Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese

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ABSTRACT

Lactobacillus spp. from Mexican Cocido cheese were shown to produce bacteriocin-like substances (BLS) active against *Staphylococcus aureus*, *Listeria innocua*, *Escherichia coli*, and *Salmonella typhimurium* by using the disk diffusion method. Crude extracts of *Lactobacillus fermentum* showed strong inhibitory activity against *Staph. aureus*, *L. innocua*, *E. coli*, and *Salmonella cholerae*. Complete inactivation of antimicrobial activity was observed after treatment of crude extracts with proteinase K, pronase, papain, trypsin, and lysozyme, confirming their proteinaceous nature. However, antimicrobial activity was partly lost for some of the crude extracts when treated with α -amylase, indicating that carbohydrate moieties were involved. The antimicrobial activity of the crude extracts was stable at 65°C for 30 min over a wide pH range (2–8), and addition of potassium chloride, sodium citrate, ethanol, and butanol did not affect antibacterial activity. However, antimicrobial activity was lost after heating at 121°C for 15 min, addition of methanol or Tween 80. Fourteen out of 18 *Lactobacillus* spp. showed antimicrobial activity against different test microorganisms, and 12 presented bacteriocin-like substances. Generation time and growth rate parameters indicated that the antimicrobial activity of crude extracts from 3 different strains was effective against the 4 indicator microorganisms. One of the crude extracts showed inhibition not only against gram-positive but also against gram-negative bacteria. Bacteriocin-like substances produced by this specific *Lactobacillus* strain showed potential for application as a food biopreservative.

Key words: bacteriocin, lactic acid bacteria, antimicrobial activity

INTRODUCTION

Lactic acid bacteria (LAB) have been used in different foods for the production of organic substances that contribute to the sensory attributes and the preservation of food (Parada et al., 2007). The preservative properties are based on the antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl, reuterin, antifungal peptides, and bacteriocins (Ponce et al., 2008; Arqués et al., 2011; Stoyanova et al., 2012; Ghanbari et al., 2013). Bacteriocins are ribosomally synthesized peptides with antimicrobial activity produced by bacteria such as LAB (*Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, and *Leuconostoc*) that are of particular interest due to their potential use as biopreservatives in the food industry (Todorov et al., 2011; Balciunas et al., 2013). The interest in the application of bacteriocins in food preservation has increased in the last years due their nontoxicity, sensitivity to proteases, general pH and heat stability, and antimicrobial effect against species of gram-positive bacteria, such as *Staphylococcus aureus*, *Clostridium tyrobutyricum*, *Listeria innocua*, *Listeria monocytogenes*, and *Bacillus cereus*. Additionally, a few bacteriocins have activity against gram-negative bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Helicobacter pylori* NCIPD 230, and *Campylobacter jejuni* (Gálvez et al., 2007; García et al., 2010; Todorov et al., 2010; Reis et al., 2012). However, only nisin (*Lactococcus lactis*) and pedocin PA-1 (*Pediococcus acidilactici*) are approved and permitted for preservation of foods (Parada et al., 2007; Pinto et al., 2011; Biscola et al., 2013). The factors affecting the bacteriocin activity in different food systems are the composition (proteins and lipids), enzymatic degradation, manufacturing process (high temperatures), physical properties, such as pH and

Received July 11, 2015.

Accepted September 2, 2015.

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additives present (Gálvez et al., 2007; Parada et al., 2007; Hartmann et al., 2011; Gao et al., 2013). Thus, the successes of using bacteriocins in the food industry for the control of different pathogens are necessary to demonstrate the antimicrobial activity of bacteriocins by in vitro studies and in food systems (Gálvez et al., 2008; Biscola et al., 2013). In dairy products, the antimicrobial effect of different bacteriocins against gram-positive bacteria has been studied, but few reports are available on the effect against gram-negative bacteria. Thus, demand for natural antimicrobial compounds has brought interest in new bacteriocins able to enhance dairy safety.

The aim of the present work was to screen for BLS production in wild *Lactobacillus*, isolated during the manufacture of artisanal Mexican Cocido cheese, with bioactivity against gram-positive and gram-negative bacteria. Mexican Cocido cheese is a semi-soft *pasta filata* cheese variety typical of the northwest of Mexico. It is made from raw milk using traditional techniques, which include acidification by using cheese whey from a previous cheese batch and renneting. A typical stage of the manufacturing is the process of cooking the curd until it reaches a dough-like consistency. Then it is placed into molds where it is shaped and cooled.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

All 18 wild *Lactobacillus* strains were obtained from the culture collection of the Dairy Laboratory at the Food Research and Development Center, A.C. (CIAD, A.C., Hermosillo, Sonora, Mexico). These strains were isolated during the making of artisanal Mexican Cocido cheese in Hermosillo, Sonora, Mexico. Briefly, at least 4 colonies were randomly picked from de Man, Rogosa, and Sharpe (MRS; BD Difco, Sparks, MD) agar count plates from different samples collected from cheese milk, whey, curd, and cheese. After purification, colonies were stored at -80°C in (80%, vol/vol) glycerol until further characterization (González et al., 2007).

Prior to each experiment, *Lactobacillus* strains were subcultured in 10 mL of MRS at 37°C (pH 6.5). Three consecutive cultures (1% inoculum) were prepared and incubated for 24, 20, and 16 h, respectively. The last culture was used as the inoculum for all the experiments (fresh culture).

Staphylococcus aureus ATCC 29213, *L. innocua* ATCC 33090, *S. typhimurium* ATCC 14028, and *E. coli* ATCC 25922 were cultured in brain heart infusion broth (BHI; BD Difco, Sparks, MD). Three subcultures were inoculated at 1% and incubated at 37°C .

The first subculture was incubated for 24 and 16 h, and the last subculture was incubated for 8 h.

Molecular Identification of LAB

Lactic acid bacteria were grown in MRS broth for 18 h. Subsequently, 1 mL of sample was taken for DNA extraction using the commercial protocol PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). The method of amplification was based in the protocol of MicroSEQ Identification Systems (Applied Biosystems). The PCR cycles were carried out in a thermocycler endpoint (Eppendorf, Hamburg, Germany). Each PCR product was purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) and then sequenced based on the MicroSeq protocol. Sequencing reactions were purified using the BigDye Xterminator protocol (Applied Biosystems). The purified samples were examined in a 3500 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed in the MicroSeq software v2.0 (Applied Biosystems), and compared with the library of MicroSeq Bacterial 500 (Applied Biosystems) to determine the identity of the strains by their similarity to reference strains.

Preparation of CE

Lactobacillus strains were grown in MRS broth and incubated for 16, 18, 24, and 72 h at 37°C in water bath shaker (Lab-Line Instruments Inc., Melrose Park, IL). Aliquots of culture medium were centrifuged at $4,000 \times g$ for 20 min, at 4°C . The crude extracts (CE) were adjusted to pH 6.5 with a 1 N NaOH, treated with catalase enzyme (1,000 U/mL) for 1 h at 25°C , and filter sterilized (0.22 μm , Millex-GV, Millipore SpA, Milan, Italy). The untreated and treated CE were stored at 4°C (Karapetyan et al., 2010).

Antimicrobial Activity Determination

Antibacterial activity was tested against *Staph. aureus* ATCC 29213, *L. innocua* ATCC 33090, *S. typhimurium* ATCC 14028, and *E. coli* ATCC 25922 by the disk diffusion method. The filter-sterilized CE were used to evaluate antimicrobial activity. An aliquot of 20 μL supernatant was applied on a sterilized cellulose disk (6 mm) placed on a BHI soft agar plate (0.8% wt/vol) inoculated with each pathogen (10^7 cfu/mL). Plates were incubated at 37°C for 24 h, and the diameters of the inhibition zones around the disk were measured (Nespolo and Brandelli, 2010). The CE with inhibition zones larger or equal to 6 mm were selected (Ponce et al., 2008).

Effect of Enzymes on Antimicrobial Activity of CE

The CE were evaluated to find out if the inhibition was due to bacteriocin-like inhibitory substances (BLS). Thus, CE were treated with papain (30 U/mg), proteinase K (30 U/mg), pronase E (3.5 U/mg), lysozyme (35,000 U/mg), α -amylase (30 U/mg), and pepsin (250 U/mg) (all from Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/mL. Enzymes were dissolved (0.2%, wt/vol) in 0.05 mol/L Tris, 5 mmol/L CaCl_2 buffer (pH 7). For pepsin testing, the enzyme was dissolved in citrate buffer (pH 3). Enzyme solutions were filter sterilized (0.22 μm , Millex-GV, Millipore SpA). The CE were incubated with the different enzyme solutions at 37°C for 2 h, after which the retention of BLS in treated samples was determined by the disk diffusion method previously described (Todorov and Dicks, 2006).

Effect of pH, Temperature, Organic Solvents, and Additives on Antimicrobial Substances

The effect of pH on the antimicrobial substance was tested by adjusting the pH of the CE to values ranging from 2 to 8 (at increments of 1 pH unit) with sterile 1 *N* NaOH or 1 *N* HCl. Incubation was for 30 min at 30°C. The samples were tested for antimicrobial activity by using the disk diffusion method. The effect of temperature on the activity of antimicrobial substances was tested by heating the cell CE at 65, 75, 100, or 121°C. Residual antimicrobial activity was tested by the well diffusion method after subjecting the CE to the different temperatures for 30 min. The effect of organic solvents and additives on the activity of antimicrobial substances was tested by adding 10% (vol/vol, final concentration) butanol, methanol, and ethanol to CE and 1% (wt/vol, final concentration) potassium chloride, sodium citrate, or Tween 80 to CE. Antimicrobial activity of CE treated with additives was evaluated by using disk diffusion method at 37°C for 5 h. Untreated CE served as controls (Biscola et al., 2013; Ghanbari et al., 2013).

Growth of Indicator Microorganisms in the Presence of Different CE

A 20- μL aliquot of CE filter sterilized supernatant (pH 6.5) was added to 150 mL of BHI culture containing different pathogens, namely, *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*. Then, mixtures were incubated for 24 h. Optical density readings at 600 nm were recorded every 2 h. Growth data were adjusted with the modified Gompertz equation (Chowdhury et al., 2007):

$$\text{Log } n = A \times \text{EXP}\{-\text{EXP}[-B \times (t)]\}.$$

This equation was applied to growth on indicator microorganisms with different CE. Log *n* = decimal logarithm of microbial counts [Log (abs)] at time *t*, *A* = average of the logarithmic increase [Log (abs)], *B* = relative growth rate at time (h^{-1}), and *t* = time (h).

Statistical Analysis

The experiments followed a complete randomized design with 3 replicates per treatment: antimicrobial activity at 24, 48, and 72 h, CE with enzymes, pH, temperature, organic solvents, and additives. Growth experiments were carried out in duplicate. Data were analyzed by one-way ANOVA with a significance level of 0.5%. Means were analyzed by the comparison test of Tukey-Kramer with a significance level of 0.05%. For statistical analyses, the NCSS statistical software version 2007 (NCSS LLC, Kaysville, UT) was used.

RESULTS AND DISCUSSION

Molecular Identification

Lactobacillus strains were identified molecularly by comparing the nucleotide sequences of the 16S r gene (500 bases) with other sequences deposited in the database of MicroSEQ bacteria. The MicroSEQ protocol states that a 97% homology with ATCC strains is enough to determine the molecular identification of bacteria. Thus, most strains were identified as *Lactobacillus fermentum* with >99% homology with *Lb. fermentum* ATCC 14931. Only J25 and J33 were identified as *Lactobacillus plantarum* and *Lactobacillus paracasei tolerans* with >99% homology to ATCC 14917 and ATCC 25599, respectively, and strains J26, J27, J31, J34, J36, and J37 were identified as *Lactobacillus pentosus* with >99% homology to ATCC 8041 (Table 1).

Antimicrobial Activity of CE from *Lactobacillus* spp. Against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*

Antimicrobial activity by LAB may be due to different metabolites such as organic acids and hydrogen peroxide and could be erroneously attributed to the production of bacteriocin-like compounds. In this study, the CE were adjusted to pH 6.5 and treated with catalase to eliminate the effect of acid or hydrogen peroxide. Thus, the inhibitory activity of the CE was not due to the production of these compounds. The CE obtained from *Lactobacillus* spp. at 16 and 18 h did not show antimicrobial activity by the disk diffusion

Table 1. Molecular identification of lactic acid bacteria (LAB)¹

Strain	Identified LAB	% Similarity
J10	<i>Lactobacillus fermentum</i>	99.72 (ATCC 14931)
J20	<i>Lactobacillus fermentum</i>	99.78
J23	<i>Lactobacillus fermentum</i>	99.78
J24	<i>Lactobacillus pentosus</i>	100 (ATCC 8041)
J25	<i>Lactobacillus plantarum</i>	100
J26	<i>Lactobacillus pentosus</i>	100
J27	<i>Lactobacillus pentosus</i>	100
J28	<i>Lactobacillus fermentum</i>	99.64
J29	<i>Lactobacillus fermentum</i>	99.77
J30	<i>Lactobacillus fermentum</i>	99.62
J31	<i>Lactobacillus pentosus</i>	100
J32	<i>Lactobacillus fermentum</i>	99.81
J33	<i>Lactobacillus paracasei tolerans</i>	99.99 (ATCC 25599)
J34	<i>Lactobacillus pentosus</i>	100
J35	<i>Lactobacillus fermentum</i>	99.78
J36	<i>Lactobacillus pentosus</i>	100
J37	<i>Lactobacillus pentosus</i>	99.99
J38	<i>Lactobacillus fermentum</i>	99.68

¹Results are based on 16Sr gene (500 bases) deposited in the database of MicroSEQ.

method. Nevertheless, most of the CE obtained at 24, 48, and 72 h presented antimicrobial activity against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*, except for extracts produced by *Lb. pentosus* strain J24, J26, J27, and J34 (Figure 1). However, CE obtained at 72 h showed the largest antimicrobial activity for each strain ($P < 0.05$); therefore, this time was used for the rest of experiments. The largest antimicrobial activity of bacteriocins at 72 h as shown in this study is in agreement with previous studies (McClerren et al., 2006; Basurto-Cadena et al., 2012). In fact, authors reported minimum antimicrobial activity at 24 h and maximum antimicrobial activity after 60 h for a *Bacillus subtilis* strain (Basurto-Cadena et al., 2012), suggesting that the antagonistic activity of proteases was lost in the first 24 h, not affecting the activity of the bacteriocins in the rest of the time. Also, McClerren et al. (2006) reported that *Bacillus halodurans* produced the bacteriocin haloduracin after 90 h of incubation. It has been reported that several mechanisms may be responsible for the decrease of bacteriocin activity of which protein aggregation, proteolytic degradation (proteases), and bacteriocin adsorption to the cells were reported (De Vuyst et al., 1996).

Among the 14 strains that presented antimicrobial activity against indicator microorganisms, *Lb. fermentum* strain J23 presented the highest antimicrobial activity against all tested microorganisms. On the other hand, *Lb. fermentum* strains J20, J28, and J32 presented the highest antimicrobial activity against *E. coli* and *S. typhimurium*. Simova et al. (2009) reported that CE of *Lactobacillus rhamnosus* obtained at 20 h presented antimicrobial activity against *L. innocua*, *Staph. aureus*, and *E. coli*, whereas *Lactobacillus bulgaricus*

presented activity against *Staph. aureus*, *E. coli*, and *S. typhimurium*. However, the antimicrobial activity reported by these authors was lower than the antimicrobial activity found in the present study. The effect of the CE against different test pathogens is very important, particularly against *Staph. aureus* and *E. coli*, because these pathogens have been previously found in Fresco cheese manufactured with raw milk (Torres-Llanez et al., 2006). *Staphylococcus aureus* has been described as a common contaminant of dairy products and was detected in milk and artisanal Mexican cheeses (Torres-Llanez et al., 2006).

Effect of Enzymes on Antimicrobial Substances Against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*

The CE from *Lactobacillus* spp. were treated with different enzymes to verify the proteinaceous nature of the inhibitory substances (Table 2). Determination of the proteinaceous nature of the inhibitory substances confirmed that the antimicrobial agents produced by *Lactobacillus* spp. were bacteriocin-like substances (BLS), except for those from *Lb. fermentum* strains J29 and J30. According to Ghanbari et al. (2013), the inactivation of antimicrobial activity by the action of proteolytic enzymes was an indication of the proteinaceous nature of BLS.

Additionally, BLS from *Lb. fermentum* strains J10, J20, J25, J28, and J32 and *Lb. pentosus* strain J36 were inhibited by α -amylase, indicating that the bacteriocins produced by *Lactobacillus* may be glycoproteins (carbohydrate moiety), which require both the glyco and the protein portion of the molecule for the activity.

This indicates that probably these bacteriocins belong to group IV in their classification, which contains carbohydrates and lipids in the molecular structure (Todorov et al., 2011). However, this fact needs to be confirmed after bacteriocin purification and structure determination. Several glycoprotein bacteriocins have been reported such as *Enterococcus faecium* DB1 and *Lactobacillus brevis* DF01, which produced bacteriocins sensitive to α -amylase (Seo et al., 2014). Other bacteriocins reported were leuconocin S produced by *Leuconostoc paramesenteroides* and carnocin 54 produced by *Leuconostoc carnosum* (Todorov et al., 2010).

Effect of Temperature, pH, Organic Solvents, and Additives on Antimicrobial Substances Against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*

Temperature Effect. The effect of temperature on the antimicrobial activity of CE against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* is shown in Table 3. In general, the antimicrobial activity was stable at 65°C for 30 min and was most unstable at 121°C for 15 min. The CE from J20, J23, J25, J28, J32, J37, and J38 showed the most stability to heat treatment, retaining 100% of antimicrobial activity after 30 min at 65 or 75°C. However, antimicrobial activity decreased to some extent at 100°C for 30 min because the residual activity of the CE was around 80 to 90% (Table 3).

This observation indicates that these bacteriocins may be used as biopreservatives in foods that are subjected to pasteurization, as is the case for dairy foods. Similar results were reported for Pringsulaka et al. (2012), bacteriocins produced by *Weissella cibaria* N23 were stable after subjecting them to 60 to 100°C for 23 min. These bacteriocins retained 50% of the antimicrobial activity at 100°C for 30 min. Conversely, bacteriocins produced by *Lb. bulgaricus* BB18 and *Lb. lactis* BCM5 showed high stability to heat treatment, retaining the antimicrobial activity after 60 min at 100°C (Simova et al., 2009). It has been reported that bacteriocin stability to heat treatment produced by different strains may be attributed to differences in ecological and environmental adaptation (Ponce et al., 2008).

pH Effect. The CE produced by *Lactobacillus* spp. were inhibitory against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* at a wide pH range (2.0–8.0; Table 3). Similar results were reported where antibacterial activity of different LAB strains presented a wide pH range (4.0–8.0) (Mezaini et al., 2009). Bacteriocin ALP57 from *Pediococcus pentosaceus* and bacteriocin ALP7 from *E. faecium* maintained antimicrobial activity against different strains at pH range from 2 to 8, but lost activity at pH 12 (Pinto et al., 2009). This is in agreement with the fact that the highest antimicrobial activity was found at the acid or neutral pH (Ponce et al., 2008). Bacteriocin stability in a wide pH range, as was shown in this study, is of technological importance

Figure 1. Antimicrobial activity of crude extracts obtained from different *Lactobacillus* against indicator strains. *Indicates significant differences between 24, 48, and 72 h for each strain ($P < 0.05$). Error bars indicate 95% confidence interval.

Table 2. Effect of enzymes on the residual antimicrobial activity (%) of crude extracts from different *Lactobacillus* strains on indicator microorganisms¹

Enzyme	Strain												
	J10	J20	J23	J25	J28	J29	J30	J31	J32	J33	J35	J36	J38
Papain	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	83 ± 0.9	76 ± 0.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pepsin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	67 ± 0.6	98 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Protease E	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	56 ± 0.9	89 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Proteinase K	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	88 ± 0.8	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α-Amylase	15 ± 0.4	65 ± 0.18	100 ± 0	22 ± 0.8	25 ± 0.6	100 ± 0	10 ± 0	100 ± 0	89 ± 0.9	100 ± 0	100 ± 0	75 ± 0.9	100 ± 0

¹Enzyme concentration 1 mg/mL. Results represent the mean value of triplicates ± SD.

Table 3. Effect of temperature, pH, organic solvents, and additives on residual antimicrobial activity (%) of crude extracts from different *Lactobacillus* strains (J10 to J38) on indicator microorganisms¹

Treatment	Strain												
	Concentration	J10	J20	J23	J25	J28	J31	J32	J33	J35	J36	J37	J38
Temperature/time													
65°C/30 min		95 ± 0.26	100 ± 0	100 ± 0	100 ± 0	100 ± 0	98 ± 0.26	100 ± 0	79 ± 0.19	100 ± 0	67 ± 0.26	100 ± 0	100 ± 0
75°C/30 min		88 ± 0.18	100 ± 0	100 ± 0	100 ± 0	100 ± 0	83 ± 0.17	100 ± 0	56 ± 0.13	89 ± 0.19	43 ± 0.19	100 ± 0	100 ± 0
100°C/30 min		22 ± 0.09	85 ± 0.19	90 ± 0.12	75 ± 0.17	89 ± 0.18	49 ± 0.38	88 ± 0.12	16 ± 0.56	49 ± 0.31	0 ± 0	95 ± 0.17	90 ± 0.23
121°C/15 min		0 ± 0	35 ± 0.21	37 ± 0.23	17 ± 0.59	28 ± 0.87	0 ± 0	15 ± 0.74	0 ± 0	0 ± 0	0 ± 0	22 ± 0.15	9 ± 0.04
pH													
2		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
3		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
4–8		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Organic solvent													
Butanol	10% (vol/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Ethanol	10% (vol/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Methanol	10% (vol/vol)	95 ± 0.48	98 ± 0.32	95 ± 0.59	90 ± 0.25	98 ± 0.32	81 ± 0.44	95 ± 0.49	96 ± 0.33	89 ± 0.21	93 ± 0.22	0.91 ± 0.15	88 ± 0.16
Additive													
Potassium chloride	1% (wt/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Sodium citrate	1% (wt/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Tween 80	1% (wt/vol)	0 ± 0	23 ± 0.79	18 ± 0.62	0 ± 0	10 ± 0.24	0 ± 0	11 ± 0.24	0 ± 0	0 ± 0	0 ± 0	16 ± 0.49	13 ± 0.29

¹Results represent the mean value of triplicates ± SD.

for the dairy industry, because they may be used in different foods, such as fermented milk products.

Organic Solvent and Additive Effects. The CE remained inhibitory to *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* after 30 min of treatment with substances such as sodium citrate and potassium chloride (Table 3). Parada et al. (2007) reported that the bacteriocin lactocin 705 against *L. monocytogenes* lost activity in the presence of sodium chloride, sodium citrate, ascorbic acid, and sodium lactate. Although the addition of anionic compounds did not affect antibacterial activity against test microorganisms, the addition of Tween 80 decreased their activity (Table 3). Additionally, CE produced by *Lactobacillus* spp. presented antimicrobial activity against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* in the presence of different organic solvents (Table 3). Similarly, lactocin RN78 and plantaricin LC74 bacteriocins produced by *Lactobacillus* spp. maintained antimicrobial activity after treatments with organic solvents such as butanol, ethanol, and methanol. The fact that bacteriocins were stable with organic solvents indicated that they were soluble in them (Ghanbari et al., 2013).

Growth Inhibition of *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* in the Presence of Different CE

Microbial growth parameters growth rate (μ) and generation time (gt) showing inhibition of indicator microorganisms by the different CE containing BLS are summarized in Tables 4, 5, 6, and 7. Coefficients of determination ($R^2 = 0.95$ to 0.99) indicated that the Gompertz model was able to describe microbial growth accurately ($P < 0.05$). Of the 12 CE that presented antimicrobial activity and contained BLS, *Lb. fermentum* strain J23 showed the longest gt and the lowest μ compared with the controls (Tables 4, 5, 6 and 7). Thus, the CE from J23 had the greatest inhibitory activity against the 4 test microorganisms and may be the one with the largest concentration of BLS. The CE from J20 and J32 showed the next longest gt and the next lowest μ compared with the controls, for *Staph. aureus* and *Salmonella*, respectively (Tables 5 and 7). Therefore, CE from *Lb. fermentum* strains J20 and J32 had the greatest inhibitory activity against these pathogens after *Lb. fermentum* strain J23. Growth inhibition of the indicator microorganism by these 3 CE is shown in Figure 2. It is important to note that CE from J23 inhibited growth of not only gram-positive bacteria but also of gram-negative bacteria. Thus, these results indicated that CE from *Lb. fermentum* strain J23 contained BLS with a wide antimicrobial spectrum.

Table 4. Microbial growth parameters, derived from modified Gompertz equation, of *Escherichia coli* and *Lactobacillus* spp.¹

Item	R ²	μ (abs/h) ²	Generation time (h)
<i>E. coli</i>	0.98	0.20 ± 0.05^g	1.26 ± 0.23^a
J10	0.98	0.19 ± 0.06^g	1.33 ± 0.34^a
J20	0.98	0.09 ± 0.003^b	2.58 ± 0.35^f
J23	0.98	0.08 ± 0.004^a	2.65 ± 0.48^f
J25	0.98	0.10 ± 0.06^d	2.05 ± 0.42^{cd}
J28	0.98	0.08 ± 0.005^a	2.54 ± 0.67^f
J31	0.99	0.10 ± 0.04^d	2.37 ± 0.31^e
J32	0.95	0.10 ± 0.07^c	1.63 ± 0.42^b
J33	0.97	0.11 ± 0.08^e	1.93 ± 0.18^{cd}
J35	0.98	0.13 ± 0.06^f	1.90 ± 0.11^{cd}
J36	0.97	0.12 ± 0.05^e	1.36 ± 0.14^a
J37	0.98	0.09 ± 0.006^b	2.30 ± 0.52^e
J38	0.97	0.12 ± 0.06^f	1.80 ± 0.31^{bc}

^{a-g}Different superscripts within a column indicate significant differences ($P < 0.05$).

¹Data represent the mean values of duplicates $\pm$ SD.

² μ = growth rate, measured in absorbance units per hour.

CONCLUSIONS

Screening of *Lactobacillus* spp. isolated and identified in artisanal Mexican Cocido cheese for antimicrobial substances showed that they presented bacteriocin-like substances not only against gram-positive bacteria but also against gram-negative bacteria. This is an important finding since bacteriocins from LAB are not usually effective against gram-negative bacteria and rather present a narrow antimicrobial spectrum. Studies are undergoing to fully characterize the bacteriocin-like substances produced by *Lb. fermentum* strains J20, J23, and J32 and evaluate their antimicrobial activity in dairy foods.

Table 5. Microbial growth parameters, derived from modified Gompertz equation, of *Staphylococcus aureus* and *Lactobacillus* spp.¹

Item	R ²	μ (abs/h) ²	Generation time (h)
<i>Staph. aureus</i>	0.99	0.19 ± 0.06^h	1.34 ± 0.34^a
J10	0.99	0.18 ± 0.04^h	1.36 ± 0.27^a
J20	0.97	0.06 ± 0.009^b	2.79 ± 0.31^g
J23	0.98	0.05 ± 0.002^a	4.18 ± 0.45^h
J25	0.99	0.17 ± 0.08^g	1.35 ± 0.31^a
J28	0.98	0.08 ± 0.004^c	1.79 ± 0.56^c
J31	0.97	0.12 ± 0.05^d	2.08 ± 0.36^d
J32	0.96	0.08 ± 0.06^c	2.63 ± 0.54^f
J33	0.96	0.17 ± 0.09^f	2.55 ± 0.29^e
J35	0.97	0.16 ± 0.05^{ef}	2.79 ± 0.43^g
J36	0.97	0.18 ± 0.04^h	1.42 ± 0.11^b
J37	0.98	0.09 ± 0.009^d	2.42 ± 0.22^e
J38	0.96	0.16 ± 0.07^e	1.36 ± 0.38^a

^{a-h}Different superscripts within a column indicate significant difference ($P < 0.05$).

¹Data represent the mean value of duplicates $\pm$ SD.

² μ = growth rate, measured in absorbance units per hour.

Figure 2. Growth inhibition of indicator microorganisms by crude extracts from different *Lactobacillus* strains. Parameters were derived from modified Gompertz equation.

Table 6. Microbial growth parameters, derived from modified Gompertz equation, of *Listeria innocua* and *Lactobacillus* spp.¹

Item	R ²	μ (abs/h) ²	Generation time (h)
<i>L. innocua</i>	0.99	0.19 ± 0.05 ^g	1.25 ± 0.22 ^a
J10	0.96	0.18 ± 0.06 ^f	1.63 ± 0.19 ^b
J20	0.97	0.09 ± 0.006 ^b	2.00 ± 0.24 ^d
J23	0.98	0.09 ± 0.005 ^a	2.15 ± 0.37 ^e
J25	0.99	0.18 ± 0.06 ^f	2.02 ± 0.34 ^d
J28	0.98	0.10 ± 0.008 ^c	2.16 ± 0.28 ^e
J31	0.99	0.12 ± 0.09 ^e	1.72 ± 0.18 ^c
J32	0.96	0.10 ± 0.06 ^c	2.16 ± 0.36 ^e
J33	0.97	0.19 ± 0.06 ^g	1.2 ± 0.11 ^a
J35	0.98	0.11 ± 0.07 ^d	1.63 ± 0.19 ^b
J36	0.99	0.19 ± 0.09 ^g	1.63 ± 0.25 ^b
J37	0.98	0.09 ± 0.008 ^a	1.78 ± 0.42 ^c
J38	0.99	0.18 ± 0.09 ^f	1.26 ± 0.37 ^a

^{a-g}Different superscripts within a column indicate significant difference ($P < 0.05$).

¹Data represent the mean value of duplicate ± SD.

²μ = growth rate, measured in absorbance units per hour.

Table 7. Microbial growth parameters, derived from modified Gompertz equation, of *Salmonella typhimurium* and *Lactobacillus* spp.¹

Item	R ²	μ (abs/h) ²	Generation time (h)
<i>S. typhimurium</i>	0.99	0.20 ± 0.06 ^f	1.25 ± 0.15 ^a
J10	0.97	0.15 ± 0.06 ^e	1.6 ± 0.15 ^c
J20	0.99	0.09 ± 0.003 ^c	2.00 ± 0.28 ^d
J23	0.99	0.054 ± 0.003 ^a	2.76 ± 0.18 ^f
J25	0.98	0.19 ± 0.04 ^f	1.26 ± 0.11 ^a
J28	0.99	0.09 ± 0.004 ^b	2.60 ± 0.22 ^e
J31	0.96	0.14 ± 0.07 ^d	1.59 ± 0.12 ^b
J32	0.99	0.08 ± 0.004 ^b	2.69 ± 0.17 ^f
J33	0.99	0.14 ± 0.04 ^d	1.59 ± 0.36 ^b
J35	0.98	0.15 ± 0.07 ^e	1.62 ± 0.35 ^c
J36	0.96	0.19 ± 0.05 ^f	1.26 ± 0.18 ^a
J37	0.99	0.09 ± 0.003 ^c	2.52 ± 0.19 ^e
J38	0.97	0.15 ± 0.09 ^e	1.26 ± 0.25 ^a

^{a-f}Different superscripts within a column indicate a significant difference ($P < 0.05$).

¹Data represent the mean values of duplicates ± SD.

²μ = growth rate, measured in absorbance units per hour.

ACKNOWLEDGMENTS

The authors thank the National Council for Science and Technology (CONACYT) of Mexico for graduate scholarships to P. Y. Heredia-Castro and J. I. Méndez-Romero. The study was supported by SAGARPA-CONACYT Project 2010-144591. The technical assistance of María del Carmen Estrada-Montoya and Ricard Reyes-Díaz is gratefully acknowledged.

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