i** (X22800); 2 h incubation, **j** (X34300), **k** (X96300): (*PG*) peptidoglycan; (*PS*) periplasmic space. *Scale bar* 200 nm

Ultrastructural studies in samples of *L. monocytogenes* cells treated with bacteriocin at 60 and 120 min were performed. At 60 min, both granulation and contraction of cytoplasmic material were observed (Fig. 4a). Formation of multimembraned vesicles within the cytoplasm was also observed (Fig. 4a–c). An increase in cell wall electron density in the area associated with the plasmatic membrane was also detected (Fig. 4d).

At 120 min, the clumping of the cytoplasmic material was more evident (Fig. 4e), with an increase in the periplasmic space and an altered pattern of the cell wall, which lost its electron density and showed a tumefaction-like aspect (Fig. 4f, g, h).

Molecular analysis

Bacteriocin nucleotide sequence identification

PCR amplifications with the primers listed in Table 1 and the genomic DNA from *L. lactis* CRL 1584 as template showed that only oligonucleotides designed for the nisin family of bacteriocins (primers NisAf1-Fw and NisBr3-Rev) enabled amplification of a DNA fragment of the expected size ($\sim$450 bp). The PCR fragment was sequenced and analyzed, the deduced amino acid sequence being identical to nisin Z. The sequence of this bacteriocin revealed a propeptide of 34 amino acids (ITSISLCTPGC KTGALMGCNMKTATCNCSIHVSK). The bacteriocin

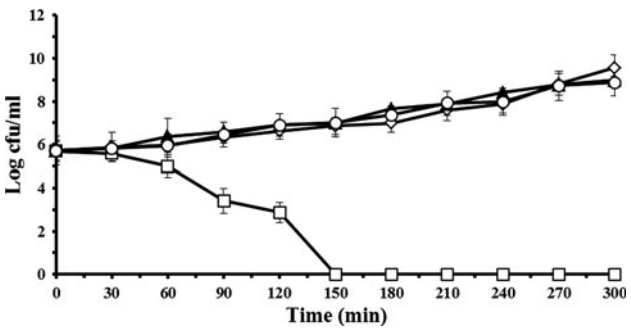
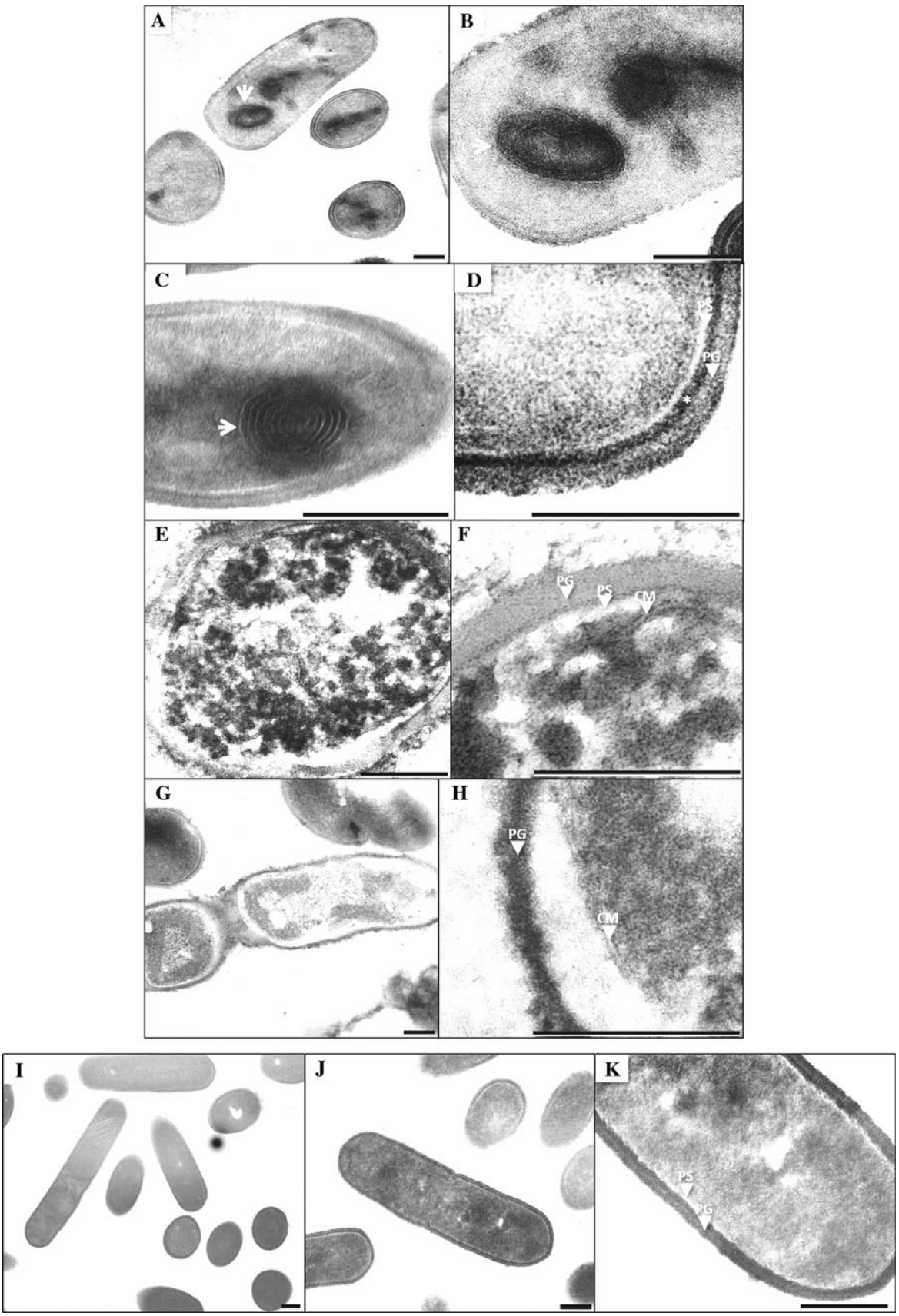


Fig. 3 Effect of bacteriocin on *L. monocytogenes* viability. *Diamond* growth control, *square* neutralized + catalase, *triangle* neutralized + catalase + chymotrypsin (bacteriocin control, negative), and *circle* chymotrypsin + heating (enzyme control, negative). All tests were performed in three separate assays ($P \leq 0.05$)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produced by *L. lactis* CRL 1584 belongs to the class I lantibiotic peptides.

Plasmid presence in L. lactis CRL 1584 and curing experiments

In order to evaluate the presence of plasmids in *L. lactis* CRL 1584, an isolation method for these extrachromosomal genetic materials was applied. The results revealed the presence of one plasmid (data not shown).

Since bacteriocin genes are frequently encoded in plasmids (Mills et al. 2006) and the ability to produce the inhibitory molecule is lost when the strains are cured, so the bacteriocin production becomes an unstable characteristic, a plasmid-curing strategy was applied to *L. lactis* CRL 1584. The plasmid-cured strains retained the ability to produce the bacteriocin during the subsequent cultures in the appropriate medium without the curing agent. Thus, the genetic determinants for the bacteriocin production are not harbored in the plasmid.

Discussion

The traditional strategies to treat or control bacterial infectious diseases in aquaculture-related activities include the use of antibiotics and antiseptics that can modify the indigenous microbiota. This increases production costs and promotes the emergence of antibiotic-resistant bacteria related to the presence of antimicrobial residues in meat. Therefore, the use of probiotics as novel and valid application alternatives is being developed (Verschuere et al. 2000; FAO/WHO 2001; Ammor et al. 2007; Jiang et al. 2009).

Lactococcus lactis is a species with potentially probiotic properties. For example, the AR21 strain has been shown to inhibit *Vibrio anguillarum*, a potential fish pathogen (Shiri Harzevili et al. 1998). More recently, other *L. Lactis* strains were proposed as probiotics in aquaculture because of their in vitro mucus adhesion, in vivo colonization ability (Sugimura et al. 2011) and immunostimulatory effect in fish species (Balcázar et al. 2007; Pérez-Sánchez et al. 2011). Some strains were found to inhibit specific fish pathogens by organic acids (Hagi and Hoshino 2009) and H_2O_2 (Sugita et al. 2007), while *L. lactis* TW34 isolated from fish intestinal tract (Sequeiros et al. 2010) and *L. lactis* from olive flounder (Heo et al. 2012) were reported to produce bacteriocin and nisin Z, respectively. These strains were able to control *L. garvieae* and *Streptococcus iniae* responsible for lactococcosis and streptococcosis in freshwater and marine fish, respectively.

There are no reports on bacteriocin production by LAB from raniculture. Therefore, in this work, a bacteriocin produced by the indigenous *L. lactis* CRL 158 from captive bullfrogs was characterized for the first time. The strain was able to produce the antimicrobial compound only in LAPTg broth, its production being associated with microbial growth and highest activity being reached at the end of the exponential growth phase, as reported for *L. lactis* TW34 (Sequeiros et al. 2010). The antimicrobial molecule has a proteinaceous nature and a synergistic effect with organic acids and H_2O_2 on *L. monocytogenes* inhibition was observed, as reported previously for *C. freundii* isolated from skin ulceration of bullfrog displaying RLS (Pasteris et al. 2011).

The bacteriocin produced by *L. lactis* CRL 1584 showed some interesting physicochemical properties such as heat-stability in crude supernatants, indicating a possible protective effect of the organic acids during heating. Also, the treatment of the bacteriocin-containing supernatants with chloroform caused a decrease in the bacteriocin activity, showing that the molecule has both polar and non-polar moieties. The presence of hydrophobic (non-polar) regions in bacteriocins could be essential for their activity against sensitive bacteria, since inactivation of microorganisms by bacteriocins depends on the hydrophobic interaction between bacterial cells and bacteriocins, as reported by Parada et al. (2007).

Similarly to the bacteriocin produced by *L. lactis* TW34, the antimicrobial molecule synthesized by the CRL 1584 strain was active within a wide pH range, this activity being higher at neutral and acidic values. When evaluating the combined effect of temperature and storage on the bacteriocin activity, a high residual activity was maintained between pH 5-6.5 at 4 and 25 °C at 3 days of storage.

Evaluation of *L. monocytogenes* viability showed that a bactericidal effect can be attributed to the bacteriocin produced by *L. lactis* CRL 1584. The antimicrobial compound caused different types of damage to *L. monocytogenes* cells that increased with the time of contact between pathogenic cells and bacteriocin. The ultrastructural variations of *L. monocytogenes* showed changes in the electron density and integrity of the cell wall, enlargement of the periplasmic space and disruption and loss of cytoplasmic material. This ultrastructural pattern was also demonstrated when *L. monocytogenes* cells were exposed to antimicrobial agents such as protamine (Johansen et al. 1996), enterocin CRL35 (Minahk et al. 2000) and divergicin M35 (Naghmouchi et al. 2007). The microvesicle formation observed in our studies could be a consequence of a cellular membrane disruption as previously reported by Johansen et al. (1996) when *L. monocytogenes* cells were exposed to protamine.

The nucleotide sequence indicates that the bacteriocin produced by *L. lactis* CRL 1584 has the same sequence as nisin Z and is not encoded in plasmidic DNA. According to

Heng et al. (2007), the bacteriocin produced by *L. lactis* CRL 1584 belongs to class I lantibiotic peptides (type A, subtype AI: nisin-like), which are characterized by their content of post-translationally modified aminoacids, including lanthionine and/or b-methyl-lanthionine, and is produced by different *L. lactis* strains (Yoneyama et al. 2008).

Although the bacteriocin from *L. lactis* CRL 1584 was active against both Gram-negative bacteria associated with RLS (Pasteris et al. 2011) and *L. monocytogenes*, further studies concerning its purification are required to determine if the inhibitory effect against *C. freundii* (Pasteris et al. 2011) is due to other bacteriocins produced by this LAB species such as lactoccins, diplococcins, lactococcin-poration complex and lactostrepticins as reported by Klaenhammer (1993). Even though bacteriocin coproduction is not a common property, it was demonstrated in a *L. lactis* subsp. *lactis* strain isolated from raw ewe's milk (Bravo et al. 2009).

On the other hand, it is also possible that the bacteriocin activity by *L. lactis* CRL 1584 against RLS-related pathogens could be enhanced by the presence of organic acids and hydrogen peroxide, the latter being produced by the LAB strain at low concentrations (Pasteris et al. 2011). The purified bacteriocin could be included in the design of probiotics for captive bullfrogs and therefore would enhance the beneficial effects of the product, especially those related to the inhibition spectra. Moreover, the designed probiotics could be also used during the ex situ breeding of other amphibians under threat of extinction, especially in those species where RLS is associated with their wildlife declination (Carey et al. 1999).

Finally, this work shows an interesting perspective to apply the purified bacteriocin for the control of *L. monocytogenes* in bullfrog carcasses.

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