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Isolation of a *Lactobacillus* strain from aguamiel and preliminary characterization of its antimicrobial components

Benítez-Serrano Juan Carlos[1]*, Martínez-Pérez Laura[1], Rosas-Murrieta Nora Hilda[2], Brambila-Colombres Eduardo Miguel[3], Hernández-Castro Rigoberto[4], Rosales-Pérez Mónica[5] and Aguilar-Alonso Patricia[3]

[1]Laboratorio de Microbiología Aplicada, Facultad de Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, México.
[2]Laboratorio de Bioquímica, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, México.
[3]Laboratorio de Investigaciones Químico Clínicas, Facultad de Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, México.
[4]Departamento de Ecología y Agentes Patógenos, Hospital General Manuel Gea González, Ciudad de México, México.
[5]Instituto Tecnológico de Estudios Superiores de Monterrey, Campus Puebla, México.

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The aim of the study was to characterize the antimicrobial components of *Lactobacillus paracasei* KSI. In the study, a *L. paracasei* KSI strain was isolated and identified from aguamiel using a 16S rRNA, hsp, recA and rpoBgenes sequencing. The antimicrobial capacity of the *L. paracasei* strain KSI was determined by agar double layer diffusion technique, while the antagonistic activity of the cell-free extract (E-KSI) was evaluated by agar well diffusion method against different bacterial strains; it demonstrated a wide spectrum of inhibition. Likewise, E-KSI showed stability at different temperatures and digestive enzymes; its activity was lost at pH>5. Subsequently, E-KSI was concentrated (10x) by evaporation increasing its antagonistic effect. Antagonism tests by thin-layer chromatography (TLC)–bioautography of the E-KSI 10x showed the presence of more than one active substances different from lactic acid, possibly of the bacteriocin type. Some of these substances were recovered by extractions with ethyl ether, ethyl acetate and n-butanol respectively. Finally, we evaluated them using antagonism tests by minimum inhibitory concentration (MIC) and microdilution. *L. paracaseistrain* KSI generates substances with antibacterial activity having a wide spectrum of inhibition; it is a promising alternative to future biotechnological applications. The strain of *L. paracasei* KSI as well as the antimicrobial components that it generates has important antagonistic properties, making them an interesting biotechnological alternative to be used as probiotic or a safe functional food.

Key words: Lactobacillus, antimicrobials, probiotics, biotechnology, food safety.

INTRODUCTION

The group of lactic acid bacteria (BAL) are microorganisms Gram-positive bacilli, do not form spores, usually immobile, anaerobic, microaerophilic or air tolerant, catalase, oxidase and benzidine negative, without

cytochromes, nor reduce nitrate to nitrite and produce lactic acid as the only or main product of carbohydrate fermentation (Carr et al., 2002). This group of microorganisms is widely distributed in nature and has been isolated from different foods (Azadnia et al., 2011) (milk and dairy products, wines, fruits, vegetables, fishery products, among others), soil, digestive tract, mouth and vagina of mammalian between other sources (Claesson et al., 2007). The type of metabolism developed by the BAL is primarily used to obtain fermented food and beverages (Chilton et al., 2015). The BAL can also play the role of food biopreservatives and bioprotective cultures (Parada et al. 2007; Benmechernene et al., 2013), because in addition to competing for nutrients, they also produce metabolites that inhibit the growth of contaminating or pathogenic microorganisms. Antimicrobial capacity starts with the production of lactic acid that participates in the progressive decrease of the pH (Erdogrul and Erbilir, 2006); during the fermentation process, the BAL can produce other substances capable of inhibiting the growth of competing bacteria, among which low molecular weight molecules have been found, the most common being: acetic acid, propionic acid, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, ethanol (Piard and Desmazeaud, 1991; Lahtinen et al., 2011); peptides with antimicrobial properties called "bacteriocins" (Chen and Hoover, 2003; Dobson et al., 2012; Shaikh et al., 2012; Ribeiro et al., 2013; Zendo, 2013; Drissi et al., 2014; Yang et al., 2014) biosurfactant compounds (Rodrigues et al., 2006; Saharan et al., 2011; Sharma and Saharan, 2014) and other compounds not yet characterized. The BAL have the category of the generally recognized as safe (GRAS) granted by the food and drug administration (FDA) of the USA (Zacharof and Lovitt, 2012), and due that the substances that produce during their metabolic processes present activity against various pathogens included bacteria, parasites, fungi and yeasts (Papagianni, 2003; Joerger, 2003; Motta and Brandelli, 2008).

The classification of the BAL was established by Orla-Jensen (1920), and was based on their morphological, metabolic and physiological characteristics. Nowadays they are classified according to the subunit 16S rRNA gene (Olsen et al., 1994; Ben-Amor et al., 2007); its characteristics possess a guanine-cytosine content (GC) less than 50%. This group comprises of microorganisms of the following genera: *Urinae*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulun*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Cintas et al., 2001; Rojas and Vargas 2008), being *Lactobacillus* the most studied genus, and some members of this genus are considered probiotics (Axelsson, 2004). The use of probiotics is given, by its influence on the intestinal microbiota and its antagonism with the pathogenic bacteria. Recent reports describe the properties of strains of *Lactobacillus* isolated from different environments: *Lactobacillus rhamnosus* isolated from human vagina (Turovskiy et al., 2009), *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus helveticus* isolated from silage (Giraldo et al., 2010), *L. paracasei* isolated from fecal matter of infants (Bendjeddou et al., 2012), *Lactobacillus sakei* D98 isolated from sake (Sawa et al., 2013), *L. plantarum* from corn silage (Amortegui et al., 2014), *L. plantarum* isolated from grapes (Chen et al., 2014), and *L. paracasie* SD1 isolated from oral cavity (Wannun et al., 2014), *Lactobacillus casei* MRTL3 all with the ability to produce substances with antagonistic properties against various pathogenic bacteria (Sharma and Saharan, 2014).

Multiple agave species grown in the semi-desert areas of Mexico, such as maguey-pulquero (*Agave atrovirens*), aguamiel is the agave sap, which is collected when the maguey is mature and is used for the production of pulque (a drink with cultural importance in Mexico) contains fructooligosaccharides that are susceptible to fermentation in the colon by colonic microorganisms; it has also been shown to have important prebiotic properties (Romero-López et al., 2015). The objective of this work was the preliminary characterization of inhibitors produced by *L. paracasei* strain KSI isolated from aguamiel, and the evaluation of its antimicrobial activity.

MATERIALS AND METHODS

Bacterial identification

The *L. paracasei* strain KSI was isolated from an "agua miel" sample and cultivated in MRS agar and incubate for 48 h at 37°C. The macroscopic characteristics of colonies were evaluated, in addition, the gram staining and catalase and oxidase test were performed (which corresponds to the characteristics of the BAL). The identification of *L. paracasei* KSI was performed by the polymerase chain reaction (PCR) using the 16S rRNA subunit, *hsp*, *recA* and *rpoB* genes. Universal primers were used for the amplification of 16S rRNA gene; E9F: 5´-GAGTTTGATCCTGGCTCAG-3´ and E939R: 5´-CTTGTGCGGGCCCCCGTCAATTC-3´; (Forney et al., 2004) and for the amplification of the genes *hsp*, *recA* y *rpoB* were designed primers hsp-F: 5'-TGAATCGTCGTAATAATGAGTTG-3' and hsp-R 5'-TTTCAATGTTGTGACCAGAC-3'; recA-F: 5'-

*Corresponding author. E-mail: agapmx@yahoo.com.mx. Tel: (+01 222) 2295500. Ext: 2861.

GACATCGTGGTTTCAACC-3' and recA-R: 5'-TATGGTCCTGAAAGTACC-3' and rpoB-F: 5'-GATCTTCCCGATGATGAC-3' and rpoB-R: 5'-TTGAAAGTATGCAAATCGGC-3', respectively. The PCR reactions were performed to a final volume of 50 µL, with 25 µL of PCR Master Mix (2X, Thermo scientific), 1 µL of E9F (25 pmol μL^{-1}), 1 µL of E939R (25 pmol μL^{-1}), 23 µL of nuclease-free water and 1 bacterial colony as a DNA template. The amplification protocol consisted of an initial denaturation: 94°C/2 min; 35 amplification cycles with denaturation: 94°C/30 s; alignment: 59.8°C/30 s; extension: 72°C/1.30 min and a final extension: 72°C/10 min. PCR products were visualized in a 1% agarose gel stained with ethidium bromide and subsequently purified from agarose gel using the purification system Zymoclean™ Gel DNA Recovery Kit (Zymo Research) following the manufacturer's specifications; finally purified products were sent to the Molecular Biology unit of the Institute of Cellular Physiology of the UNAM for sequencing.

Evaluation of the inhibitory activity of *L. paracasei* KSI by agar double layer diffusion technique

L. paracasei KSI was cultured in MRS broth at 37°C / 48 h / 150 rpm, after the incubation time, 1 µL of the culture (3×10^{10} cells) was placed in a well of 1 mm diameter, previously generated with a sterile capillary tube, in MRS and APT agar plates. The agar plates were incubated at 37°C for 48 h and later the bacteria were removed. Finally, the plates were covered with 10 mL of soft agar Müller Hinton, previously inoculated with 1 mL of the indicator strain: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 respectively, all of them adjusted to the concentration of 0.5 of the Mc Farland standard (CLSI, 2017). The plates were solidified in sterile conditions and incubated at 37°C / 24 h for the interpretation.

Obtaining cell-free extract of *L. paracasei* KSI and test of antagonism by agar well diffusion assay

L. paracasei KSI was cultivated in 100 mL MRS broth and incubated at 37°C/ 48 h/ 150 rpm. After incubation, the culture was centrifuged at 3396 g/ 4°C/30 min, the cell-free extract (E-KSI) was recovered and sterilized by ultrafiltration with 0.22 µm membranes. The pH value was determined to carry out the tests of antagonism by diffusion in agar to be evaluated against the indicator strains, which were cultivated on AST agar plates at 37°C /24 h, to prepare an inoculum adjusted to the 0.5 of Mc Farland standard. The inoculum was cultivated on Müller Hinton agar plates and glass cylinders were placed to generate wells in which 150 µL of E-KSI (0.63 µg/µL of total protein) which was subjected to different conditions were deposited, subsequently the plates were incubated at 37°C / 24 h to observe areas of inhibition (Valgas et al., 2007).

Evaluation of the stability of the cell-free extract (E-KSI)

To determine the stability of the cell-free extract (E-KSI), different treatments were performed. Thermal stability was evaluated at 121°C/15 min; 100°C/30 min; 37, 25, 4, -10 and -80°C for a week, respectively. For pH stability, different pH conditions were adjusted to pH = 5 with acetate buffer (0.1 mol L^{-1}), pH= 6.8 with Tris (0.1 mol L^{-1}), pH=7.4 with PBS (0.1 mol L^{-1}) and pH=8 with Tris (0.1 mol L^{-1}). Finally the stability of the E-KSI was evaluated with the digestive enzymes: proteinase K, lysozyme, trypsin and chymotrypsin, which were used at a final concentration of 1 mg mL^{-1}. Samples were incubated at 37°C for 24 h, after incubation time each of the enzymes was inactivated at 100°C for 15 min. Each one of the treated samples was evaluated by test of antagonism by agar well diffusion assay.

Evaluation of the E-KSI (10x) through antagonism tests by thin-layer chromatography (TLC)–bioautography

For obtaining of E-KSI (10x), the strain of *L. paracasei* KSI was incubated in 100 mL of MRS broth at 37°C/48 h/150 rpm recovering the supernatant by centrifugation, which was evaporated at 65°C at reduced pressure, until the almost dry product was obtained, the evaporated solvent was also recovered. The concentrated product was rehydrated to a final volume of 10 mL (10x) with sterile distilled and deionized water, while the recovered solvent did not receive any subsequent treatment. All the products were sterilized by filtration with 0.22 µm membranes to evaluate antagonistic activity. For analysis of E-KSI (10x) a thin layer chromatography (TLC) using a stationary phase of plates silica gel (SiO_2) was performed. Samples were analyzed and compared with lactic acid using a mobile phase of a mixture of ethyl acetate: hexane (9:1) and methanol: acetic acid (9:1), and the developed was performed by UV light, iodine and ninhydrin, respectively. While for bioautography (Mehrabani et al., 2013), silice plates were placed at 65°C for eluents evaporation. Subsequently, the chromatographic plates were placed on Müller Hinton agar plates (modified from Mehrabani). Finally, the plates were covered with 10 mL of soft agar Müller Hinton, previously inoculated with 1 mL of the indicator strain, adjusted to the concentration of 0.5 of the Mc Farland standard. The plaques were cultivated and incubated at 37°C/24 h for interpretation.

Obtaining antimicrobial components from E-KSI (10x) by extraction with different organic solvents

Aliquots of E-KSI (10x) were mixed with equal volumes (1:1) of isopropyl alcohol, butyl alcohol, ethyl acetate, hexane, chloroform and ethyl ether, respectively. Subsequently the samples were centrifuged and organic fractions were separated and evaporated to obtain the dry product which was weighed and reconstituted in sterile deionized water. The residual aqueous phase was reconstituted and both products were tested by agar well diffusion assay.

E-KSI 10x active fractions evaluation by minimum inhibitory concentration (MIC)

For MIC test (Balouiri et al., 2016), a 96 well microplate and aliquots of E-KSI (10x) extracted with ethyl ether, ethyl acetate and butyl alcohol was used. Serial dilutions were made with each E-KSI (10x) extraction. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 cultures adjusted to 0.5 of Mc Farland standard were added and finally incubated at 37°C/24 h for interpretation.

RESULTS

Isolation and identification of *L. paracasei* KSI

After 48 h incubation in MRS agar there were isolated in beige colony with regular edges, convex, with creamy consistency, negative to catalase and oxidase, and microscopically observed as Gram positive bacilli. The

Table 1. Evaluation of the antagonistic capacity of *L. paracasei KSI.*

Indicator strain	Agar double layer diffusion technique	Agar well diffusion assay			
		E-KSI	MRS broth pH= 4	E-KSI (10X)	Solvents
	Inhibition halo (mm)				
E. coli ATCC 25922	34.0	13	-	22	-
P. aeruginosa ATCC 27853	35.0	13	-	21	-
S. aureus ATCC 25923	32.0	-	-	21	-

The assays shown were performed in triplicate, obtaining the same results for each test.

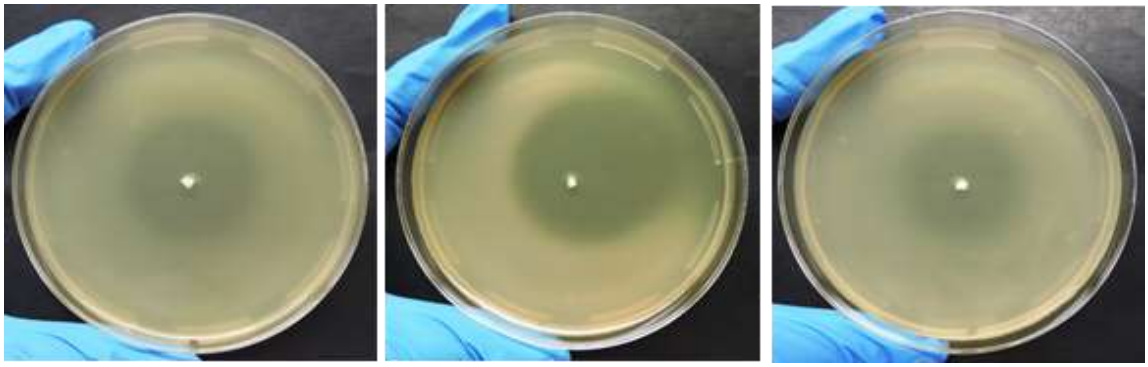

Figure 1. Evaluation of the inhibitory activity of *L. paracasei* KSI by agar double layer diffusion technique. From left to right, *E. coli ATCC 25922, P. aeruginosa ATCC 27853 y S. aureus ATCC 25923.*

molecular identification was performed by PCR amplification and sequencing of 16S rRNA (957 bp), *hsp* (452 bp), *recA* (578 bp) and *rpoB* (3495) genes. The amplicons were sequenced in both directions and the sequences were compared in the GenBank database for homology search using the Basic Local Alignment search Tool (Blastn). The 16S ribosomal subunit gene showed a 100% of identity with strains of *L. rhamnosus*, *L. casei* and *L. paracasei*, respectively, while the *hsp*, *recA* and *rpoB* genes showed a 100% of identity and similarity with different strains of *L. paracasei*. Thus the isolated strain was called *L. paracasei* KSI.

Evaluation of the antagonistic capacity of *L. paracasei* KSI

The evaluation of the antagonistic capacity of the strain of *L. paracasei* KSI was evaluated in a solid medium by agar double layer diffusion technique, showing an important inhibitory effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 y *S. aureus* ATCC 25923 strains on MRS agar (Table 1, Figure 1), but not on APT agar where inhibition halos were not observed. To evaluate the antagonistic capacity generated by the strain in liquid medium, we used the test of antagonism by agar well diffusion assay and cell-free extract (E-KSI) recovered at 48 h with pH 4 and MRS broth at pH 4 as control. The results show that there is a lower inhibitory effect in the test of antagonism by agar well diffusion assay compared to the agar double layer diffusion technique against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains, but no inhibitory effect against *S. aureus* ATCC 25923 strain, it was also observed that the pH did not participate in the antagonist effect (Table 1, Figure 2A). However, when the E-KSI (10x) was evaluated, it was observed that an increase in inhibitory effect against the three indicator strains (Table 1, Figure 2B), without the solvents present in the culture medium participated in this effect (Table 1, Figure 2C).

Stability evaluation of E-KSI in different conditions of temperature, pH and digestive enzymes

After evaluating the inhibitory action of E-KSI extract in a liquid medium its stability was determined at different temperatures (121, 100, 37, 25, 4, -10 and -80°C) and pH values (pH = 5, pH= 6.8, pH=7.4 and pH=8), as well as its sensitivity to digestive enzymes (proteinase K, lysozyme, trypsin and chymotrypsin, respectively). After each treatment, diffusion tests were performed and compared with *E. coli* ATCC 25922, *P. aeruginosa* 27853 and *S. aureus* 25922, respectively. The results show the

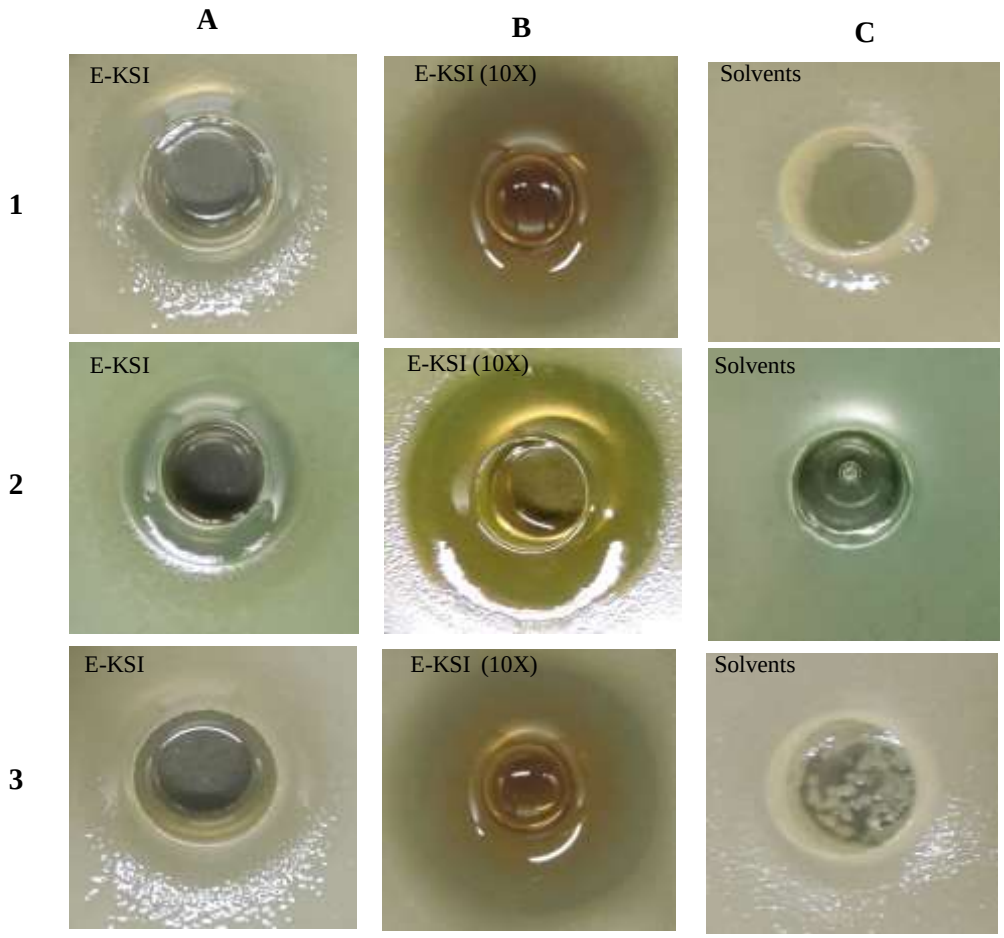

Figure 2. Test of antagonism by agar well diffusion assay of E-KSI. Rows, 1: *E. coli ATCC 25922, 2: P. aeruginosa ATCC 27853 and 3: S. aureus ATCC 25923.*Columns, A: *E-KSI*, B: *E-KSI (10X)* and C: Solvens.

thermal stability of E-KSI in the temperature ranges evaluated, however, the activity is lost at pH values higher than 5. Finally, the extract maintains its antagonistic effect after being treated with enzymatic digestion (Table 2).

Evaluation of E-KSI (10X) by thin-layer chromatography (TLC)–bioautography and MIC

For antagonism test bioautography, firstly it compared the migration profile of E-KSI (10x) with the lactic acid profile (8.5%) using thin-layer chromatography. For thin-layer chromatography we used two elution systems, one with less polar characteristics (ethyl acetate: hexane (9:1)) and another with more polar characteristics (methanol: acetic acid (9:1)), observing that the E-KSI (10x) has a migration profile different from lactic acid and that E-KSI (10x) presents a diversity of compounds that was separated by this technique. Similarly, when it observed the sample of E-KSI (10x) eluted with the methanol: acetic acid mixture, the presence of polar molecules that react when revealed with ninhydrin was demonstrated, which suggested protein structures or biogenic amines (Figure 3). The bioautography assay showed an inhibition area along the migration profile of E-KSI (10X) different from lactic acid profile, indicating more than one component of the extract promotes an inhibitory effect (Figure 4). From E-KSI (10x) there obtained the active fractions of the organic extractions made with n-butanol (But), ethyl acetate (EA) and ethyl ether (EE) but not with hexane and chloroform (Table 3 and Figure 4), which were evaluated first by antagonism tests by agar well diffusion assay method (Figure 5) and later by MIC.

Minimum inhibitory concentration (MIC)

For the MIC test the extractions obtained with the organic solvents were used. The stock solutions obtained were

Table 2. Evaluation of E-KSI submitted to different treatments by agar diffusion assay.

Treatment on the extract KSI (E-KSI)	Inhibitory activity (mm)		
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923
Temperature			
-80°C	13.0	13.0	-
-10°C	13.0	13.0	-
4°C	13.0	13.0	-
25°C	13.0	13.0	-
37°C	13.0	13.0	-
100°C	13.0	13.0	-
121°C	13.0	13.0	-
Enzymes digest			
Trypsin	13.0	13.0	-
Chymotrypsin	13.0	13.0	-
Proteinase K	13.0	13.0	-
Lisozime	13.0	13.0	-
pH			
4.0	13.0	13.0	-
5.0	-	-	-
6.8	-	-	-
7.4	-	-	-
8.8	-	-	-

The assays shown were performed in triplicate, obtaining the same results for each test.

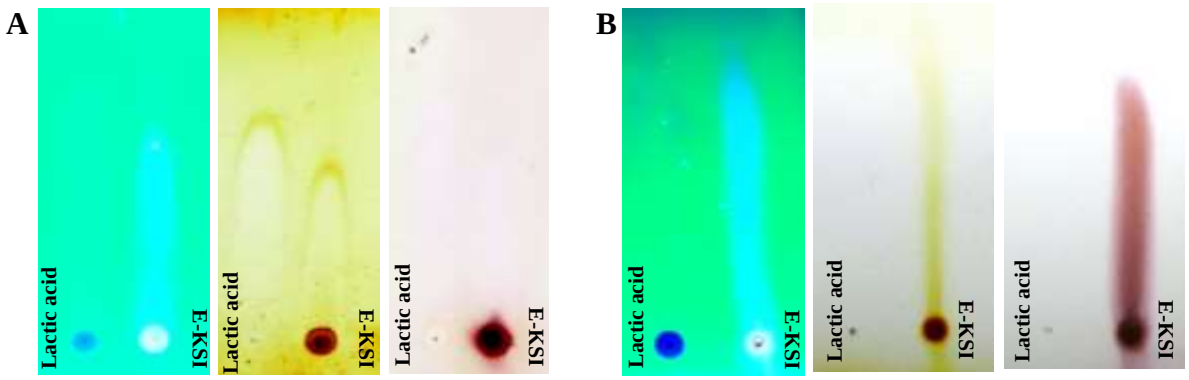

Figure 3. Analysis of E-A5 (10X) using thin-layer chromatography (TLC). A. TLC using as eluent ethyl acetate: hexane (9:1). B. TLC using as eluent methanol: acetic acid (9:1); revealed from left to right with UV, iodine and ninhydrin, respectively.

14 mg 500 μL^{-1} from ethyl ether extraction, 34 mg 500 μL^{-1} from the ethyl acetate and 122 mg 500 μL^{-1} from n-butanol. The MIC of recovered fraction with ethyl ether was 350 µg for *E. coli* and *S. aureus*, respectively, whereas for *P. aeruginosa* was 700 µg. Besides, the MIC from recovered fraction of ethyl acetate was 850 µg for *E. coli* and 425 µg for *S. aureus* and *P. aeruginosa,* respectively. Finally the MIC for the fraction obtained from n-butanol was 1525 µg for three strains.

DISCUSSION

Currently, the antimicrobial capacity of bacteria with probiotic potential is still being explored, one of the most studied genera is *Lactobacillus*. Today the identification of these microorganisms is based on their metabolic characteristics and sequencing of the 16S rRNA subunit, it also demonstrate its low phylogenetic to differentiate the species level and poor discriminatory power for some

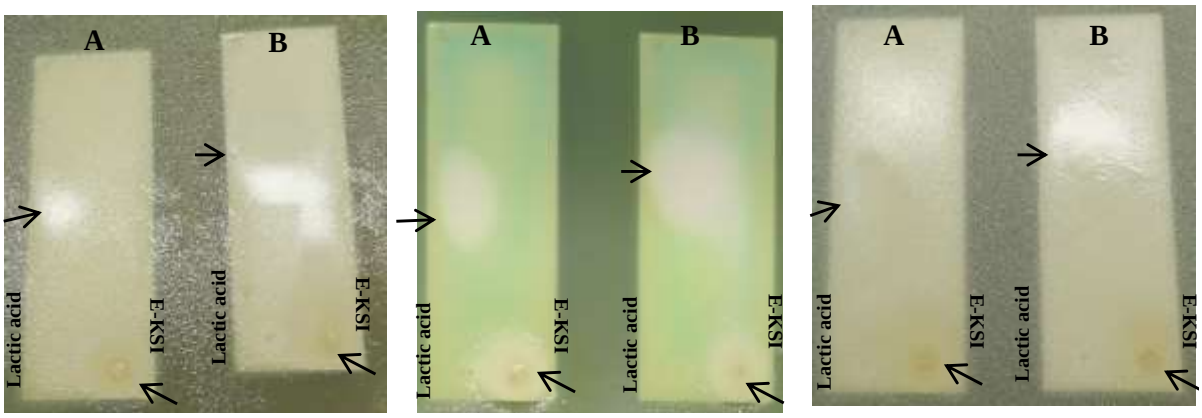

Figure 4. Analysis of E-KSI (10X) through essays of autobiography. From left to right, *E. coli ATCC 25922, P. aeruginosa ATCC 27853* and *S. aureus ATCC 25923*. A. TLC eluted with ethyl acetate: hexane (9:1). B: TLC eluted with methanol: acetic acid (9:1), the arrows indicate the halos of inhibition present. Eluted samples with methanol: acetic acid has inhibition along the bleed.

Table 3. Evaluation of E-KSI (10x) submited to extractions with organic solvents by agar diffusion assay method.

Treatment on the extract E-KSI	Inhibitory activity (mm)		
	E. coli **ATCC 25922**	***P. aeruginosa*** **ATCC 27853**	***S. aureus*** **ATCC 25923**
Extraction of E-KSI 10x (organic phase)			
MRS control (-)	-	-	-
n-Butanol (But)	18.0	20.0	18.0
Ethyl acetate (EA)	18.0	20.0	18.0
Hexane (Hex)	-	-	-
Chloroform (CF)	-	-	-
Ethyl ether (EE)	12.0	14.0	14.0
Extraction of E-KSI 10x (organic aqueous)			
E-KSI 10x control (+)	22.0	21.0	22.0
n-Butanol (But)	15.0	14.0	-
Ethyl acetate (EA)	16.0	16.0	15.0
Hexane (Hex)	16.0	18.0	18.0
Chloroform (CF)	16.0	17.0	18.0
Ethyl ether (EE)	17.0	17.0	16.0

The assays shown were performed in triplicate, obtaining the same results for each test.

genera (Janda and Sharon, 2007). So it was necessary to analyzer diverse housekeeping genes (*hsp*, *recA* and *rpoB*), which contribute to a more efficient bacterial identification at the level of genus, species and subspecies (Bou et al., 2011).

On the other hand, the importance of these bacteria has lately focused on their ability to generate antagonistic effect or produce substances for this purpose against competing bacteria, mainly pathogenic. In this study the ability of the *L. paracasei* KSI strain to generate antibacterial substances in both solid and liquid media was demonstrated. However, there was difficulty in recovering the antimicrobial substances from the solid medium in comparison with the liquid medium when extractions with organic solvents were related (data not shown).

Studies for the production of antimicrobial substances generally focus on *in vitro* tests with the use of complex media designed for the proliferation of BAL (Garsa et al., 2014), as medium MRS. In this study, the effectiveness of APT agar (designed for the efficient proliferation of Lactobacillus) was evaluated for this same purpose, without obtaining positive results. This result verifies that the components of the culture medium are important for

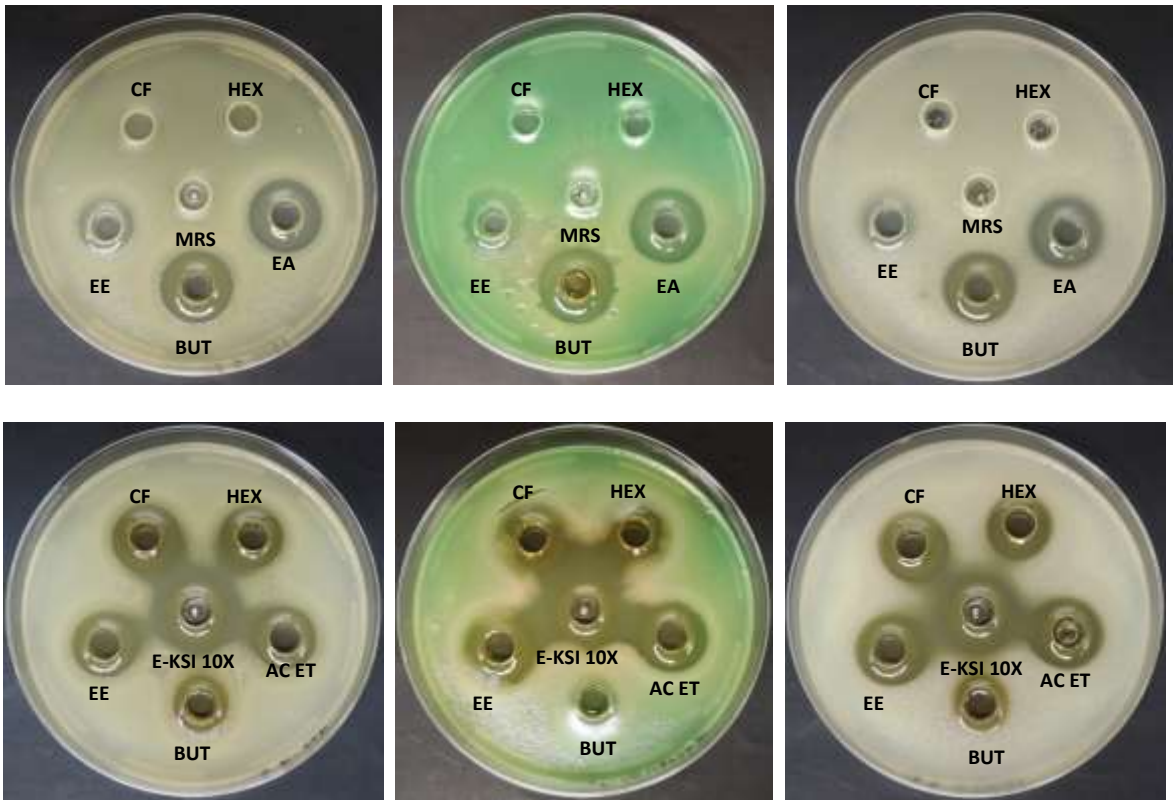

Figure 5. Antagonism tests of E-KSI extractions wth different organic solvents. From left to right, *E. coli ATCC 25922, P. aeruginosa ATCC 27853* and *S. aureus ATCC 25923*. Plates from the top: Extractions of E-KSI 10X (organic phase). Plates from the bottom: Extractions of E-KSI 10X (organic aqueous).

the production of antimicrobial substances, in such a way that for their generation *in vivo*, the nutritional conditions suitable for the microorganism are required (Klaenhammer et al., 2007).

The ability of BALs to secrete bacteriocin-like protein substances in liquid culture media is widely demonstrated (Bodaszewska-Lubas et al., 2012). The study of protein characteristics of these substances was demonstrated by evaluation of stability at different temperatures, pH and digestive enzymes sensitivity. This study showed that *L. paracasei* KSI strain produced stable thermo-substances in liquid medium results was similar to Chen et al. (2014) who evaluated the thermal stability of the Plantaricin Y bacteriocin (isolated from a strain of *L. plantarum*), which were resistant to the activity of the digestive enzymes with which they were treated. On the other hand, in the results obtained by Wannun et al. (2014), the cell-free extract obtained from *L. paracasei* SD1 lost activity when treated with proteinase K and lysozyme.

When evaluating the stability of the pH changes, we observed that antagonistic activity is lost when the value exceeds a pH = 5, different from that observed by Bendjeddou et al. (2012), which evaluated the activity of Paracaseicin A bacteriocin of strain BMK2005 of *L. paracasei*, losing activity at pH = 7 and decreasing its inhibitory capacity with the action of proteolytic enzymes.

However, it is observed that adjusting E-KSI to a pH = 4 value recovers its inhibitory activity, which reveals the cationic characteristics of the substances it contains. When the extract was concentrated by evaporation, an increase in the inhibitory effect was observed. On the other hand, the participation of lactic acid in the antagonist effect was analyzed since this is one of the main metabolites generated by the BAL and it was demonstrated that high concentrations of this are required to present the same inhibitory effect as the extract.

From the E-KSI (10X), active fractions were recovered by extraction with ethyl ether, ethyl acetate and n-butanol, which implies that E-KSI contains molecules with different polarities (possibly of the biosurfactant type), as observed in thin-layer chromatography and the bioautography tests. All recovered fractions were evaluated by agar diffusion antagonism assays, in the same way the residual aqueous phase generated from each extraction was evaluated and also showed an antagonistic effect when it was evaluated. The results obtained were similar to that reported by Shiba et al.

(2013) that was able to recover active fractions of bacteriocins type from extractions made with n-butanol from cell-free extracts obtained from cultures of strain *L. brevis* FPTLB3, however, it did not recover active fractions with non-polar solvents. The above demonstrates the presence of more than one active molecule different from lactic acid, possibly of the bacteriocins type, as suggested by the ninhydrin reaction observed in TLC. Finally, it was determined that molecules extracted with ethyl ether required lower concentration when evaluated by minimum inhibitory concentration (MIC) test.

In conclusion the strain of *L. paracasei* KSI is capable of generating several molecules with antagonistic activity against *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *S. aureus* ATCC 25923, in both liquid and solid media. Therefore the results presented in this study offers a promising alternative as probiotic potential.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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