Identification and antimicrobial activity of Lactobacillus strains of poultry origin

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This study was developed as part of the process for the selection of Lactobacillus strains with possible probiotic activity. Their objectives were to identify isolated strains of the digestive tract of broiler chickens and to discover the production of antimicrobial substances in front of Gram-positive and Gram-negative bacteria for their selection as probiotic strains. Six bacterial isolates (C7, C10, C11, C19, C44 and C65) were identified from the 16S-23S intergenic region sequence of the ribosomal RNA. The production of antimicrobial substances of these strains was determined by the substance diffusion method in agar. All strains were identified as *Lactobacillus salivarius* and were capable of inhibiting growth in the bacteria used as indicators, mainly by acid production. The inhibition by the production of possible bacteriocins was only found in the C7, C19 and C65 strains in the presence of *Escherichia coli*. These results confirm the possible utilization of the studied bacteria as candidates to probiotic microorganisms.

Key words: *Lactobacillus spp., Lactobacillus salivarius, antimicrobial activity*

Lactobacilli of the digestive tract of birds are part of the beneficial bacterial community. They actively participate in the fermentative processes, show inhibitory activity in the presence of pathogen microorganisms, neutralize enterotoxines, synthesize vitamins, stimulate the immune response and improve the absorption of nutrients contained in the diet (Patterson and Burkholder 2003). Thus, the isolation and later the characterization and selection of these beneficial microorganisms allow having at our disposal a natural biological product that supplied to animals favor the equilibrium of their gastrointestinal ecosystem and health in general (Rosmini *et al*. 2004).

Native lactobacilli develop different mechanisms for the inhibition of pathogen microorganisms, among which are the competition for the colony and nutrient sites and the production of toxic compounds. These processes are not mutually exclusive, and the inhibition can involve one, various or all the above mentioned mechanisms (Patterson and Burkholder 2003, Higgins *et al*. 2008 and Juárez and Molina 2010).

The main antimicrobial substances produced during the metabolic processes of lactobacilli, as well as of other lactic acid bacteria, are the organic acids, the hydrogen peroxide, enzymes and bacteriocins (Price and Lee 1970, Stanier *et al*. 1996, Aymerich *et al*. 200 and Powell *et al*. 2007).

This experiment was aimed at identifying Lactobacillus strains from the digestive tract of broilers and to determine the production of antimicrobial substances of these strains in front of microorganisms potentially pathogens, for their selection as candidates to probiotic strains.

Materials and Methods

Identification of strains. Six *Lactobacillus spp*. strains (C7, C10, C11, C19, C44 and C65) which were isolated from the digestive tract of broilers by Rondón *et al*. (2008) were cultivated by exhaustion in MRS (Man, Rogosa and Sharpe) agar at 37 º C, under anaerobiosis conditions for 48 h. They were identified from the 16S-23S intergenic spacer region sequence, according to the methodology described by Tannock *et al*. (1999). The DNA of each strain was amplified with the specific primers: 16-1A, 5'-GAATCGCTAGTAATCG-3 and 23-1B, 5'-GGGTCCCCCATTCGGA-3' (Life Technologies). The strain *Lactobacillus casei* ATCC 334 was also used as pattern and a negative control revealing all the PCR (Polymerase Chain Reaction) compounds, but lacking the DNA mold.

The sequence process was realized at the Sequence Laboratory of the University of León, Spain, with the aid of the automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

Reading and analysis of the sequences were completed from the observation of the electrophoregrams. Sequences of nucleotides obtained were compared to all database entries of the non-redundant nucleotide (nr/nt) collection of the GenBank + EMBL + DDBJ + PDB sequences in the program: BLASTN 2.2. 19+ (Altschul *et al*. 1997)1. For the identification the microorganisms showing similar homology percentage with the problem

sequence were considered and the phylogenetic trees were constructed with the recorded strains in these database. Later the nucleotide sequences of the strains were aligned with the CLUSTALW2 program. From these results, the homology dendrogram between the sequences of the strains identified was obtained.

Production of antimicrobial substances. To determine the antimicrobial activity of the strains, the diffusion in agar technique proposed by Schillinger and Lucke (1989) was applied. For that, the collection strains (ATCC, American Type Culture Collection) and the reference strains of the Microbiology Laboratory of the Veterinary Faculty of the University of León, Spain, were used.

Treatment of the producing strains. The C7, C10, C11, C19, C44 and C65 were cultivated in MRS culture medium at 37ºC, under static conditions, for 18 h (10 Log CFU. mL-1). From these cultures they were inoculated (1:10 v/v) in flasks containing 45 mL of MRS culture medium, which were maintained under the same experimental conditions. Samples were taken at 0, 6, 12, 18 and 24 h of incubation, centrifuged at 15000 rpm at 5ºC (MSM HIGH SPEED 18) for 10 min and the supernatant were sterilized through cellulose acetate filters, with 0.22 µm pores (Minisart, Satorius 600 kPa max).

Each supernatant was processed in three variants: V1 - Unmodified supernatant; V2 – Modified supernatant with the addition of NaOH 0.5 N (until pH 6.5) and pronase E (MerK) at 1% to eliminate the action of acids and bacteriocins and V3 – Supernatant modified with the addition of NaOH 0.5N (until pH 6.5) and catalase (Merck) at 0.1% to eliminate the action of acids and hydrogen peroxide.

Treatment of indicator strains. The indicator strains *Staphylococcus aureus* (ATCC 29737), *Enterobacter cloacae* (ATCC 13047), Klebsiella (ATCC 11296), *Staphylococcus epidermidis* (ATCC 12228), *Proteus vulgaris* (ATCC 13315), *Salmonella typhimurium* (ATCC 14028), *E. coli* 0157:H7, *Listeria inmocua*, *Listeria monocytogenes* and *Salmonella enteritis*, were used. All the indicator bacteria were inoculated in nutrient enriched culture medium (HISPANLAB, Spain) and were incubated in sieve with controlled temperature (UNITRONIC 320 OR) for 18 h, at 37ºC.

Development of the diffusion technique in agar. From the indicator strain cultures, 200 µL were taken which were inoculated in tubes with 20 mL of nutrient agar (with 10% of Ion-Agar, OXOID) and poured in dishes for their solidification. In each dish containing the indicator strains, wells of 5 mm diameter were opened where 60 µl of the samples of the producing strains, positive controls (MRS culture medium + lactic acid 1N until reaching pH 3) and negative controls (MRS culture medium) were deposited. The dishes were maintained at 5ºC for 4 h for better diffusion of the substances in agar. Later, they were incubated for 24 h at 37ºc until discovering the growth and the appearance of the inhibition halos. The diameter of the halos was measured with a millimeter rule. To each value the diameter of the wells was substracted.

Data were analyzed according to a simple classification design. The statistical program INFOSTAT, version 1.0 (Belzarini *et al*. 2001) was used for data processing. In the necessary cases, Duncan's (1955) multiple range test was used.

Results and Discussion

The taxonomic identification of the six strains by sequence confirmed the results of Rondón *et al*. (2008), with the application of the API 50CHL Test to these strains, since all were identified as *Lactobacillus salivarius* sub-species salivarius. This species is considered a GRAS (Generally Recognized as Safe) microorganism and employed for the production of probiotic additives (Escalante 2001 and Neville and O'Toole 2010b).

Tilsala-Timisjarvi and Alatossava (1997) and Tannock *et al*. (1999) validated the utilization of the intergenic region between the 16S-23S segments of the ARNr for the identification of the species of Lactobacillus genus, since it is considered a region highly preserved, but of great variability for this genus. Also, this is a fast method that allows the molecular characterization of lactobacilli, without using specific-primer species to perform the PCR.

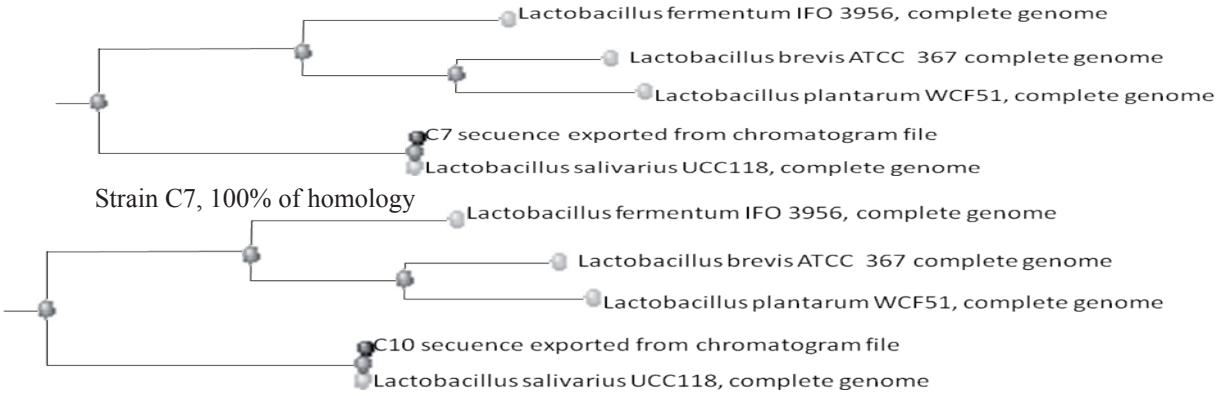
In figure 1 are shown the phylogenetic trees which confirm the relationship between the sequences of the six strains, when compared to others recorded in the database. In all cases it was observed homology with the sequence of the *Lactobacillus salivarius* strain UCC118 (NC. 0079291). This strain is characterized by its probiotic potential. Dunne *et al*. (1999) isolated it from the ileum-cecal region of the human digestive tract and confirmed that it has de capacity of inhibiting different microorganisms as Listeria, *E. coli*, Staphylococcus, Enteroccus, Pseudomonas and Bacillus (Dunne *et al*. 2001).

Once aligned the sequences, the dendrogram demonstrating the homology among the strains under study was obtained (figure 2). Although all belong to the same species, there were differences in the homology of C7, C11, C19 and C10 sequences with C44 and C65, which indicates that they are different strains.

Rogosa *et al*. (1952) described *Lactobacillus salivarius* with two sub-species: salivarius and salicinius. Recently, Li *et al*. (2006) realized a multiphase analysis

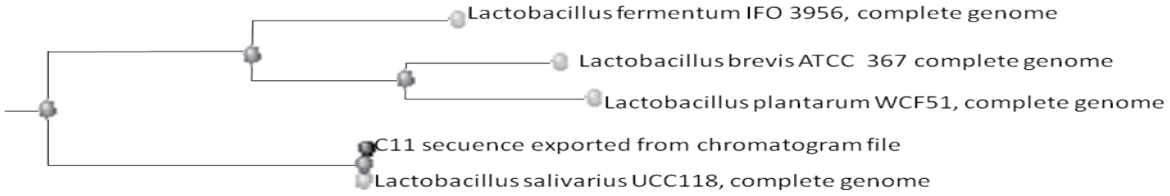
[1]Available in web page of NCBI (National Center of Biological Information). Group of BLAST applications (http://Blast.ncbi.nlm.nih.gov/Blast.cgi.)

[2]Available in: http://clustalw.genome.jp/. Kyoto University Bioinformatic Center

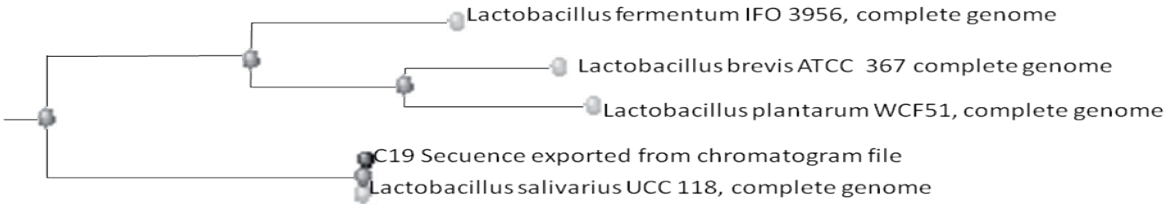


Strain C7, 100% of homology

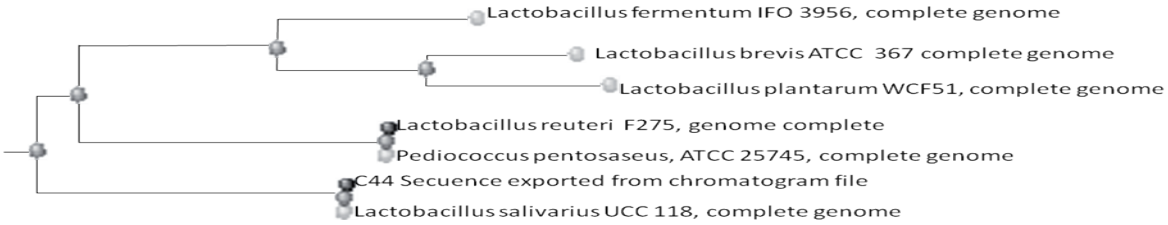
Strain C10, 100% of homology



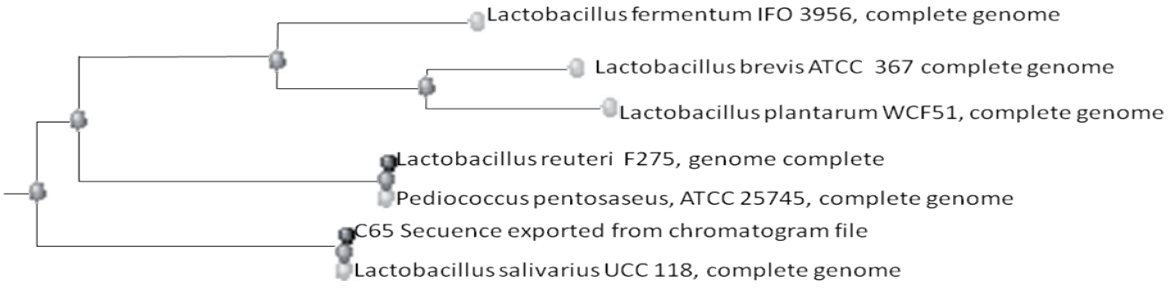
Strain C11, 100% of homology



Strain C19, 96% of homology



Strain C44, 100% of homology



Strain C65, 100% of homology

Figure 1. Phylogenetic trees of C7, C10, C11, C19, C44 and C65 strains confirming the homology with other sequences deposited in the Genbank.

of the characteristics of the two sub-species. These authors indicated that it was improper to make a division between its members, since in all strains characterized there were no notable differences among the 16A ARNr genic sequences and the 16S-23S ARNr intergenic segments.

Results from strain identification through molecular methods coincide with those reported by Garriga *et al*:

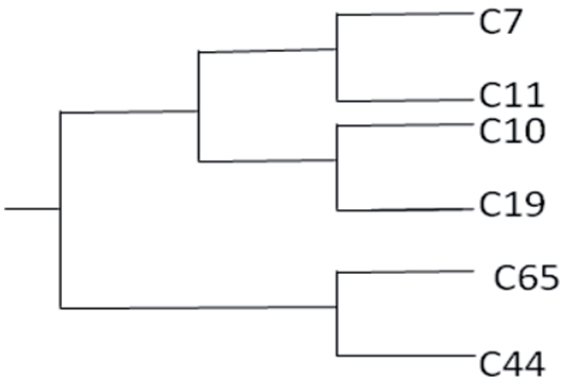


Figure 2. Dendrograme representing the homology among *Lactobacillus salivarius* strains.

(1998), who isolated strains of lactic acid bacteria of chicken cecum, for finally select eight of *Lactobacillus salivarius*. These showed the best attributes as probiotics. *L. salivarius* is considered by some authors the dominant species in chicken intestine and cecum (Pascual *et al*. 1996, Gusils *et al*. 1999 and Neville and O'Toole 2010a).

Results from the dynamics of antimicrobial action of the six strains in front of different Gram-positive and Gram negative strains are shown in table 1. From 6 h, there was in the wells with V1 the formation of inhibition halos in the indicator strains, excepting *Staphylococcus spp.*. which in this time was not inhibited in front of none of the producing strains. This latter is perhaps due to the fact that the concentration of antimicrobial substances in the medium was not sufficient to inhibit the growth of this microorganism.

Also, at 6 h with the unmodified (V1) supernatants the highest inhibition halos for the C7, C19 and C65 strains were observed in the presence of 0157:H7 *E. coli*. It must be borne in mind that in this variant the supernatant contains all possible substances capable of inhibiting the indicator strains, as organic acids, hydrogen peroxide and bacteriocins. In this time inhibition halos of *E. coli* were observed in front of supernatants treated with NaOH and catalase (V3) of the strains C7 (9.42 mm), C19 (7.8 mm) and C65 (9.33). This indicates the action of substances different than organic acids and the hydrogen peroxide, possibly bacteriocins. These results are related to those found for variant V2 (treated supernatant with NaOH and pronase E), where there was no inhibition. This demonstrates that in V3 the substances inhibiting *E. coli* had a protein nature. However, this hypothesis needs to be corroborated with more specific methods to find bacteriocins.

For the remaining strains (C10, C11 and C44) there were no halos in the wells where the variants V2 and V3 were deposited. This demonstrates that these strains inhibit the growth of the potential pathogen strains, due to the action of the organic acids they produce, mainly lactic acid (Rondón *et al*. 2008).

At 12, 18 and 24 h, inhibition halos were observed in all the indicator strains, due to the acid production (no bacterioricins, nor hydrogen peroxide), since these were not produced in the wells with variants V2 and V3. At 24 h, the C7 and C65 strains showed inhibition halos of greater diameter ($P < 0.001$) than the remaining ones. As carbohydrate fermentation of the medium occurs, acid concentration increases, pH decreases and the inhibition halo is bigger.

From the previous results it is demonstrated that the evaluated strains exert mainly the antimicrobial activity by the production of organic acids and inhibit microorganisms that can potentially be pathogens in animals. Similar results obtained Reque *et al*. (2000), when a *Lactobacillus fermentum* LPB strain was used isolated from the intestinal content of chickens, which *in vitro* inhibited *E. coli*, *S. aureus* and *S. thyphimurium* by pH decrease of the medium.

The organic acids produced by the lactic bacteria act in sensible bacteria on entering their walls, providing that the acid molecule is in undissociated form. Once these acids penetrate this barrier, they dissociate in their two components: the anion, which modifies the cell genetic material and the cation that acidify the cytoplasm. This submits the cell to a great energy decline for its neutralization (Segura and De Bloss 2000, Murry *et al*. 2004 and Mayanagi *et al*. 2009).

Traditionally, bacteriocins are considered as peptides biologically active, with bactericide properties against species closely related to the producing strain. However, this concept was modified, since bactericide actions were also found against strains phytogenetically distanced (Sablon *et al*. 2000).

Regarding bacteriocin production, other authors as Audisio and Apella (2006), Lima *et al*. (2007) and Shin *et al*. (2011) observed *in vitro* this capacity in *Lactobacillus salivarius* strains. In this study *E. coli* 0157:H7 inhibition was produced by possible bacteriocins that C7, C19 and C65 strains created. This inhibition was only noticed at 6h, when the pH of the cultures was between 5 and 5.5, that is, at the beginning of the exponential phase. These results do not coincide with Desmazeaud

Table 1. Dynamics of the antimicrobial action of *Lactobacillus salivarius* strains regarding the collection strains.

Indicator strains	Producing strains (V1) (Halo diameter, mm)						
	C7	C10	C11	C19	C44	C65	SE ± Sig
Six hours							
Escherichia coli	13.7^{d}	5.7^{b}	5.26^{a}	11.67^{c}	5.26^{a}	13.43^{d}	0.10***
Listeria innocua	6.03^{c}	5.3^{b}	NI	5^{a}	NI	6.1^{c}	0.09***
Listeria monocytogenes	7.03^{b}	NI	NI	6.00^{a}	NI	7.27^{b}	0.11***
Salmonella enteritidis	7.30^{d}	4.70^{a}	5.00^{b}	6.67^{c}	5.03^{b}	7.23^{d}	0.08***
Staphylococcus aureus	NI	NI	NI	NI	NI	NI	-
Enterobacter cloacae	9.37^{d}	7.30^{b}	7.07^{a}	8.30^{c}	7.03^{a}	9.30^{d}	0.07***
Klebsiella spp.	7.67^{c}	4.33^{a}	5.30^{b}	7.30^{c}	4.70^{a}	7.40^{c}	0.15***
Staphylococcus epidermidis	NI	NI	NI	NI	NI	NI	-
Proteus vulgaris	8.30^{d}	7.30^{ab}	7.03^{a}	8.03^{cd}	7.70^{bc}	8.27^{d}	0.16***
Salmonella typhimurium	7.50^{d}	4.33^{a}	5.03^{b}	6.03^{c}	4.67^{ab}	7.27^{d}	0.13***
Twelve hours							
Escherichia coli	10.53^{d}	8.27^{a}	8.30^{a}	10.00^{bc}	9.73^{b}	10.30^{cd}	0.13***
Listeria innocua	12.77^{e}	12.17^{d}	5.27^{a}	11.03^{c}	9.03^{b}	12.73^{e}	0.17***
Listeria monocytogenes	10.30^{e}	9.27^{c}	8.03^{a}	10.03^{d}	9.00^{b}	10.10^{de}	0.07***
Salmonella enteritidis	9.57^{b}	7.03^{a}	6.67^{a}	7.03^{a}	6.73^{a}	9.30^{b}	0.12***
Staphylococcus aureus	8.63^{d}	7.27^{b}	5.30^{a}	8.03^{c}	5.03^{a}	8.43^{d}	0.11***
Enterobacter cloacae	12.40^{b}	11.00^{ab}	11.27^{a}	12.20^{b}	10.73^{a}	12.29^{b}	0.25**
Klebsiella spp.	12.70^{e}	10.67^{b}	11.30^{c}	12.03^{d}	10.30^{a}	12.40^{e}	0.11***
Staphylococcus epidermidis	7.40^{b}	7.03^{ab}	6.67^{a}	7.03^{ab}	6.70^{a}	7.30^{b}	0.12**
Proteus vulgaris	13.43^{c}	12.03^{a}	12.03^{a}	12.67^{b}	12.03^{a}	13.37^{c}	0.09***
Salmonella typhimurium	9.27^{c}	7.00^{b}	6.67^{a}	7.03^{b}	6.70^{a}	9.17^{c}	0.09***
Eighteen hours							
Escherichia coli	13.80^{d}	13.27^{c}	12.03^{b}	12.30^{b}	11.03^{a}	13.73^{d}	0.11***
Listeria innocua	14.03^{e}	13.13^{d}	12.70^{c}	12.30^{b}	11.73^{a}	13.83^{e}	0.12***
Listeria monocytogenes	12.17^{d}	11.70^{c}	10.73^{a}	11.70^{c}	11.03^{b}	12.07^{d}	0.08***
Salmonella enteritidis	13.23^{d}	9.67^{b}	9.70^{b}	10.27^{c}	8.30^{a}	13.03^{d}	0.10***
Staphylococcus aureus	9.17^{d}	8.70^{c}	7.70^{a}	8.90^{cd}	8.30^{b}	9.03^{d}	0.10***
Enterobacter cloacae	14.37^{e}	13.70^{c}	13.03^{b}	14.03^{d}	12.73^{a}	14.17^{de}	0.07***
Klebsiella spp.	14.67^{d}	12.03^{a}	13.30^{b}	14.00^{c}	12.03^{a}	14.60^{d}	0.06***
Staphylococcus epidermidis	8.63^{d}	8.00^{b}	7.73^{a}	8.30^{c}	8.27^{c}	8.43^{cd}	0.08***
Proteus vulgaris	17.23^{d}	14.00^{a}	14.30^{b}	14.70^{c}	14.03^{a}	17.03^{d}	0.07***
Salmonella typhimurium	13.23^{d}	9.73^{c}	9.30^{b}	10.00^{c}	8.30^{a}	13.13^{d}	0.08***
Twenty four hours							
Escherichia coli	15.00^{d}	14.33^{c}	13.70^{b}	14.30^{c}	12.30^{a}	14.93^{d}	0.05***
Listeria innocua	15.03^{d}	14.03^{c}	13.00^{b}	14.03^{c}	12.67^{a}	14.87^{d}	0.07***
Listeria monocytogenes	13.73^{e}	13.00^{d}	12.73^{c}	12.30^{b}	12.03^{a}	13.63^{e}	0.05***
Salmonella enteritidis	14.27^{d}	10.27^{a}	10.70^{b}	11.00^{c}	10.30^{a}	14.10^{d}	0.08***
Staphylococcus aureus	10.70^{d}	10.00^{c}	9.30^{a}	9.70^{b}	9.70^{b}	10.53^{d}	0.07***
Enterobacter cloacae	15.30^{e}	14.00^{c}	13.73^{b}	14.27^{d}	13.00^{a}	15.30^{e}	0.06***
Klebsiella spp.	14.73^{e}	12.70^{a}	13.67^{c}	14.27^{d}	13.30^{b}	14.70^{e}	0.07***
Staphylococcus epidermidis	10.27^{d}	9.70^{b}	9.33^{a}	10.03^{c}	9.70^{b}	10.17^{d}	0.04***
Proteus vulgaris	17.99^{e}	16.03^{a}	16.33^{b}	17.03^{d}	16.70^{c}	18.03^{e}	0.04***
Salmonella typhimurium	14.03^{d}	10.70^{c}	10.30^{b}	10.73^{c}	9.73^{a}	14.00^{d}	0.06***

NI – No inhibition V1 Supernatant without treatment

^{abcde}Means with different letters within each row differ for $P < 0.05$ (Duncan 1955).

(1997) and Powell *et al*. (2007) who pointed out that the biosynthesis of bacteriocins occur at the end of the exponential phase of the microbial growth. On the other hand, Avonts *et al*. (2004) demonstrated that this property is not equally manifested for all the strains, since they noted that *Lactobacillus acidophilus* IBB801, L. gasseri K7 and L. johnsonii La 1 showed the peak of activity for bacteriocin production at the end of the exponential phase of growth. While *L. casei* Imunitas and *L. casei* YIT 9029 generated the bacteriocins during the first hours of the growth phase, as it took place in this research study.

Previous disagreements indicate the need of assessing this test, preceded by the development of the production dynamics of antimicrobial substances. With this study, samples are taken of the compounds generated at the different phases of microbial growth (Rondón *et al*. 2007).

Barret *et al*.)2007) observed bacteriocin synthesis bicomposed by *Lactobacillus salivarius* (salivaricin P), which inhibited *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp bulgaricus, *Listeria innocua* and *Pediococcus pentosaceus*. However, growth of *Staphylococcus aureus* or of other *Lactobacillus salivarius* strains was not prevented.

All seems to indicate that the origin of the disagreeing results could be due to the presence or not of plasmids in the cells. These mediate or intervene in bacteriocin synthesis, though this capacity is specifically manifested in the strains having them (Silva *et al*. 2002, Stern *et al*. 2006 and Nazef *et al*. 2008).

From the results of this study it was determined that C7, C10, C19, C44 and C65 strains show genetic characteristics coincident with *Lactobacillus salivarius* species. C7, C19 and C65 strains displayed the best characteristics for the production of antimicrobial substances, on inhibiting microorganisms with pathogen potential, due to the high acid production and possible bacteriocins in front of *Escherichia coli*. These characteristics recognize their selection as candidates to probiotic strains.

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