Meat Science xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Lactic acid bacteria isolated from artisanal dry sausages: Characterization of antibacterial compounds and study of the factors affecting bacteriocin production

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ARTICLE INFO

Article history:
Received 28 June 2010
Received in revised form 3 October 2010
Accepted 6 November 2010
Available online xxxx

Keywords:
Biopreservation
Lactic acid bacteria
Artisanal dry sausages
Bacteriocin
Food additives

ABSTRACT

Lactic acid bacteria (LAB) were isolated from artisanal dry sausages sampled from north-eastern region of Chaco, Argentina. Among 141 isolates, 27 showed antimicrobial activity against *Listeria innocua, Staphyloccus aureus* or *Brochothrix* spp. One isolate, identified as *Lb. curvatus/sakei*, produced bacteriocin like substances (BLIS). These BLIS were heat stable, effective after refrigerated storage and freeze/thaw cycles and even active against pathogens when produced under refrigeration at 3% NaCl concentration. The influence of several factors on production of BLIS was assessed in MRS broth added with: EDTA, ascorbic acid, KCl, potassium sorbate, sodium citrate, 3 and 6% NaCl, Tween 20 or Brij 35. These additives showed different effects towards the effectiveness of the bacteriocin produced by *Lb. sakei/curvatus* against *L. innocua* and *S. aureus*. Conditions that provided high cell density favored high bacteriocin production. BLIS production by this LAB strain was greatly influenced by NaCl concentration and the presence of surfactants.

1. Introduction

Consumers have been consistently concerned about possible adverse health effects from the presence of chemical additives in their foods. As a result, consumers are drawn to natural and "fresher" foods with no chemical preservatives added. This perception, coupled with the increasing demand for minimally processed foods with long shelf life and convenience, has stimulated research interest in finding natural but effective preservatives. Bacteriocins, peptides ribosomally synthesized by lactic acid bacteria (LAB), may be considered natural preservatives or biopreservatives that fulfill these requirements. Biopreservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life (Schillinger, Geisen, & Holzapfel, 1996). Three approaches are commonly used in the application of bacteriocins for biopreservation of foods (Schillinger et al., 1996): (1) Inoculation of food with LAB that produce bacteriocin in the products. (2) Addition of purified or semi-purified bacteriocins as food preservatives. (3) Use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing. Recent studies indicated that bioprotective LAB isolated from meat and meat products can contribute to the safety of fermented meat products through bacteriocin production (Castellano, Holzapfel, & Vignolo, 2004; Dicks, Mellett, & Hoffman, 2004). Antimicrobial activity of bacteriocins in foods is affected by its levels, type and number of microorganisms, condition of application, interaction/inactivation by food components, and pH and temperature of product (Naidu, 2000). Some previous studies have helped clarify the effect of specific conditions from food environments on the production of bacteriocins (Leroy & De Vuyst, 1999a, 1999b, 2003; Motta & Brandelli, 2003). Bacteriocin titres can dramatically change by altering environmental conditions and optimum production may require a certain combination of influencing factors (Leal-Sánchez, Jiménez-Díaz, Maldonado-Barragán, Garrido-Fernández, & Ruiz-Barba, 2002). Regarding the complexity of food environments, a better knowledge of the interactions of these factors in bacteriocin production is needed (Delgado, Brito, Peres, Noe´-Arroyo, & Garrido-Fernández, 2005).

According to the latest trends in food preservation, the improvement of microbiological safety of certain foods can be attained with the help of bacteriocin-producing strains of LAB isolated from the same food which needs to be preserved. LAB isolated from these sources might be considered to be the best candidates for biopreservation since they are well adapted to the conditions provided in those foods and should therefore be more competitive than LAB from other sources (Ammor, Tauveron, Dufour, & Chevallier, 2006). Moreover, bacteriocin-producing strains of LAB can also be applied to other food systems. The absolute necessity to find new sources of these natural antimicrobial compounds and, consequently, to define bacteriocin effectiveness against foodborne pathogens in the presence of certain additives motivated this study. In

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doi:10.1016/j.meatsci.2010.11.006

order to achieve these goals LAB were isolated from artisanal dry sausages. These products, being fermented by autochthonous strains, comprised a natural reservoir of potential bacteriocin-producing strains. Hence, the aims of this work were the preliminary characterization of a bacteriocin like substance produced by a LAB strain isolated from an artisanal meat product and the study of the factors that affect its production and antimicrobial activity in a laboratory media supplemented with food additives as an approach to a future application in food systems.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The following spoilage and pathogenic bacterial strains were considered in the screening for antagonistic activity: *Listeria innocua* ATCC 33090 — in lieu of *Listeria monocytogenes*, because of their similar response to stress factors (Friedly et al., 2008)-, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* FBUNT, *Staphylococcus aureus* FBUNT, *Escherichia coli* FBUNT (these last three strains were obtained from clinical isolates and were identified by the Microbiology Department of Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (FBUNT), Argentina), *Lactobacillus plantarum* ATCC 8014 and *Brochotrix thermosphacta* 396 and *B. thermosphacta* 405. These latter strains were isolated from cooked meat products in our laboratory. All strains were maintained as frozen stocks at -30 °C, Prior to being used as bacterial lawns in the screening assay, all the indicator strains were recovered in Brain Heart Infussion (BHI, Biokar Diagnostics, France) at the convenient temperature for each one (at 25 °C: *B. thermosphacta* 396 *and B. thermosphacta* 405*;* at 30 °C: *L. innocua* ATCC 33090 and *P. aeruginosa* FBUNT; at 37 °C: *S. aureus* ATCC 6538, *S. aureus* FBUNT and *E. coli* FBUNT), with the exception of *L. plantarum* ATCC 8014 which was recovered in MRS broth at 30 °C.

2.2. Isolation of lactic acid bacteria

Fourteen different types of dry fermented products were collected throughout the north-eastern region of the province of Chaco, Argentina. The products were purchased from small-scale facilities producing traditional dry sausages without the addition of starter cultures. In all the cases, sausage casing was aseptically removed and 10 g of the sample derived as cross section was homogenized with 90 ml of sterile solution containing 0.1% (w/v) peptone (Britania, Argentina) and 0.85% (w/v) NaCl (Anedra, Argentina) using a domestic blender (Braun, Germany). Ten-fold serial dilutions of the meat homogenate were prepared in peptone water to spread plate the sample on de Man Rogosa Sharpe agar (MRS, Biokar Diagnostics, France) with 1 g/l of sorbic acid (Sigma-Aldrich, USA) to inhibit yeast and molds growth. After incubation for 72 h at 30 °C, ten colonies were randomly picked from each plate. Attention was given to choose colonies with different macroscopic morphology. Isolates were reinoculated in MRS broth, incubated at 30 °C and checked for purity by streaking on MRS agar. Plates with pure cultures were used to test for cell morphology by phase contrast microscopy, Gram stain and catalase formation. Gram positive and catalase negative strains were selected. These isolates were maintained as frozen stocks in MRS broth supplemented with 10% (v/v) glycerol at −18 °C during a month. Before experimental use, all LAB strains were recovered in MRS broth and were incubated at 30 °C.

2.3. Screening for antagonistic activity

Detection of antagonistic activity in LAB strains was initially screened by means of an agar well diffusion assay (AWDA) (Schillinger & Lücke, 1989). MRS agar was used for *Lb. plantarum* while BHI agar was used for the rest of the indicator microorganisms. Briefly, Petri dishes were overlaid with 15 ml of molten agar (1% agar), inoculated with 30 μl of an overnight culture of the indicator microorganism, in which wells were formed. Wells, of 5 mm in diameter and of 30 μl in capacity, were formed by carving the agar with a cork borer. Afterwards, 30 μl of an overnight culture of the putative inhibitor strain were placed in each well. The plates were then incubated aerobically for 24 h at a temperature conductive to growth of the indicator microorganism and were subsequently examined for zones of inhibition. Inhibition was recorded as negative if no zone was observed around the agar well. Each antagonistic activity was related to the area (mm^2) of the inhibition zone displayed (Ammor et al., 2006). A positive control for antimicrobial activity, i.e. a bacteriocin-producing *Lb. plantarum* strain, was included.

2.4. Characterization of the antibacterial compounds

Isolates exhibiting antagonistic activities against the indicator microorganisms were investigated for their antibacterial compounds. These isolates were grown overnight at 30 °C in MRS broth. In each case, overnight bacterial culture was harvested by centrifugation (4000 × *g*, 10 min at room temperature) to obtain a cell-free supernatant (CFS) followed by a filtration through a 0.22 μm-pore-size cellulose acetate filter (Sartorius, Goettingen, Germany). Samples were adjusted to pH 6.5 with 1 N of NaOH (Merck, Argentina) to rule out acid inhibition. Inhibitory activity from hydrogen peroxide was ruled out by the addition of catalase (300 IU/ml) (C9322, Sigma-Aldrich Chemie, Steiheim, Germany). The antagonistic activities of these samples were determined for each isolate by the AWDA as described above using *L . innocua* ATCC 33090 and *S. aureus* FBUNT as indicator microorganisms. Sensitivity of the antimicrobial compounds to proteolytic enzymes was investigated by the addition of Trypsin (T 1426, Sigma-Aldrich Chemie, Steiheim, Germany) and Proteinase K (P6556, Sigma-Aldrich Chemie, Steiheim, Germany) at a final concentration of 1 $mg.ml^{-1}$ to the culture supernatants. The samples were incubated for 3 h at 37 °C and immediately after, the residual activity was determined by the AWDA as described previously for the two indicators mentioned above.

2.5. Genetic identification of bacteriocin-producing LAB strain

The microbial isolate from which the CFS maintained its antibacterial activity throughout the characterization of antibacterial compounds was selected for genetic identification. Total cellular DNA was isolated according to De los Reyes-Gavilán, Limsowtin, Tailliez, Sechaud, and Accolas (1992). Oligonucleotide primers (PLB16, 5′- AGAGTTT-GATCCTGGCTCAG-3′; and MLB16, 5′-GGCTGCTGGCACGTAGTTAG-3′) were used to amplify the variable (V1) region of the 16 S ribosomal RNA gene, as described by Kullen, Sanozky-Dawes, & Klaenhammer (1999). Primers were synthesized by The Great American Gene Company (Ramona, CA, USA). Briefly, 100 ng of genomic DNA were amplified in a total volume of 50 μl of PCR reaction mixture containing 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 $MgCl_2$, 1.0 μM of each primer, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3, and 1 U Taq polymerase (Promega, Madison, WI, USA). The PCR tubes were placed in a DNA Thermal cycler 480 (Perkin-Elmer, Norwalk, CT, USA) and the reaction started by denaturation for 5 min at 94 °C followed by 30 cycles at 94 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C for 30 s. A final 10 min incubation at 72 °C was allowed for the completion of primer extension after the last cycle. PCR products were analyzed by electrophoresis on a 1.2% agarose gel at 70 V for 2 h, followed by a 30 min staining in 0.5 μg/ml ethidium bromide solution and a 15-min de-staining in distilled water, with a final visualization and photography under UV light. Amplicons were excised from the gel and purified using a Prep-A-gene Kit (Bio-Rad). Purified PCR products were resuspended in 20 ml of TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0), and sequenced at CERELA (Centro de Referencia para Lactobacilos, CONICET, Tucumán, Argentina). DNA homology searches were performed on line with the BLAST program (Altschul, Gish, Miller, Myers, & Lipman, 1990). The sequence of the partial 16 S rRNA of *Lb. sakei* and *Lb. curvatus* were submitted to

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the GenBank database (Accession numbers AF429524 and AY375292, respectively).

2.6. Determination of LAB growth for maximum bacteriocin production

The time of incubation at which the selected LAB strain exhibited the maximum bacteriocin production was determined as follows: sterile flasks containing 50 ml of MRS broth were inoculated with 1% (v/v) bacteriocin-producing LAB strain and incubated at 30 °C. Then, 1-ml aliquots were retrieved after 0, 1.5, 3, 4.5, 6, 9, 12, 15, 24 and 36 h in order to proceed with the bacterial count and to monitor the production of bacteriocin along the bacterial growth. Cell numbers were determined by spread plating on MRS agar and incubating the plates at 30 °C for 72 h. Bacteriocin production was determined from 6 h of storage by the AWDA previously described. Arbitrary units (AU) per ml were calculated as $AU = (1000/v)/d$; being v: volume seeded in the well and d: dilution (Kouakou et al., 2009).

2.7. Effect of thermal treatments on bacteriocin activity

The thermal treatments tested were chosen based on their usual levels in foods and in their processing operations. All of them were performed using the CFS of the selected strain, obtained as described above. The effect of heating temperatures on bacteriocin stability was determined in the CFS subjected to treatment at 70 °C/15 min, 85 °C/15 min, 85 °C/30 min and 100 °C/15 min in a water bath (BioElec, Argentina). Immediately after each treatment, samples were cooled under refrigeration and residual activity was determined by the AWDA, as previously described. A positive control, consisting of freshly prepared extracellular extract, was tested in parallel.

To test the stability of CFS during three freeze–thaw cycles, it was frozen at −20 °C during 24 h and thawed for 20 min at 5 °C. In all instances, a positive control, consisting of freshly prepared CFS was tested in parallel.

The effect of extended storage at low temperature (5 °C) on bacteriocin stability was also evaluated by placing CFS in an incubator device at 5 ° C for 7, 14 and 21 days. In all cases, the remaining bacteriocin activity was determined by the AWDA. A positive control, consisting of freshly prepared extracellular extract, was tested in parallel.

2.8. Effect of pH on bacteriocin activity

The pH ranges tested were chosen so as to imitate food environmental conditions. To check the pH stability of the bacteriocins, 1 ml portions of the CFS of the selected strain, obtained as described above, were adjusted to pH values of 3.5, 4.5, 5.5 and 6.5 by adding the appropriate volumes of 4 N HCl or 4 N NaOH. The pH values were measured by means of a glass electrode attached to a pHmeter (Oakton®, Eutech Instruments, Singapore). Then, the samples were sterilized by filtration through 0.22 μm (Sartorius, Germany), incubated for 2 h at 30 °C and antimicrobial activity was determined by the AWDA described above. Negative controls, aimed at elucidating the possible role of acid pH values in the inhibition of *L. innocua* and *S. aureus*, were prepared by testing portions of non-inoculated MRS broth whose pH values were adjusted to 3.5, 4.5, 5.5 and 6.5. CFS from the bacteriocin-producer *Lactobacillus plantarum* ATCC 8014 was used as positive controls.

2.8.1. Growth and bacteriocin like inhibitory substance production in the presence of NaCl at 5 °C

The selected strain was tested on its potential to grow in MRS broth at low temperature (5 °C) combined with salt concentrations occurring in the water phase of cooked meat products (3% or 6% NaCl). The selected strain was inoculated at a level of 10^6–10^7 CFU/ml in 5 ml of MRS broth containing 0, 3% and 6% of NaCl and stored at 5 °C. Each day, during 6 weeks at that temperature, growth was followed by visually examining the turbidity of the broth. The tubes that showed turbidity after storage were centrifuged, filter-sterilized and then, the supernatants were used in the AWDA in order to determine bacteriocin activity, as described above.

2.9. Effect of several food additives on bacteriocin activity

The growth of, and bacteriocin production by, the collected strain was assessed using MRS broth supplemented with each one of the following additives: 0.075 g kg^{-1} disodium salt of ethylendiamine tetraacetic acid (EDTA) (Anedra, Argentina), 0.500 g kg^{-1} ascorbic acid (Anedra, Argentina), 10 or 30 g kg^{-1} potassium chloride (Anedra, Argentina), 1 g kg^{-1} potassium sorbate (Sigma-Aldrich, USA), 3 g kg^{-1} sodium citrate (Anedra, Argentina), 30 or 60 g kg^{-1} sodium chloride, 10 g kg^{-1} Tween®20 (P1379, Sigma-Aldrich, USA) and 10 g kg^{-1} Brij®35 (B4184, Sigma-Aldrich, USA). The additives were added to MRS broth, the pH was adjusted to 6.4 ± 0.2 with drops of 4 N HCl if necessary. This mixture was placed in tubes and sterilized by autoclaving. Tubes containing 15 ml of the different formulations were inoculated at 1% from an overnight culture of the collected strain and incubated at 30 °C. Then, 1 ml aliquots were retrieved after 0, 3, 6, 9, 12, 15, 24 and 36 h to determine bacterial count and the production of bacteriocin during the bacterial growth, as it was previously described. The additives were selected so that they could mimic several environmental conditions found in meat products. Bacteriocin production was determined from 6 h of storage by the AWDA previously described using *L. innocua* and *S. aureus* as indicator microorganisms.

2.9.1. Statistical analyses

All experiments were carried out in duplicate and replicated twice and the data shown are the means of the replicates. Statgraphics *Plus* for Windows, Version 4.0, was used for the statistical study of the results. A variance analysis (ANOVA) was also applied to establish whether significant differences ($p<0.05$) existed between the values obtained for the means of every trial conducted.

3. Results

3.1. Isolation of lactic acid bacteria

From the 14 different dry sausages assayed, 141 strains were isolated. All isolates were Gram positive, catalase negative and oxidase negative confirming the selectivity of MRS supplemented with sorbic acid. In general, the isolated bacteria presented a rod-like morphology. Those characteristics suggested the correspondence of the isolates with the group of LAB.

3.2. Screening for antagonistic activity

None of the isolates showed antimicrobial activity against the Gram negative bacteria considered in this study, i.e. *E. coli FBUNT* and *P. aeruginosa FBUNT*. However, twenty seven isolates showed antimicrobial activity against at least one of the following indicator microorganisms: *L. innocua* ATCC 33090, *S. aureus* FBUNT, *Lb. plantarum* ATCC 8014 and *Brochothrix* spp. 396 and 405. Inhibitory spectra of these isolates is presented in Table 1, where it can be seen that almost all of the isolates, i.e. twenty one strains, showed antimicrobial activity towards *L. innocua* ATCC 33090. Finally, many isolates presented antimicrobial activity against one or both *Brochothrix* spp. strains.

3.3. Characterization of the antibacterial compounds

Among the isolates that exhibited antagonistic properties against target microorganisms, only three kept their antimicrobial activity in

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Table 1
Inhibitory spectra of LAB isolates exhibiting antibacterial activity.

Isolate	Indicator strain						
	L. innocua ATCC 33090	*S. aureus FBUNT*	*L. plantarum ATCC 8014*	*Brochothrix* spp. 1	*Brochothrix* spp. 2	*P. aeruginosa FBUNT*	*E. coli FBUNT*
1	+	+	−	−	−	−	−
2	+	−	−	−	++	−	−
3	+	+	−	+	++	−	−
4	−	−	−	−	+	−	−
5	+	−	−	−	−	−	−
6	+	−	−	−	−	−	−
7	+	−	−	−	−	−	−
8	++	−	−	+	+	−	−
9	+	−	−	−	−	−	−
10	−	−	−	−	+	−	−
11	+	−	−	+	−	−	−
12	+	−	−	−	−	−	−
13	+	−	−	−	−	−	−
14	+	−	−	−	−	−	−
15	+	−	−	−	−	−	−
16	+	−	−	−	−	−	−
17	+	−	−	−	+	−	−
18	++	+	+	−	−	−	−
19	+	−	−	+	+	−	−
20	−	−	−	−	+	−	−
21	−	−	−	−	+	−	−
22	+	−	−	−	+	−	−
23	−	−	−	−	+	−	−
24	+	−	−	−	−	−	−
25	+	−	−	−	+	−	−
26	−	−	−	+	++	−	−
27	+	−	−	+	++	−	−

+: <50 mm^2; ++: <100 mm^2.

the CFS. From this group of three strains, isolate 3 and isolate 24, lost their antimicrobial activity when samples were treated with catalase. Moreover, the inhibitory substance was not sensitive to proteolytic enzymes showing that, in both cases, inhibition was due to the production of hydrogen peroxide. Isolate 18 kept its antibacterial activity against *L. innocua* and *S. aureus* in the CFS and it was also sensitive to proteolytic enzymes. Therefore, the CFS from this strain was considered to contain bacteriocin-like substances and it was the one chosen for further studies, including genotypic identification.

3.4. Genetic identification of bacteriocin-producing LAB strain

The fragment of the rRNA gene amplified from strain 18 not only exhibited 100% homology with respect to *Lactobacillus sakei* ATCC 15578 (GenBank accession number AF429524) but also with respect to *Lactobacillus curvatus* (accession number AY375292). Difficulties in correctly identifying strains of the *Lb. sakei*/*curvatus* group have been reported before (Champomier-Vergès, Chaillou, Cornet, & Zagorec, 2002; Vermeiren, Devlieghere, & Debevere, 2004) and are further discussed. The strain was named as *Lb. sakei*/*curvatus* ACU-1 (Austral Chaco University).

3.5. Determination of LAB growth for maximum bacteriocin production

Maximum inhibitory activity against *L. innocua* was found at 24 h of incubation at 30 °C, which corresponded to the stationary phase (Fig. 1). Likewise, maximum inhibitory activity of strain ACU-1 against *S. aureus* was detected after 15 h of incubation, coinciding with the beginning of the stationary phase of growth (Fig. 1). In both cases, after time mentioned no further increase of inhibitory activity was found. Moreover, a decrease of 50% in the antimicrobial activity of the bacteriocin-producing strain *Lb. sakei*/*curvatus* ACU-1 against *S. aureus* was observed at 36 h of incubation.

3.6. Effect of thermal treatments on bacteriocin activity

Treatment of the extracellular extracts of this strain at either 70 °C/15 min, 85 °C/30 min or at 100 °C/15 min did not elicit any loss of antimicrobial activity with respect to either *L. innocua* or *S. aureus*.

The results of bacteriocin stability throughout the refrigerated storage time showed that the maximum inhibitory activity remained constant up to 21 days when the supernatant was stored at 5 °C. In addition, 100% of the initial activity was observed after the three freeze-thaw cycles to which the CFS was subjected.

3.7. Effect of pH on bacteriocin activity

The CFS obtained from a bacterial culture grown at 30 °C during 24 h was used to perform this trial since this condition led to maximum bacteriocin production as it is exposed elsewhere in this work. The antimicrobial activity of the different CFS is summarized in Table 2. It can be observed that the pH of the growth media exerted a

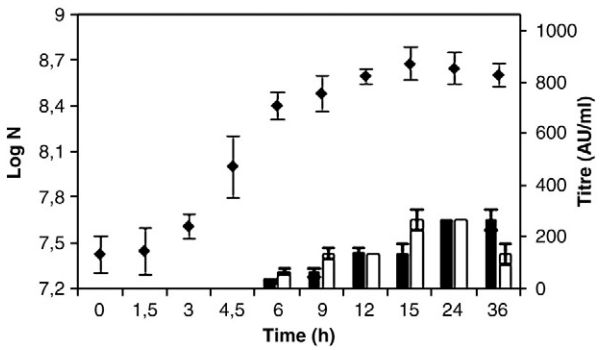

Fig. 1. Bacteriocin production and growth kinetics of *Lb. curvatus*/*sakei* ACU-1 growing in MRS broth. Dark column: titre of bacteriocin against *L. innocua* ATCC 33090. Light column: titre of bacteriocin against *S. aureus* FBUNT.

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Table 2
Influence of different pH on bacteriocin activity of free cell supernatant from *Lb. sakei/curvatus* ACU-1. Data showed correspond to the diameters (mm) of the inhibition zones displayed in the Agar Well Diffusion Assay.

pH treatment	Indicator strain	
	L. innocua ATCC 33090	*S. aureus* FBUNT
Control (final pH value: 4.23)*	$11.25^{a} \pm 0.25$	$11.00^{a} \pm 0.00$
3.50	$10.00^{a} \pm 0.00$	$10.00^{a} \pm 0.00$
4.50	$9.25^{a} \pm 0.00$	$9.50^{a} \pm 0.00$
5.50	6.00 ± 0.00	6.00 ± 0.00
6.50	0	0

* Cell free supernatant from *Lb. sakei/curvatus* ACU-1 culture grown in MRS broth without adjusted pH. Mean values in the same column with the same letter are not significantly different ($P>0.05$).

significant influence ($p<0.05$) on the antimicrobial activity of the CFS against both indicator microorganisms. Thus, the bacteriocin-producing strain exhibited a broad pH range of activity against *L. innocua* as well as *S. aureus*. It should be pointed out that the antimicrobial activity increased as pH values decreased from 6.5 to 3.5 (Table 2). Maximum antimicrobial activity values were recorded at pH 3.5 and 4.5, showing no significant differences between them ($p>0.05$). Although the bacteriocin was effective within the pH range 3.5–5.5, it became completely ineffective at pH 6.5. It is also remarkable that the effect of pH on bacteriocin activity was inherent to each target microorganism. As expected, non-inoculated MRS broth whose pH values were adjusted to 3.5, 4.5, 5.5 and 6.5 showed no antimicrobial activity.

3.8. Growth and BLIS production in the presence of NaCl at 5 °C

Lb. sakei/curvatus ACU-1 was able to grow at 5 °C combined with 3% and 6% NaCl. Nevertheless, its antimicrobial activity was displayed by those CFS grown with 3% NaCl neither by those grown with 6% NaCl. The antimicrobial activity found was effective against both indicator microorganisms.

3.9. Effect of several food additives on bacteriocin activity

Various food additives were tested for their influence on the growth of *Lb. sakei/curvatus* ACU-1 and the production of the bacteriocin in MRS broth. Bacteriocin production was monitored along bacterial growth being the results presented in Fig. 2. No modifications on the pattern of bacterial growth curves were found for any of the additives tested compared to the control system (Figs. 1 and 2, panels A to I), except for 6% NaCl which showed a decrease on bacterial counts of the bacteriocin-producing strain (Fig. 2, panel J). Regarding bacteriocin activity, the presence of the different additives tested had several effects which were also dependent upon the sensibility of each indicator microorganism. Table 3 exposes the percentage of titre variation with respect to the control system for the maximum value reached during storage. Both indicator microorganisms showed a lower titre than the control system when 3% KCl, or 3 and 6% NaCl were added to the nutritive broth. As an illustration, bacteriocin activity against *L. innocua* was reduced eight times in the presence of 6% NaCl. Meanwhile, it was not recorded against *S. aureus*, being this additive the one that exerted the most drastic effect on bacteriocin activity. On the contrary, the presence of the two surfactant agents, Tween 20 and Brij 35, enhanced bacteriocin activity against *L. innocua* and *S. aureus*. Moreover, the presence of EDTA and ascorbic acid duplicated the value of the titre of the bacteriocin against *S. aureus*, while potassium sorbate reduced it. To sum up, the additives tested showed different effects on the activity of the bacteriocin produced by *Lb. sakei/curvatus* ACU-1 against the target foodborne bacteria selected for this trial.

4. Discussion

An amount of 141 strains had been isolated in this study, however, only 27 showed antimicrobial activity against at least one of the following indicator microorganisms: *L. innocua* ATCC 33090, *S. aureus* FBUNT, *Lb. plantarum* ATCC 8014 and *Brochothrix* spp. 396 and 405. None of the isolates showed antimicrobial activity against the Gram negative bacteria *E. coli* and *P. aeruginosa*. These findings are in accordance with De Martinis and Freitas (2003), Mathieu, Suwandhi, Rekhif, Millière, and Lefevre (1993), among others. It may be attributed to the lipopolysaccharide layer of the cell wall, which protects the cell membrane, i.e. the site of action of bacteriocins (Stevens, Sheldon, Klapes, & Klaenhammer, 1991). Regarding antimicrobial activity towards *L. innocua* ATCC 33090, twenty one strains were found to inhibit its growth. As this strain was chosen in lieu of the foodborne pathogenic bacteria *L. monocytogenes*, it could be possible that these isolates also show antibacterial activity against this pathogen.

In the last decades, several bacteriocinogenic strains of LAB were suggested as starter cultures to control foodborne pathogens in meat and meat products, including dry fermented sausages (Cintas, Casaus, Fernández, & Hernández, 1998; Mataragas, Drosinos, & Metaxopoulos, 2003; Työppönen, Markkula, Petäjä, Suihko, & Mattila-Sandholm, 2003). Moreover, bacteriocins produced by LAB could contribute to the safety of fermented meat products (Castellano et al., 2004; Dicks et al., 2004; Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007; Kouakou et al., 2009). Hence, the twenty one isolates that showed listericidal activity constitute a natural reservoir of strains that could be used as starter cultures in the local manufacture of fermented meat products. Finally, it is remarkable that many isolates presented antimicrobial activity against one or both *Brochothrix* spp. strains, a frequent spoilage microorganism of meat products. Thus, they could be added to the formulation of protective cultures which would enhance microbial safety of cooked meat products.

Despite the fact that the isolates showed antimicrobial activity against almost the entire indicator strains tested, it was found that their antibacterial activity was due to the production of organic acids, hydrogen peroxide or catalase. According to Ammor et al. (2006), this phenomenon might be related to the fact that the bacteriocin-like substance would remain attached to the wall of the bacteriocin-like producing cell and thus the inhibition would not be effective unless the bacteriocin-like producing cell and the indicator strain are in contact.

One isolate (18) kept its antimicrobial activity against *L. innocua* and *S. aureus* in the cell-free supernatant and it was also sensitive to proteolytic enzymes. On the whole, the cell-free supernatant from this strain contained bacteriocin-like substances leading to a genetic-procedure identification of the strain.

The identification of the selected strain showed 100% homology with respect to *Lactobacillus sakei* ATCC 15578 (GenBank accession number AF429524) as well as to *Lactobacillus curvatus* (accession number AY375292). Difficulties in correctly identifying strains of the *Lb. sakei/curvatus* group have been reported before (Champomier-Vergès et al., 2002; Vermeiren et al., 2004). Identification of these organisms was hampered not only because of the similar phenotypic reactions possessed by them but apparently also because of the heterogeneity within the species (Koort, Vandamme, Schillinger, Holzapfel, & Björkroth, 2004). The phenotypic and genotypic diversity within *Lb. curvatus* and *Lb. sakei*, as well as the close relatedness of the two species, have been revealed by many studies (Berthier & Ehrlich, 1999). Phenotypically, *Lb. curvatus* and *Lb. sakei* were often split into sub-groups (Klein et al., 1996; Samelis, Tsakalidou, Metaxopoulos, & Kalantzopoulos, 1995; Schillinger & Lücke, 1987) when physiological and/or biochemical data were analyzed. The main criteria used to differentiate *Lb. sakei* from *Lb. curvatus* varied among laboratories, which resulted in the assignment of a strain to either *Lb. sakei*, *Lb.*

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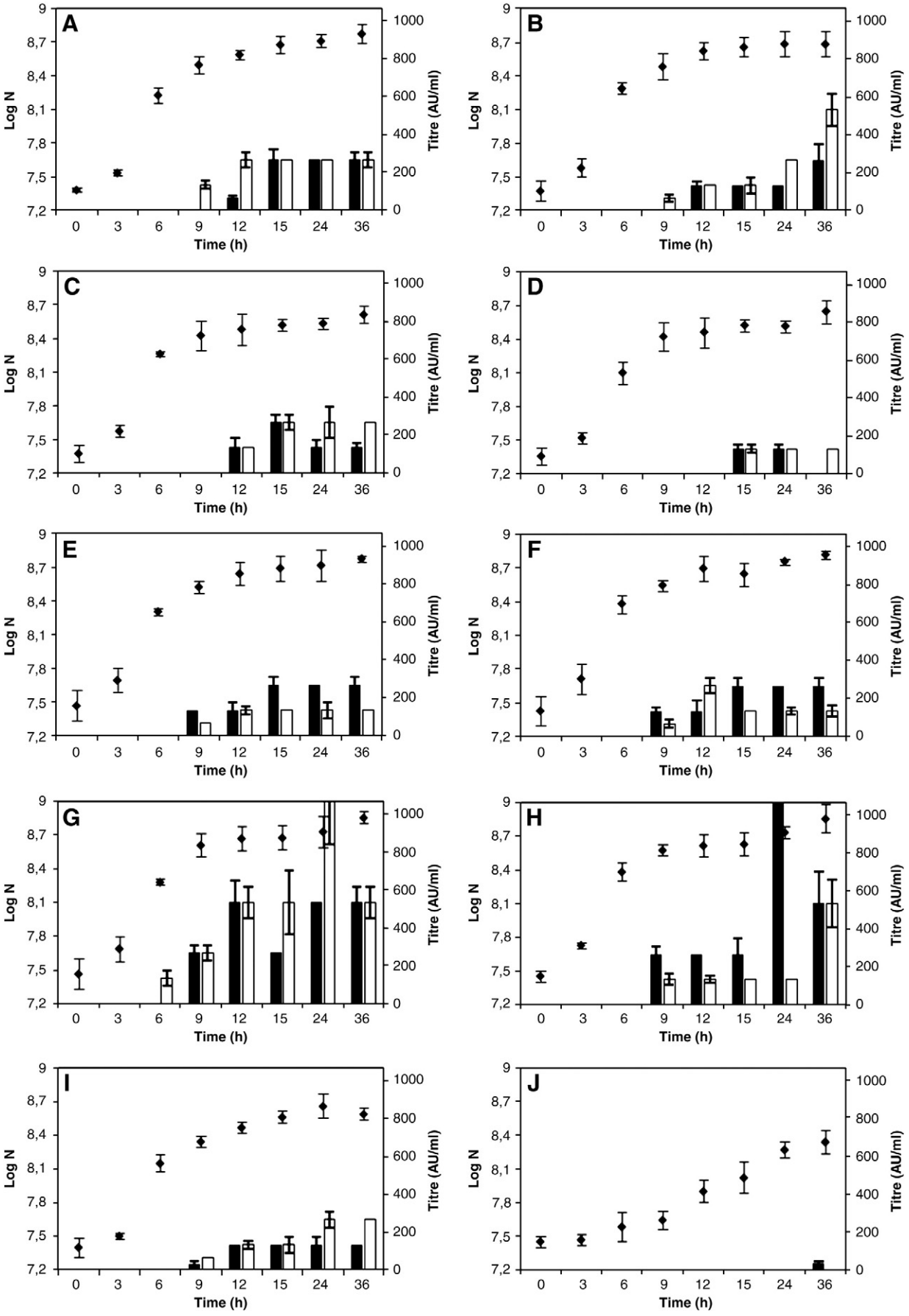

Fig. 2. Bacteriocin production and growth kinetics of *Lb. curvatus/sakei* ACU-1 growing in MRS broth with different additives. A: EDTA; B: Ascorbic Acid; C: 1% KCl; D: 3% KCl; E: Potasssium Sorbate; F: sodium citrate; G: Tween 20; H: Brij 35; I: 3% NaCl; J: 6% NaCl. Dark column: titre of bacteriocin against *L. innocua* ATCC 33090. Light column: titre of bacteriocin against *S. aureus* FBUNT.

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Table 3
Percentage of titre variation with respect to the control system for the maximum value reached during storage.

Additive	Titre variation (%)	
	L. innocua ATCC 33090	*S. aureus* FBUNT
Control system (without additives)	0	0
EDTA	0	(+)100
Ascorbic Acid	0	(+)100
KCl (1% w/v)	0	0
KCl (3% w/v)	(−)50	(−)50
Potassium sorbate (PS)	0	(−)50
Sodium citrate	0	0
Tween 20	(+)100	(+)400
Brij 35	(+)400	(+)100
NaCl (3% w/v)	(−)50	0
NaCl (6% w/v)	(−)83	−*

The positive and negative signs indicate increase and reduction towards control system, respectively. The titres of antimicrobial activity were performed in two independent experiments by duplicate.
* No antimicrobial activity was detected.

curvatus, a group of atypical *Lb. sakei*/*Lb.curvatus*, or no assignment at all, depending on the criteria used (Döring, Ehrhardt, Lücke, & Schillinger, 1988; Klein et al., 1996; Montel, Talon, Fournaud, & Champomier, 1991; Samelis et al., 1995; Schillinger & Lücke, 1987). On account of both this hurdle and the scope of the present study, it had been decided to name after the isolate 18 as *Lb. sakei*/*Lb. curvatus* ACU-1 considering the need for further research to define bacterial identity.

In order to make commercial use of bacteriocins economically feasible, optimization of yield during production is necessary. As a first attempt, the findings of the relationships between the growth rate of strain ACU-1 and maximum bacteriocin production gave a valuable piece of information. Maximum levels of antimicrobial activity against *L. innocua* and *S. aureus* were detected during the stationary phase of growth. Similar trends were reported by Leroy and De Vuyst (2003) for a bacteriocinogenic *Lb. sakei* strain isolated from a Spanish fermented dry sausage. In this study, the production of BLIS from *Lb. sakei*/*Lb. curvatus* ACU-1 reached maximum activity (266 AU/ml) after 15-24 h of incubation at 30 °C, and the highest bacterial count was observed after 15 h of incubation (Fig. 1) which is in agreement with the bacteriocin production data from LAB (Cheikhyoussef et al., 2009; Ghrairi, Frere, Berjaud, & Manai, 2008; Liu, Lv, Li, Zhou, & Zhang, 2008). This observation leads to the idea of a production dependent upon the cell number, which suggests the secondary metabolite production mechanism (Cheikhyoussef et al., 2009; Ondaa, Yanagidab, Tsujia, Shinoharab, & Yokotsuka, 2003; Todorov & Dicks, 2009).The BLIS titre at 36 h decreased by 50% which is in keeping with bacteriocins production by *Lactococcus* (Ondaa et al., 2003) and *Bifidobacterium* (Cheikhyoussef et al., 2009). This decrease in the titre may be due to the activity of proteases during this growth phase (Ondaa et al., 2003; Zamfir, Callewaert, Cornea, & De Vuyst, 2000).

Among other environmental conditions, a bacteriocin should resist pH variations so as to be used as a potential antimicrobial agent. The pH variations (3.5-6.5 range) had a significant influence on the antimicrobial activity of the bacteriocin-like substance produced by *Lb. sakei*/*Lb. curvatus* ACU-1 strain against both indicator microorganisms. Maximum inhibitory effects were registered at pH values 3.5 and 4.5, findings that are in agreement with previous reports referring to bacteriocinogenic LAB from meat products which described the highest antimicrobial activity at acidic pH values (Schneider et al., 2006; Vignolo, de Kairuz, de Ruiz Holgado, & Oliver, 1995). Besides, pH effect was dependent upon the indicator microorganism tested which had also been observed by other authors (Campos, Rodríguez, Calo-Mata, Prado, & Barros-Velázquez, 2006; Castellano, Farias, Holzapfel, & Vignolo, 2001). Although the bacteriocin-like substance was effective within the pH range 3.5–5.5, it failed to inhibit bacterial growth at pH 6.5. Campos et al. (2006) referred to LAB bacteriocins as having a stronger effect at acidic pH values. The maintenance of the antimicrobial activity in the pH range exposed above suggests a promising application of this bacteriocin-like substance in acidic foods.

Although it has been described earlier that only a limited number of bacteriocinogenic strains are able to grow at low temperatures (Hugas, 1998), the bacteriocin-producing strain *Lb. sakei*/*curvatus* ACU-1 was tested on its potential to grow in MRS broth at low temperature (5 °C) combined with salt concentrations occurring in the water phase of cooked meat products.

Lb. sakei/*curvatus* ACU-1 was able to grow at 5 °C combined with 3% and 6% NaCl coinciding with a work from Vermeiren et al. (2004) where most strains belonging to the *Lactobacillus sakei*/*curvatus* group could grow at low temperatures and high salt concentrations. Likewise, *L. sakei* is known to be one of the most psychrotrophic species of lactobacilli since some strains grow at 2–4 °C (Champomier-Vergès et al., 2002). Therefore, the production and effectiveness of the bacteriocin-like substance was tested in these conditions. Bacteriocin production was decreased by 3% NaCl, however, it could not be detected in MRS broth containing 6% NaCl. This is in keeping with the results of Himelbloom, Nilsson, and Gram (2001) which showed a decreasing bacteriocin production with increasing NaCl concentrations, not being detected in fish juice medium containing 6 and 7% NaCl. As far as it is known, this phenomenon might be related to the extent of bacterial growth. Delgado et al. (2005) postulated that bacteriocin production in the presence of NaCl is closely related to the number of growing cells in the medium, thus, if 6% NaCl conditioned bacterial growth, it would affect bacteriocin production.

A number of factors must be taken into consideration when choosing a bacteriocinogenic strain for bacteriocin *in situ* or *ex situ* production. Among several factors, presence and concentration of food additives are master keys for an optimum performance of a bacteriocinogenic strain in a producing medium. In the present study various food additives were tested for their influence on the growth of *Lb. sakei*/*curvatus* ACU-1 and the production of the bacteriocin in MRS broth. Even though a negative effect of 6% NaCl was observed on bacterial counts, no changes were found in the rest of the additives tested compared to the control system. Regarding bacteriocin production, food additives may interact with the bacteriocins themselves, or with their target, i.e. the bacterial cytoplasmic membrane. The activity of bacteriocins is the result of hydrophobic and electrostatic interactions of these amphiphilic, positively charged peptides with the microbial membrane (Gänzle, Weber, & Hammes, 1999). Hence, the quantity of the response may be specific for the combinations of bacteriocins, food additives and target organisms.

EDTA is used in food products to prevent oxidation, it also has antimicrobial activity and potentiates the activity of antimicrobials, especially against Gram-negative microorganisms (Belfiore, Castellano, & Vignolo, 2006). In the present study, EDTA had no effect on bacteriocin activity against *L. innocua*, but exerted a stimulatory effect on the activity of the bacteriocin against *S. aureus*. Since EDTA is known to disturb the highly ordered structure of the outer membrane and thus allows access of hydrophobic molecules to the cytoplasmic membrane (Vaara, 1992), it can be assumed that, in this case, it would be able to sensitize *S. aureus* membrane but not *L. innocua* one. Because bacteriocins do not act equally against target species, many researchers have examined the affinity of bacteriocins to specific species and strains. The phospholipid composition of the target strains and environmental pH influence the MIC values (Chen, Ludescher, & Montville, 1997). Instead of pore formation occurring indiscriminately, it appears that “docking molecules” on the target cell membrane facilitate the interaction with the bacteriocin, thereby increasing the effectiveness of the bacteriocin (Cleveland, Montville, Nes, & Chikindas, 2001). Equally important, ascorbic acid influenced bacteriocin activity in the same way exposed for EDTA.

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Potassium chloride is the most closely related compound to sodium chloride, especially with regard to its physical and functional properties (Ravishankar & Juneja, 2000). As a 1:1 mixture of sodium and potassium chloride when fed to human subjects showed a reduction of sodium intake by 44–55% (Mickelson, Nakadani, Gill, & Frank, 1972). In the present study, the presence of 1% KCl did not affect bacteriocin activity while 3% KCl halved its activity, emulating the effect of the same concentration of NaCl found on bacteriocin activity against *L. innocua*. It has been suggested that the decrease in bacteriocin production in the presence of salt is due to interference of sodium chloride molecules with binding of the induction factor, which is essential for bacteriocin production, to its receptor (Nilsen, Nes, & Holo, 1998).

Potassium sorbate is widely used as antimicrobial. Its high reactivity becomes important in food systems where it can participate in reactions with different food components (Gerschenson, Campos, Rojas, & Binstok, 2000). In this work, the presence of potassium sorbate in MRS broth either affected bacteriocin effectiveness (against *L. innocua*) or halved its antibacterial activity (against *S. aureus*). Nevertheless, Castro, Rojas, Campos, and Gerschenson (2009) found that nisin activity against *Lb. fructivorans* was enhanced in the presence of potassium sorbate in acidic food model systems.

Remarkably, both non-ionic surfactants used, namely Tween 20 and Brij 35, enhanced bacteriocin activity in a great extent. Although the effects were strain dependent, they reinforce the importance of the addition of this type of emulsifiers to the growth media of bacteriocinogenic bacteria. Indeed, Vignolo et al. (1995) indicated that Tween 80 was a critical factor in lactocin 705 production. Non-ionic detergents may mimic the effect of various food constituents to induce production of bacteriocins, and they are known to stimulate secretion of protein by affecting membrane fluidity (Reese & Maguire, 1969). Tween-treated cultures also increased the supernatant activity relative to total activity, probably by desadsorption and disaggregation of the bacteriocin as reported in some purification protocols (González, Arca, Mayo, & Suárez, 1994; Ten Brink, Minekus, van der Vossen, Leer, & Huis in't Veld, 1994), but not by sensitization of the target cells as total activities remained at the same level (Aymerich, Artigas, Garriga, Monfort, & Hugas, 2000). Furthermore, the presence of surfactants, which are included in sauces and dressings formulations, may counteract the antagonistic effect exerted by a lipid phase against the antimicrobial activity of bacteriocins (Bhatti, Veeramachaneni, & Shelef, 2004; Castro et al., 2009).

The results presented in this study could contribute to characterize the antimicrobial activity of the indigenous flora of artisanal meat products manufactured in the north-eastern region of the province of Chaco, Argentina. In addition, the bacteriocinogenic strain isolated and identified as *Lb. curvatus/sakei* ACU-1showed a regular production of bacteriocin like substances in culture broth and yielded different bacteriocin activity in the presence of the additives tested. It has been shown that bacteriocin like substances production by this LAB strain was greatly influenced by NaCl concentration and the presence of surfactants while it was influenced to a lesser extent by the presence of EDTA, KCl, potassium sorbate and sodium citrate. This fact highlights the importance of considering additives interactions when evaluating antimicrobial activity and productivity. In further studies bacteriocin activity will be assessed in sausages that were used for isolation of the producing strain. This information will help specify whether such strain can be of industrial interest.

Acknowledgements

The authors acknowledge the financial support from Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Tecnológicas de la República Argentina and Agencia Nacional de Investigaciones Científicas y Tecnológicas de la República Argentina. Besides, the authors wish to thank Dr. María Elisa Cayré and María Isabel Aznar for valuable discussions.

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Please cite this article as: Castro, M.P., et al., Lactic acid bacteria isolated from artisanal dry sausages: Characterization of antibacterial compounds and study of the factors affecting bacteriocin production, *Meat Science* (2010), doi:10.1016/j.meatsci.2010.11.006