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Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS

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ABSTRACT

Lactobacillus (*Lb.*) *plantarum* ST71KS was isolated from homemade goat feta cheese and identified using biochemical and molecular biology techniques. As shown by Tricine-SDS-PAGE, this lactic acid bacterium produces a bacteriocin (ST71KS) with an estimated molecular weight of 5.0 kDa. Bacteriocin ST71KS was not affected by the presence of α-amylase, catalase and remained stable in a wide range of pH and after treatment with Triton X-100, Triton X-114, Tween 20, Tween 80, NaCl, SDS, urea and EDTA. This bacteriocin also remained active after being heated at 100 °C for 2 h and even after 20 min at 121 °C; however, it was inactivated by proteolitic enzymes. Production of bacteriocin ST71KS reached 6400 AU/mL during stationary growth phase of *Lb. plantarum* cultivated in MRS at 30 °C and 37 °C. Bacteriocin ST71KS displayed a bactericidal effect against *Listeria monocytogenes* strains 603 and 607 and did not adsorb to the producer cells. *Lb. plantarum* ST71KS harbors two bacteriocin genes with homology to plantaricin S and pediocin PA-1. These characteristics indicate that bacteriocin ST71KS is a class IIa bacteriocin. The peptide presented no toxic effect when tested *in vitro* with kidney Vero cells, indicating safe technological application to control *L. monocytogenes* in foods.

1. Introduction

Increasing demands by consumers for natural and chemical-free products has led the food industry to search for novel and alternative strategies for food biopreservation (Cosentino et al., 2012). Among these alternatives, it is important to highlight the use of bacteriocins, defined as ribosomally synthesized antimicrobial peptides that exhibit antagonism mainly against Gram-positive bacteria (Cotter et al., 2005).

Lactic acid bacteria (LAB) are a promising group of bacteriocin-producing microorganisms due to their GRAS (generally recognized as safe) status, which indicates their safe and easy application as food preservatives (Nishie et al., 2012).

Lactobacillus (*Lb.*) *plantarum* is used as a starter culture in various food fermentations contributing to organoleptic properties, flavor and texture (Todorov and Franco, 2010). Numerous studies have reported bacteriocin-producing *Lb. plantarum* strains (Todorov, 2009), whereas some researchers have evaluated the application of different plantaricins to control the growth of the foodborne pathogen *Listeria* (*L.*) *monocytogenes* (Atrih et al., 2001; Todorov, 2008). *L. monocytogenes* has been detected in a great variety of foods (Gandhi and Chikindas, 2007; Newell et al., 2010) and it is the causative agent of listeriosis, a disease particularly dangerous to certain risk groups such as pregnant women, the elderly, newborns and immunocompromised patients (Newell et al., 2010).

Although many studies have evaluated a wide range of bacteriocins produced by *Lb. plantarum* strains that are active against specific pathogens, few have been studied in a complete and organized manner (Todorov, 2009). A deeper characterization of bacteriocins is essential for their successful application in foods, with regards to the target organism and the conditions in which the bacteriocins are active, which must be similar to those encountered in the food (Kaur et al., 2011).

The aim of this work was to characterize the bacteriocin produced by *Lb. plantarum* ST71KS, a LAB isolated from a homemade goat feta cheese, in terms of antimicrobial spectrum of activity,

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molecular weight, mode of action, presence of genes involved in the bacteriocin production and assessment of cytotoxicity potential. The influence of heat and denaturing agents (pH, enzymes, and detergents) on its activity will also be described.

2. Materials and methods

2.1. Isolation and identification of Lb. plantarum ST71KS

Bacteriocinogenic LAB were isolated from homemade goat feta cheese samples produced in Belogratchik, Bulgaria. Twenty-five grams of each cheese were homogenized with 225 mL of saline solution [0.85% (m/v) NaCl] in a Stomacher (Laboratory Blender Stomacher 400, Seward, England). Serial decimal dilutions were prepared in sterile saline solution and aliquots plated on MRS (Difco, BD, Franklin Lakes, NJ, USA) supplemented with 1% (m/v) agar and incubated for 48 h at 30 °C. LAB were enumerated and plates presenting 15–20 colonies were covered with 10 mL of BHI (Oxoid, Basingstoke, UK) supplemented with 1% (m/v) agar and 10^6 CFU/mL of *L. monocytogenes* 607. Plates were incubated for 24 h at 37 °C and single colonies displaying an inhibition zone were selected, grown on MRS agar plates for purification, and examined for colony morphology, microscopic characteristics, production of catalase and acids.

Catalase negative Gram-positive rods that produced acid were tested for their ability to grow in skim milk at 10 °C and 45 °C, in MRS broth at pH 4.4 and 9.6, and in the presence of 6.5% (m/v) of NaCl (Harrigan, 1998). These bacteria were screened for bacteriocin production by agar-spot-test method (Todorov, 2008) against members of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Listeria*, and *Pediococcus*.

The bacteriocin-producing isolates were identified by API50CHL system (BioMerieux, Marcy-l'Etoile, France). The DNA of the microorganisms was extracted with the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and further evaluated by using the random amplification of polymorphic DNA (RAPD) PCR technique with primers OPL-14 (5′-GTG ACA GGC T-3′) and OPL-20 (5′-TGG TGG ACC A-3′) (Todorov et al., 2010a). The amplified products were separated by electrophoresis in 1.4% (m/v) agarose gels and band patterns were analyzed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

Based on results of RAPD-PCR and size of the inhibition zone, isolate ST71KS was selected for future studies. The microorganism was identified using PCR species-specific primers (Torriani et al., 2001) and further confirmed by amplification of 16S rDNA with primers F8 and R1512 (Felske et al., 1997). The amplified fragments were purified (QIAquick PCR Purification Kit – Qiagen, Hilden, Germany), sequenced, and compared to sequences available at GenBank using BLAST (Basic Local Alignment Search Tool).

2.2. Biochemical characterization of Lb. plantarum ST71KS bacteriocin

One milliliter of a cell-free supernatant obtained from a 24 h culture of strain ST71KS, prepared as described by Todorov (2008), was homogenized by use of Vortex Mixer (Phoenix Luferco Equipamentos Científicos Ltda, Araraquara, SP, Brazil) with each one from the following solutions (1 mg/mL): α-amylase, catalase, proteinase K, pronase, trypsin, pepsin and papain (all from Sigma–Aldrich). Homogenates were incubated at 30 °C for 30 min and then heated at 95–97 °C for 5 min. In a separate set of experiments, the pH of 10 mL of cell-free supernatants was adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 or 12.0 with 1 mol/L HCl or 1 mol/L NaOH and incubated at 30 °C for 1 h. Another batch of cell-free supernatants was treated with solutions (10 mg/mL) of Triton X-100, Triton X-114, NaCl, SDS (Sigma–Aldrich), Tween 20, Tween 80, urea or EDTA (Merck, Darmstadt, Germany) and incubated for 30 min at 30 °C. The effect of temperature on bacteriocin ST71KM was determined by incubating cell-free supernatants at 30 °C, 37 °C, 45 °C, 60 °C, 80 °C and 100 °C for both 30 min and 2 h, and at 121 °C for 20 min. The pH of all samples was adjusted to 6.0 prior to bacteriocin ST71KM activity test, determined using *L. monocytogenes* 607 as the indicator strain (Todorov, 2008).

2.3. Partial purification of bacteriocin produced by Lb. plantarum ST71KM

Lb. plantarum ST71KS was cultivated in one liter of MRS broth for 18 h at 30 °C and the cell-free supernatant was obtained by centrifugation of the culture suspension at 12,000*g* for 15 min at 4 °C (Sorvall® Instruments, Du Pont, model RC5C, Newtown, USA). The pH of the supernatant was corrected to 6.0, and heated at 80 °C for 30 min for inactivation of extracellular proteases and hydrogen peroxide. Bacteriocin was precipitated by addition of ammonium sulfate to the cell-free supernatant to obtain 60% saturation and stirred for 4 h at 4 °C. After an additional centrifugation for 1 h at 12,000*g* at 4 °C, the resulting pellet was re-suspended in 100 mL of 25 mM ammonium acetate buffer (pH 6.5), and loaded on an activated SepPakC_{18} cartridge (Waters, Millipore, MA, USA), which was washed with 20% and 40% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). The bacteriocin was eluted with 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). The active fraction was dried under vacuum (Speed-Vac, Savant, France) and the bacteriocin fraction was re-suspended in sterile distilled water and filtered using 0.22 μm pore size filter units (Millipore, Carrigtwahill, Co. Cork, Ireland). Antimicrobial activity against *L. monocytogenes* 603 and *L. monocytogenes* 607 was determined as previously described by Todorov (2008).

2.4. Estimation of molecular weight of bacteriocin ST71KS

The molecular weight of the partially purified bacteriocin produced by *Lb. plantarum* ST71KM was determined by Tricine-SDS-PAGE as described by Schägger and Von Jagow (1987). The acrylamide gels that were loaded in duplicate with the samples were then divided in two parts; one half was fixed and stained with Coomassie Blue R250, whereas the other half was over-laid with BHI agar containing *L. monocytogenes* 603 or *L. monocytogenes* 607 (ca. 10^6 CFU/mL) and incubated at 37 °C for 24 h (Powell et al., 2007).

2.5. Mode of action of bacteriocin produced by Lb. plantarum ST71KS

2.5.1. Growth of Lb. plantarum ST71KS and production of bacteriocin

Lb. plantarum was cultivated (2%, v/v) in glass flasks containing 150 mL of MRS broth for up to 24 h at 30 °C and 37 °C. The growth of the microorganism was monitored measuring the optical density at 600 nm (UV/Visible Spectrophotometer, Ultrospec 2000, Pharmacia Biotech, Cambridge, UK) and assessment of the pH of the bacterial suspension (pHmeter Láctea, LCP-210, São Paulo, SP, Brazil) at one-hour time-intervals. The bacteriocin production was evaluated at a two-hour basis and the activity against *L. monocytogenes* 603 and *L. monocytogenes* 607 and expressed as Arbitrary Units per milliliter (AU/mL), according to Todorov (2008).

2.5.2. Survival of target microorganisms

Two glass flasks containing 50 mL of BHI broth were inoculated with 2% (v/v) of *L. monocytogenes* 603 or *L. monocytogenes* 607

(previously grown in BHI broth at 30 °C for 24 h), and incubated at 30 °C without agitation. Aliquots were taken from every flask and used to measure the absorbance at 600 nm hourly up to 12 h. Once the tested microorganisms had reached the exponential growth phase (appr. 3 h), partially purified bacteriocin ST71KS was added to one of each pair of flasks (20%, v/v). The flasks with no added bacteriocin were used as controls.

2.5.3. Cell lysis, measured by extracellular levels of β-galactosidase

The cells in 10 mL of 18 h cultures of *L. monocytogenes* 603 and *L. monocytogenes* 607 were harvested (8000*g*, 15 min at room temperature), washed twice with sterile saline solution and re-suspended to the same initial volume with sterile saline solution (ca. 10^8 CFU/mL). Cell suspensions were individually treated for 5 min at 25 °C with equal volumes of the partially purified ST71KS bacteriocin (item 2.3) and 0.2 mL of a 0.1 mol/L ONPG (O-nitrophenyl-β-D-galactopyranoside, Sigma–Aldrich) solution prepared in sterile distilled water was added. After 10 min at 37 °C, cells were harvested (8000*g*, 15 min at room temperature) and the supernatant submitted to absorbance readings at 420 nm. Results were displayed as percentage of cell lysis, using water (negative control) as the base-line.

2.5.4. Reduction of viable cells of target microorganisms in the presence of bacteriocin ST71KS

L. monocytogenes 603 and *L. monocytogenes* 607 were cultivated (2%, v/v) for 18 h at 30 °C in 50 mL of BHI broth, harvested (5000*g* for 5 min at 4 °C), washed twice with sterile saline solution and re-suspended in 10 mL of sterile saline solution. In parallel, *Lb. plantarum* ST71KS was cultivated (2%, v/v) in 50 mL of MRS broth for 24 h at 30 °C, harvested (5000*g*, 5 min, 4 °C) and the supernatant was heated at 80 °C for 10 min and then filter-sterilized (0.22 μm pore size filter-units, Millipore). Aliquots of one milliliter of the cell suspensions and one milliliter of the bacteriocin preparation (or sterile water for the negative control) were homogenized and the number of viable cells was determined before and after incubation during 1 h at 4 °C, 25 °C, 30 °C, and 37 °C. Enumeration of *L. monocytogenes* colonies was performed by seeding aliquots of every dilution on the surface of BHI supplemented with 1% (m/v) agar, followed by incubation at 37 °C for 48 h.

2.6. Evaluation of the adsorption of bacteriocin ST71KS to the producer cells

This test was performed as previously described by Yang et al. (1992). Briefly, *Lb. plantarum* ST71KS (2%, v/v) was cultivated in 50 mL of MRS broth at 30 °C for 24 h and the pH of the culture was adjusted to 6.0. Cells were then harvested (8000*g*, 15 min, 4 °C) and washed with 0.1 mol/L sterile phosphate buffer solution (pH 6.5). Afterwards, cells were re-suspended in 10 mL of a solution of 100 mM of NaCl (pH 2.0), stirred during 1 h at 4 °C and centrifuged again (8000*g*, 15 min, 4 °C). The supernatant obtained was neutralized to pH 7.0 and tested for activity using the agar spot-test (Todorov, 2008).

2.7. Identification of genes involved in the production of bacteriocin ST71KS

The presence of genes encoding known bacteriocins produced by *Lb. plantarum* (plantaricin S, plantaricin NC8, plantaricin W, pediocin PA-1 and nisin) was evaluated by PCR with specific primers for these genes. Genomic DNA was extracted from *Lb. plantarum* ST71KS using a DNA extraction kit (Zymo Research, USA). PCR reactions were conducted according to Remiger et al. (1996), Maldonado et al. (2003), Holo et al. (2001), Todorov and Dicks (2009) and de Kwaadsteniet et al. (2008), respectively for the detection of plantaricin S, plantaricin NC8, plantaricin W, pediocin PA-1 and nisin. The PCR products were visualized in 1.0% (m/v) agarose gel stained with ethidium bromide (0.5 μg/μL). Amplicons obtained from positive PCR reactions were further purified by use of QIAquick® PCR Purification Kit (Qiagen) and then sequenced at the Human Genome Research Center, University of São Paulo (Brazil). Analysis of the partial gene(s) sequences was conducted by using the GenBank database and the BLAST algorithm.

2.8. Evaluation of bacteriocin ST71KS cytotoxicity

Cytotoxicity was assessed using monkey kidney Vero cells as previously described by Wachsman et al. (2003). The results were calculated by regression analysis and expressed as CC_{50}, which corresponds to the concentration of bacteriocin (μg/mL) needed to lower the cell viability to 50%.

3. Results and discussion

Studies describing the isolation and identification of strains of *Lb. plantarum* from feta cheeses are very limited since this food matrix is normally considered unsuited for the growth of this microorganism due to its high content of sodium chloride. Nevertheless, seven isolates with antimicrobial activity were obtained from the goat feta cheese samples; however, RAPD-PCR and physiological and biochemical characteristics indicated that they were replicas of the same strain (data not shown). PCR with species-specific primers (Torriani et al., 2001), and 16S rDNA sequencing (data not shown) indicated that this strain is *Lb. plantarum*, which received the designation *Lb. plantarum* ST71KS. This strain showed antimicrobial activity against 16 of 19 *L. monocytogenes* strains tested, 2 from 8 tested *Enterococcus faecium*, 1 from 1 *Lactobacillus delbrueckii*, and only 1 from 9 *Lactobacillus paracasei* (data not shown).

Treatment with proteinase K, pronase, trypsin, pepsin and papain resulted in complete loss of activity of bacteriocin ST71KS, but the bacteriocin was not affected by α-amylase or catalase. These results suggest that the activity of bacteriocin ST71KS is not dependent on glycosylation and that the antimicrobial activity is not related to H_2O_2. Similar results have been reported for other bacteriocins produced by *Lactobacillus* spp. (Klaenhammer, 1993; Todorov, 2009).

Activity of bacteriocin ST71KS remained the same at pH 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 and treatment with Triton X-110, Triton X-114, Tween 20, Tween 80, SDS, NaCl, urea and EDTA had no effect on the activity. Similar behaviors were reported for the majority of bacteriocins produced by other *Lb. plantarum* (Todorov, 2009).

Bacteriocin ST71KS (pH 6.0) was revealed to be heat tolerant and remained stable after 2 h up to 100 °C. However, a slight decrease in activity was observed upon heat treatment at 121 °C for 20 min (data not shown). This finding is consistent with results reported for a number of bacteriocins produced by *Lactobacillus* spp. and *Enterococcus* spp. (Van Reenen et al., 1998; Todorov and Dicks, 2005; Todorov et al., 2005).

The Tricine-SDS-PAGE technique indicated that the molecular weight of bacteriocin ST71KS was estimated to be less than 5 kDa. The antimicrobial activity of the peptide was confirmed by the inhibition zones against both *L. monocytogenes* 603 and *L. monocytogenes* 607 observed in the position corresponding to bacteriocin ST71KS band in the Tricine-SDS-PAGE gel. The molecular size of bacteriocin ST71KS was bigger than most bacteriocins previously described for the genus *Lactobacillus* (De Vuyst and Vandamme, 1994). However, Todorov and Dicks (2005) reported that *Lb. plantarum* ST23LD, a strain isolated from spoiled olives,

produced two bacteriocins (ST23LDa and ST23LDb) with approximate sizes of 3.0 kDa and 14.0 kDa. Based on the strong anti-*Listeria* activity, it is feasible that bacteriocin ST71KS is a class IIa bacteriocin, as this is an important characteristic for those molecules (De Vuyst and Vandamme, 1994). Further purification and amino-acid sequencing of this bacteriocin are necessary to confirm this classification of bacteriocin ST71KS.

As shown in Fig. 1, *Lb. plantarum* ST71KS displayed good growth at both 30 °C and 37 °C in MRS broth. Furthermore, bacteriocin production increased during the logarithmic phase of growth at both temperatures, reaching a maximum of 6400 AU/mL and remaining stable during the stationary phase (Fig. 1). Optimal production of bacteriocin ST71KS was recorded during stationary phase of growth (Fig. 1), which may suggest that the peptide is a secondary metabolite. Similar findings were reported for bacteriocin ST23LD (Todorov and Dicks, 2005) and bacteriocin ST4V (Todorov et al., 2005). The pH of the cultures decreased from 6.6 ($t = 0$) to approximately 4.0 after 18 h and remained constant afterwards up to the end of the experiment (Fig. 1).

After addition of bacteriocin ST71KS (final activity of 1066 AU/mL) to the cultures of *L. monocytogenes* 603 or *L. monocytogenes* 607, bactericidal effect against both strains was clearly observed (Fig. 2) and an increase (50% and 25%, respectively) in the extracellular levels of β-galactosidase in both cultures was observed. These results suggest that bacteriocin ST71KS acts by a pore formation mechanism, corroborating with the hypothesis that this is a class IIa bacteriocin (Cotter et al., 2005). The determination of extracellular levels of β-galactosidase for studies on the mode of action of bacteriocins has been explored for several strains of *Lb. plantarum* (Todorov et al., 2007).

According to these results, the populations of *L. monocytogenes* 603 and *L. monocytogenes* 607 in the cell suspensions remained

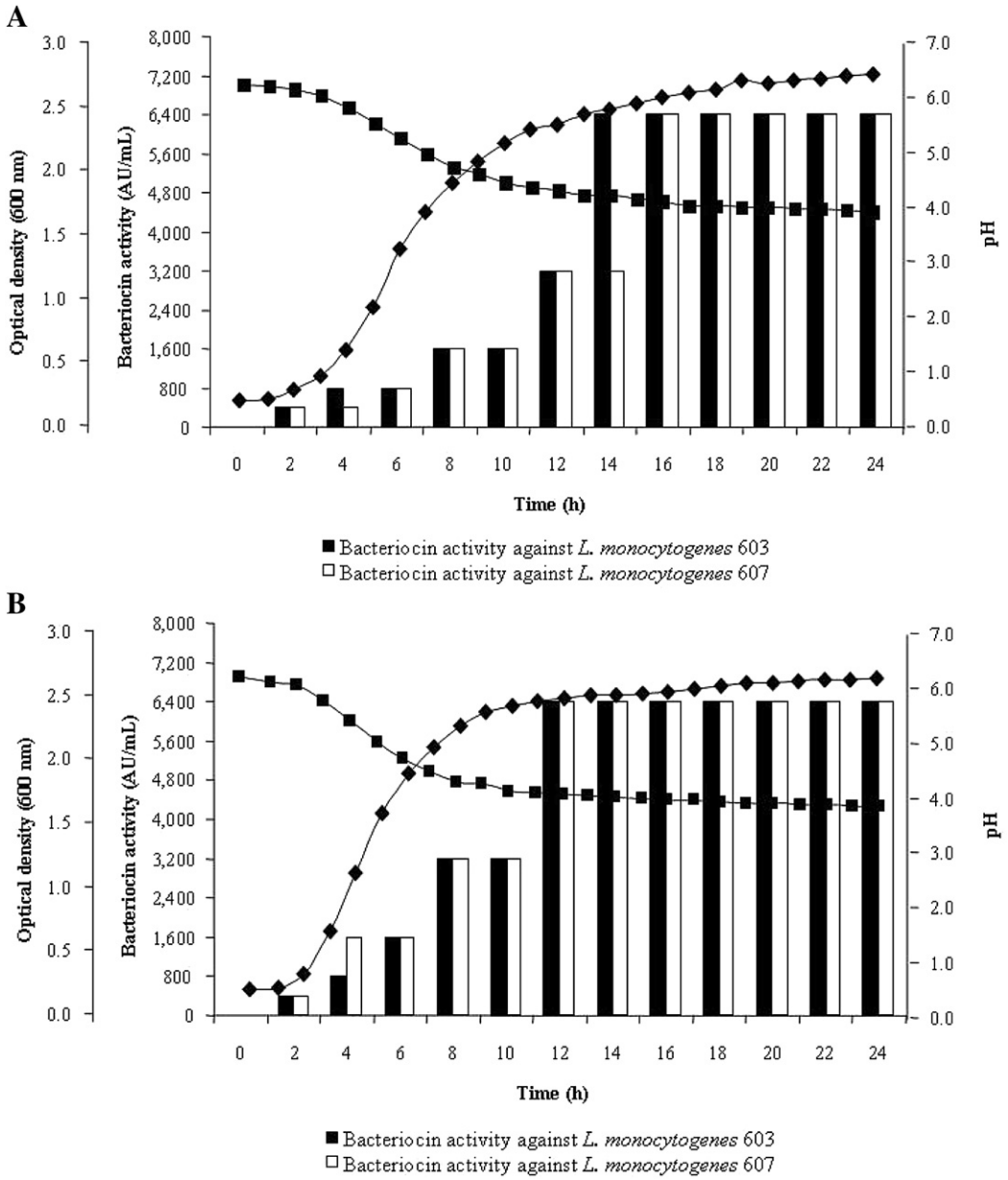

Fig. 1. Production of bacteriocin by *Lb. plantarum* ST71KS in MRS broth at 30 °C (**A**) and 37 °C (**B**). Antimicrobial activity against *L. monocytogenes* 603 and *L. monocytogenes* 607 is expressed as Arbitrary Units per milliliter (AU/mL) (bars). Results assessed for optical density (OD) (◆) and pH (■) are also indicated. Standard deviations were not shown since values were less than 5%.

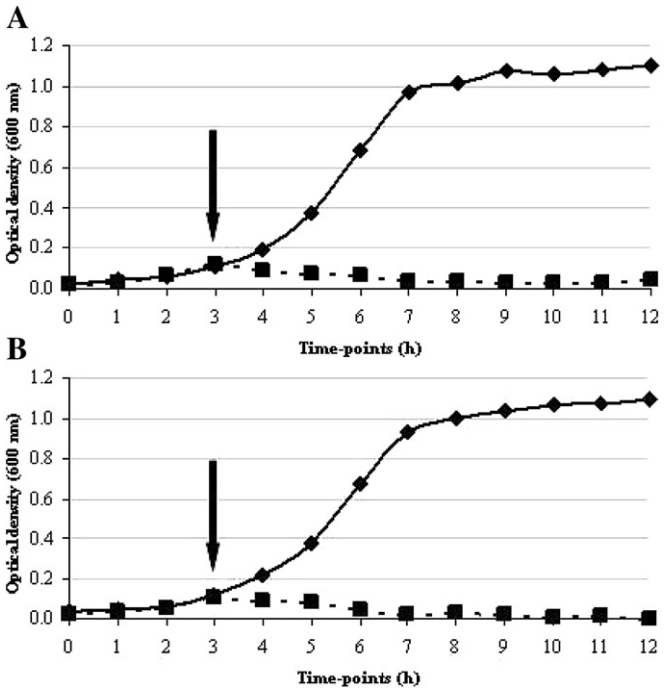

Fig. 2. Effect of bacteriocin ST71KS on *L. monocytogenes* 603 (**A**) and *L. monocytogenes* 607 (**B**) growth. (-◆-): control (no added bacteriocin) and (···■···): growth of *L. monocytogenes* 603 and *L. monocytogenes* 607 in the presence of bacteriocin ST71KS, added at time-point 3 h (↓). Standard deviations were not shown since values were less than 5%.

high (>9.2 log CFU/mL) after 1 h when not treated with bacteriocin ST71KS, at all temperatures tested (4 °C, 25 °C, 30 °C and 37 °C) (data not shown). However, when treated with the bacteriocin, both microbial populations decreased after 1 h, ranging from 6.5 to 6.8 log CFU/mL, according to the temperature tested. It is necessary to underline that these high concentrations of pathogen contamination is very unlikely to occur in foods but a two log reduction in the contamination level in cheeses is important from a public health standpoint.

Bacteriocin ST71KS did not adsorb to the producer cells. Similar observations have been recorded for bacteriocins produced by other *Lb. plantarum*, such as *Lb. plantarum* ST23LD and *Lb. plantarum* ST341LD (Todorov and Dicks, 2005) and *Lb. plantarum* C19 (Atrih et al., 2001). However, the opposite has been reported for bacteriocin ST5Ha (Todorov et al., 2010b), which exhibited a strong adhesion to the producer cells, indicating that adsorption of bacteriocins to the producer cell surface is strain and bacteriocin dependent.

Among five tested genes encoding plantaricin S, plantaricin NC8, plantaricin W, pediocin PA-1 and nisin, *Lb. plantarum* ST71KS was positive only for plantaricin S and pediocin PA-1 (data not shown). The obtained amplicons were purified, sequenced and displayed high homology (>98%) when compared to sequences previously deposited in the GenBank for plantaricin S and pediocin PA-1. It has been previously reported that pediocin PA-1 is a class IIa bacteriocin effective against *L. monocytogenes* by recognition of lipid II cell-surface receptor and its mode of action is based in the pore formation in the cell membrane of the target microorganism (Cotter et al., 2005). However, the detection of the two genes does not guarantee that *Lb. plantarum* ST71KS expresses one or both of these two bacteriocins. This needs to be confirmed after purification and determination of their molecular masses by mass spectrometry and the amino-acid sequences of the active bacteriocin(s).

The *in vitro* cytotoxicity assay performed with Vero cells and ST71KS bacteriocin resulted in a CC_{50} above 1200 μg/mL, indicating that cytotoxicity is low. Possibly, application of this bacteriocin in semi-purified or purified preparations in foods will not have a negative effect for the consumers. Determination of the cytotoxicity is an important parameter in the characterization of bacteriocins in order to recommend their application for food biopreservation or as an alternative to antibiotics in medical practice. Only a few bacteriocins have been previously characterized regarding their cytotoxicity (Wachsman et al., 2003; Todorov et al., 2005, 2007, 2010b).

In conclusion, bacteriocin ST71KS, produced by *Lb. plantarum* ST71KS, is effective in the control of *L. monocytogenes*, an important foodborne pathogen, and does not present a cytotoxic activity. Together, these characteristics make bacteriocin ST71KS a promising tool to be used for food biopreservation. Although *Lb. plantarum* ST71KS harbors genes that encode for production of plantaricin S and pediocin PA-1, further studies are required to understand under which circumstances both bacteriocins are produced, which could consequently increase its applicability.

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