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Characterization of a Bacteriocin Produced by *Enterococcus gallinarum* CRL 1826 Isolated from Captive Bullfrog: Evaluation of its Mode of Action against *Listeria monocytogenes* and Gram-Negatives

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Abstract

Enterococcus gallinarum CRL 1826 isolated from an American bullfrog (*Lithobates catesbeianus*) skin inhibits the growth of *Citrobacter freundii*, *Pseudomonas aeruginosa* (bullfrog pathogens) and *Listeria monocytogenes* by a synergistic effect between organic acids and a bacteriocin-like molecule. This bacteriocin, named enterocin CRL 1826, showed a proteinaceous nature, heat stability and polar characteristics. Its production followed kinetics of primary metabolites synthesis reaching a maximum of 61,400 AU/mL. The minimum inhibitory and minimum bactericidal concentrations were 2,640 and 5,280 AU/mL, respectively, against *L. monocytogenes*. The addition of 120,000 AU/mL of enterocin to growing *L. monocytogenes* and Gram-negative (*P. aeruginosa* and *C. freundii*) bacteria showed bactericidal and bacteriostatic effects, respectively. However, enterocin derived-peptides had bactericidal effect only against Gram-negatives.

Enterocin produced cell envelope damages and efflux of citosolic content on *L. monocytogenes*, while enterocin derived-peptides showed granulation and contraction of cytoplasm material on *P. aeruginosa* and increase in the periplasmic space and empty cells appearance on *C. freundii*.

Enterocin CRL 1826 is the first bacteriocin described for *E. gallinarum* from raniculture. It could be used as a biopreservative while the derived-peptides represent an alternative to control multi-drug resistant Gram-negatives. The antimicrobial spectrum and the stability of enterocin and its derived-peptides indicate that they could be applied in different biotechnological areas.

Keywords: *Enterococcus gallinarum*; Raniculture; Bacteriocin; Probiotics; Biopreservatives

Introduction

Lactic Acid Bacteria (LAB) isolated from aquatic environments produce a range of antagonistic molecules such as organic acids, hydrogen peroxide, diacetyl, and bacteriocins [1]. The characterization of these compounds supports the selection of beneficial LAB. Thus, some species have been proposed as probiotics to restore beneficial microbial populations that could help to control potentially pathogenic microorganisms in general aquaculture [2,3] and raniculture [4-6].

Probiotic products are generally designed with single or mixed beneficial bacterial strains, mainly LAB, but they could also include different bioactive compounds such as prebiotics (inulin) or bacteriocins [7]. Postbiotic metabolites (lactic and acetic acids and bacteriocins) produced by LAB strains have been extensively studied as feed additive to achieve high productivity and better laying hens' health while reducing in-feed antibiotics [8].

Bacteriocins are ribosomally-synthesized antimicrobial peptides or proteins produced by different bacterial genera. They are usually active against genetically close species and have been grouped into four classes according to their genetic and biochemical characteristics and mode of action [9]. In recent years, bacteriocins have attracted increasing interest for their use as biopreservatives in food industry, Nisin and Pediocin PA, being commercially available as Nisaplin and ALTA 2341, respectively. They are used as food additives [10] according to their GRAS (Generally Regarded as Safe) characteristics [11-13]. More recently, the effectiveness of Nisin A to reduce spoilage bacteria in high-fat chilled dairy dessert, a milk-based pudding, was reported [14].

Listeria is a ubiquitous bacterium extremely dangerous for high risk human populations. *Listeria monocytogenes* is the most pathogenic species associated with aquaculture products such as raw, smoked and fermented fish and crab meat [15,16]. It is a frequent inhabitant isolated from different frog species including the American bullfrog (*Lithobates catesbeianus*) [17] which meat is considered a delicacy in the international gastronomy. Thus, this pathogenic microorganism could be transferred as food-borne bacteria during bullfrogs' meat manufacturing.

Enterocins are bacteriocins produced by *Enterococcus* species that are able to inhibit Gram-positives such as *L. monocytogenes* [18-23] and some Gram-negatives in a lesser degree [24-26].

In previous studies, we demonstrated that Gram-negative bacteria are responsible for Red-Leg Syndrome (RLS) outbreaks in raniculture: Also, enterococci were isolated from the autochthonous microbiota

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associated with *L. catesbeianus* hatchery environments [4,6,27]. *Enterococcus gallinarum* CRL 1826 isolated from the bullfrog skin inhibited the growth of *L. monocygenes* Scott A by a synergistic effect among lactic acid, hydrogen peroxide and a bacteriocin-like metabolite [6]. Preliminary studies have indicated that bacteriocin containing culture supernatants treated with proteolytic enzymes inhibited the growth of RLS-related pathogens (*Pseudomonas aeruginosa* and *Citrobacter freundii*). Taking into account that enteroccoci are considered no GRAS species [28,29], *E. gallinarum* CRL 1826 cannot be included in probiotics for raniculture. However, the natural products (bacteriocin/s) could be used as biopreservative to reduce *L. monocytogenes* prevalence or as a bioactive ingredient for probiotics formulation.

Thus, the present study aimed to characterize the bacteriocin from *E. gallinarum* CRL 1826 culture supernatants and to evaluate its mode of action against a food-borne bacterium (*L. monocytogenes*) and indigenous RLS-related pathogens, as a potential bioactive agent to be included as biopreservative during the manufacture of bullfrog carcasses and probiotics' formulation, respectively.

Materials and Methods

Bacterial strains and culture conditions

Enterococcus gallinarum CRL 1826 was isolated from the ventral skin of healthy *L. catesbeianus* and identified by phenotypic and genotypic tests [6]. The strain was grown on MRS broth [30], LAPT g broth [31] pH 6.8 and Brain Heart Infusion (BHI) broth pH 7.4, for 10 h at 37°C in a 5% CO_2-enriched chamber (microaerophilic conditions).

Pseudomonas aeruginosa 1047 and *Citrobacter freundii* CFb isolated from raniculture were grown in nutritive broth (in g/L: pluripeptone, 5; meat extract, 3), pH 6.9 for 7 h while *Listeria monocytogenes* Scott A was cultured in BHI broth, for 6 h. All cultures were incubated at 37°C in microaerophilic conditions [6,32]. Bacterial strains were stored at -20°C in their specific growth media supplemented with 20% (w/v) glycerol.

Characterization of the bacteriocin produced by *Enterococcus gallinarum* CRL 1826

One-hundred milliliters of 10 h cultures of *E. gallinarum* CRL 1826 grown in MRS, LAPTg and BHI broth were centrifuged (3,000 *g* at 4°C, 20 min) and 3 mL fractions of crude (untreated), neutralized (NS) and neutralized+catalase (treated) supernatants were used to determine the chemical nature of the bacteriocin-like metabolite. Its activity was evaluated by the agar-well diffusion assay according to Pasteris et al. [4]. Thus, soft BHI agar (0.7% w/v) plates were inoculated with 1×10^5 CFU/mL *L. monocytogenes*. Then, 100 µL of untreated, NS, and treated supernatants were added to the plates in which 10 mm holes had been previously punched. Fractions of crude supernatants were formerly adjusted to pH 7.0 with 1 N NaOH (NS) and NS samples were supplemented with 0.5 mg/mL catalase (1 h at 25°C) to abolish the inhibitory effect due to organic acids and H_2O_2, respectively [4].

The antimicrobial titer was defined as the reciprocal of the highest two fold dilution able to produce a clear zone of inhibition and was expressed as arbitrary units per milliliter of culture supernatant (AU/mL).

Chemical nature of the bacteriocin

Two-milliliter fractions of treated supernatants from MRS cultures were supplemented with 1 mg/mL pepsin, α-chymotrypsin, trypsin, lipase and α-amylase. Enzymes were suspended in their specific buffer solutions according to the suppliers' indications [32]. Positive and negative controls were performed with enzyme solutions and treated fractions diluted with sterile water, respectively. Enzymatic treatments of cell-free supernatants were carried out for 1 h at 37°C except for α-chymotrypsin that were incubated for 1 h at 25°C [32].

Physicochemical characterization of the bacteriocin produced by the lactic acid bacterium

Effect of organic solvents and filtration on bacteriocin activity: Five-milliliter fractions of untreated and treated supernatant were supplemented with different organic solvents: 10 and 20% (v/v) hexadecane, ethyl acetate, chloroform and ethanol [4,5].

Moreover, 2-mL fractions of untreated and treated supernatants were filtered throughout 0.22 µm Millipore membranes (Sigma-Aldrich). The collected untreated samples were neutralized with 1 N NaOH before the quantification of bactericion activity [32].

Temperature stability of the bacteriocin: Five-milliliter fractions of untreated and treated supernatants were exposed to 60, 80 and 100°C for 10, 20 and 30 min and to 121°C (autoclave) for 1, 5, 10, 15 and 30 min previous to residual bacteriocin activity determination. Samples without treatment were used as control.

Growth of *Enterococcus gallinarum* CRL 1826 and kinetics of the bacteriocin production

Growth of the LAB strain was evaluated in MRS broth for 34 h in the conditions stated above. Samples were taken at different time intervals for growth determinations [optical density λ= 540 nm and number of colony-forming units (CFU/mL)]. Cultures were centrifuged (3,000 *g* at 4°C, 15 min) to obtain crude supernatants which were used for pH determinations [4-6]. Treated supernatants were then used for bacteriocin quantification against *L. monocytogenes* [32].

Storage conditions: effect of pH and temperature on bacteriocin stability

To study the stability of the bacteriocin during storage, untreated and treated cell-free supernatants fractions were kept at -20°C and the residual bacteriocin activity was determined up to 42 days.

The combined effect of pH and temperature on the bacteriocin stability during storage was evaluated. Thus, 30-mL fractions of the LAB strain supernatants containing bacteriocin were heated at 80°C for 30 min adjusted to pH values between 2 and 9 by using sterilized 1 N HCl and NaOH solutions and kept at 4 and 25°C for 7 days. Every day samples were removed, neutralized and treated with catalase previous to bacteriocin quantification [32].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the bacteriocin

Culture supernatants of *E. gallinarum* CRL 1826 were obtained according to the protocol described above, concentrated 10 times in a SAVANT (SpeedVac® Concentrators), and filtered throughout 0.22 µm Millipore membranes. Sterilized supernatants were adjusted to pH 7.0 with 5 N NaOH and the bacteriocin quantification was carried out by using *L. monocytogenes* as indicator strain [32].

The MIC of the bacteriocin was determined by the method of dilution in BHI broth following the guidelines of the Clinical Laboratory Standard Institute (CLSI) [33], while the MBC was determined according to the CLSI [34] recommendations.

Mode of action of the bacteriocin on pathogen cells

The effect of the bacteriocin on indigenous RLS-related pathogens (*C. freundii* and *P. aeruginosa*) and *L. monocytogenes* was evaluated following the guidelines of the CLSI [34]. Thus, 200-mL LAB cultures (6 h at 37°C) were harvested; cells were washed twice with sterile Phosphate-Buffered Saline (PBS, pH 7.0) and suspended in order to get approximately 1×10^8 CFU/mL. Concentrated supernatants were obtained as indicated above and 4.75 mL of **a**-treated supernatant and **b**-treated supernatant + trypsin were supplemented with 0.2 mL BHI broth to reach the nutritional conditions suitable for pathogens growth and then inoculated with the pathogen cell suspensions to obtain 5×10^6 CFU/mL. The bacteriocin concentration in each treatment was 120,000 AU/mL.

Treated supernatant + trypsin were heated at 121°C for 10 min to abolish the enzyme activity on bacterial cell wall components.

All the samples were incubated at 37°C in microaerophilic conditions and the number of viable cells (CFU/mL) was determined every hour during 6 h. The ultrastructural cell damages were analyzed in *L. monocytogenes* cells treated with bacteriocin for 30 min and on RLS-related pathogens treated with bacteriocin derived-peptide: 1.5 h for *P. aeruginosa* and 2.5 h for *C. freundii*.

Transmission electron microscopy (TEM)

Pathogenic cells obtained under each of the experimental conditions described above were harvested, suspended in MRS 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. Finally, cells were processed and observed by TEM [35].

Chemicals, Millipore membranes and enzymes used were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO (USA). The components for LAPTg preparation were supplied by Britania laboratories (Buenos Aires, Argentina) while MRS and BHI media were obtained from Merck (Germany).

Statistical analysis

The results correspond to the media of three independent assays. Statistical treatments were performed using INFOSTAT software (2008 student version). For bacteriocin thermal sensitivity 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). The significant differences among the residual bacteriocin activity under storage conditions were determined by applying the non-parametric analysis of variance (Kruskal Wallis test, 95% confidence interval).

Results

Chemical nature and thermal stability of the bacteriocin produced by *Enterococcus gallinarum* CRL 1826

The antimicrobial activity of treated (neutralized + catalase) supernatants from the *E. gallinarum* strain cultures was abolished when fractions were subjected to the action of pepsine, chymotripsin and trypsin, indicating that the inhibitory metabolite has a proteinaceus nature. Amylase and lipase did not affect the bacteriocin activity (Table 1). Thus, the bacteriocin was named enterocin CRL 1826. The LAB strain was able to produce enterocin in all the media assayed in this work: MRS, LAPTg and BHI broth. The highest inhibitory activity values were detected in MRS (data no shown), then, samples from MRS cultures were used for the bacteriocin characterization. Therefore, the effect of some physicochemical factors on enterocin stability was evaluated. No differences in the activity were detected when organic solvents were added to both untreated and treated culture supernatants. Their supplementation with 10 and 20% (v/v) hexadecane did not modify the bacteriocin activity, while the addition of 10 and 20% (v/v) ethyl acetate decreased it by 25%; and 10 and 20% (v/v) chloroform by 45 and 69%, respectively. Also, ethanol inhibited the enterocin activity by 78% (Table 1). Filtration did not affect enterocin activity, independently of the supernatant fraction evaluated.

The effect of heating on enterocin CRL 1826 stability was determined and no significant differences ($P>0.05$) between treated and untreated supernatants were observed (Figure 1A). The antimicrobial molecule was stable up to 80°C for 30 min as its residual activity did not differ significantly from the control ($P>0.05$). At 100°C a significant diminution (59 ± 13%) in the inhibitory activity was observed (Figure 1B). Moreover, at 121°C the loss of the enterocin activity was significantly affected ($P\leq0.05$) and depended on pH values. After 15 min treatment, 50 and 20% residual activity were detected in untreated and treated supernatants, respectively (Figure 1B).

Growth and kinetics of the enterocin production by *Enterococcus gallinarum* CRL 1826

The growth of *E. gallinarum* CRL 1826 in MRS broth under microaerophilic conditions and the enterocin production using *L. monocytogenes* as indicator strain are shown in Figure 2. After 4 h, the LAB strain grew exponentially during 6 h, while the number of viable cells increased 2.5 $\log_{10}$ units and the pH decreased about 1.0 unit at 10 h culture. The enterocin synthesis started at the beginning of the exponential growth phase (40 AU/mL), reaching a maximum of 61,440 AU/mL at the end of this phase (6 h). Then, enterocin activity remained stable until 16 h culture; however, at 22 h it diminished up to 25,600 AU/mL, title that was kept until the end of the assay (34 h) (Figure 2).

Effect of pH and temperature of storage on enterocin stability

No inactivation of the enterocin CRL 1826 was observed when untreated and treated supernatants of the LAB strain were stored at -20°C for 42 days (data not shown).

When crude supernatants were adjusted to pH between 2 and 9 and stored at 4 and 25°C for 7 days, the pH did not exert significant effect ($P>0.05$) on the residual enterocin activity (Figure 3A). On the other hand, a significant decrease ($P\leq0.05$) in bacteriocin activity was observed during the storage of untreated and treated supernatants,

Supernatant	Residual activity (%)*
Neutralized + catalase (N+C)	100**
N+C + pepsin	0
N+C + trypsin	0
N+C + α-chymotrypsin	0
N+C + lipase	100
N+C + α-amylase	100
10 and 20% hexadecane	100
10 and 20% ethyl acetate	75
10% chloroform	55
20% chloroform	31
10 and 20% ethanol	22

Table 1: Effect of chemical treatments on bacteriocin activity; *Percentage of the inhibitory activity on *L. monocytogenes* growth; **100%=61,440 AU/mL (control).

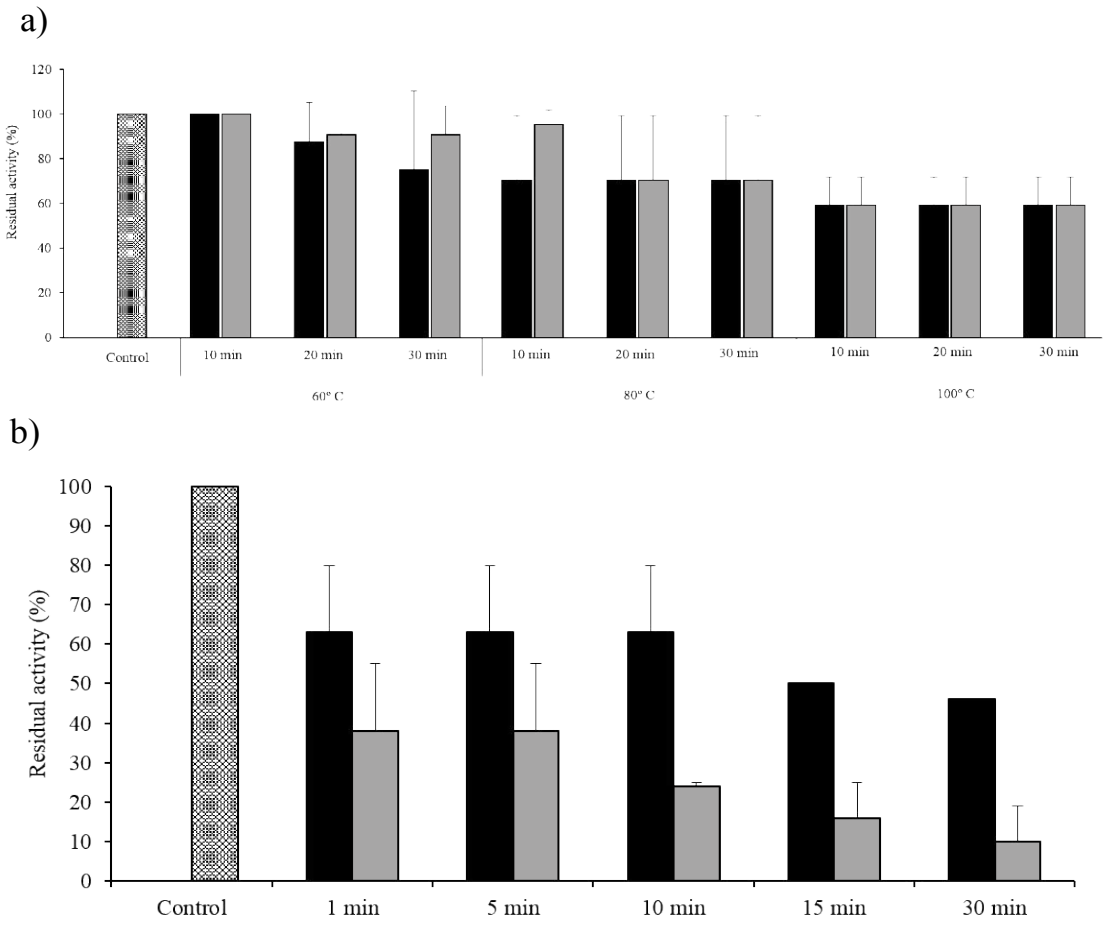

Figure 1: Effect of temperature and time on enterocin CRL 1826 activity (a) 60, 80 and 100°C for 10, 20 and 30 min (b) 121°C for 1, 5, 10, 15 and 30 min. Results are expressed as % of residual activity (100% = 61,440 AU/mL). Black bars: crude supernatants; gray bars: neutralized supernatants.

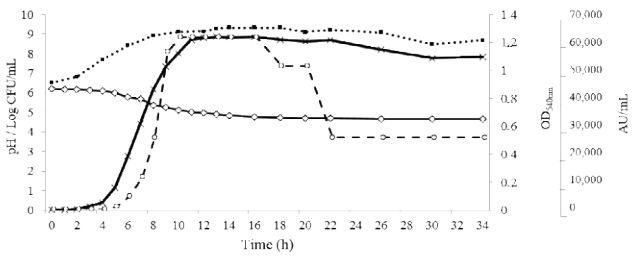

Figure 2: Growth and bacteriocin production by *Enterococcus gallinarum* CRL 1826
Log CFU/mL ·····■····· AU/mL - -○- - O.D. —×— pH —◇—

indicated by the factor time according to the Kruskal Wallis analysis (Figure 3A).

Although the statistical analysis of the data did not show significant differences between both temperatures of storage, a dissimilar profile in the mean values of the residual enterocin activity was observed when supernatants were stored at 4 and 25°C (Figure 3B and 3C). Therefore, at 4°C the highest enterocin activity was detected at pH 4 and 5, while at 25°C the highest activity was obtained when supernatants were stored at neutral pH (6.5 ± 0.5).

MIC, MBC, mode of action of the enterocin on pathogenic bacteria and studies of cell damage

The MIC of enterocin CRL 1826 against *L. monocytogenes* was 2,640 AU/mL while the MBC resulted in 5,280 AU/mL. The effect of the addition of 120,000 AU/mL enterocin on *L. monocytogenes*, *C. freundii* and *P. aeruginosa* cells was studied. Therefore, neutralized supernatants exerted a bactericidal effect on *L. monocytogenes* and no viable cells were detected at 60 min co-incubation (Figure 4). However, when samples of treated supernatants were supplemented with trypsin before its addition to pathogenic cells, a bacteriostatic effect was observed. The same effect was also detected for *C. freundii* and *P. aeruginosa* when cells were co-incubated with neutralized supernatants (data not shown). When these supernatants were then treated with trypsin, a bactericidal effect was observed and no cell counts were detected at 2 and 3 h incubation for *P. aeruginosa* and *C. freundii*, respectively.

When analyzing the control samples, *L. monocytogenes* grew poorly (0.22 log units) while *P. aeruginosa* and and *C. freundii* grew to 0.46 and 0.59 log units, respectively.

Ultrastructural studies in *L. monocytogenes* cells treated with bacteriocin and both *P. aeruginosa* and *C. freundii* treated with bacteriocin derived-peptides were performed. *Listeria* revealed that the predominant features of morphological alterations concern the partial

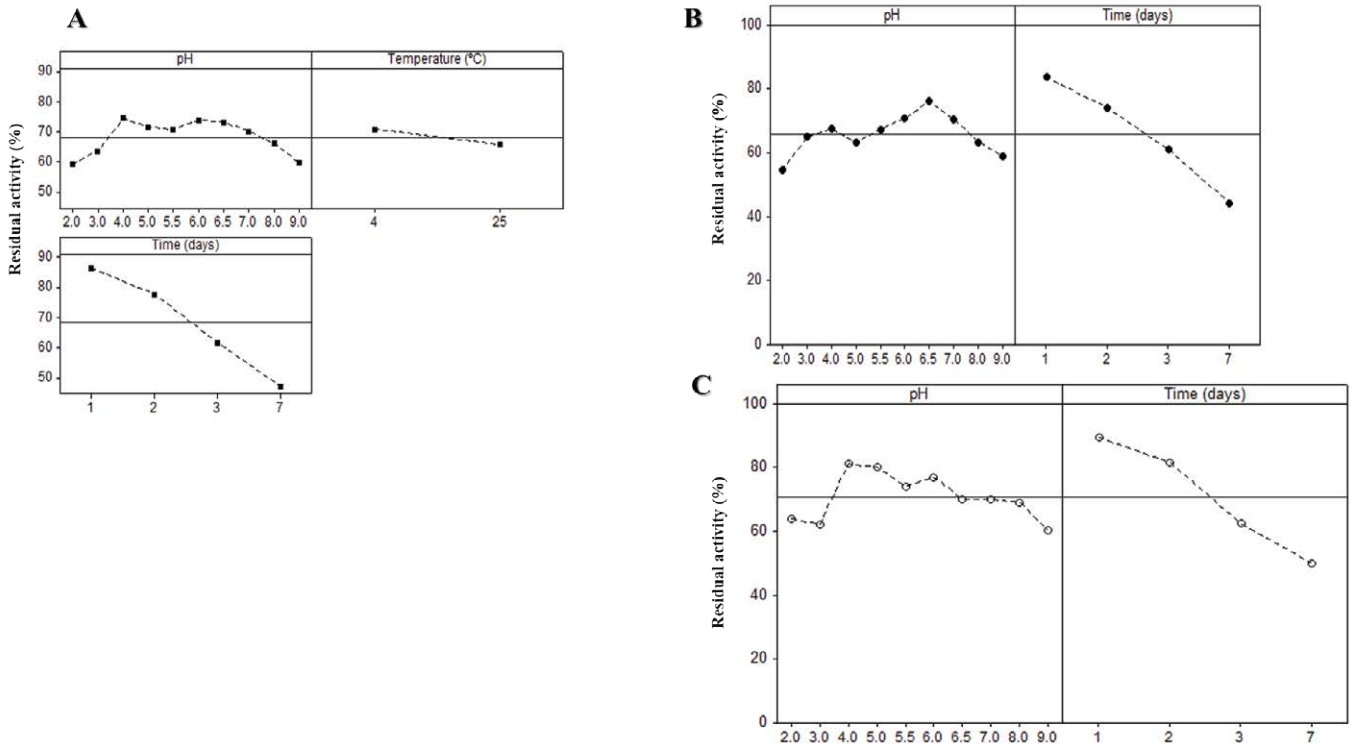

Figure 3: Main effects of pH, temperature and time on enterocin CRL 1826 stability during storage (a) Global position; (b) 4°C; (c) 25°C. Similar letters indicates no significant differences ($P \leq 0.05$) by using Kruskal Wallis test between initial and final points.

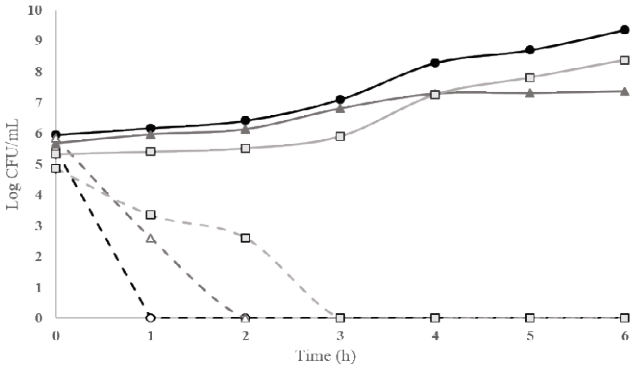

Figure 4: Growth inhibition of pathogenic bacteria by both enterocin and enterocin-derived peptides fractions
L. monocytogenes control (—●—) and neutralized supernatant + catalase (– ◇– ·)
P. aeruginosa control (—▲—) and neutralized supernatant + catalase + tripsine (- -△- -)
C. freundii control (—□—) and neutralized supernatant + catalase + tripsine (– ·□– ·)
Samples containing enzymes were treated at 121°C, 3 min before dead curves performance.

dissolution of the cell content (diminution in the cytosolic electron density) and damage of the cell envelope which enabled an efflux of cell material (Figure 5A and 5B). *Pseudomonas* showed both granulation and contraction of cytoplasm material (Figure 5C and 5D) while *Citrobacter* showed an increase in the periplasmic space and empty cells appearance (Figure 5E and 5F).

Discussion

Enterocins are antimicrobial substances with a potential use as biopreservatives in food, feed, and also as alternative therapies instead of antibiotics for human and animals [36]. Some enterocins were reported to be active against *L. monocytogenes* and their potential antilisterial activity was also shown in a murine model of pregnancy-associated listeriosis [37]. Moreover, few enterocins have been found to be effective against Gram-negatives, an unusual characteristic among bacteriocins from LAB species [24,25].

E. gallinarum CRL 1826 is an autochthonous LAB strain isolated from bullfrog skin in hatchery conditions [6]. The synergistic effect of a bacteriocin-like molecule, hydrogen peroxide and organic acids against *L. monocytogenes* Scott A was previously demonstrated [6] and preliminary studies showed that bacteriocin derived-peptides were effective against *C. freundii* and *P. aeruginosa* (RLS-related pathogens for raniculture). On the basis of these observations, the aim of this work was to go further on the characterization of the bacteriocin produced by *E. gallinarum* CRL 1826 from cell-free supernatants, supported by their potential biotechnological applications. The results indicate that the inhibitory molecule has a proteinaceus nature and therefore it was named enterocin CRL 1826.

Most of the enterocins have been reported to be produced by *E. faecium* and *E. faecalis* strains [26,38,39], although some bacteriocinogenic *E. mundtii* [40-42], *E. casseliflavus* [43], *E. hirae* [44], *E. avium* [45] and *E. durans* [23] strains were described. With respect to *E. gallinarum* strains, Jennes et al. [46] characterized for the first time the enterocin 012 produced by *E. gallinarum* 012 isolated from the intestinal tract of ostrich. To our knowledge, there are no reports on bacteriocins produced by this LAB species isolated from

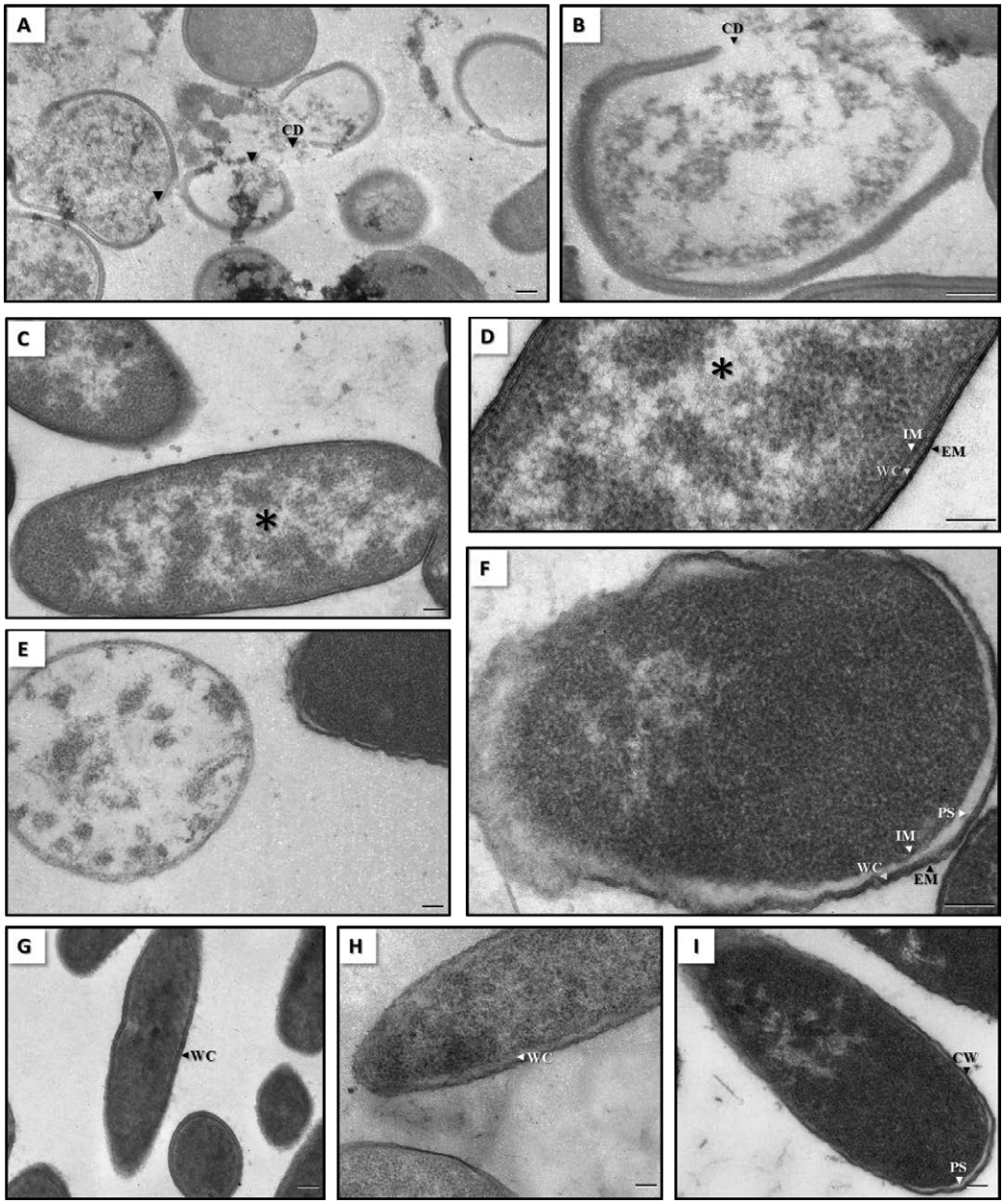

Figure 5: Transmission electronic microscopy of pathogens incubated with enterocin and enterocin-derived peptides fractions. *L. monocytogenes* treatment: A- 22,800X, B- 56,600X, control: G- 22,800X. *P. aeruginosa* treatment: C- 22,800X, D- 56,600X, control: H- 22,800X. *C. freundii* treatment: E- 22,800X, F- 56,600X, control: I- 22,800X. IM: Internal Membrane; EM: External Membrane; WC: Wall Cell; PS: Periplasmic Space; CD: Cellular Disruption. *Diminution of electron density. Scale bar: 200 nm.

aquaculture-related activities, thus enterocin CRL 1826 represents the first bacteriocin produced by *E. gallinarum* CRL 1826 isolated from captive bullfrogs.

Enterocin CRL 1826 inhibited the growth of *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactococcus lactis* and *L. garvieae* strains isolated from a bullfrog hatchery (data no shown) as well as *L. monocytogenes* Scott A. Overall, bacteriocins produced by LAB are effective against closely related Gram-positives species [9] and most of the enterocins are able to inhibit the *Listeria* species growth since they are phylogenetically associated with the genus *Enterococcus*. However, enterocin´s inhibition on *P. aeruginosa*, *P. putida, Salmonella* spp., *Salmonella* Typhimurium, *Escherichia coli*, *Acinetobacter baumanii*, *Klebsiella pneumoniae, Proteus* spp., *P. mirabilis, Citrobacter* spp., *Enterobacter* spp., *Vibrio* spp., *Shigella* spp., was also reported [26,46-50].

Enterocin CRL 1826 showed kinetics of primary metabolites synthesis since its production started at the beginning of the log growth phase, reaching a maximum of 61,400 AU/mL at the end of this phase. Then, the bacteriocin activity decreased. This diminution can be attributed to the presence of proteolytic enzymes in the culture supernatants or to the low pH values that allows the bacteriocin to be absorbed to the bacterial wall cell [51]. Similar results were reported for the enterocins produced by *E. mundtii* [42] and *E. faecium* [52,53].

However, Jennes et al. [46] reported a different behavior for the enterocin 012 synthesis that started in the middle log phase, reaching a first maximum at the end of this phase and a second one with the highest enterocin activity during the stationary growth phase.

Enterocin CRL 1826 was heat stable and polar. Its inhibitory activity was preserved in a pH range between 2 and 9 for 48 h and was not affected when stored for 42 days at -20°C. These properties were reported for mundticin KS [40] and enterocin QU2 [41] produced by *E. mundtii,* and the bacteriocins produced by *E. faecium* JCM 5804^T [54] and GM-1 [48]. Therefore, enterocin CRL 1826 could be classified into Class II bacteriocins: heat stable peptides with antilisteria activity [10].

The MIC and MBC of enterocin CRL 1826 were 2,640 and 5,280 AU/mL, respectively by using *L. monocytogenes* and the addition of 120,000 AU/mL enterocin CRL 1826 on food-borne growing cells showed bactericidal effect which was switched to bacteriostatic when enterocin-containing supernatants were previously treated with trypsin. However, the released-peptides from enterocin showed bactericidal effect on Gram-negatives from raniculture which must be identified once enterocin CRL 1826 is purified. Saavedra et al. [55] reported that derived-peptides from the C-terminal domain diminished the enterocin CRL 35 activity; while those from middle N-terminal domain improved it when used combined. However, none of them showed activity individually. Later, Salvucci et al. [37] demonstrated the inhibitory activity of N-terminal released peptides from mesentericin Y105, pediocin PA-1, sakasin P, piscicolin 126 and listeriocin 743A.

Enterocin produced cell envelope damages and efflux of cell material on *L. monocytogenes*. This effect was reported by purified enterocin E1A, a bacteriocin produced by *Streptococcus faecium* E1 on a *L. monocytogenes* strain [56]. With respect to enterocin-derived peptides, they produced granulation and contraction of cytoplasm material on *P. aeruginosa* and increase in the periplasmic space and empty cells appearance on *C. freundii*. It represents the first report regarding the mode of action of bacteriocin derived-peptides on Gram negatives, since it has only been previously reported for plantaricin MG on *Salmonella* Typhimurium [57], EDTA plus bacteriocin-like substance produced by *Bacillus* sp. P34 on *Escherichia coli* and *S.* Typhimurium [58] and lipase plus enterocin on other *E. coli* and *S.* Typhimurium strains [59].

Listeriosis is one the main diseases associated with industrial food processing; therefore it is placed in the high social and economic relevance diseases [60]. *L. monocytogenes* is a normal inhabitant of amphibian species including *L. catesbeianus* [17], thus bullfrog legs may not meet appropriate microbiological standards by virtue of methods of collection and preparation, and then cross-contaminations are of main concern. These products are intended for the direct use of the final consumer; therefore decontamination with enterocin CRL 1826 would increase meat safety.

To maintain the organoleptic characteristics and nutritional properties of food during conservation, the use of bacteriocinogenic LAB strains offers potential alternative applications to diminish the utilization of chemicals preservatives and intensity of heat treatments. Therefore, Nisaplin and Alta 2341 are used as food additives [10].

Taken into account that *E. gallinarum* CRL 1826 is not appropriated to be included in a multi-strain probitiotic for raniculture, enterocin CRL 1826 would represent an interesting bioactive compound to be combined with probiotics to control/prevent the RLS outbreaks. The use of nisin in aquaculture products has been reported to control botulism in fish vacuum packed and *L. monocytogenes* in smoked salmon and crab meat [61]. Bacteriocins can be added as concentrated preparations to preserve food, as additives or ingredients to extend the shelf life, or they can be produced *in situ* by starters, adjunct or protective cultures [62]. Therefore, enterocin CRL 1826 would be used as a biopreservative since it was stable in a range of pH and temperatures and able to inhibit *L. monocytogenes* after 1 h co-incubation.

Finally, the enterocin derived-peptide represents veterinary/pharmaceutical alternatives to control multidrug-resistant Gram-negatives such as *P. aeruginosa* and *C. freundii* [63].

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