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agrocol_fabog@unal.edu.co
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Gutiérrez-Cortés, Carolina; Suárez, Héctor; Buitrago, Gustavo; Díaz-Moreno, Consuelo
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Isolation and evaluation of the antagonist activity of lactic acid bacteria in raw cow milk

Aislamiento y evaluación de la actividad antagonista de bacterias ácido lácticas en leche cruda de vaca

Carolina Gutiérrez-Cortés[1], Héctor Suárez[1], Gustavo Buitrago[2], and Consuelo Díaz-Moreno[1]*

ABSTRACT

Lactic acid bacteria (LAB) are considered as a good alternative to reduce the risk of food borne diseases in food industry. In addition to the improvement effects on the organoleptic characteristics of fermented foods from the LAB metabolites, they can inhibit the growth of microorganisms responsible of the food spoilage. This work is an advance on the biodiversity exploration of natural additives in food. Isolation, identification and screening of potential antimicrobial activity of LAB were the aims on this work. Species of *Lactobacillus (Lb. casei, Lb. brevis, Lb. paracasei,* and *Lb. plantarum*) and *Pediococcus acidilactici* were identified and their antagonism against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 7644 was demonstrated.

Key words: antagonistic bacteria, raw milk, *Lactobacillus*, isolation, pathogen inhibition.

RESUMEN

Las bacterias ácido lácticas (BAL) son una alternativa en la industria de alimentos para la reducción del riesgo que representan las enfermedades transmitidas por algunos alimentos. Los metabolitos típicos de las BAL, además de sus reconocidos efectos sobre las características organolépticas de los alimentos fermentados, pueden inhibir el crecimiento de microorganismos responsables del deterioro e incluso la aparición de patógenos. Este trabajo es un avance en la exploración de la biodiversidad y en la búsqueda de aditivos naturales con aplicación en la industria de alimentos. Los principales logros de este trabajo fueron el aislamiento, identificación y evaluación de la actividad antimicrobiana de BAL. Se identificaron especies de *Lactobacillus (Lb. casei, Lb. brevis, Lb. paracasei* y *Lb. plantarum*) y *Pediococcus acidilactici* que presentaron antagonismo frente a *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 y *Listeria monocytogenes* ATCC 7644.

Palabras clave: bacterias antagónicas, leche cruda, *Lactobacillus*, aislamiento, inhibición de patógenos.

Introduction

Foodborne diseases are the most serious and expensive issues in food industry. According to the USDA (United States Department of Agriculture) annually in USA, 48 million people suffer foodborne illnesses and 3000 are reported as deadly cases (FDA and Administration, 2013). New meals, manufacturing processes, and the growing demand for minimally processed products (ready-to-eat) increase the possibility of microbiological contamination. Alternative food preservation technology such as bio-preservation is a reliable option to extent the shelf-life and to enhance the hygienic quality, minimizing the impact on the food nutritional and organoleptic properties (García *et al*., 2010). Bio-preservation uses the antimicrobial potential of non-pathogen microorganisms or their metabolites to inhibit the growth of pathogens or spoilage related microorganisms (Nath *et al*., 2014; Settanni and Corsetti, 2008; Smid and Lacroix, 2013).

Different strains of microorganisms with potential use as bio-preservative agents have been reported (Ghanbari *et al*., 2013; Henning *et al*., 2015; Hwanhlem *et al*., 2014). Dairy products are one group of foods commonly used to obtain strains with antagonistic features. The most important microorganisms with antagonistic characteristics and potential use in food industry are lactic acid bacteria (LAB). They have been traditionally associated to food and are considered safe (García *et al*., 2010). LAB are Gram positive bacteria, non sporulating, anaerobic facultative, catalase and coagulase negative, tolerant to acidic conditions and with low content of guanine and cytosine in their DNA. LAB belong to the group of Firmicutes, Lactobacillales order and the most representative genera are *Aerococcus,*

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[1] Instituto de Ciencia y Tecnología de Alimentos, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Bogota (Colombia).
[2] Instituto de Biotecnología (IBUN), Facultad de Ciencias, Universidad Nacional de Colombia, Bogota (Colombia).
* Corresponding author: amcdiazmo@unal.edu.co

Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus, and *Weissella* and in phylum Actinobacteria with genera *Atopobium* and *Bifidobacterium* (Giraffa, 2012). Many LAB are considered as probiotics or live microorganisms, that consumed in adequate amounts, confer a health benefit on the host (Fijan, 2014).

LAB are successful habitat competitors due to their ability as competitive exclusion (Settanni and Corsetti, 2008) which consists on releasing antimicrobial substances that are able to affect the development of other microorganisms (Smid and Lacroix, 2013). The main product of LAB metabolism is lactic acid but they can produce other organic acids and compounds as hidrogenus peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanediona), reuterin and bacteriocins (Ammor *et al.*, 2006; Khan *et al.*, 2010; Nath *et al.*, 2014). Organic acids, especially lactic acid, are metabolites produced as a result of sugar metabolism. They are released to the environment reducing its pH, inhibiting the development of some populations of undesirable microorganisms (Okano *et al.*, 2010). Hydrogen peroxide (H_2O_2) has an oxidizing effect over sulfhydryl groups of membrane proteins and over lipids, also damaging the cell wall of some other microorganisms (Finnegan *et al.*, 2010). Additionally, H_2O_2 reacts with O_2, forming CO_2 reducing free O_2 and creating an anaerobic environment that can reduce the development of anaerobic populations (Šušković *et al.*, 2010). Diacetyl is associated to a characteristic smell of some dairy products, but it also has antagonistic activity when interacts with the cell membrane of some bacteria altering some metabolic ways (Lanciotti *et al.*, 2003). Bacteriocins are antimicrobial peptides produced by Gram positive and negative bacteria. In general, those peptides have low molecular weight and are heat stables, they do not affect the producer cells due immunity mechanisms (Cotter *et al.*, 2005; Karumathil *et al.*, 2016).

In this work, were isolated and identified LAB with antimicrobial activity against *Escherichia coli, Staphylococcus aureus,* and *Listeria monocytogenes,* three of the most important foodborne pathogens. These results are very important for the possibility of their application on biopreservation of foods.

Experimental procedures

Isolation and identification

Lactic acid bacteria were isolated from raw milk obtained from the Veterinarian Medicine Faculty of the Universidad Nacional de Colombia. Briefly, milk was incubated at 37°C during 48 h in order to allow fermentation and to obtain bacteria strains that survive on acid milk. 11 ml of fermented milk were added to 99 ml of sterile peptone water (0.1%) and tenfold dilutions until 10^{-7} were made. 1 ml of dilutions 10^{-5}, 10^{-6} and 10^{-7} were poured in petri dishes and covering with MRS agar (Man, Rogosa and Sharpe) Oxoid, petri dishes were incubated at 37°C during 48 h. In order to obtain more diversity in the isolation, colonies with different morphologies were plated onto the surface of MRS agar. After incubation at 37 °C during 24 h, Gram stain was performed. Isolates were stored at -20°C on cryovials (CRIOBANK®).

In order to know some features of the isolates some biochemistry tests were performed. Gas production test in Durham tubes on MRS broth; growth in MRS broth at different temperatures (10, 30, 37 and 45°C) and tolerance to NaCl 6.5% tests were performed. Growth was measure as optic density (O.D) on spectrophotometer (Genesys, USA) at 600 nm. Catalase and oxidase were also tested (Muñoz *et al.*, 2012). Isolates were partially identified on the basis of their biochemical features according to fermentation ability by using API 50CHL (Api System S.A., Bio-Merieux, France) (Todorov *et al.*, 2013).

To obtain the molecular identification, isolates were activated in 5 mL of MRS broth (Oxoid, UK) and incubated at 37°C during 48 h. After incubation the DNA was extracted using the kit PureLink™ Genomic DNA Mini Kit (Invitrogen) following manufacture instructions. Extraction was verified by electrophoresis in agarose gel 1.4% (w/v) at 70 Volts, 400mA during 30 minutes and the DNA was stained with SybrSafe. Concentration was measured by spectrophotometry using Nanodrop (Thermo Scientific, UK). Genera and specie were determined by amplification and sequencing of 16S ribosomal subunit by PCR technique using a Veriti® (Life Technologies, UK) thermo cycler. Conditions of PCR were: 94°C for 5 min, 30 times 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min and 72°C for 7 min. Reaction mixture contained 38.2 µL of water, 5 µL of buffer 1X, 1.5 µL $MgCL_2$ 1.5 Mm, 0.4 µL DNTs 0.2 Mm, 1.25 of primers (27F: 5' AGAGTTTGATCMTGGCTCAG 3', 1492R: 5' TACGGYTACCTTGTTACGACTT 3'), 0.4 µL Taq and 2 µL of DNA (Doi *et al.*, 2013). Amplification products were separated by electrophoresis in 1.2% agarose gel. Amplified samples were sent to the Instituto de Genética (SSiGMol) in the Universidad Nacional de Colombia (Bogota). Samples were purified and sequenced in bidirectional way. The sequences were compared to those deposited in GenBank, using the BLAST algorithm

(http://www.ncbi.nlm.nih.gov/BLAST) and phylogenetic tree was constructed using the MEGA 7.0.14 software.

Antimicrobial activity screening

Screening to find isolates with antagonistic features was performed using the spot-on-lawn method described by Schillinger and Lucke (1989) with some modifications. Briefly, overnight cultures of the isolates were spotted onto the surface of agar plates with MRS (1.2% agar) and incubated for 24 h at 30°C to allow colonies to develop and produce their metabolites. Approximately 5 x 10^7CFU/mL of the indicator strains important in food industry (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Listeria monocytogenes* ATCC 7644) were inoculated into 100 ml of soft TSA (Trypticase soy) agar (containing 0.7% agar), and poured over the plate in which the isolated LAB were grown. After incubation at 37°C during 24 h, diameter of inhibition zones was measured from the edge of the zone with a caliper, and expressed in mm.

Results

64 lactic acid bacteria were isolated from raw cow milk. 23.1% of all isolates were Gram positive and 76.9% of them presented rod shape, minor proportion presented sphere shape. Rods were large and short, some of them thinner than others, they were arranged on pairs or short chains. Most of cocci were arranged on tetrads and some of them on pairs. 24 isolates were chosen for further analysis according to their antimicrobial activity potential.

The isolates growth at 6.5% of NaCl and at temperatures of 10, 30, 37 and 45°C (Fig. 1) showed that the optimal temperature for all isolates was 30°C followed by 37°C, some of them grew at 10°C and at 45°C was the least suitable temperature for growth. 46.13% of isolates were tolerant to 6.5% of NaCl. Those tests allow knowing the technological potential of isolates in food industry applications. Gas production was negative for all cases.

The isolates that presented rod morphology (19 of them) were tested with API 50CHL (Api System S.A., Bio-Merieux, France) designed as *Lactobacillus* sp. according with the manufacturer, in order to determine phenotypic characteristics showed in biochemistry profiles (fermentative profiles). Tab. 1 shows the percentage of isolates that were able to use each sugar on the API CHL 50 panels. All of the isolates fermented N-acetyl glucosamine, glucose, ribose and fructose and none of them was able to use D-fucose, L-fucose, D-arabitol, erythritol, D-arabinose, L-xylose, adonitol, methyl-D-xiropyranose or glycerol. Galactose and gluconate were used almost for all isolates, except for the identified at molecular level as *Pediococcus*, this result can be explained because some strains of this genera do not use the sugar sources mentioned (Vos *et al.*, 2009). Arbutinine, aesculine, maltose and salicin were consumed by 89.5% of the isolates. Sugars consumed for one strain were rhamnose, metyl-D-manopiyanoside, starch, glycogen o xylitol. Partial identification according to biochemical profiles gave as a result that isolates belong to genera *Lactobacillus* spp. with species *Lb. brevis* 16%, *Lb. plantarum* 21%and *Lb. paracasei spp. paracasei* 63%.

Molecular identification

DNA extraction was performed to 19 isolates. Molecular weight marker of 1000 pb was used in order to know size of the product amplified. This size was approximated to 1400 pb according to observed bands. Chromax software was used to observe, interpreter, and depurate sequences obtained from the Genetics Institute (Universidad Nacional de Colombia, Bogota). Results were analyzed and compared to data in GenBank using BLAST (Basic Local Alignment Search Tool), phylogenetic tree was made using Mega 7.0.14 program (Fig. 2).

TAB 1. Percentage of positive isolates of each carbon source on API CHL 50.

Sugar	%	Sugar	%	Sugar	%	Sugar	%	Sugar	%
Glycerol	0	Rhamnose	4,5	Mannose	86	Xilitol	4,5	Sorbose	27
Erythritol	0	Dulcitol	9,1	Cellobiose	73	Gentiobiose	59	Salicine	91
D-arabinose	0	Inositol	41	Maltose	91	Turanosa	68	Glycogen	4.5
L-arabinose	27	Mannitol	68	Lactose	77	Lixosa	23	Esculin	91
Ribose	100	Sorbitol	68	Melobiose	36	Tagatose	64	Starch	4.5
D-xylose	55	Methyl-D-manopiranoside	4,5	Sucrose	64	D-fucose	0	Arbutine	91
L-xylose	0	Methyl-D-glucopiranoside	41	Trehalose	82	L-fucose	0	Rafinose	9.1
Adonitol	0	N-acetylglucosamine	100	Inulin	27	D-arabitol	0	Amigdaline	64
Metil-D-xiropiranosa	0	Glucose	100	Melezitose	73	5-ketogluconate potassium	64	2-cetogluconato potassium	59
Galactose	95	Fructose	100	L-arabitol	14	Gluconate	95		

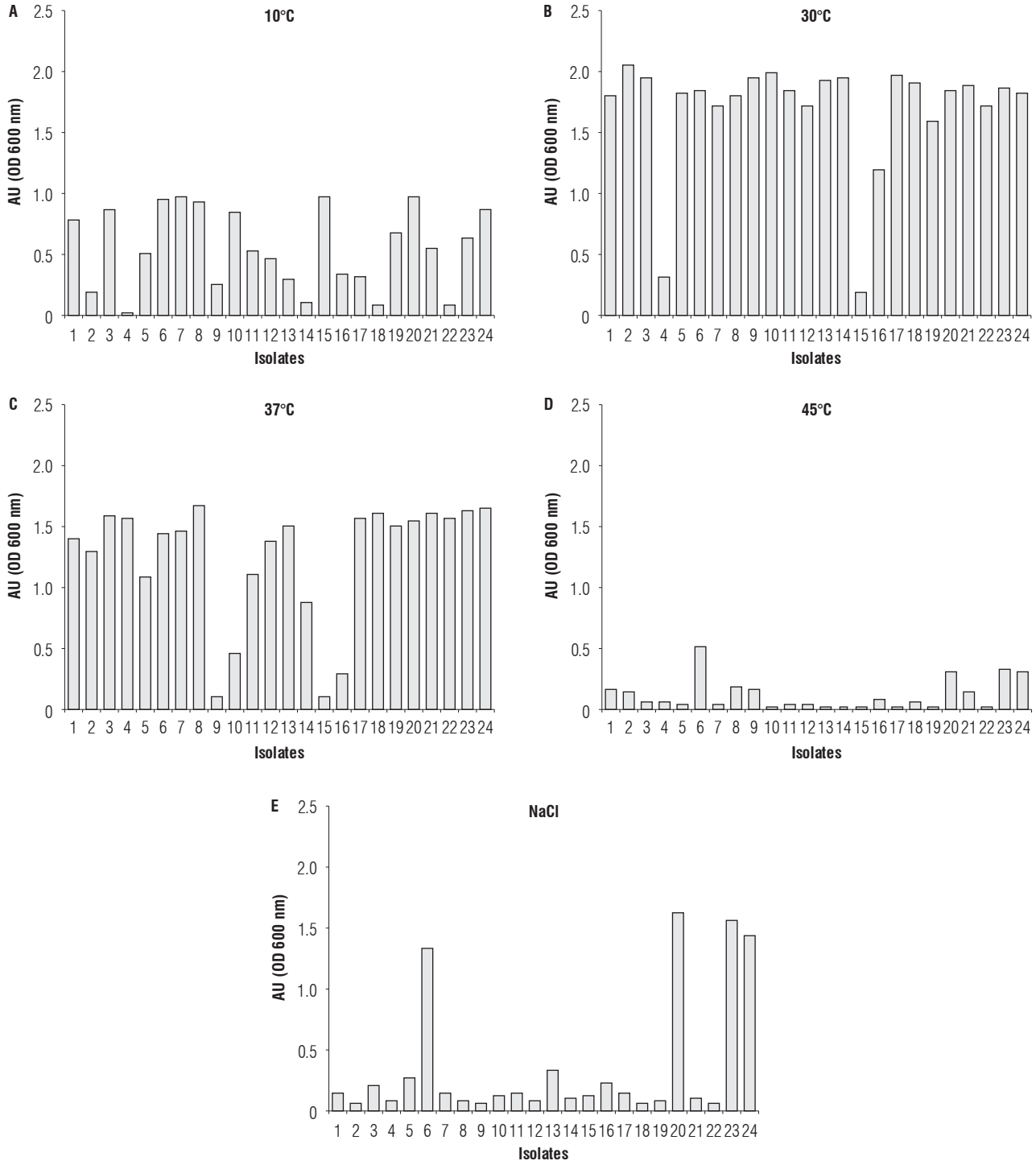

FIGURE 1. Growth of isolated LAB at different conditions of incubation,(A) 10°C, (B) 30°C, (C) 37°C, (D) 45°C, and (E) with 6.5% of NaCl. Numbers represent the isolated LAB; AU = absorbance units.

13 of the isolates were congregated on *Lb. casei/paracasei* cluster with a similarity percentage of 66% (isolates AL3, AL5, AL7, AL8, AL9, AL10, AL11, AL12, AL13, AL14, AL15, AL16 and AL18). Six isolates remaining were associated with 100% of similarity with *Lb. plantarum* (isolates AL1, AL2, AL4 and AL6), and *Lb. brevis* with 99% of similarity (isolates AL17 and AL19). According to the bioprospection concept about the obtaining of bioactive products from nature, in this case for applications in the food industry, the results showed the possibility to considerate the raw milk as an important source of antagonistic *Lactobacillus* strains with potential application on biopreservation. The

above depends mainly of the natural behavior of bacteria due that the strains express different survivor strategies, as the release of antagonist substances as response to population density (Cornforth and Foster, 2013).

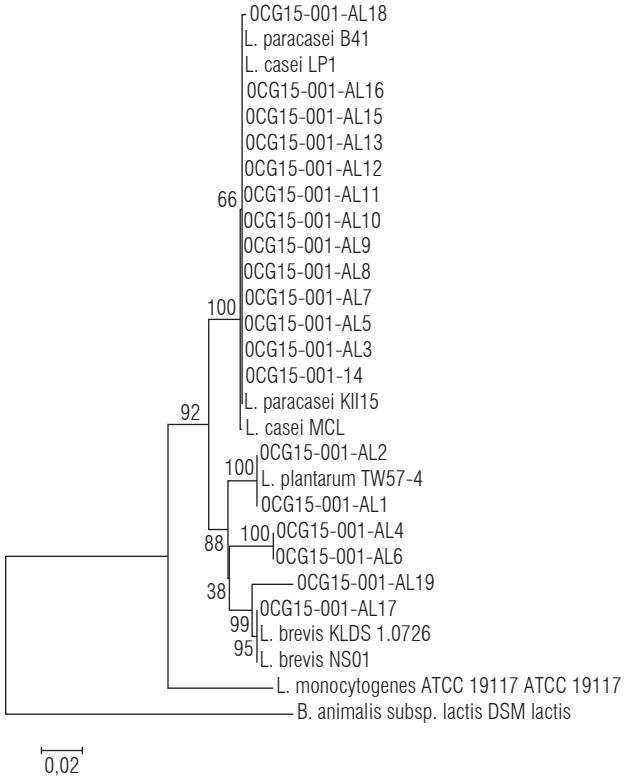

FIGURE 2. Phylogenetic tree of the isolates.

Antimicrobial activity evaluation

Fig. 3 shows the growth of the isolated BAL on MRS agar (1.2%) after incubation for 24 h at 30°C and the inhibition zones obtained after a second incubation with *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644.

Tab. 2 shows inhibition zones in millimeters against indicator strains.

Discussion

Biochemistry tests made by using API® CHL50 (BioMerieux, France) showed metabolic characteristics of the isolates, this information allowed to estimate sugar fermentation profile allowing growth media optimization. According to the results obtained on apiweb™, 11 strains corresponded to *Lb. paracasei*, 9 of them were identified at molecular level as *Lb. casei*, one of them as *Lb. casei/paracasei*, and one as *Lb. paracasei*. The close related species were *Lb. casei, Lb. paracasei,* and *Lb. casei/paracasei* and they were all identified by the apiweb™ as *Lb. paracasei*. On the other hand, those profiles are not enough to identified microorganisms, so it is necessary to use molecular techniques to obtain more robust results. Tab. 3 shows that names obtained by API CHL 50® to isolates AL2, AL4 and AL8 did not correspond to the identification obtained by sequencing and contrasted with databases.

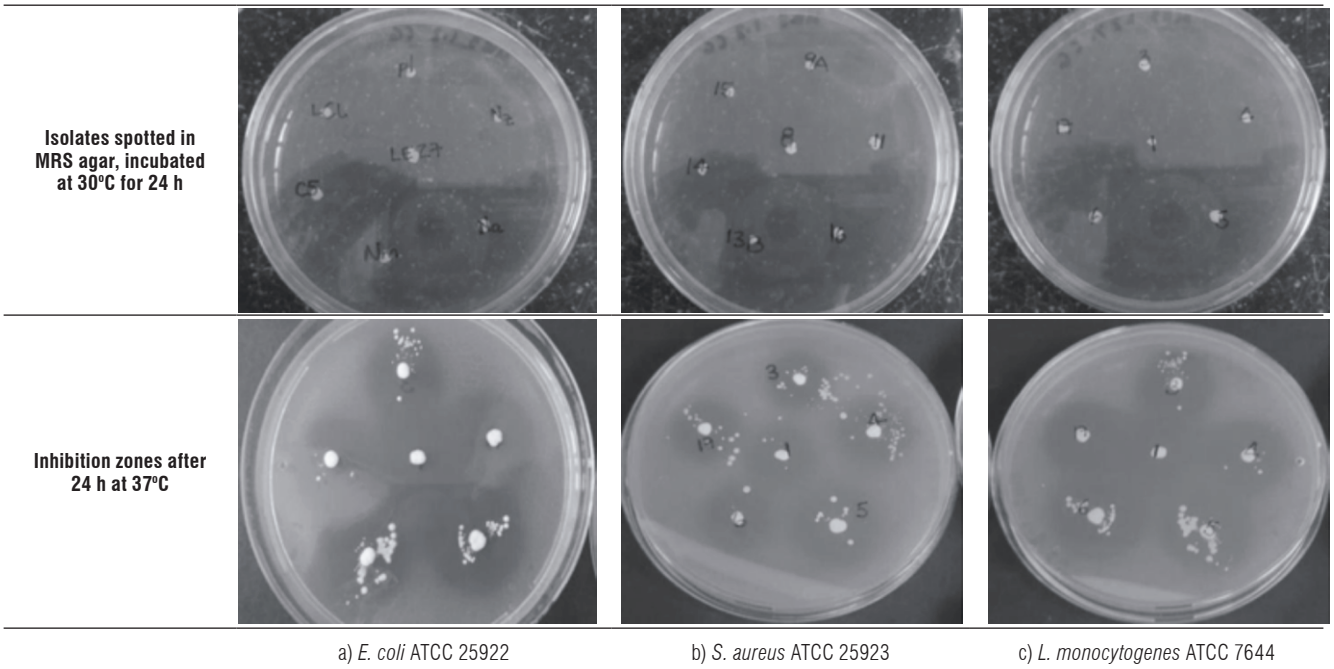

FIGURE 3. Antimicrobial activity screening. Upper part shows the growing of isolated LAB, the lower shows inhibition zones against *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 performed by the isolated strains.

TABLE 2. Inhibition zones obtained on the screening.

	Inhibition zones (mm)					
	S. aureus		*E. coli*		*Listeria*	
	Diameter	SD	Diameter	SD	Diameter	SD
AL1	20	± 0.3	17	± 0.3	23	± 0.0
AL2	23	± 0.0	21	± 0.0	19	± 0.3
AL3	28	± 0.7	25	± 0.3	16	± 0.3
AL4	21	± 0.7	17	± 1.4	14	± 0.7
AL5	20	± 0.7	22	± 0.3	20	± 0.3
AL6	25	± 0.3	19	± 0.3	17	± 0.7
AL7	19	± 0.3	23	± 0.3	18	± 0.7
AL8	19	± 0.7	18	± 0.3	15	± 0.0
AL9	23	± 0.3	19	± 0.3	18	± 0.3
AL10	22	± 0.0	21	± 0.3	21	± 0.3
AL11	22	± 0.0	18	± 0.3	21	± 0.7
AL12	19	± 0.7	18	± 0.3	15	± 0.7
AL 13	13	± 0.7	17	± 0.3	10	± 0.7
AL15	10	± 0.7	12	± 0.3	11	± 0.7
AL17	23	± 0.0	22	± 0.7	20	± 0.7
AL18	17	± 0.7	15	± 1.0	10	± 0.3
AL20	25	± 0.3	24	± 0.3	21	± 0.3
AL21	25	± 0.0	19	± 0.3	24	± 0.3
AL23	23	± 0.3	25	± 0.3	18	± 0.3
AL24	23	± 0.7	19	± 0.3	13	± 0.3

Two out of the 19 strains identified at molecular level corresponded to *Pediococcus* sp. The rest were verified as belonging to *Lactobacillus* sp. (Tab. 3). Five different species of lactobacillus were identified as *Lb. casei*, *Lb. brevis*, *Lb. paracasei*, *Lb. casei/paracasei*, *Lb. plantarum*. One species of *Pediococcus* was identified as *P. acidilactici*. Other researches focused on isolated of native LAB from different foods have shown that dairy products are an important source of this kind of microorganisms (dos Santos *et al.*, 2014). For instance, 319 strains were isolated from different products made with raw buffalo milk on Gansu province (China), authors amplified and sequenced DNA from those isolates and compared results with GenBank database finding genera as *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Enterococcus* and *Weissella.* Major proportion of isolated corresponds to *Lb. casei* and *Lb. helveticus* (Bao *et al.*, 2012). Sixty different isolates from five Spanish cheeses made without starter microorganisms were identified as *Lactococcus lactis* (Alegría *et al.*, 2010). Davati *et al.* (2015) isolated 64 LAB from camel milk and using Amplified Ribosomal DNA Restriction Analysis (ARDRA) found 12 different profiles. In the same study, identification by amplification and sequencing of 16S region of DNAr were performed and *P. pentosaceus*, *E. faecium* cepa Y-2, *E. faecium* cepa JZ1-1, *E. faecium* cepa E6, *E. durans*, *E. lactis*, *Lc. mesenteroides*, *Lb. casei* and *W. cibaria* were found.

TABLE 3. Comparison of culture and molecular identification of isolated LAB.

	API CHL 50 identification	Molecular identification	ID (%)
AL1	*Lactobacillus plantarum*	*Lactobacillus plantarum*	100
AL2	*Lactobacillus brevis*	*Lactobacillus plantarum*	100
AL3	*Lactobacillus paracasei/ casei*	*Lactobacillus paracasei*	100
AL4	*Lactobacillus brevis*	*Pediococcus acidilactici*	99.8
AL5	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	100
AL6	Did not performed	*Pediococcus acidilactici*	99.6
AL7	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.9
AL8	*Lactobacillus plantarum*	*Lactobacillus casei*	100
AL9	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.1
AL10	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	100
AL11	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.9
AL12	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.9
AL13	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	100
AL14	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	100
AL15	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.8
AL16	*Lactobacillus paracasei/ casei*	*Lactobacillus casei*	99.9
AL17	*Lactobacillus brevis*	*Lactobacillus brevis*	99.8
AL18	*Lactobacillus paracasei/ casei*	*Lactobacillus casei/paracasei*	99
AL19	*Lactobacillus brevis*	*Lactobacillus brevis*	98.2
AL22	*Lactobacillus paracasei/ casei*	Unrealized	

Other authors obtained and characterized morphologically isolates from paddy rice silage. API CHL50 tests to make the partial identification were performed. Also, analyses of 16S rRNA and recA sequences were made to obtain genera *Lactobacillus, Lactococcus, Pediococcus, Enterococcus,* and *Leuconostoc. P. pentosaceus* was the more abundant microorganism, and the low was *Lb. casei* (Ni *et al*., 2015).

Species most commonly isolated from raw milk belong to genera *Enterococcus* (*E. faecalis* and *E. faecium*), *Lactobacillus* (*Lb. delbrueckii subsp. Lactis, Lb. helveticus, Lb. hilgardii. Lb. fermentum, Lb. gasseri* and *Lb. rhamnosus*), *Lactococcus* (*Lc. lactis subsp. lactis* and *subsp. Cremoris*), *Leuconostoc* (*Ln. mesenteroides* subsp. *mesenteroides*), *Pediococcus* and *Streptococcus* (*St. Uberis* and *St. thermophilus*) (Neviani *et al*., 2013). Also, *Lactobacillus* sp. and *Streptococcus* sp. genera has been reported from raw cow milk (Elgadi *et al*., 2008). Other authors identified *Enterococcus* spp. and some members of *Lactococcus* spp., also they reported a less amount of *Lactobacillus* strains in contrast with the present work (Franciosi *et al*., 2009). Other authors isolated 7 species of *Lb. plantarum* from donkey milk. Results obtained on RAPD-PCR spectrum showed that isolates were replicates of the same strain, for this reason they evaluated one single strain named *Lb. plantarum* LP08AD. Bacteriocin LP08AD was identified and characterized at biochemistry and molecular level, also antimicrobial activity was measure against *L. monocytogenes*, *E. faecium*, *Lb. curvatus, Lb. fermentum* and *P. acidilactici* (Murua *et al*., 2013).

All strains presented antagonistic activity against *L. monocytogenes*, *E. coli* and *S. aureus* showing a potential application on research related with production and recovery of antimicrobial metabolites with broad spectrum against foodborne pathogens. Similar results have been reported with isolated strains belonging to genera *Pediococcus, Enterococcus, Leuconostoc* and *Lactobacillus* showed antimicrobial activity against *S. aureus*, *Bacillus cereus* and *E. coli* (Davati *et al*., 2015). In other study, 17 of the 60 of *Lc. lactis* isolated presented antimicrobial activity against Gram positive bacteria from the *Lc. lactis* cluster (different subspecies), *Lb. sakei* CECT 906T, *Lb. plantarum* LL 441, *L. innocua* 86/26 and *S. aureus* CECT 86T (Alegría *et al*., 2010).

Bioprospecting as a tool in the search for natural substances with potential applications in food is booming worldwide due to the need of developing food products with natural features that allow to replace the use of synthetic additives. This work showed the importance of raw milk as source of LAB with antagonistic against pathogen bacteria features and showed the needed to focus the research in the evaluation of the antimicrobial activity of the native LAB to characterize metabolites responsible of this activity with application in biopreservation of food.

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