World J Microbiol Biotechnol (2017) 33:186
DOI 10.1007/s11274-017-2353-z

ORIGINAL PAPER

Nisin Z produced by *Lactococcus lactis* from bullfrog hatchery is active against *Citrobacter freundii*, a red-leg syndrome related pathogen

Gabriel Quintana[1] · Maria V. Niederle[1] · Carlos J. Minahk[1] · Gianluca Picariello[2] · María E. F. Nader-Macías[3] · Sergio E. Pasteris[1]

Received: 29 November 2016 / Accepted: 21 September 2017

Abstract *Lactococcus lactis* subsp. *lactis* CRL 1584 isolated from a bullfrog hatchery produces a bacteriocin that inhibits both indigenous *Citrobacter freundii* (a Red-Leg Syndrome related pathogen) and *Lactobacillus plantarum*, and *Listeria monocytogenes* as well. Considering that probiotics requires high cell densities and/or bacteriocin concentrations, the effect of the temperature on *L. lactis* growth and bacteriocin production was evaluated to find the optimal conditions. Thus, the growth rate was maximal at 36 °C, whereas the highest biomass and bacteriocin activity was achieved between 20 and 30 °C and 20–25 °C, respectively. The bacteriocin synthesis was closely growth associated reaching the maximal values at the end of the exponential phase. Since bacteriocins co-production has been evidenced in bacterial genera, a purification of the bacteriocin/s from *L. lactis* culture supernatants was carried out. The active fraction was purified by cationic-exchange chromatography and then, a RP-HPLC was carried out. The purified sample was a peptide with a 3353.05 Da, a molecular mass that matches nisin Z, which turned out to be the only bacteriocin produced by *L. lactis* CRL 1584. Nisin Z showed bactericidal effect on *C. freundii* and *L. monocytogenes*, which increased in the presence L-lactic acid + H_2O_2. This is the first report on nisin Z production by *L. lactis* from a bullfrog hatchery that resulted active on a Gram-negative pathogen. This peptide has potential probiotic for raniculture and as food biopreservative for bullfrog meat.

Keywords *Lactococcus lactis* · Raniculture · Bacteriocin · Probiotics · Biopreservatives

Introduction

According to the its Generally Regarded As Safe (GRAS) status (Reid et al. 2003), some lactic acid bacteria (LAB) species are used as probiotics to be applied in different ecological niches that include human (Reid et al. 2017), and ectothermic animals such as fish (Ljubobratovic et al. 2017) and amphibians (Kueneman et al. 2016; Pereira et al. 2016). They are also used in the industrial manufacture of fermented food products due to its potential to inhibit the growth of the normal biota, food-spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes, Bacillus cereus, Clostridium perfringens, C. difficile* and *Staphylococcus aureus*. The inhibitory activity was attributed to a wide variety of metabolic end-products: organic acids, hydrogen peroxide, bacteriocins (Le Lay et al. 2015; Uraipan and Hongpattarakere 2015; Gutiérrez et al. 2016; Ünlü et al. 2016), named postbiotics.

Postbiotics produced by LAB have been extensively studied as feed additive to achieve high productivity (Loh et al. 2014). Given the rising concerns regarding resistance of bacterial pathogens to antibiotics, the search for new and alternative antimicrobial compounds is of main interest. Bacteriocins are ribosomally synthesized antimicrobial peptides of bacterial origin that show heterogeneity in regards to activity

✉ Sergio E. Pasteris
pasteris@fbqf.unt.edu.ar

[1] Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, UNT, Chacabuco 461, T4000ILI San Miguel de Tucumán, Argentina

[2] Istituto di Scienze dell'Alimentazione – CNR, Via Roma 64, 83100 Avellino, Italy

[3] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, CP: T4000ILC San Miguel de Tucumán, Argentina

Published online: 27 September 2017

spectrum, mode of action, molecular size and biochemical properties. They are usually active against closely-related species and have been grouped into four classes according to their genetic and biochemical characteristics and mode of action (Cui et al. 2012).

Bacteriocins produced by LAB attract considerable interest as natural and nontoxic food preservatives and as therapeutics, whereas the bacteriocin-producing LAB are considered potential probiotics for food, human and veterinary applications (Jiménez et al. 2015).

Among the LAB, *L. lactis* is a well-known species considered safe based on its use as starter culture for human and animals fermented products. As a matter of fact, it is extensively consumed by humans in a daily basis. Last but not least, *L. lactis* has no relationship with any human infectious process (Araújo et al. 2015). Strains from this species were reported to produce several bacteriocins including nisin A and its natural variants Z, Q, U and F (de Kwaadsteniet et al. 2008; van Staden et al. 2012; Biscola et al. 2013; Lianou and Samelis 2014; Araújo et al. 2015; Samelis et al. 2014; Sequeiros et al. 2015), lactococcins A, B, M and Z (Ishibashi et al. 2015), lacticin LC14 and 481 (Lasta et al. 2012; Knerr and van der Donk 2013; Tabanelli et al. 2014; Mirkovic et al. 2016) that were able to inhibit specific pathogenic bacteria for human and animals. However, only nisin (commercially available as Nisaplin) is currently approved as food additive (Cotter et al. 2005) according to its GRAS status (EFSA 2006).

Lactococcus lactis subsp. *lactis* CRL 1584 is an indigenous strain isolated from an American bullfrog (*Lithobates catesbeianus*) hatchery and was selected as a potential probiotic for raniculture according to its beneficial properties that include the growth inhibition of *C. freundii* (a Red Leg Syndrome-RLS-related pathogen) and *L. monocytogenes*. Interestingly, the bacteriocin/s present/s in the supernatants of *L. lactis* subsp. *lactis* CRL 1584 had a synergistic inhibitory effect on pathogens when combined with lactic acid and hydrogen peroxide (Pasteris et al. 2011). Moreover, this strain has interesting surface properties, technological characteristics and it proved to be safe in both in vitro and in vivo assays (Pasteris et al. 2009a, 2017; Montel Mendoza et al. 2013). The chemical characterization of the bacteriocin in cell-free supernatants indicates that it has a proteinaceous nature, polar moieties and heat stability. Its synthesis was associated to the bacterial growth, the maximal production (2100 AU/mL) being detected at the end of the exponential phase at 37 °C. The bactericidal effect on *L. monocytogenes* was associated to clumping of the cytoplasmic material, increase of the periplasmic space and cell wall modification (Pasteris et al. 2013).

Taken into account that a probiotic product must be designed with high cell density, studies on bacterial growth parameters and bacteriocin production were performed. Since bacteriocins co-production was previously reported for *L. lactis* strains (Fernández et al. 2004; Bravo et al. 2009; Mirkovic et al. 2016), a careful purification is required to ensure the kind and number of peptides synthetized by the LAB strain. Finally, studies on the activity of the purified bacteriocin on *C. freundii*, a Gram-negative responsible for RLS outbreaks in bullfrog cultures, and *L. monocytogenes*, a common spoilage bacteria of bullfrog meat, were carried out.

Materials and methods

Bacterial strains and culture conditions

Lactococcus lactis subsp. *lactis* CRL 1584 was isolated from an American bullfrog (*Lithobates catesbeianus*) hatchery and selected according to its beneficial properties demonstrated in both in vitro and in vivo tests (Pasteris et al. 2009a, 2011, 2017). 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) (Pasteris et al. 2013).

Both, *Citrobacter freundii* CFb, an RLS-related pathogen, and *Lactobacillus plantarum* CRL 1651 isolated from raniculture (Pasteris et al. 2006, 2009a), as well as *Listeria monocytogenes* Scott A were grown in Brain Heart infusion broth, pH 6.9, for 7 h in microaerophilia (Pasteris et al. 2013). The microorganisms were stored at −20 °C in their specific growth media supplemented with 20% (v/v) glycerol.

Growth of *L. lactis* CRL 1584 at different temperatures and quantification of bacteriocin activity

Lactococcus lactis growth and bacteriocin production at different temperatures in LAPTg broth at pH 6.8 were evaluated. Thus, 100 mL LAPTg was inoculated (1.5% v/v) with a pre-adapted LAB culture and incubated at 15, 20, 25, 30 and 37 °C for 24 h in microaerophilia. At different time intervals, samples were taken for growth parameters determinations: optical density (OD at $\lambda = 540$ nm) every hour and number of viable cells (CFU/mL) every 2 h. In the last case, serial dilutions were plated on LAPTg agar (1.5% w/v) and incubated for 24–48 h at 37 °C before cell count determinations.

Kinetics of bacteriocin production was followed according to Pasteris et al. (2009a, b). Briefly, samples from *L. lactis* CRL 1584 cultures were removed every 2 h, centrifuged (3000×*g*, 10 min, 4 °C) and crude supernatants were stored at 4 °C for 24 h. Then, soft BHI agar (0.7% w/v) plates were inoculated with 1×10^5 CFU/mL of *L. monocytogenes* or *Lb. plantarum*. One milliliter of each supernatant fraction

was adjusted to pH 6.5 with 1M NaOH and 0.5 mL of each fraction was supplemented with 0.5 mg/mL catalase (1 h at 25 °C). These treatments avoid the inhibitory effect of organic acids and H_2O_2, respectively. Then, 100 µL of each treated fraction and the serial double-dilutions in neutralized distilled water was seeded into holes of 10 mm diameter and allowed to diffuse at room temperature. Then, plates were incubated at 37 °C for 24 h. The antimicrobial titer was defined as the reciprocal of the highest double dilution that produced a clear zone of inhibition of 1 mm and was expressed as arbitrary units per milliliter (AU/mL).

Finally, pH determinations of LAB supernatants were carried out with a digital pHmeter (Altronix).

Purification of bacteriocin/s produced by *L. lactis* CRL 1584

Sample preparation

Six-hundred milliliters LAPTg broth were inoculated (1.5% v/v) with the LAB strain and incubated for 7 h at 25 °C in microerophilia. Cells were removed by centrifugation at 3100×*g* for 20 min at 4 °C and the first supernatant (FS) was collected and filtered through a 0.45 µm pore-size membrane. Proteins and peptides were precipitated with 40% saturated ammonium sulfate. The pellets were harvested (6100×*g* for 20 min at 4 °C) and the second supernatant (SS) was precipitated with additional ammonium sulfate up to 70% under continuous stirring for 12 h at 4 °C. The third supernatant (TS) was obtained as indicated above and stored at 4 °C for further analysis. The pellet obtained from fractions 40 and 70% (P40 and P70%, respectively) were dissolved in 12 mL neutralized distilled water and stored at 4 °C. Finally, residual bacteriocin activity was determined in all fractions (FS, SS, TS, P40 and P70%) against *L. monocytogenes* and *Lb. plantarum* strains.

Partial purification of P40% fractions by using cationic exchange chromatography

P40% samples and SP Sepharose Fast Flow (GE Healthcare) columns were used for the purification. As a first approach, the optimal pH for the antimicrobial molecule/s- cationic-exchange resin interaction was analyzed. Two columns containing 2 mL SP-Sepharose were prepared. One of them was equilibrated at pH 8.5 by using 20 mM Tris–HCl buffer and the second one was equilibrated at pH 5.5 by using 20 mM sodium acetate-acetic acid buffer. Afterward, P40% samples were diluted 20 times in the appropriate buffer, passed through the respective column and eluted with 6 mL of 50, 100, 200, 300 and 500 mM NaCl-buffer. The collected samples were evaluated for bacteriocin/s activity by using *Lb. plantarum* CRL 1651 as indicator strain. Elution was monitored at λ = 220, 254 and 280 nm.

Large-scale purification of the bacteriocin/s

A 60-mL column containing 15 mL SP-Sepharose was equilibrated with 10 mM acetic acid-sodium acetate buffer, pH 5.5. P40% samples were diluted 20-times with the same buffer and passed through the column. The SP-Sepharose was then washed with 120 mL of 10 mM acetic acid-sodium acetate buffer pH 5.5 and eluted with 50, 100, 200 and 300 mM NaCl-buffer. Bacteriocin/s activity was determined in all samples. Column was regenerated by washing with 1 M NaCl.

Purification of the bacteriocin/s by RP-HPLC

The final purification step was carried out with a µBondapak C_{18} (Waters) reverse phase HPLC column. Active sample from the SP-Sepharose was injected and a non-lineal gradient was performed between bi-distilled water 0.1% trifluoroacetic acid (TFA) and methanol supplemented also with 0.1% TFA. The flow rate was set at 0.5 mL/min and elution was followed at λ = 220 and 280 nm, collecting 500 µL fractions. Bacteriocin activity was determined in each fraction as described above. Samples with antimicrobial activity were stored at − 20 °C for 12 h and later freeze-dried at a condenser temperature of − 50 °C at 110 militorr chamber pressure (Heto-FD4freeze-dryer, Heto-Holten, Denmark) for 24 h. Moreover, nisin was purified from the commercial product (Sigma-Aldrich) by using the same procedure (data not shown).

Evaluation of the purification process

To determine the purity degree of the samples, electrophoresis in polyacrylamide gels was carried out. Active samples were diluted with SDS-containing buffer, boiled 10 min and electrophoresed at 160 V for 3 h. Then, gels were washed with methanol/acetic acid/distilled water (40:10:50). Afterward, one of the gels was washed with decreasing ethanol concentrations (50, 30 and 10%) and finally washed with plenty of sterile distilled water. A petri plate containing 7 mL BHI agar (1%) was prepared. Then, 10 mL BHI agar (0.7%) containing ~ 10^2 CFU/mL of *Lb. plantarum* CRL 1651 (indicator strain) was poured onto the BHI agar and left 10–15 min at room temperature. After that, the gel was placed onto the petri plate, incubated at 37 °C for 24 h and examined for inhibition zones (Acuña et al. 2012). The other gel that was maintained in methanol/acetic acid/distilled water was stained with Coomassie Blue for 24 h, washed four-times and kept in this solution for 24 h at 25 °C.

Characterization of L. lactis CRL1584 bacteriocin/s by MS analysis

The HPLC active fraction was lyophilized and dissolved in 100 µL of 0.1% TFA. Prior to MS analysis, a 10 µL aliquot was further desalted by C18 Zip Tip® micro-columns (Millipore, Bedford, CA, USA). Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS assays with sinapinic acid as the matrix were carried out on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N_2 laser ($\lambda = 337$ nm). The mass spectra were acquired in the reflector linear ion mode using the Delayed Extraction (DE) technology. The instrument operated at an accelerating voltage of 25 kV. External calibration was carried out with commercial standards. Raw data were processed with Data Explorer 4.0 software.

Mode of action of antagonistic metabolites produced by *L. lactis* CRL 1584

To determine if nisin displayed a synergistic effect with lactic acid and hydrogen peroxide on *C. freundii* and *L. monocytogenes*, pathogenic cells were incubated with different combinations of purified bacteriocin and organic compounds. Briefly, pathogens were grown as indicated above and cells were collected by centrifugation (3000×*g*, 15 min at 4 °C). Bacteria were washed twice and resuspended in 20 mL PBS solution pH 7.0. Then, the following assays were performed, taken into consideration the end-concentrations of lactic acid and hydrogen peroxide produced by this LAB strain (Pasteris et al. 2011):

- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 480 AU/mL nisin
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 480 AU/mL nisin + 0.01 mM H_2O_2
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 480 AU/mL nisin + 5 g/L L-lactic acid
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 480 AU/mL nisin + 5 g/L L-lactic acid + 0.01 mM H_2O_2
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 0.01 mM H_2O_2
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 5 g/L L-lactic acid
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* + 5 g/L L-lactic acid + 0.01 mM H_2O_2.
- 10^5 CFU/mL *C. freundii* or *L. monocytogenes* (control)

The viability of the pathogens was evaluated every 2 h during the first 12 h of incubation and then at 24 h. Serial dilutions of each sample was performed and bacteria were plated onto BHI 1% agar. Petri plates were incubated at 37 °C for 24–48 h before cell counts.

In order to verify the synergistic effect of nisin with the organic compounds produced by the LAB strain, the cited assays were carried out with purified commercial nisin alone and combined with L-lactic acid and H_2O_2.

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

Transmission electron microscopy (TEM)

Cells of *C. freundii* treated with 7.5 nM of nisin alone or combined (L-lactic acid/hydrogen peroxide) obtained from the above experimental conditions were harvested, suspended in LAPTg broth supplemented with 3% glutaraldehyde (1:1 v/v) and incubated for 30 min at room temperature; then they were centrifuged at 3000×*g* at 4 °C for 5 min and suspended in 3% glutaraldehyde. Finally, they were fixed in osmium tetroxide and observed by TEM (Pasteris et al. 2011).

Results

Growth of *L. lactis* CRL 1584 at different temperatures and quantification of bacteriocin activity

When the temperature of incubation of *L. lactis* CRL 1584 increased from 15 to 36 °C, the bacterial growth rate increased as well, with maximal values ($\mu = 0.59\ h^{-1}$) at 36 °C. Regarding bacteriocin production, there was a clear correlation with the bacterial growth (Fig. 1), the highest specific inhibitory activities (3.72 and 3.70 AU/10^7 cells) being detected at the end of the exponential growth phase at 20 and 25 °C, respectively (Table 1). Taken into account that at 15 °C bacteriocin activity was low, the kinetics of bacteriocin production was not evaluated at this temperature. When *L. lactis* CRL 1584 was grown at 20 °C, the stationary phase was reached after 14 h. In these conditions, 3200 and 5300 AU/mL were detected by using *L. monocytogenes* and *Lb. plantarum*, respectively. In this period of time, the biomass increased by 3 log units (Fig. 1) and the pH of the cultures decrease 2 units.

At 25 °C the LAB strain reached the stationary phase after 8 h, the bacteriocin activity being 3800 and 6200 AU/mL against *L. monocytogenes* and *Lb. plantarum*, respectively. The biomass increased by 3.5 log units (Fig. 1) and the pH values were similar to those of LAB cultures grown at 20 °C.

Lactococcus lactis CRL 1584 grown at 30 °C showed similar parameters to those observed at 25 °C, with the exception of bacteriocin activity. Indeed, it was

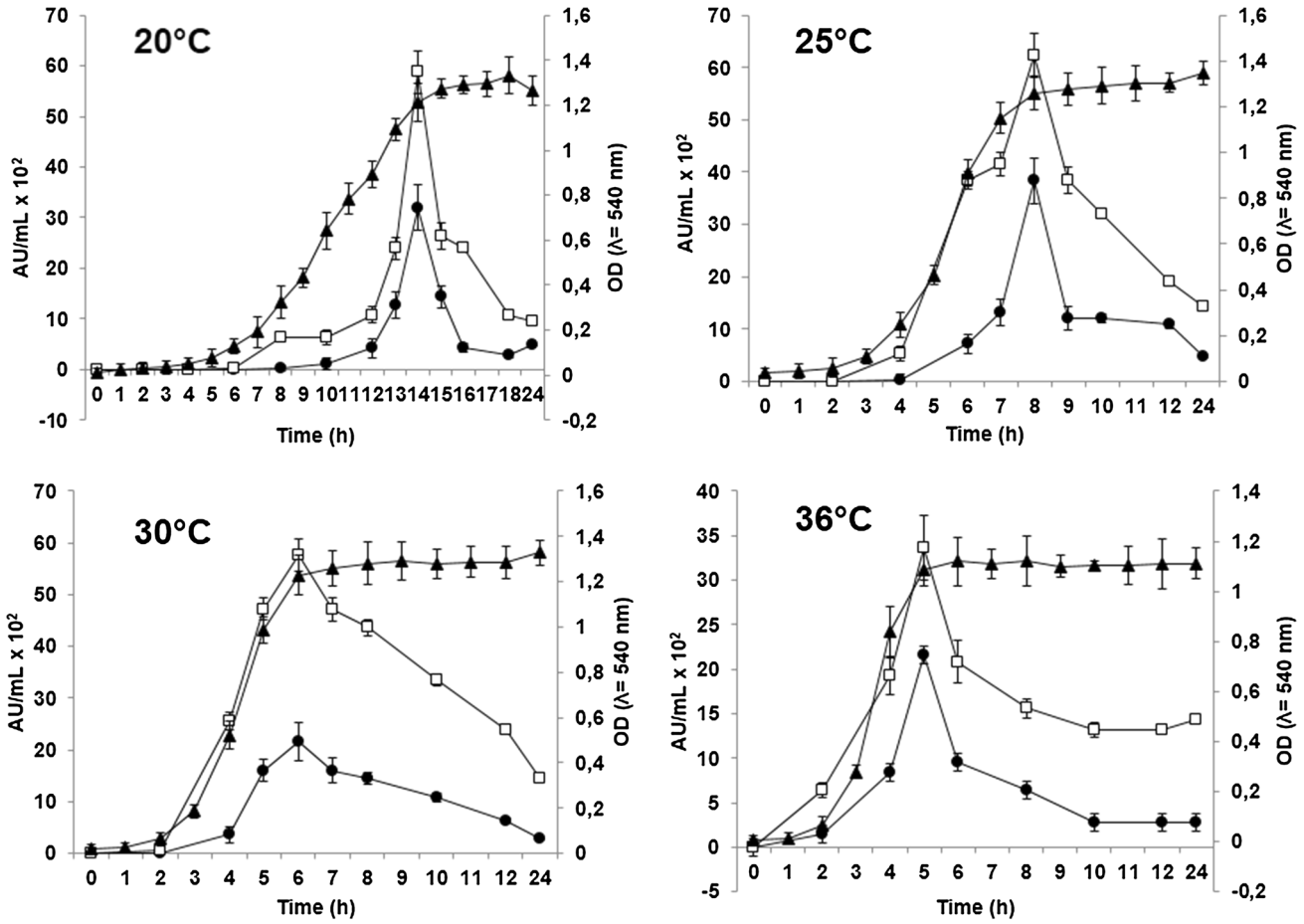


Fig. 1 Growth and bacteriocin production by *L. lactis* CRL 1584, (filled triangle) OD; (open square) AU/mL with *Lb. plantarum*; (filled circle) AU/mL with *L. monocytogenes*

Table 1 Growth rate and specific bacteriocin production by *L. lactis* CRL 1584 at different temperatures of incubation

Temperature (°C)	Growth rate μ (h^{-1})	Specific activity (AU/10^7 cells)		
		Middle exponential phase	End of exponential phase	Stationary phase
15	0.111	1.04	0.92	0.60
20	0.241	1.54	3.72	0.55
25	0.368	2.58	3.70	1.50
30	0.531	1.02	1.80	0.40
36	0.591	1.60	0.90	0.60

significantly lower (2100 AU/mL) when using *L. monocytogenes* as indicator strain (Fig. 1). Howerver, at 36 °C the LAB strain produced 3300 and 2100 AU/mL bacteriocin, measured with *Lb. plantarum* and *L. monocytogenes*, respectively (Fig. 1). In these conditions, the biomass and pH modifications were similar to 25 °C.

Based on the results, 25 °C was selected as the optimal temperature for bacteriocin production.

Purification of bacteriocin/s produced by *Lactococcus lactis* CRL 1584

The antimicrobial activities of 40 and 70% ammonium sulfate samples are shown in Fig. 2. As it can be seen, P40% fractions turned out to have 3800 AU/mL when *L. monocytogenes* was used as indicator strain. However, P70% showed only a residual activity of about 1080 AU/mL. Importantly, no activity was detected in both SS and TS samples. All fractions were also tested against *Lb. plantarum* CRL 1651. Using this strain as the sensitive microorganism, P40% fractions displayed 4920 AU/mL. Once again, residual bacteriocin activity was observed in P70% (2080 AU/mL) and SS (490 AU/mL) fractions (Fig. 2). These results led us to pursue the bacteriocin/s purification with the P40% fraction.

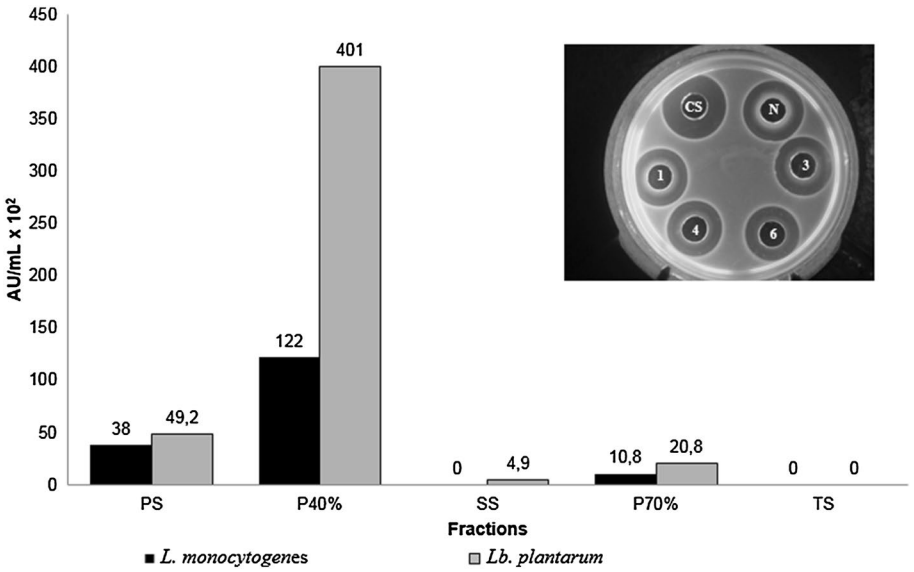


Fig. 2 Bacteriocin activity of fractions post-precipitation of culture supernatants from *L. lactis* CRL 1584 against *L. monocytogenes* and *Lb. plantarum*. Insert figure. *CS* crude supernatant, *N* neutralized supernatant; 1, 3, 4 and 6 represent the dilutions 1/2; 1/3; 1/4 and 1/6 by using *L. monocytogenes* as indicator strain

Partial purification of P40% bacteriocin fractions

Samples from P40% fractions were partially purified by using cationic-exchange chromatography. One milliliter samples were collected and purification was followed spectrophotometrically (λ = 220, 254 and 280 nm). Based on the antimicrobial tests, activity was only found in fractions 22, 23 and 24, which were eluted with 100 mM NaCl (Fig. 3a). Afterward, larger SP-Sepharose columns were used in order to scale-up the bacteriocin purification. Fractions eluted with 50, 200 and 300 mM NaCl-buffer did not display any antimicrobial activity (Fig. 3b).

Purification of the bacteriocin/s by RP-HPLC and MALDI-TOF analysis

Samples obtained so far were separated by SDS-PAGE. As observed in Fig. 4a, lane 1 (from crude supernatant), lane 3 (from P40%) and lane 4 (eluted with 100 mM NaCl-buffer) showed antimicrobial activity. As expected, no activity band was observed in the supernatant post ammonium sulfate precipitation (lane 2). Active fraction eluted from SP-Sepharose was then subjected to RP-HPLC and a prominent peak was observed at 34 min of elution, which turned out to have a strong antimicrobial activity (Fig. 4b). Indeed, the titer was 480 AU/mL when *Lb. plantarum* CRL 1651 was used as indicator strain. Finally, it was submitted to mass

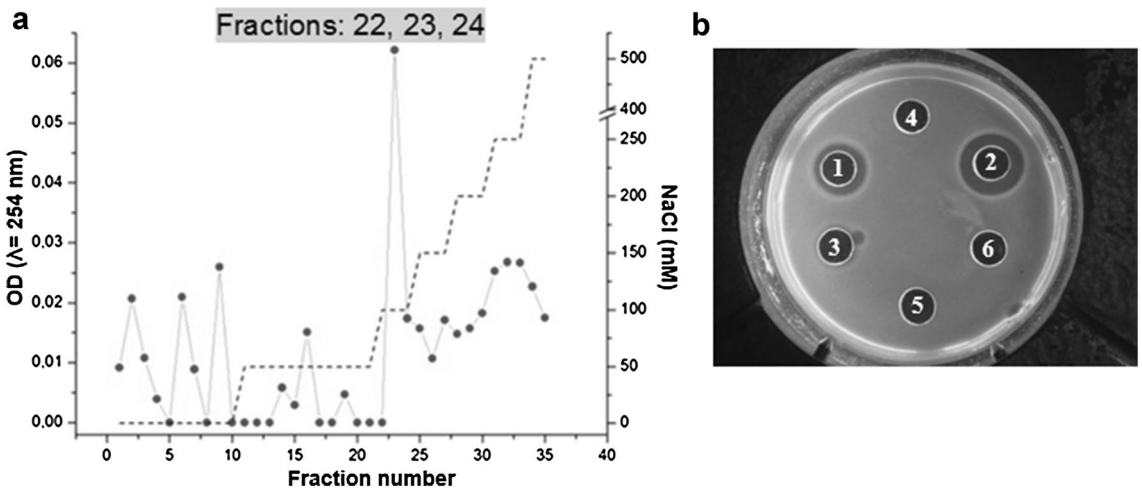


Fig. 3 Absorbance of fractions eluted from the cation exchange column (**a**) and determination of the bacteriocin activity (**b**). (*b*) (1) 30 mL eluted by using 100 mM NaCl-buffer (2) 30 mL eluted by using 100 mM NaCl-buffer and (3) 60 mL eluted by using 100 mM NaCl-buffer. 4, 5 and 6 samples eluted with 50, 200 and 300 mM NaCl-buffer

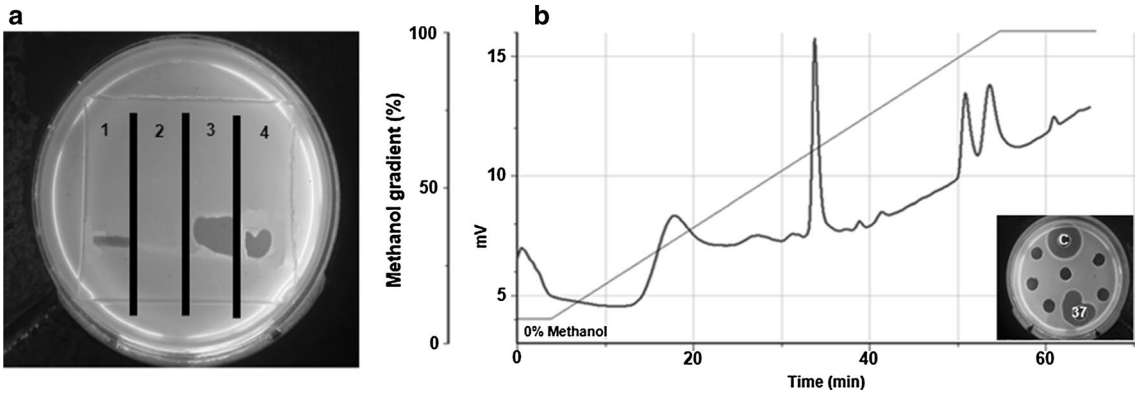


Fig. 4 Biological activity of the antimicrobial fractions (**a**) and purification of bacteriocin/s by RP-HPLC (**b**). (1) First supernatant, (2) second supernatant, (3) precipitate 40%, (4) 100 mM NaCl-buffer. *c* control, 37 number of fraction

spectrometry and MALDI-TOF unequivocally showed a signal of 3353.05 Da that matched nisin Z. However, no other peptide could be found in this sample, strongly suggesting that nisin would be the only bacteriocin produced by *L. lactis* CRL 1584 (data not shown).

Moreover, the purification process of nisin from the commercial product allowed getting a 15 nM nisin solution.

Mode of action of antagonistic metabolites produced by *L. lactis* CRL 1584 against pathogenic bacteria

Studies on the effect of the purified bacteriocin alone or in combination with lactic acid and hydrogen peroxide at their physiological concentrations on the viability of *C. freundii* y *L. monocytogenes* strains, were carried out.

When cells of *C. freundii* were treated with purified nisin alone or combined (Fig. 5a), a reduction of cell viability was observed with similar values in samples from Bacteriocin + Cells (B + C) and B + Hydrogen Peroxide + C (B + HP + C) in which an ~ 2 log units drop was detected after 24 h incubation. However, the highest reduction in *C. freundii* viability was found when lactic acid was also added: B + lactic acid + C (B + LA + C) (3.5 log units) and B + HP + LA + C (3 log units).

The effect of lactic acid and hydrogen peroxide alone and combined on the viability of this bacterium was also determined. Therefore, in samples from *C. freundi* treated with HP a diminution in cell viability (2.5 log units) was observed at 8 h of co-incubation, being stronger in HP + C during the first 4 h. For combined metabolites, a decrease in 3 log units was detected during the first 10 h of co-incubation.

When the purified and lyophilized nisin Z individually and combined with the LAB metabolites was added to *L. monocytogenes* cell suspensions, a diminution in 1 log

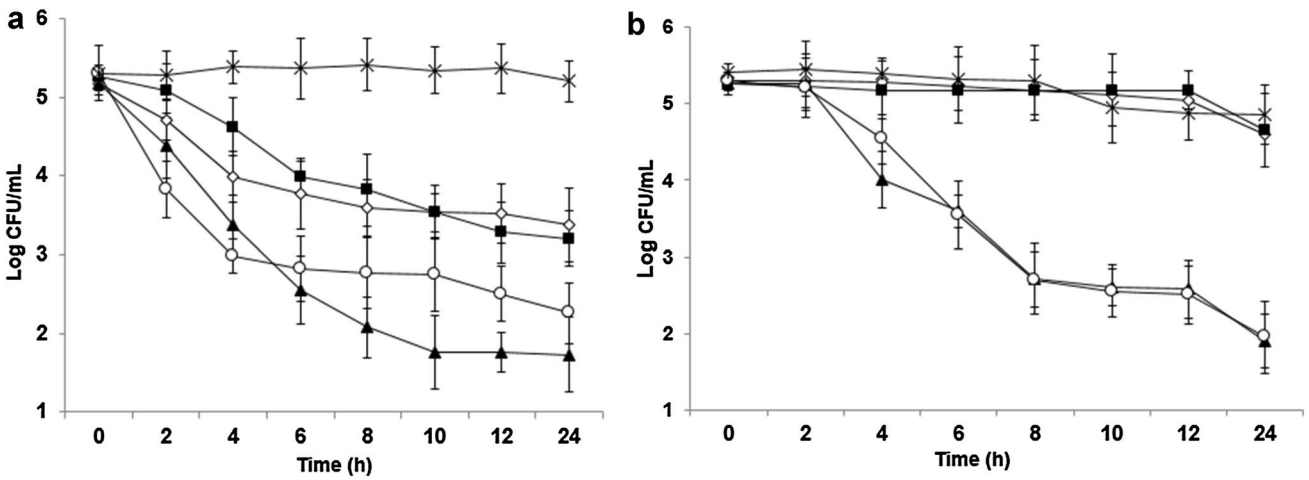
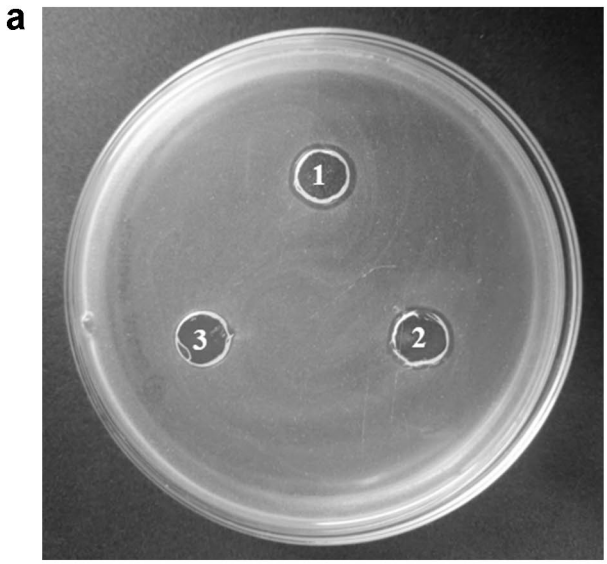
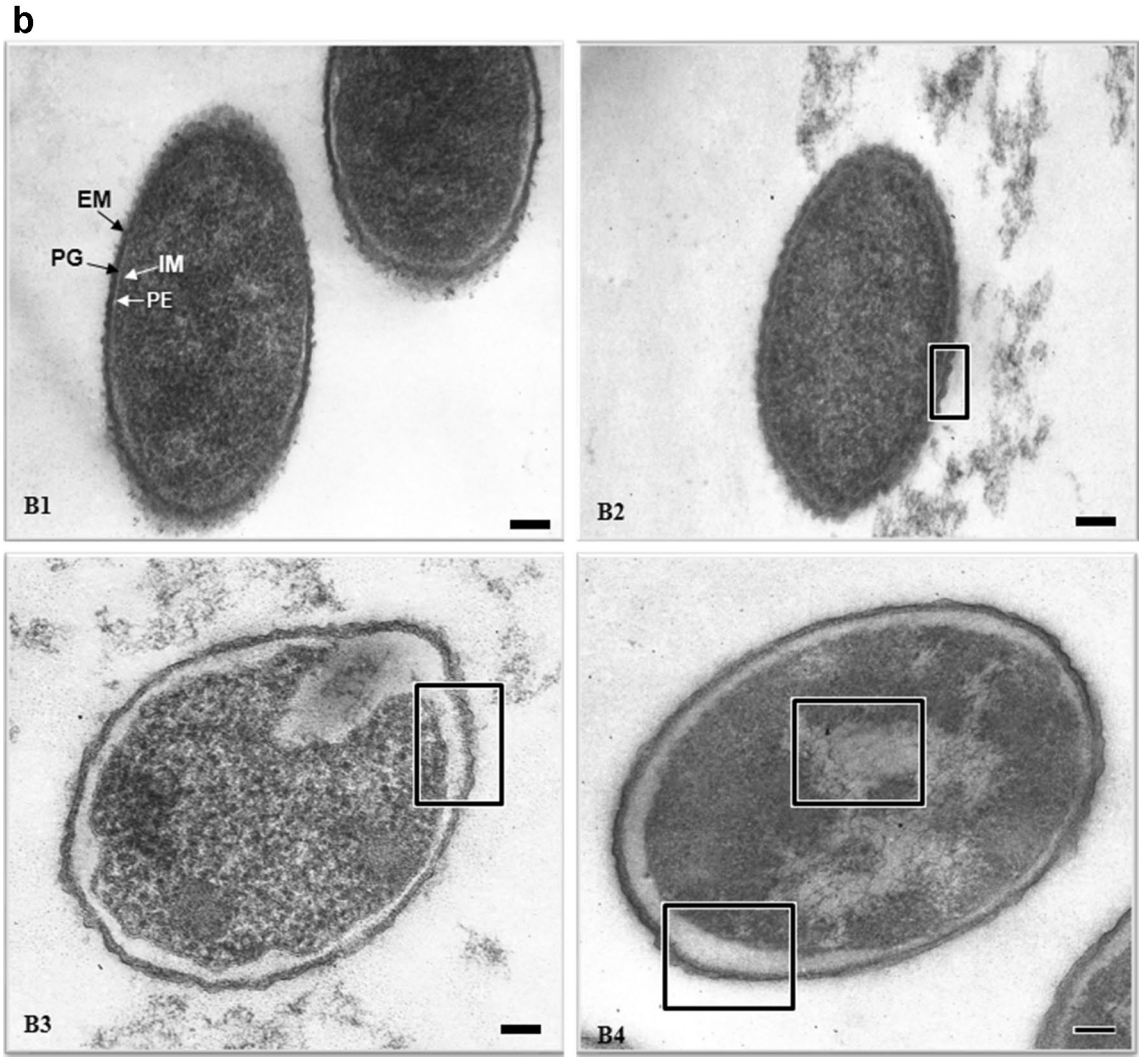


Fig. 5 Effect of nisin alone and combined with lactic acid and hydrogen peroxide on pathogenic bacteria viability. (**a**) *C. freundii*. (**b**) *L. monocytogenes*, (cross) control; (open diamond) Bacteriocin + cells; (filled *square*) Bacteriocin + H_2O_2 + cells; (filled triangle) bacteriocin + L-lactic acid + cells; (open *circle*) bacteriocin + L-lactic acid + H_2O_2 + cells


a
1
2
3


b
EM
PG
IM
PE
B1
B2
B3
B4

◂**Fig. 6** Effect of commercial purified nisin on *C. freundii* cells. **a** Inhibitory activity on *C. freundii*: (1) 15 nM nisin, (2) 7.5 nM nisin, (3) 3.75 nM nisin. **b** Transmission Electronic Microscopy of *C. freundii* cells (50,080). Scale bar: 200 nm. (*B1*) Control: *EM* outer membrane, *PG* peptidoglycan, *IM* inner membrane, *PE* periplasmic space, (*B2*) 7.5 nM nisin, (*B3*) 7.5 nM nisin + 0.01 mM H_2O_2, (*B4*) 7.5 nM nisin + 5 g/L L-lactic acid. Rectangles show the main cell alteration areas

unit of cell viability was observed at the end of the assay in samples from B + C and B + HP + C. However, samples containing lactic acid (B + LA + C and B + HP + LA + C), showed a decrease of 3.5 log units after 24 h of co-incubation (Fig. 5b).

The assays of agar-well diffusion by using purified nisin from the commercial product against *C. freundii* were carried out. As shown in Fig. 6a, 7.5 nM nisin is enough to inhibit the growth of the Gram-negative bacterium. Moreover, when cells of *C. freundii* were treated with 7.5 nM nisin and their combinations with L-lactic acid or hydrogen peroxide, killing curves were similar to those obtained by using 480 AU/mL of nisin from *L. lactis* CRL 1584 (data not shown).

The purified commercial nisin produced ultrastructural changes on *C. freundi* (Fig. 6b). Therefore, when cells were exposed to 7.5 nM nisin, invaginations along the whole cellular surface were detected (Fig. 6B2); while with nisin + H_2O_2 or nisin + L-lactic acid, cytoplasm contractions, enhancement of the periplasmic space and invaginations along the cell wall were observed (Fig. 6B2, B3). It is interesting to point out that *C. freundii* treated with H_2O_2 or lactic acid showed an enhancement of cellular size.

Discussion

Lactococcus lactis is a LAB used in the manufacture of fermented dairy products such as milk and cheese (Dijkstra et al. 2014; De Angelis et al. 2015). This species has been isolated from different ecological niches including soils (Yanagida et al. 2008), plants (Ishibashi et al. 2015) and intestine surface (Takanashi et al. 2014), suggesting its adaptability to different environments. Moreover, *L. lactis* strains were also isolated from the intestinal tract of both freshwater and saltwater fishes (Itoi et al. 2009; Sequeiros et al. 2010), although some strains were related to infectious diseases in aquaculture (Wang et al. 2008; Chen et al. 2012).

Many *L. lactis* strains have been proposed as probiotics in aquaculture based on their tolerance to the gastrointestinal conditions (Takanashi et al. 2014), ability to adhere to mucus, to colonize the gut (Sugimura et al. 2011; Lukić et al. 2012) and also to the immunostimulatory effect on fish species (Pérez-Sánchez et al. 2011). Regarding their antagonist activities, some strains were able to inhibit fish pathogens by the action of organic acids (Hagi and Hoshino 2009) and H_2O_2 (Sugita et al. 2007). Interestingly, *L. lactis* TW34, isolated from fish gastrointestinal tract (Sequeiros et al. 2010, 2015), and a strain isolated from olive flounder (Heo et al. 2012) were shown to produce bacteriocins. These strains were able to control *L. garvieae* and *Streptococcus iniae* responsible for lactococcosis and streptococcosis, respectively in freshwater and saltwater fishes.

Lactococcus lactis subsp. *lactis* CRL 1584 is a native strain from bullfrog hatchery that inhibits *C. freundii*, a RLS-related pathogen, and *L. monocytogenes* Scott A by a synergistic effect among lactic acid (5 g/l), hydrogen peroxide (0.01 mM) and bacteriocin (2100 AU/mL). These features, combined with its hydrophilic properties, which would favor the colonization of the intestinal mucus, support the potential use of the LAB strain as a probiotic for American bullfrog culture (Pasteris et al. 2009b, 2011, 2017).

In this work, we evaluated the growth parameters of *L. lactis* CRL 1584 and the bacteriocin production. Moreover, purification steps were carried out in order to know whether or not other bacteriocins were co-produced. The analysis of the activity against pathogenic bacteria was carried out to evaluate its potential use in a probiotic product or as biopreservative for bullfrog meat.

The bacteriocin production was tightly associated to the growth as reported in *L. lactis* TW34 (Sequeiros et al. 2010), 194-K (Ustiugova et al. 2012), Z11 (Enan et al. 2013) and 69 (Biscola et al. 2013). *L. lactis* CRL 1584, like the strains studied by other authors, reached maximum values of bacteriocin activity at the end of the exponential growth phase and then decreased. It is important to note that *Lb. plantarum* was more sensitive than *L. monocytogenes*.

The highest bacteriocin concentration was detected when *L. lactis* was incubated at 20 and 25 °C. Interestingly, the lowest bacteriocin activity was detected at 36 °C. In all cases, the secreted antimicrobial activity peaked at the end of the exponential phase and then slowly decreased over time during stationary phase. Thus, we may suggest that under the incubation conditions used in this work, bacteriocin would form aggregates as reported for pediocin N5p (Manca Nadra et al. 1998), acidocin CH5 (Chumchalová et al. 2004) and a bacteriocin produced by *L. lactis* subsp. *lactis* LL171 (Kumari et al. 2012). Actually, it has been demonstrated that bacteriocin activity increased when culture supernatants were treated with non-ionic detergents, which might act as dispersing agents that can release bacteriocin monomers from the peptide aggregates (Diop et al. 2007).

In the case of *L. lactis* CRL 1584, purification was an important requirement since previous studies indicated that this strain would produce nisin Z and the culture supernatants of the LAB strain inhibited *C. freundii* (Pasteris et al. 2011, 2013). However, nisin is well-known for inhibiting only Gram-positive bacteria and is ineffective

against Gram-negatives. Indeed, nisin can only act on Gram-negative bacteria upon addition of chelating agents (Delves-Broughton 2005). Thus, we assumed that there were other bacteriocins that were being co-produced, as it was previously demonstrated in *L. lactis* from sheep milk (Bravo et al. 2009), *Enterococcus faecium* (Ibarguren et al. 2010) and *Bacillus subtilis* (Compaoré et al. 2013).

Under our experimental conditions, no bacteriocin co-production could be detected which would indicates that nisin inhibits *C. freundii*, a RLS-related pathogen responsible for mass mortality in bullfrog hatchery (Pasteris et al. 2006). So, we decided to evaluate the mechanism of action of the purified nisin (480 AU/mL) from *L. lactis* CRL 1544, individually or combined with the other antagonistic metabolites produced by the LAB strain (Pasteris et al. 2011) on pathogenic bacteria. Thus, a bactericidal effect on *C. freundii* and *L. monocytogenes* was demonstrated, which increased when metabolic end-product were added, being higher with lactic acid since acidic conditions favored the nisin action (Tokarskyy and Marshall 2008).

The synergistic effect observed with pure nisin Z was corroborated by using purified commercial nisin, which displayed a similar activity against *C. freundii*, with and without the physiological concentrations of L-lactic acid/hydrogen peroxide produced by *L. lactis*. These results are supported by the TEM evaluations that showed cytosolic alterations in the presence of the organic compounds.

In conclusion, nisin can be an excellent alternative as biopreservative of frog legs and carcasses and could be used individually or combined with other bacteriocins as proposed for the control of pathogens in milk (Arauz et al. 2009; Arqués et al. 2011). It could also be included in the formulation of probiotic products for bullfrog breeding to prevent epizootics associated with a Gram-negative bacterium (*C. freundii*) and potentially in amphibians endangered species to repopulate devastated environments. Thus, this work contribute to the knowledge not only for biotechnology but also for ecology.

Acknowledgements This research was supported by grants from Consejo de Investigaciones de la Universidad Nacional de Tucumán (26/D 414 and 26/D 528), Consejo de Nacional de Investigaciones Científicas y Técnicas (PIP 744 and 063) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 2998).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Acuña L, Picariello G, Sesma F, Morero RD, Bellomio A (2012) A new hybrid bacteriocin, Ent35-MccV, displays antimicrobial activity against pathogenic gram-positive and gram-negative bacteria. FEBS Open Bio 2:12–19

Arauz LJ, Jozala AF, Mazzola PG, Junior AP, Penna TCV (2009) Nisin biotechnological production and application: a review. Trends Food Sci Technol 20:146–154

Araújo C, Muñoz-Atienza E, Pérez-Sánchez T, Poeta P, Igrejas G, Hernández PE, Herranz C, Ruiz-Zarzuela I, Cintas LM (2015) Nisin Z production by *Lactococcus lactis* subsp. *cremoris* WA2-67 of aquatic origin as a defense mechanism to protect rainbow trout (*Oncorhynchus mykiss*, Walbaum) against *Lactococcus garvieae*. Mar Biotechnol 17(6):820–830

Arqués JL, Rodríguez E, Nuñez M, Medina M (2011) Combined effect of reuterin and lactic acid bacteria bacteriocins on the inactivation of food-borne pathogens in milk. Food Control 22:457–461

Biscola V, Todorov SD, Capuano VS, Abriouel H, Gálvez A, Franco BD (2013) 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. Meat Sci 93(3):607–613

Bravo D, Rodríguez E, Medina M (2009) Nisin and lacticin 481 coproduction by *Lactococcus lactis* strains isolated from raw ewes milk. J Dairy Sci 92(10):4805–4811

Chen MH, Hung SW, Shyu CL, Lin CC, Liu PC, Chang CH, Shia WY, Cheng CF, Lin SL, Tu CY, Lin YH, Wang WS (2012) *Lactococcus lactis* subsp. l*actis* infection in Bester sturgeon, a cultured hybrid of *Huso huso* × *Acipenser ruthenus*, in Taiwan. Res Vet Sci 93(2):581–588

Chumchalová J, Stiles J, Josephsen J, Plocková M (2004) Characterization and purification of acidocin CH5, a bacteriocin produced by *Lactobacillus acidophilus* CH5. J Appl Microbiol 96(5):1082–1089

Compaoré CS, Nielsen DS, Ouoba LI, Berner TS, Nielsen KF, Sawadogo-Lingani H, Diawara B, Ouédraogo GA, Jakobsen M, Thorsen L (2013) Co-production of surfactin and a novel bacteriocin by *Bacillus subtilis* subsp. *subtilis* H4 isolated from Bikalga, an African alkaline *Hibiscus sabdariffa* seed fermented condiment. Int J Food Microbiol 162(3):297–307

Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3:777–788

Cui Y, Zhang C, Wang Y, Shi J, Zhang L, Ding Z, Qu X, Cui H (2012) Class IIa bacteriocins: diversity and new developments. Int J Mol Sci 13:16668–16707

de Kwaadsteniet M, ten Doeschate K, Dicks LMT (2008) Characterization of the structural gene encoding nisin F, a new lantibiotic produced by a *Lactococcus lactis* subsp. *lactis* isolate from freshwater catfish (*Clarias gariepinus*). Appl Environ Microbiol 74(2):547–549

De Angelis M, Campanella D, Cosmai L, Summo C, Rizzello CG, Caponio F (2015) Microbiota and metabolome of un-started and started Greek-type fermentation of Bella di Cerignola table olives. Food Microbiol 52:18–30

Delves-Broughton J (2005) Nisin as food preservative. Food Australia 57(12):525–527

Dijkstra AR, Alkema W, Starrenburg MJ, Hugenholtz J, van Hijum SA, Bron PA (2014) Fermentation-induced variation in heat and oxidative stress phenotypes of *Lactococcus lactis* MG1363 reveals transcriptome signatures for robustness. Microb Cell Fact 13:148

Diop MB, Robin DD, Emmanuel T, Abib N, Jaqueline D, Philippe T (2007) Bacteriocin producers from traditional food products. Biotechnol Agron Soc Environ 11:1370–1377

EFSA (2006) Opinion of the AFC panel related to the use of nisin (E 234) as a food additive. Available from http://www.efsa.europa.eu/en/science/afc/afc_opinions/1392.html

Enan G, Abdel-Shafi S, Ouda S, Negm S (2013) Novel antibacterial activity of *Lactococcus lactis* subspecies *lactis* z11 isolated from zabady. Int J Biomed Sci 9(3):174–180

Fernández A, Horn N, Gasson MJ, Dodd HM, Rodríguez JM (2004) High-level coproduction of the bacteriocins nisin A and lactococcin A by *Lactococcus lactis*. J Dairy Res 71(2):216–221

Gutiérrez S, Martínez-Blanco H, Rodríguez-Aparicio LB, Ferrero MA (2016) Effect of fermented broth from lactic acid bacteria on pathogenic bacteria proliferation. J Dairy Sci 99(4):2654–2665

Hagi T, Hoshino T (2009) Screening and characterization of potential probiotic lactic acid bacteria from cultured common carp intestine. Biosci Biotechnol Biochem 73(7):1479–1483

Heo WS, Kim EY, Kim YR, Hossain MT, Kong IS (2012) Salt effect of nisin Z isolated from marine fish on the growth inhibition of *Streptococcus iniae*, a pathogen of streptococcosis. Biotechnol Lett 34(2):315–320

Ibarguren C, Raya RR, Apella MC, Audisio MC (2010) *Enterococcus faecium* isolated from honey synthesized bacteriocin-like substances active against different *Listeria monocytogenes* strains. J Microbiol 48(1):44–52

Ishibashi N, Seto H, Koga S, Zendo T, Sonomoto K (2015) Identification of *Lactococcus*-specific bacteriocins produced by lactococcal isolates, and the discovery of a novel bacteriocin, lactococcin Z. Probiotics Antimicrob Proteins 7(3):222–231

Itoi S, Yuasa K, Washio S, Abe T, Ikuno E, Sugita H (2009) Phenotypic variation in *Lactococcus lactis* subsp. lactis isolates derived from intestinal tracts of marine and freshwater fish. J Appl Microbiol 107(3):867–874

Jiménez JJ, Diep DB, Borrero J, Gútiez L, Arbulu S, Nes IF, Herranz C, Cintas LM, Hernández PE (2015) Cloning strategies for heterologous expression of the bacteriocin enterocin A by *Lactobacillus sakei* Lb790, *Lb. plantarum* NC8 and *Lb. casei* CECT475. Microb Cell Fact 14(1):166

Knerr PJ, van der Donk WA (2013) Chemical synthesis of the lantibiotic lacticin 481 reveals the importance of lanthionine stereochemistry. J Am Chem Soc 135(19):7094–7097

Kueneman JG, Woodhams DC, Harris R, Archer HM, Knight R, McKenzie VJ (2016) Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. Proc Biol Sci 283(1839)

Kumari A, Akkoç N, Akçelik M (2012) Purification and partial characterization of bacteriocin produced by *Lactococcus lactis* ssp. *latics* LL171. World J Microbiol Biotechnol 28(4):1647–1655

Lasta S, Ouzari H, Andreotti N, Fajloun Z, Mansuelle P, Boudabous A, Sampieri F, Sabatier JM (2012) Lacticin LC14, a new bacteriocin produced by *Lactococcus lactis* BMG6.14: isolation, purification and partial characterization. Infect Disord Drug Targets 12(4):316–325

Le Lay C, Fernandez B, Hammami R, Ouellette M, Fliss I (2015) On *Lactococcus lactis* UL719 competitivity and nisin (Nisaplin®) capacity to inhibit *Clostridium difficile* in a model of human colon. Front Microbiol 6:1020

Lianou A, Samelis J (2014) Addition to thermized milk of *Lactococcus lactis* subsp. *cremoris* M104, a wild, novel nisin A-producing strain, replaces the natural antilisterial activity of the autochthonous raw milk microbiota reduced by thermization. J Food Prot 77(8):1289–1297

Ljubobratovic U, Kosanovic D, Vukotic G, Molnar Z, Stanisavljevic N, Ristovic T, Peter G, Lukic J, Jeney G (2017) Supplementation of lactobacilli improves growth, regulates microbiota composition and suppresses skeletal anomalies in juvenile pike-perch (*Sander lucioperca*) reared in recirculating aquaculture system (RAS): a pilot study. Res Vet Sci 115:451–462

Loh TC, Choe DW, Foo HL, Sazili AQ, Bejo MH (2014) Effects of feeding different postbiotic metabolite combinations produced by *Lactobacillus plantarum* strains on egg quality and production performance, faecal parameters and plasma cholesterol in laying hens. BMC Vet Res 10:149

Lukić J, Strahinić I, Jovčić B, Filipić B, Topisirović L, Kojić M, Begović J (2012) Different roles for lactococcal aggregation factor and mucin binding protein in adhesion to gastrointestinal mucosa. Appl Environ Microbiol 78(22):7993–8000

Manca de Nadra MC, Sandino de Lamelas D, Strasser de Saad AM (1998) Pediocin N_5p from *Pediococcus pentosaceus*: adsorption on bacterial strains. Int J Food Microbiol 39(1–2):79–85

Mirkovic N, Polovic N, Vukotic G, Jovcic B, Miljkovic M, Radulovic Z, Diep DB, Kojic M (2016) *Lactococcus lactis* LMG2081 produces two bacteriocins, a nonlantibiotic and a novel lantibiotic. Appl Environ Microbiol 82(8):2555–2562

Montel Mendoza G, Pasteris SE, Otero MC, Nader-Macías ME (2013) Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. J Appl Microbiol 116(1):157–166

Pasteris SE, Bühler MI, Nader-Macías ME (2006) Microbiological and histological studies in farmed-bullfrog (*Rana catesbeiana*) displaying red-leg syndrome. Aquaculture 251:11–18

Pasteris SE, Roig Babot G, Otero MC, Nader-Macías ME (2009a) Beneficial properties of lactic acid bacteria isolated from a *Rana castesbeiana* hatchery. Aquac Res 40:1605–1615

Pasteris SE, Vera Pingitore E, Roig Babot G, Otero MC, Bühler MI, Nader-Macías ME (2009b) Characterization of the beneficial properties of lactobacilli isolated from bullfrog (*Rana catesbeiana*) hatchery. Antonie Van Leeuwenhoek 95:375–385

Pasteris SE, Guidoli MG, Otero MC, Bühler MI, Nader-Macías ME (2011) In vitro inhibition of *Citrobacter freundii*, a red-leg syndrome associated pathogen in raniculture, by indigenous *Lactococcus lactis* CRL 1584. Vet Microbiol 151:336–344

Pasteris SE, Vera Pingitore E, Ale CE, Nader-Macías ME (2013) Characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CRL 1584 isolated from a *Lithobates catesbeianus* hatchery. World J Microbiol Biotechnol 30(3):1053–1062

Pasteris SE, Montel Mendoza G, Llanos RJ, Pucci Alcaide F, Nader-Macías MEF (2017) Preliminary assessment of in vivo safety of potentially probiotic lactic acid bacteria for American bullfrog culture. Aquac Res 48(5):2157–2172

Pereira SA, Jerônimo GT, da Costa Marchiori N, de Oliveira HM, Owatari MS, Jesus GFA, Garcia P, do Nascimento Vieira F, Martins ML, Mouriño JLP (2016) Autochthonous probiotic *Lactobacillus* sp. in the diet of bullfrog tadpoles *Lithobates catesbeianus* improves weight gain, feed conversion and gut microbiota. Aquac Nutr. doi:10.1111/anu.12458

Pérez-Sánchez T, Balcázar JL, Merrifield DL, Carnevali O, Gioacchini G, de Blas I, Ruiz-Zarzuela I (2011) Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection. Fish Shellfish Immun 31(2):196–201

Raibaud P, Gapin JV, Ducluzeau R, Mocquot G, Oliver G (1963) Le genre *Lactobacillus* dans le tube digestif du Rat.II Caractères de souches hétérofermentaires isolées de rats “Holo” et “Gnotoxeniques”. Ann Microbiol 124:2223–2235

Reid G (2017) Probiotic use in an infectious disease setting. Expert Rev Anti Infect Ther 15(5):449–455

Reid G, Sanders ME, Rex Gaskins H, Gibson GR, Mercenier A, Rastall R, Roberfroid M, Rowland I, Cherbut C, Klaenhammer TR (2003) New scientific paradigms for probiotics and prebiotics. J Clin Gastroenterol 37(2):105–118

Samelis J, Lianou A, Pappa EC, Bogovič-Matijašić B, Parapouli M, Kakouri A, Rogelj I (2014) Behavior of *Staphylococcus aureus* in culture broth, in raw and thermized milk, and during processing

and storage of traditional Greek Graviera cheese in the presence or absence of *Lactococcus lactis* subsp. *cremoris* M104, a wild, novel nisin A-producing raw milk isolate. J Food Prot 77(10):1703–1714

Sequeiros C, Vallejo M, Marguet ER, Olivera NL (2010) Inhibitory activity against the fish pathogen *Lactococcus garvieae* produced by *Lactococcus lactis* TW34, a lactic acid bacterium isolated from the intestinal tract of a Patagonian fish. Arch Microbiol 192:237–245

Sequeiros C, Garcés ME, Vallejo M, Marguet ER, Olivera NL (2015) Potential aquaculture probiont *Lactococcus lactis* TW34 produces nisin Z and inhibits the fish pathogen *Lactococcus garvieae*. Arch Microbiol 197(3):449–458

Sugimura Y, Hagi T, Hoshino T (2011) Correlation between in vitro mucus adhesion and the in vivo colonization ability of lactic acid bacteria: screening of new candidate carp probiotics. Biosci Biotechnol Biochem 75(3):511–515

Sugita H, Ohta K, Kuruma A, Sagesaka T (2007) An antibacterial effect of *Lactococcus lactis* isolated from the intestinal tract of Amur catfish, *Silurus asotus* Linnaeus. Aquac Res 38:1002–1004

Tabanelli G, Montanari C, Bargossi E, Lanciotti R, Gatto V, Felis G, Torriani S, Gardini F (2014) Control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin forming lactococci. Int J Food Microbiol 190:14–23

Takanashi S, Miura A, Abe K, Uchida J, Itoi S, Sugita H (2014) Variations in bile tolerance among *Lactococcus lactis* strains derived from different sources. Folia Microbiol 59(4):289–293

Tokarskyy O, Marshall DL (2008) Mechanism of synergistic inhibition of *Listeria monocytogenes* growth by lactic acid, monolaurin, and nisin. Appl Environ Microbiol 74(23):7126–7129

Ünlü G, Nielsen B, Ionita C (2016) Inhibition of *Listeria monocytogenes* in hot dogs by surface application of freeze-dried bacteriocin-containing powders from lactic acid bacteria. Probiotics Antimicrob Proteins 8(2):102–110

Uraipan S, Hongpattarakere T (2015) Antagonistic characteristics against food-borne pathogenic bacteria of lactic acid bacteria and bifidobacteria isolated from feces of healthy Thai infants. Jundishapur J Microbiol 8(6):e18264

Ustiugova EA, Timofeeva AV, Stoianova LG, Netrusov AI, Katrukha GS (2012) Characteristics and identification of bacteriocins produced by *Lactococcus lactis* subsp. *lactis* 194-K. Prikladnaia Biokhimiia Mikrobiologiia 48(6):618–625

van Staden AD, Brand AM, Dicks LM (2012) Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus* in vivo. J Appl Microbiol 112(4):831–840

Wang PC, Lin YD, Liaw LL, Chern RS, Chen SC (2008) *Lactococcus lactis* subspecies *lactis* also causes white muscle disease in farmed giant freshwater prawns *Macrobrachium rosenbergii*. Dis Aquat Organ 79(1):9–17

Yanagida F, Srionnual S, Chen YS (2008) Isolation and characteristics of lactic acid bacteria from koshu vineyards in Japan. Lett Appl Microbiol 47(2):134–139