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Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitic cows

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ABSTRACT

Bovine mastitis (BM) is a costly disease in dairy cattle production. The prevention and treatment of mastitis is performed by applying antimicrobial products that negatively affect milk quality. In the last years, the use of probiotic microorganisms to prevent infections in humans and animals has being aggressively studied.

Samples from teat canal and milk (foremilk and stripping) were taken from healthy and mastitic mammary quarters. A screening of the surface properties and antagonistic substances production of lactic acid bacteria (LAB) isolated from the mammary gland was performed to select potential probiotic strains to prevent mastitis. Somatic cell count, physico-chemical and microbiological studies were carried out. Pre-selected microorganisms were genetically identified.

Compared with stripping milk, foremilk showed lower levels of fat and higher levels of pH, density, microorganism numbers, lower percentage of strains with mean and high hydrophobicity and mean autoaggregation and higher number of strains able to produce hydrogen peroxide and bacteriocins. The other parameters analyzed were not statistically significant. One hundred and two LAB strains were isolated. Most of them had low degrees of hydrophobicity and autoaggregation. No correlation between these properties was found. Antagonistic metabolites were mainly produced by strains isolated from healthy quarters. Most of the pre-selected strains were identified as *Streptococcus bovis* and *Weissella paramesenteroides*. Three bacteriocin-producers were found and their products partially characterized.

The results of this work are the basis for the further design of a specie-specific probiotic product able to prevent BM.

1. Introduction

Bovine mastitis (BM) can be defined as an inflammation of one or more quarters of the udder usually caused by bacterial infection (FAO, 1989) and is considered to be one of the most expensive diseases in dairy farming and the dairy industry. Miles et al. (1992) calculated that the average cost of BM per cow per year due to reduced milk production, discarded milk, replacement cost, extra labor, treatment and veterinary services in the United States was $ 125. Further, antibiotic therapy frequently produces residues in milk that could contribute to microbial resistance (Duarte et al., 2005), contamination of the human food supply and a decrease in milk quality, making

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milk unacceptable for industrial processes. BM can also affect the reproduction rates of cows, although the mechanism that lead to embryonic loss is still not completely understood (Hansen et al., 2004).

The control of BM is principally based on prevention (Rodrigues and Ruegg, 2005). The prevention and treatment of BM are carried out by the application of systemic or local therapies (pre- and post-milking teat dipping). Drug concentrations must also decrease to safe levels before the milk is harvested for human consumption (Gehring and Smith, 2006).

Alternatives to antibiotics developed during the last decade include products containing, iodophores (Foret et al., 2006), hydrogen peroxide (Leslie et al., 2006), bismuth, bacteriocins such as lacticin (Crispie et al., 2005) and nisin (Cao et al., 2007). Recently, a novel approach using probiotic microorganisms to prevent infections in animals is being widely studied (Otero et al., 2006), mainly in the gastrointestinal tract (Alexopoulus et al., 2004). Saarela et al. (2000) suggested that probiotic strains must be isolated from the same ecosystem where they will be applied. Based on the host specificity exerted by the members of the indigenous microbiota (Zoetendal et al., 2006), the aim of the present work was to study the autochthonous lactic microbiota of the udder with different health status and to select potentially beneficial strains to be included in a probiotic product to prevent mastitis in cows.

2. Materials and methods

2.1. Sampling

Milk samples were collected from 51 clinically healthy adult Holstein dairy cows from dairy farms of Northwestern Tucumán (Argentina). Quarter foremilk samples were aseptically collected immediately before milking and at the end of milking (stripping milk). The teat ends were cleaned with 70% ethanol and individual paper towels and allowed to dry. The first few streams of milk derived from the four mammary quarters were collected in sterile plastic tubes. Each animal was machine milked, and at the end of milking, another sample was taken in a different sterile plastic tube. The samples were refrigerated and transported to the laboratory, and processed immediately.

Six different samples were obtained from the teat end. Teat end was cleaned with 70% ethanol as described above. Before milking, teat canals were scraped with a sterile cotton swab and the scrapings placed in MRS (De Man, Rogosa and Sharpe-Merck) broth, pH 5.5. Samples were immediately refrigerated and transported on ice to the lab. MRS tubes were later incubated at 37 °C for at least 24 h.

2.2. Analysis of milk samples

Determination of fat content, solids not fat (SNF), protein and milk density of milk samples were performed with an ultrasonic milk analyzer (Ekomilk – Milkana Kam 98-2A).

Somatic cell count (SCC) was determined by the microscopic method according to the International IDF Standard 148A:1995. A milk sample was considered as coming from an infected quarter when SCC was $\geq$300,000 cells/ml (Deluyker et al., 2005).

2.3. Microbiological analysis

The quantification of microorganisms was performed by the serial dilutions method using 0.1% (v/v) peptone as dilution media, aliquots plated on Plate Count Agar (Britania, Argentina) and incubated at 37 °C for 24 h.

For the enumeration of enterobacteriae, staphylococci and LAB, milk samples were centrifuged at 600 × *g* in an Eppendorf 5415 D centrifuge for 10 min. Supernatant was discarded and peptone water was added to obtain 1/10 initial volume. For enterobacteria enumeration, 100 μl of the pellet were plated on MacConkey Agar (Britania, Argentina) and incubated at 37 °C for 24 h. For staphylococci quantification, 100 μl of the pellet was spread on the surface of agarized media (Manitol Salt Agar-Britania) and incubated at 37 °C for 24 h. The number of LAB was determined by using two different culture media: LBS (Lactobacillus selective agar-Merck) agar and MRS agar. Plates were incubated in a 5% CO_2 atmosphere at 37 °C for 48 h.

2.4. Isolation of LAB and phenotypical identification

The isolation of LAB from milk samples was performed by centrifugation of 10 ml of milk. Pellets were streaked out with a sterile loop on LBS, MRS agar and modified Brain Heart Infusion (modified BHI) agar containing sodium azide (0.8 g/l for inhibition of Gram (−) microorganisms) and crystal violet (0.0008 g/l to inhibit Gram (+) microbiota). Plates were incubated in a 5% CO_2 atmosphere at 37 °C for 24 h (modified BHI) and 48 h (LBS and MRS agar). For the isolation of LAB, morphological different colonies were subcultured in MRS broth and incubated at 37 °C for 24 h. Strains were stored in milk yeast extract at −20 °C.

Samples obtained from the teat canal received in MRS broth pH 5.5 were incubated at 37 °C during 24–48 h. Later, cultures were streaked out with a sterile loop on MRS agar. Plates were incubated as described previously for the isolation and storage of microorganisms.

Phenotypic identification was performed by morphological and phenotypic characteristics: Gram staining, catalase reaction, nitrate reduction, indol production, CO_2 production from glucose and gluconate.

2.5. Surface properties

To determine the surface characteristics of the isolated LAB strains, two types of assays were performed: hydrophobicity and autoaggregation. LAB were subcultured three times in LAPTg broth (1% yeast extract, 1.5% peptone, 1% tryptone, 1% glucose, 0.1% Tween 80). The third culture was centrifuged for 5 min at 9.300 × *g*. Supernatants were discarded and the pellets washed three times with sterile saline solution. The washed pellets were used to evaluate the surface properties.

For the assessment of the degree of surface hydrophobicity, the microbial adhesion to hydrocarbons method

(MATH) (Otero et al., 2004) was applied with hexadecane (Sigma, USA). Strains were classified as Low, Middle and High according to their degree of hydrophobicity.

For the assessment of the autoaggregation capability, the method described previously by Ocaña and Nader-Macías (2002) was applied. Strains were classified as Low, Middle and High according to their autoaggregative capability.

2.6. Production of antagonistic metabolites

2.6.1. Screening of hydrogen peroxide production in TMB agar plates

Strains that were subcultured three times at 37 °C were streaked out with a sterile loop on MRS or LAPTg agar-TMB (3,3′,5,5′-tetramethylbenzidine) plates to obtain isolated colonies. The technique used was described previously by Juárez Tomás et al. (2004), and allows identifying strains able to produce H_2O_2 in plates containing 1 mM TMB (Sigma, USA) and type II peroxidase (Sigma, USA) 2 U/ml. Plates were incubated microaerophically at 37 °C during 48 h, then exposed to air for 10 min. Colonies that produced hydrogen peroxide turn blue or brown. Strains were classified according to the intensity of their colour as non-producers, low producers or high producers of H_2O_2.

2.6.2. Screening of production of antimicrobial substances in culture-supernatants

LAB supernatants from the third culture from LAPTg broth were tested against different strains (nine of them isolated from infected quarters) by using the plate diffusion technique (Juárez Tomás et al., 2004).

Staphylococcus aureus ATCC 29740, *Streptococcus agalactiae* ATCC 27956, *S. dysgalactiae* ATCC 27957, *Escherichia coli* (two different strains), *S. uberis*, *S. dysgalactiae* (isolated by INTA-Rafaela) strains were provided by Dr. Calvinho (INTA-Rafaela), *S. aureus* was provided by Dr. Bogni (Universidad Nacional de Río Cuarto, Argentina). Coagulase negative staphylococci was isolated in our laboratory. All these strains were isolated from infected quarters. *Listeria monocytogenes* Scott A was provided by Dr. Pasteris (Universidad Nacional de Tucumán) and *Listeria innocua* 7 by the Unité de Recherches Laitiéres et Génetique Appliqueé, INRA France. *Enterococcus faecium* CRL 988 (ATCC19434), *Lactobacillus plantarum* CRL 691 and *Enterococcus faecalis* CRL 341 were obtained from the CRL Collection Culture. Strains were subcultured twice in LAPTg broth at 37 °C (except *L. monocytogenes* and *L. innocua* that were incubated at 30 °C) until the late exponential growth phase. They were then diluted with sterile peptone water to obtain a concentration of 10^7–10^8 CFU/ml in each plate.

Aliquots of the supernatant were neutralized with sterile 2N NaOH. Another aliquot of neutralized supernatants was treated with catalase (1000 U/ml) for 30 min at 25 °C. Supernatants were stored at 4 °C until the plates with pathogens were examined. Plates that showed contamination were replated.

Briefly, 100 μl of each indicator strain was plated in 1% LAPTg agar. Holes were made with sterile straws and 30 μl of each supernatant were placed into different holes. Plates were left for 4 h at room temperature until supernatants diffused and later incubated at 37 °C for 24 h, except for *L. monocytogenes* and *L. innocua* that were incubated at 30 °C. The diameter of the inhibition halo was determined.

2.6.3. Quantification of organic acids

To determine the concentration of the organic acids produced, the supernatants of the third subculture from LAPTg were analyzed by using HPLC. A KNAUER Smartline chromatographer with a column for organic acids (Bio-Rad HPX-87H 300 mm × 7.8 mm) was employed at 41 °C. The flow-rate was 0.6 ml/min. The eluent used was H_2SO_4 5 mM (pH 2).

2.7. DNA isolation, primers and PCRs conditions

DNA was isolated according to the method described previously by Pospiech and Neumann (1995). All the oligonucleotides employed (Table 1) were synthesized by Genbiotech (Buenos Aires, Argentina). MLB and PLB primers were used to amplify the variable (V1) region of the 16S ribosomal RNA gene (Hébert et al., 2000). Bacteriocinogenic microorganisms were analyzed by PCR using degenerated oligonucleotides designed to amplify lantibiotic genes LanBFwd-LanBRev and LanCFwd-LanCRev (Wirawan et al., 2006), and enterocin A structural gene EntAFwd-EntARev (Saavedra et al., 2004). DNAs with positive amplification with Lan primers were amplified with nisin primers: NisAf1-NisAr2, NisAf2-NisBr3, NisAf1-NisBr3 (designed according to the nisin biosynthesis genes). Amplification of DNA was carried out in a Biorad MyCycler™ thermal cycler. Each 50 μl reaction contained PCR Buffer (1x) (Invitrogen, Brazil), 2.5 mM $MgCl_2$ (Invitrogen, USA), 0.2 mM dNTPs (Invitrogen, USA), DNA, primers (1 μM), Taq DNA polymerase (2.5 U) (Invitrogen, Brazil) and miliQ water. PCRs were performed under the following condition: 4 min at 94 °C of initial denaturation, 30 cycles consisting of 30 s of denaturation at 94 °C, 45 s of annealing at the temperature required for each pair of primers and 30–45 s at 72 °C and a final extension at 72 °C for 7 min.

Amplicons were purified by using a GFX PCR DNA and Gel Band Purification Kit (GE Biosciences). Molecular cloning of the PCR fragments was performed by using

Table 1
PCRs primers employed in this work.

Primer name	Sequence (5′–3′)
MLB	GGCTGCTGGCACGTAGTTAG
PLB	AGAGTTTGATCCTGGCTCAG
LanB Fwd	TATGATCGAGAARYAKAWAGATATGG
LanB Rev	TTATTANRCANATGNAYDAWACT
LanC Fwd	TAATTTAGGATWNSYNMAYGG
LanC Rev	ACCWGKNNNNCCRTRRCACCA
NisAf1	AAAATGAGTACAAAAGATTTYAAC
NisBr3	TGCATAACATCATAGAGTTTAGG
NisAf2	TTCACGTAAGCAAATAACCA
NisAr3	TGGTTATTTGCTTACGTGAA
EA Fwd	AAATATTATGGAAATGGAGTGTAT
EA Rev	GCACTTCCCTGGAATTGCTC

K = T/G, M = A/C, R =A/G, W = A/T, and Y = C/T.

the Topo TA Cloning kit (Invitrogen, USA), according to the manufacturer's instructions. Clones were analyzed by colony PCR and those that were positive for any pair of primers were re-amplified, purified and sequenced. DNA sequences were analyzed by using appropriate BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

2.8. Statistical analysis

The experiments were performed in duplicate and means and standard deviations were calculated. The statistical analysis of the data was performed by using the software MINITAB (version 14). ANOVA was applied to analyze the differences between the portions of the milk samples. The correlation between the probiotic properties was determined by calculating the Pearson coefficient.

3. Results

3.1. Samples characterization

The results obtained from the physico-chemical analysis of different milk parameters indicate that foremilk and stripping milk exhibited significant differences ($p < 0.05$) in their characteristics (Table 2). Foremilk had lower fat content and higher values of pH and density than stripping milk.

Most of the milk samples were obtained from healthy quarters ($n = 87$). Remaining milk samples were obtained from quarters with subclinical mastitis ($n = 19$).

3.2. Composition of the udder microbiota

The determination of the total population of enterobacteria, staphylococci and LAB performed in MacConkey agar, MSA (Manitol Salt Agar), MRS and LBS agar, respectively (Fig. 1). The number of mesophilic microorganisms resulted in acceptable values in all the cases (lower than 100.000 CFU/ml). The mean value of the different bacterial groups was higher in foremilk than in stripping milk (Table 2). The number of staphylococci was higher than the number of enterobacteria, while the mean number of LAB was significantly higher in MRS agar than in LBS agar. However, there was no statistical difference between the two milk fractions in this culture medium.

3.3. Isolation of LAB

One hundred and two LAB strains were isolated from the 87 milk and 6 teat canal samples. Strains isolated from the teat canal were considered strains cultured from foremilk. Isolated LAB came from foremilk and a lower number (38%) from strippings (Table 2). 56% of the isolated LAB strains were cocobacilli, while 31% were cocci, 8% bacilli and 5% diplococci.

3.4. Surface properties

The values of hydrophobicity and autoaggregation obtained from individual samples are shown in the matrix plot (Fig. 2). Most of the isolated strains (85.3%) showed low hydrophobicity values (L); 6.9% were classified as strains with middle hydrophobicity (M) and 7.8% were strains with high hydrophocity (H) values. Most of the strains were microorganisms with low hydrophobicity independent of the milk fraction from which they were isolated (Table 2). Nevertheless, the percentage of strains with middle and high hydrophobicty values was higher in strippings (Fig. 3a).

With reference to the autoaggregation capabilities of all the isolated strains, 89.2% of them showed low values, while only 10.8% exhibited mean values of autoaggregation. No isolated strains showed a high autoaggregative pattern. By comparing the degree of autoaggregation between the two milk fractions, those strains isolated from stripping milk and with mean autoaggregation index were 11.6% higher than the LAB isolated from foremilk (Fig. 3b). The low values of the Pearson coefficients obtained from

Table 2
Differential characteristics between foremilk and stripping milk samples.

Characteristic property	Foremilk samples	Stripping milk samples
pH[a]	6.89 ± 0.20	6.71 ± 0.11
Fat content [g/100 ml][a]	1.91 ± 1.20	6.05 ± 1.71
Density [g/ml][a]	1.0030 ± 0.0003	1.0020 ± 0.0006
Protein [g/100 g]	3.08 ± 0.26	3.07 ± 0.28
SNF [g/100 g]	8.31 ± 0.70	8.16 ± −075
SCC [cells/ml]	166124 ± 252173	124224 ± 175907
Number of microorganisms	Higher	Lower
Distribution of the isolated LAB[b]	62%	38%
Strains with mean and high hydrophobicity[c]	11.1%	20.6%
Strains with mean autoaggregative capability[c]	7.9%	15.4%
Strains able to produce any antagonistic metabolite[c]	47.6%	30.8%
Distribution of the 42 strains able to produce any antagonistic metabolite	71.4%	28.6%
Strains able to produce hydrogen peroxide[c]	36.5%	12.8%
Number of bacteriocinogenic strains	3	0
Strains able to inhibit the tested pathogens by lactic acid production[c]	17.7%	20.5%

[a] Differences statistically significant.
[b] Referred to the 102 isolated strains.
[c] Percentage referred to each fraction.

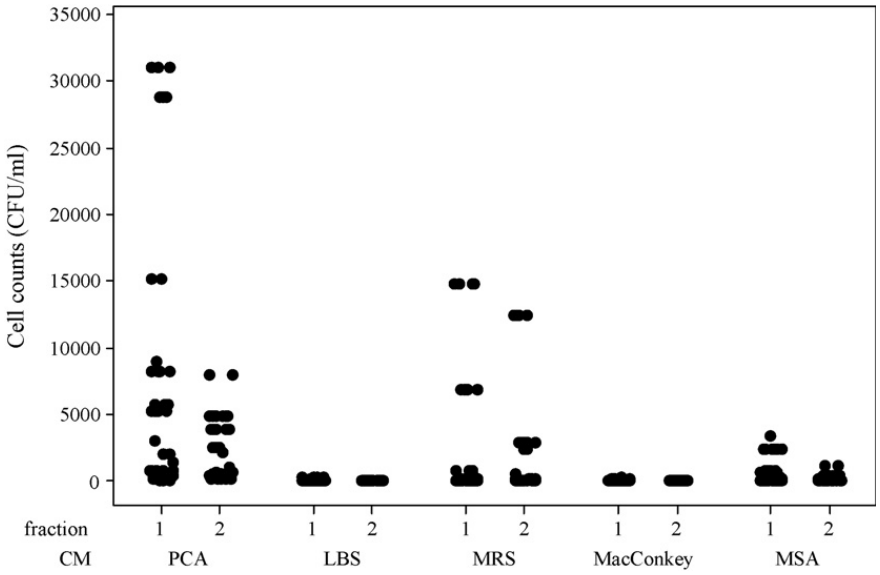

Fig. 1. Individual value plot of the number of microorganisms isolated in different culture media: Plate Count Agar (PCA), Lactobacillus Selective Media (LBS), De Man, Rogosa ans Sharpe (MRS), Mac Conkey, Manitol Salt Agar (MSA), discriminated by fractions (foremilk and stripping). CM: culture media. (1) Count of microorganisms in foremilk samples. (2) Count of microorganisms in stripping milk samples.

the analysis of the surface properties showed that there was no correlation between both surface properties analyzed (Pearson correlation: 0.215).

3.5. Production of antagonistic metabolites

Among the isolated strains, 42 produced at least one antagonistic metabolite and were pre-selected to study organic acids production and their genetical identification. From these, 64.3% of the strains were cocobacilli, 16.7% were cocci, 14.2% were rods and 4.8% diplococci. Most of these strains (71.4%) were microorganisms isolated from foremilk.

When testing for the production of hydrogen peroxide according to the colour in TMB agar plates, most of the strains (71.6%) were non producers while 14.7% were low producers; 4.9% were moderate producers and only 8.8% were classified as high producers. With regard to the milk fraction and hydrogen peroxide production, the results indicate that in the group of strains from foremilk, 36.5% of them were considered as hydrogen peroxide producers (low, moderate or high).

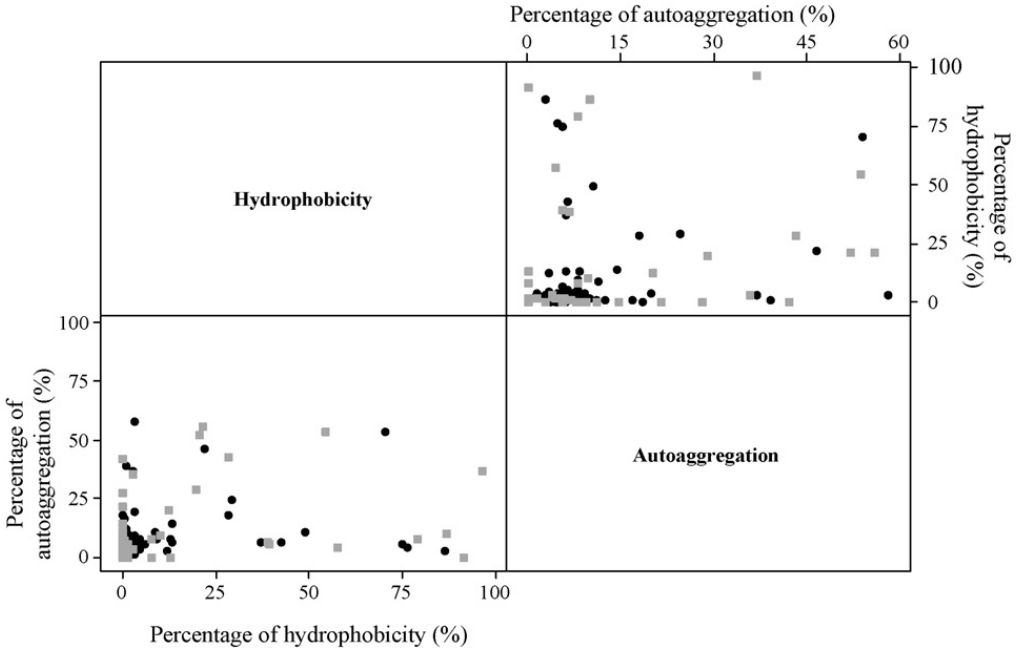

Fig. 2. Relationship between the degree of hydrophobicity and capability of autoaggregation of isolated LAB discriminated by fractions. (●1) Foremilk samples. (■2) Stripping milk samples.

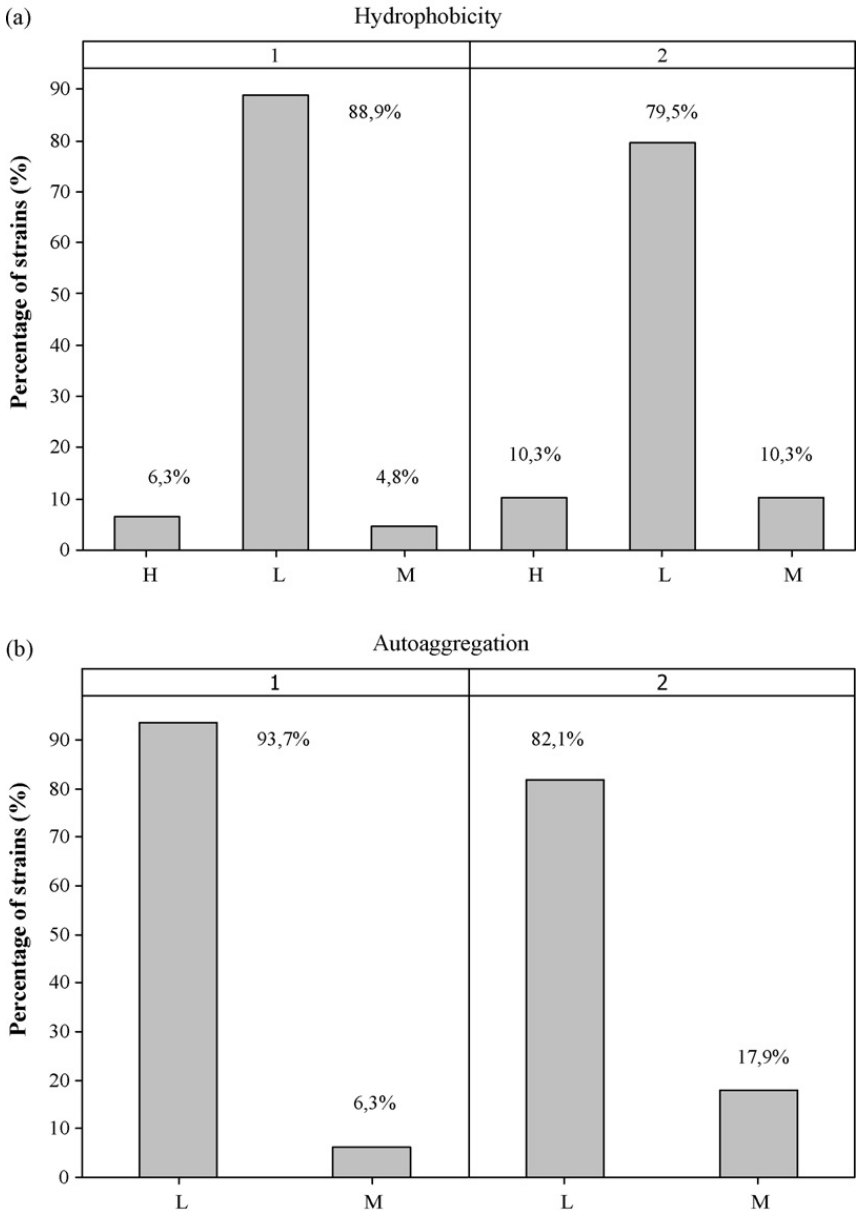

Fig. 3. (a) Charts of Hydrophobicity of the strains according to the milk fractions. (b) Charts of Autoaggregation of the strains according to the milk fractions. H: high. L: low. M: middle. (1) Strains isolated from foremilk samples. (2) Strains isolated from stripping milk samples. Most of the isolated LAB strains corresponded to low degree of hydrophobicity and low capability of autoaggregation strains.

From the strains isolated from strippings, only 12.8% produced hydrogen peroxide (Table 2). Those strains classified as high producers were isolated from foremilk (Fig. 4a).

When determining the degree of production of antagonistic substances in all the isolated strains by using the plate diffusion test, only 18.6% showed inhibition to some of the assayed pathogens by production of organic acids. Considering only this group of strains, 47.4% of them were able to inhibit only one pathogen, 31.6% inhibited two pathogens and 21.0% inhibited more than two pathogens. These LAB strains were able to inhibit the tested pathogens by organic acids production. The percentages of LAB that showed inhibitory properties are presented in Table 3. Three strains produced antagonistic metabolites classified as bacteriocins. One of these inhibited five of the pathogens isolated from infected quarters. The complete spectrum of the three bacteriocinogenic strains is shown in Table 4.

The antagonistic metabolites were mainly produced by strains isolated from healthy quarters (Fig. 4b), particularly those that produce bacteriocin were isolated from healthy quarters.

Most of the hydrogen peroxide and bacteriocin-producers were found in foremilk. Considering the milk fraction and the capability to produce antagonistic substances, strains able to produce hydrogen peroxide and bacteriocions isolated from foremilk, represented the highest percentage (Table 2). LAB isolated from stripping milk produced mainly organic acids (Fig. 4c).

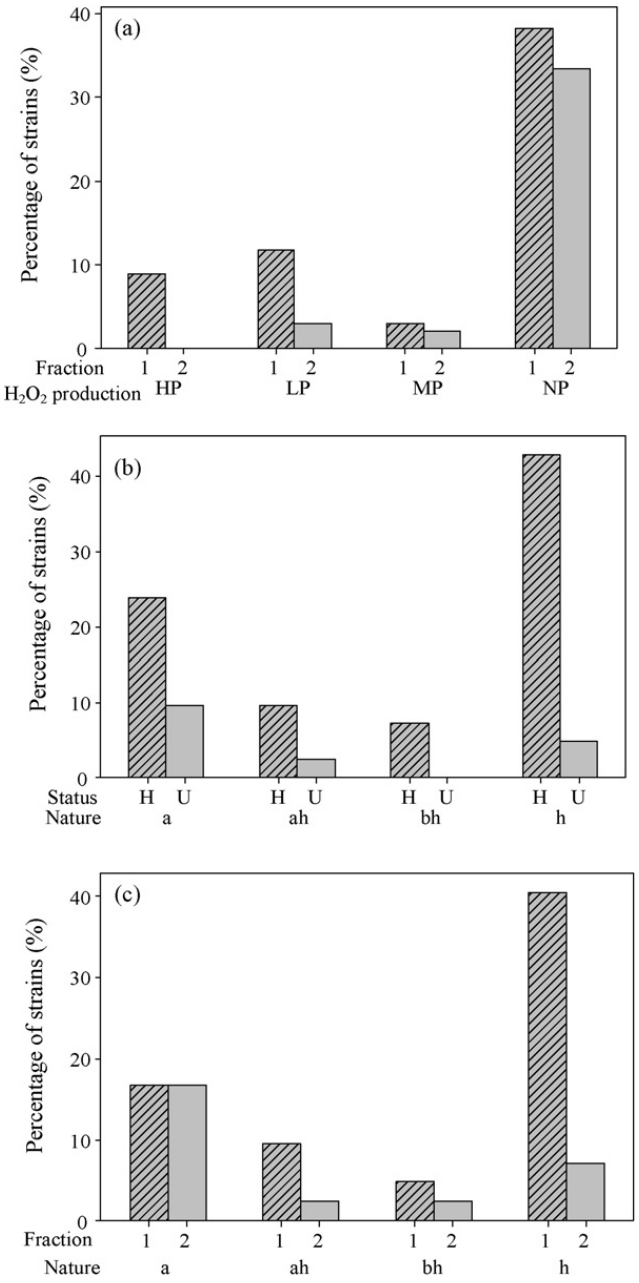

Fig. 4. (a) Production of hydrogen peroxide in TMB-agar plates by the one hundred and two isolated LAB according to the milk fraction where the strains were isolated. (b) Production of inhibitory substances of the 42 pre-selected strains isolated from quarters with different health status. (c) Production of antagonistic metabolites by the 42 pre-selected LAB, discriminated by the fraction where they were isolated. (1) Strains isolated from foremilk samples. (2) Strains isolated from stripping milk samples. HP: High producers. LP: Low producers. MP: moderate producers. NP: no producers. H: healthy quarters. U: unhealthy quarters with subclinical mastitis. a: organic acids production. ah: organic acids and hydrogen peroxide production. bh: bacteriocin and hydrogen peroxide production. h: hydrogen peroxide production.

Some strains were able to produce only lactic acid, at levels between 1.8 and 10.0 g/l, while other strains produced lactic and acetic acid, which allows one to determine the heterofermentative nature of these strains.

3.6. Genetic identification of pre-selected strains

Most of the pre-selected strains that were identified by 16S gene sequencing corresponded to *Streptococcus bovis* (38.1%), *Weissella paramesenteroides* (14.3%) and *Lactococcus lactis* subsp *lactis* (9.5%). The other isolated species were *Enterococcus hirae*, *E. mundtii*, *E. saccharominimus*, *Lactobacillus brevis*, *E. faecium*, *E. saccharolyticus*, *L. mucosae*, *L. plantarum*, and *L. reuteri*. Three strains were taken out from the list of pre-selected microorganisms after they were identified as *S. agalactiae* and *Lactococcus garvieae*.

3.7. Identification of bacteriocin coding genes

Strains that produced bacteriocin-like inhibitory substances were analyzed by PCR using primers designed to amplify lantibiotic biosynthesis genes involved in the

Table 3
Percentage of the 102 LAB strains isolated from milk samples producing inhibitory substances against pathogens.

Pathogens	% LAB strains showing inhibitory properties	Type of inhibitory substance
S. aureus	1.0	Bacteriocin
S. dysgalactiae	8.8	Organic acid
L. monocytogenes Scott A	2.9	Bacteriocin/organic acid
S. uberis	7.8	Bacteriocin/organic acid
Coagulase-negative *Staphylococcus*	1.0	Bacteriocin
E. coli	3.9	Organic acid
S. agalactiae ATCC 27956	2.9	Bacteriocin/organic acid

postranslational modification of the bacteriocin (*lan*B and *lan*C genes). Positive amplification was observed with the DNA from *L. lactis* subsp *lactis* CRL 1655. A second amplification with degenerated nisA genes and the sequencing of the amplicons indicated that *L. lactis* CRL 1655 possessed lantiobiotic biosynthesis genes, and that the bacteriocin structural gene was associated with nisin Z, a variant of nisin A.

On the other hand, *E. faecium* CRL 1657 did not show amplification with Lan primers, but a 126 bp amplification fragment was observed with EntAFwd-EntARev primers. A similar band was observed with the DNA from strain ATCC 19434 which produced Enterocin A. Sequencing of this amplicon confirmed that strain CRL 1657 produced an antimicrobial peptide almost identical to enterocin A.

DNA from *E. mundtii* CRL 1656 did not exhibit amplification with either Lan or EntA primers.

3.8. Criteria for selection

We adopted the next criteria to select potentially probiotic LAB: strains not reported as pathogens of mastitis, health status of the quarters where the isolation was performed, inhibition of at least one of the tested pathogens, high hydrophobicity and autoaggregation phenotype. The selected strains presented at least three of the mentioned properties. The five LAB selected for further studies are summarized in Table 5. They will be used for further studies.

4. Discussion

Mastitis is an important health problem that affects the dairy farm and the dairy industry. This is one of the most persistent and expensive diseases in dairy cows which is usually treated or prevented with intramammary antibiotic formulations (Calvinho et al., 2002; Gentilini et al., 2002). Although the application of these therapies to control mastitis has been very effective, they present some disadvantages including the appearance of residues in the milk of treated cows, and also the transmission of antibiotic resistance to both members of the indigenous microbiota or potential pathogens (Calvinho et al., 2002; Gentilini et al., 2002).

At the same time, probiotic products for animal use, specially directed to the gastrointestinal tract, have been developed during the last years, (Alexopoulus et al., 2004). One of the parameters to take into account in designing a probiotic is the origin of strains (Saarela et al., 2000). Strains for bovine use should preferably be of bovine origin. In this sense, there has been some evidence of the host-specificity of some members of the indigenous microbiota to colonize specific hosts (Zoetendal et al., 2006). The isolation and identification of microorganisms of the mammary gland of adult cows has been performed by our group, in an area with no references of the design of a species-specific probiotic product. In this work we presented the isolation of one hundred and two indigenous LAB from milk and teat canal samples, the screening of the beneficial properties and the selection of potentially probiotic strains. The isolation was performed in the same ecological niche where the product will be

Table 4
Antibacterial spectra from the supernatants of the three bacteriocinogenic strains.

Indicator strain	Lactic acid bacteria		
	Lactococcus lactis subsp. *lactis* CRL 1655	*Enterococcus mundtii* CRL 1656	*Enterococcus faecium* CRL 1657
Staphylococcus aureus ATCC 29740	+	–	–
Streptococcus dysgalactiae ATCC 27957	–	–	–
Listeria monocytogenes Scott A	+	+	+
Streptococcus uberis	+	–	–
Staphylococcus coagulasa negativa	+	–	–
Staphylococcus aureus	+	–	–
Streptococcus dysgalactiae	–	–	–
Escherichia coli 3952	–	–	–
Escherichia coli 3511	–	–	–
Streptococcus agalactiae ATCC 27956	+	–	–
L. innocua 7	+	+	+
Enterococcus faecium ATCC19434	+	+	+
Lactobacillus plantarum CRL 691	+	+	+
Enterococcus faecalis CRL 341	+	+	+

potentially applied. Other authors reported the effect of BAL in the treatment of mastitis but they tested microorganisms isolated from another origin (Greene et al., 1991) or substances produced by LAB such as bacteriocins (Crispie et al., 2005; Cao et al., 2007). The objective of the isolation of this specific niche is supported by the host specificity and ecological niche specificity showed by different research groups (Ocaña et al., 1999; Otero et al., 2006; Zoetendal et al., 2006). In this way, our main interest was directed to the isolation of microorganisms with some probiotic potential, with the possibility to be included into the GRAS (Generally Regarded as Safe) group to be used in animals without any type of risk. The present work also includes a study about the indigenous microbial populations of the udder and teat canal with different health status. Enterobacteria were only found in samples taken from unhealthy quarters. Catalase (+) Gram (+) cocci, constituted the predominant microbiota of the mammary gland of the healthy quarters. The number of total mesophilic microorganisms, lactic acid bacteria and staphylococci was higher in foremilk than in stripping milk because environmental microorganisms enter the teat canal and can be transferred into the foremilk (Mantere-Alhonen, 1995). Some reports (Woodward et al., 1987; Gill et al., 2006) were published on the identification of microorganisms from the teat canal, either in culture media or by 16S RNA gene sequencing from a scrapping, but this is the first study to identify and characterize LAB isolated from milk before and after milking.

To evaluate different beneficial properties of bacteria that could potentially be used as probiotics, we applied microbial adhesion to hydrophobic solvents tests (hexadecane) (Otero et al., 2004) and the bacterial autoaggregation phenotype (Ocaña and Nader-Macías, 2002). They were used to predict the possibilities of a higher adhesion and formation of a protective biofilm at the mucosa, once administered to the host. Most of the LAB strains showed low degrees of hydrophobicity and low capabilities of autoaggregation. However, most of the LAB with middle and high degrees of hydrophobicity were found in stripping milk samples. These samples showed a higher fat content, and one possible explanation could be that the fat globules could provoke the adhesion of those microorganisms with some degree of hydrophobicity on their surface (Ly et al., 2006). Strains with average degrees of autoaggreagation were also found in stripping milk. One possible explanation is that the strains with autoaggregative capability adhered to the external surface of the epithelia, and then were released at the end of the milking. Cesena et al. (2001) reported the differences in the colonization of an autoggregating *Lactobacillus crispatus* and its nonaggregating mutant. There was no correlation between the two surface properties studied (Pearson correlation: 0.215). Some previous results by our group found bovine vaginal BAL strains with low degrees of hydrophobicity and even hydrophilic autoaggregative strains that possess glycoproteins or glycosylated peptides on their surface (Otero et al., 2006). These are probably both characteristics for strains isolated from cows, because very high hydrophobic strains were detected when screening bacteria isolated from the human vagina (Ocaña et al., 1999; Ocaña and Nader-Macías, 2002).

It is known that LAB are able to inhibit pathogenic microorganisms by organic acids, hydrogen peroxide and bacteriocin production (Juárez Tomás et al., 2004). We evaluated the inhibition against relevant autochthonous udder pathogens and other collection strains and also peroxidehydrogen production by using a simple test. Most of the isolated LAB from unhealthy quarters produced some type of organic acid that was able to inhibit the tested pathogen, while most of the LAB isolated from healthy cows produced hydrogen peroxide. In some way, these results are similar to those obtained from the healthy vaginal tract of adult women, where there are increased evidences that the LAB present in this ecosystem are those that produce hydrogen peroxide that may provide a protective effect against establishment of infection (Patterson et al., 2008).

The pre-selected LAB were mainly able to inhibit growth of the mastitis causing pathogens *S. dysgalactiae*, *S. uberis* and *E. coli*. *L. monocytogenes* is another pathogen inhibited by a smaller number of LAB strains. This microorganism is a rare casual agent of mastitis, but it is significant due to the severity of the clinical symptoms and mortality associated to the infection (Mantere-Alhonen, 1995; Ruegg, 2003). In Argentina the most prevalent microorganisms in subclinical mastitis are *S. aureus* and *Streptococcus* spp, while in clinical mastitis they are *S. aureus*, *S. agalactiae*, CNS, *S. dysgalactiae, S. uberis* and coliforms (Calvinho and Tirante, 2005). We found microorganisms capable of inhibiting growth of the most prevalent mastitis agents of Argentina.

The development of non-antibiotic formulations for the prevention of mastitis in cows has the potential to reduce the dependence on antibiotics for prophylactic therapies in the future. An alternative to antibiotic therapies is the use of bacteriocin such as nisin and lacticin, both produced by *L. lactis*, that were tested as active ingredients of teat dips for prevention of mastitis (Crispie et al., 2005; Cao et al., 2007). We found a nisin producer and two another bacteriocinogenic strains active against *L. monocytogenes* that will be used for future studies to design probiotic products. Further studies are being performed used for their characterization. This is a potential area for technological applications, because some studies indicate that the use of broad spectrum bacteriocins produced by LAB may provide valuable alternatives to antibiotics for the prevention or treatment of BM.

In this work we selected strains taking into account general guidelines (Saarela et al., 2000; ISAPP) and some beneficial properties. We discarded strains by tracking isolated from unhealthy quarters or previously described as mastitis etiologic agents: two *S. agalactiae* strains (Calvinho and Tirante, 2005), and *L. garvieae* (Collins et al., 1983). We finally chose five LAB strains for further *in vitro* (safety, functional and technological aspects) and *in vivo* studies.

Table 5
Individual properties of the strains with some beneficial properties isolated from mammary gland of cows, and pre-selected by these properties.

Strain †	Surface Properties				Inhibitory Substances production					Origin of Samples		
	Hydrophobicity[a]		Autoaggregation[b]		H_2O_2 production[c]	Lactic Acid [g/l][d]	Acetic Acid [g/ml][d]	Inhibited pathogens[f]	Inhibitory substance[f]	Origin	Fraction	Health status
	(%)	Group	(%)	Group								
Streptococcus bovis CRL 1710	13,18	L	14,29	L	lp	5,1643	0,2713	-	-	Milk	I	h
Lactobacillus brevis CRL 1711	37,31	M	6,25	L	lp	1,3778	0,5090	-	-	Milk	I	h
Enterococcus hirae CRL 1712	1,73	L	0,00	L	np	8,1950	0,3362	7, 9	A	Milk	II	h
Enterococcus mundtii CRL 1713	0,00	L	8,33	L	np	6,7051	0,3085	9	A	Milk	II	h
Streptococcus bovis CRL 1715	1,67	L	4,94	L	lp	6,3804	0,3329	3, 7, 9	A	Milk	II	h
Lactobacillus plantarum CRL 1716	96,87	H	51,95	M	np	5,6703	0,6135	4	A	Milk	II	h
Streptococcus bovis CRL 1717	1,61	L	3,90	L	lp	5,7038	0,3443	-	-	Milk	I	h
Streptococcus bovis CRL 1718	3,13	L	3,16	L	hp	6,0553	0,3079	-	-	Milk	I	h
Weissella paramesenteroides CRL 1719	3,33	L	1,37	L	hp	3,3885	0,5123	-	-	Milk	I	h
Enterococcus saccharominimus CRL 1720	1,32	L	4,41	L	np	4,5209	0,3799	7	A	Milk	II	h
Weissella paramesenteroides CRL 1721	1,56	L	5,88	L	np	1,7570	0,5175	2, 7, 8, 9	A	Milk	II	h
Enterococcus saccharominimus CRL 1722	1,56	L	8,75	L	lp	5,6501	0,2688	2	A	Milk	I	h
Weissella paramesenteroides CRL 1723	1,56	L	1,41	L	p	2,9394	0,4245	-	-	Milk	II	h
Lactobacillus perolens CRL 1724	75,09	H	5,55	L	np	10,0208	0,5694	2, 4	A	Milk	I	h
Streptococcus bovis CRL 1725	1,67	L	2,60	L	lp	7,3773	0,3690	7	A	Milk	I	h
Lactococcus lactis subsp *lactis CRL 1655*	13,51	L	14,37	L	p	6,0571	0,5122	1, 4, 5, 6, 10	B	Milk	I	h
Streptococcus bovis CRL 1726	2,30	L	1,23	L	hp	6,4095	0,3777	-	-	Milk	I	h
Lactobacillus mucosae CRL1727	1,67	L	10,00	L	p	4,0452	0,5301	-	-	Milk	I	h
Streptococcus bovis CRL 1728	1,67	L	0,00	L	hp	6,6549	0,3745	-	-	Milk	I	h
Streptococcus bovis CRL 1729	3,45	L	3,52	L	lp	5,7617	0,3524	2, 4, 7	A	Milk	I	h
Streptococcus bovis CRL 1730	2,59	L	5,00	L	lp	6,7978	0,3664	-	-	Milk	I	h
Streptococcus bovis CRL 1731	5,00	L	6,10	L	lp	5,7553	0,4633	-	-	Milk	I	h
Enterococcus hirae CRL 1732	1,67	L	7,87	L	nl	7,0150	0,3218	2, 7	A	Milk	I	h

Weissella paramesenteroides CRL 1733	0,00	L	7,69	L	hp	1,8689	0,3939	-	-	Milk	I	h
Lactobacillus reuteri CRL 1734	3,39	L	9,29	L	lp	5,3861	0,5602	-	-	Scraped	I	u
Enterococcus saccharolyticus CRL 1735	0,00	L	18,50	L	np	2,9332	0,4245	2	A	Scraped	I	u
Lactococcus lactis subsp *lactis* CRL 1736	1,67	L	4,22	L	np	6,5264	0,5067	2, 4, 7	A	Scraped	I	u
Streptococcus bovis CRL 1737	3,51	L	20,00	L	np	7,9032	0,3569	2, 7	A	Milk	I	u
Streptococcus bovis CRL 1738	1,73	L	5,19	L	np	6,9572	0,2818	2, 4	A	Milk	I	u
Streptococcus bovis CRL 1739	4,70	L	3,50	L	p	4,0982	0,2730	10	A	Milk	I	u
Weissella paramesenteroides CRL 1740	3,20	L	5,36	L	lp	2,7306	0,5144	-	-	Scraped	I	u
Weissella paramesenteroides CRL 1741	4,86	L	6,29	L	hp	3,0242	0,5080	-	-	Scraped	I	h
Streptococcus bovis CRL 1742	0,00	L	21,67	L	np	7,3822	0,3467	4, 10	A	Milk	II	h
Streptococcus bovis CRL 1743	12,81	L	0,00	L	np	5,9594	0,4677	4	A	Milk	II	h
Lactococcus lactis subsp *lactis* CRL 1744	0,00	L	5,54	L	lp	7,1763	0,3917	-	-	Milk	II	h
Enterococcus mundtii CRL1656	0,00	L	11,11	L	p	6,4923	0,4824	3	B	Milk	II	h
Enterococcus faecium CRL1657	4,29	L	7,80	L	lp	5,6917	0,4235	3	B	Scraped	I	h
Streptococcus bovis CRL 1745	13,19	L	6,25	L	hp	5,6432	0,4935	-	-	Scraped	I	h
Lactococcus lactis subsp *lactis* CRL 1746	8,06	L	8,06	L	lp	5,1326	0,4144	-	-	Milk	II	h

L: low. M: middle. H: high. np: no producer. lp: low producer. p: producer. hp: high producer. A: organic acid. B: bacteriocin. h: healthy. u: unhealthy. I: foremilk sample. II: Stripping milk sample. 1: *Staphylococcus aureus* ATCC 29740. 2: *Streptococcus dysgalactiae* ATCC 27957. 3: *Listeria monocytogenes* Scott A. 4: *S. uberis*. 5: Coagulase-negative *Staphylococcus*.6: *S. aureus*. 7: *S. dysgalactiae*. 8: *Escherichia coli. 9: Escherichia coli. 10: S. agalactiae* ATCC 27956.

[a]All the experiments were performed with the third culture of the analyzed strain. The five LAB selected for further studies are grey coloured.

[b]Determined by MATH in hexadecane (Otero et al., 2004).

[c]Autoagregative capability (Ocaña and Nader-Macías, 2002).

[d]Production of H_2O_2 in TMB-plates (Juárez Tomás et al., 2004).

[e]Determined by using HPLC technique.

[f]Determined by using a pH meter.

[g]Analyzed by using the plate diffusion technique (Juárez Tomás et al., 2004).

5. Conclusion

The results obtained from this work will serve as the basis for further study. The strains with probiotic potentiality and bacteriocin-producer strains could be administered to cows as a species-specific probiotic product.

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