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Advances in the Design of a Multi-Strain Homologous Probiotic Formula for Cattle

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Abstract

Microorganisms colonizing different mucosas can exert diverse type of beneficial effects if they are included in probiotic formula. Then, the objective of this work was to complete the set of probiotic-related characteristics of different lactic acid bacteria strains previously isolated from different bovine mucosas to further include them in a probiotic product. The studies include some complementary assays related with their surface-adhesive characteristics (autoaggregation, biofilm formation, hydrophobicity, production of exopolisaccharides), production of inhibitory substances against pathogenic bacteria (hydrogen peroxide, bacteriocins). Also, the strains compatibility to go further in the design of a multi'strain probiotic formula to be used either to pregnant cows through the vaginal tract (for metritis prevention), later to the newborn calves (for diarrhea prevention), and to the mammary gland of the cows (for mastitis prevention). The formula will be integrated by strains from the three bovine tracts, being host' specific and able to promote the mucosal colonization and the beneficial effect in different ages and physiological states of bovines.

Introduction

The microbiome of different human or animal tracts is constituted by a wide set of microorganisms (bacteria, viruses, archaea or lower eukaryotes) in ecological balance that inhabit a specific environment, such as the gastrointestinal, reproductive, respiratory tracts, mucous surfaces or other [1,2]. With the emergence of sequences based molecular methods that allowed the analysis of complex microbial communities [3], our understanding of the key functions that the microbiome plays in the illness, health, growth and development of the specific host has increased extensively [4]. In such a way that the microbioma is being currently considered as an organ, since it provides metabolic activities, coding capabilities and fulfills a wide variety of physiological functions that include immunomodulation and prevention of infections in humans and animals, beside others [5,6]. Factors such as birth mode, environmental conditions and\or genetic polymorphisms, or host-specificity determine the bacterial genera presence or its dominance in the microbioma, being modified along the time and in many situations, indicating also the host-specific dependence [5,7-11]. Eventhough, different researches have shown remarkable similarities in the dominant bacterial species in the tracts and mucous membranes of different hosts [12-14].

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Referred to the microbiome of bovine tracts, it can be affected by endogenous or exogenous factors, which allow the entry and proliferation of pathogenic or potentially pathogenic microorganisms that generate infections. Traditionally, antibiotics and antimicrobials are the therapies currently applied; however, the unintended consequences of their administration as residues in meat and milk, and spread of bacterial resistance with the risk of vertical transmission to other members of the microbiota, have forced to search alternative and innovative strategies to reduce the massive use of antibiotics against infectious diseases [15,16,24]. Then, the application of natural products demonstrating positive effects was proposed, including probiotics, prebiotics, enzymes, natural extracts and organic acids. In recent years, probiotics for small ruminants have been authorized in the European Union for the first time, both for young animals (lambs and kids), adults (sheep and dairy goats), and milk buffaloes, since previously only probiotics for cattle were authorized [17]. Probiotic microorganisms are defined as "viable microorganisms that are administered to the host in adequate quantities to produce a beneficial physiological effect" [18-20]. Most of the bacteria used as probiotic in animals are lactic acid bacteria (LAB) sustained by their GRAS status (generally recognized as safe), being *Lactobacillus* one of the most frequently used supported by the EFSA qualification of this genus as QPS (Qualified Presumption of Safety). Referred to their use in cattle, different scientists have shown that the application of an adequate combination of probiotics could effectively counteract an endometrial infection by *E. coli* [21-23]. Pellegrino et al. [16] showed that the intramammary administration of LAB during the drying period in dairy cows stimulates locally and systemically the immune system, and could be used as an alternative therapy for the treatment and/or prevention of mastitis. Maldonado et al [25] demonstrated that oral administration of a *Lactobacillus* mixture decreased the incidence of diarrhea in calves, because those of infectious origin are the main causes of mortality in calves during the first weeks of life.

It must be pointed out that the protective effect of LAB can be exerted by different mechanisms, including suface-adhesion related properties (hydrophobicity, self and co-aggregation), production of Exopolysaccharides (EPS), biofilm formation, or the capability to produce antagonistic substances (organic acids as lactic or acetic, hydrogen peroxide or bacteriocins) to protect against pathogens [26]. The enzymes produced by LAB in food additives for ruminants are of main interest to catalyze degradative reactions during digestion, either components of the vegetal cell wall they receive (cellulases, xylanases, E-glucanases, pectinases, etc.), as well as its content (amylases, proteases). The efficient digestion of complex substrates in the rumen requires the combined action of many enzymes, subject of main interest when selecting LAB that express some type of hydrolytic activity to favor the degradation of the food consumed by animals. Some other mechanisms proposed are the competition for nutrients or the inmunemodulation.

Our research group have isolated, quantified, identified by pheno and genotypic methods different groups of cultivable microorganisms from cattle tracts, with special interest in lactic bacteria, which are predominant in some of these mucosas and historically used as probiotics [27-29,45]. They were administered to animals, at the same tract of isolation supported by the host and mucose specificity of the lately described microbioma [16,24,25,28-33]. Some of the beneficial properties were evaluated at that moment by applying the techniques available at the time of isolation.

As we are interested in go further in the design of a probiotic formula homologous to the host, to be applied to different bovine tracts, the objective of this work is to complement the evaluation of the beneficial properties of the strains previously selected from different cattle mucose, and the compatibility between them, in such a way to have available scientific criteria for the next formulation step.

Materials and methods

Microorganisms and culture conditions

The microorganisms used in this study were selected previously by our research group and are shown in Table 1. Strains of lactic acid bacteria (LAB) were isolated from different bovine ecosystems; vagina and mammary gland of adult cows, and oral cavity and gastrointestinal tract of calves, and the first set of beneficial characteristics were previously determined [26,28,32].

Table 1: Origin of isolation of selected beneficial LAB strains evaluated

Microorganism		Origin
Lb. gasseri	**CRL 1461**	Bovine vagina*
Lb. delbrueckii	**CRL 1460**	Bovine vagina*
Lb. gasseri	**CRL 1412**	Bovine vagina*
Lb. gasseri	**CRL 1421**	Bovine vagina*
Lb. fermentum	**CRL 1580**	Bovine vagina
Lb. fermentum	**CRL 1577**	Bovine vagina
Lb. fermentum	**CRL 1576**	Bovine vagina
Lb. fermentum	**CRL 1575**	Bovine vagina
Lb. fermentum	**CRL 1574**	Bovine vagina
Lb. fermentum	**CRL 1573**	Bovine vagina
Lb. fermentum	**CRL 1571**	Bovine vagina
Lb. fermentum	**CRL 1570**	Bovine vagina
Lb. fermentum	**CRL 1568**	Bovine vagina
Lb. fermentum	**CRL 1567**	Bovine vagina
Lb. fermentum	**CRL 1566**	Bovine vagina
Lb. gasseri	**CRL 1556**	Bovine vagina
Lb. fermentum	**CRL 1557**	Bovine vagina
Lb. fermentum	**CRL 1558**	Bovine vagina
Lb. fermentum	**CRL 1559**	Bovine vagina
Lb. fermentum	**CRL 1560**	Bovine vagina
Lb. fermentum	**CRL 1561**	Bovine vagina
Lb. fermentum	**CRL 1562**	Bovine vagina
Lb. fermentum	**CRL 1563**	Bovine vagina
Lb. fermentum	**CRL 1564**	Bovine vagina
Lb. fermentum	**CRL 1565**	Bovine vagina

Lb. plantarum	CRL 1716	Bovine mammary gland$^{\gamma}$
Lb. perolens	CRL 1724	Bovine mammary gland$^{\gamma}$
Lc. lactis subsp lactis	CRL 1655	Bovine mammary gland$^{\gamma}$
P. pentosaceus	CRL 1831	Bovine mammary gland$^{\gamma}$
W. cibaria	CRL 1833	Bovine mammary gland$^{\gamma}$
E. hirae	CRL 1835	Bovine mammary gland$^{\gamma}$
E. hirae	CRL 1842	Bovine mammary gland$^{\gamma}$
E. mundtii	CRL 1656	Bovine mammary gland$^{\gamma}$
E. faecium	CRL 1657	Bovine mammary gland$^{\gamma}$
Lb. johnsonii	CRL 1693	Faecal matter calves^{+}
Lb. murinus	CRL 1695	Faecal matter calves^{+}
Lb. mucosae	CRL 1696	Faecal matter calves^{+}
Lb. amylovorus	CRL 1697	Faecal matter calves
Lb. salivarius	CRL 1702	Faecal matter calves^{+}
E. faecium	CRL 1703	Oral cavity calves

CRL: Centro de Referencia para Lactobacilos Culture Collection.
*Probiotic properties published by Otero et al (2006).
γ Probiotic properties published by Espeche et al (2012).
+Probiotic properties published by Maldonado et al (2012).

The microorganisms were stored in milk-yeast extract (13% non-fast milk, 1% yeast extract) (Britania, Argentine) at -20 °C, inoculated at 2% in MRS broth (de Man et al., 1960) (Biokar, France) at 37 °C for 24 h, and subcultured twice every 12 h. The third subculture was washed with sterile saline (0.85% Na Cl), centrifuged (7.000 rpm, 5 min, Presvac, Argentine) and the supernatant discarded.

Bacterial surface properties

Degree of hydrophobicity and auto-aggregation test

Surface hydrophobicity was evaluated and quantified by determining the variation of optical density at 600 nm (OD_{600} nm) of cell suspensions in physiological solution after partition with organic solvents [34] adding xylene and toluene, as modified by Ocaña et al [35-37]. The pellet was washed twice with saline (0.85% NaCl), centrifuged and resuspended to an $OD_{600\ nm}$ = 0.60 ± 0.06 (Spectronic 20, Bausch and Lomb, Rochester, New York, USA). The exact value of initial $OD_{600\ nm}$ (OD0) was recorded. 0.6 ml of hydrophobic solvent (xylene and toluene) (Cicarrelli, Argentine) was added to the tubes with 3.6 ml of bacterial suspension (solvent-bacterial suspension ratio: 1/6). They were vortexed for 1 minute and the final OD_{600nm} (OD_f) determined again after 15 min agitation. The values were recorded as OD_f.

The percentage of hydrophobicity was calculated applying the following formula [38]: Eq.

(A) % Hydrophobicity: $[(OD0 - OD_f) / OD_0] \times 100$ (1)

The percentage of hydrophobicity of the strains was classified as low (0-35%), medium (36% -70%) and high (71-100%).

The autoaggregation was determined applying the technique described by Vandevoorde et al. [38] and modified by Ocaña and Nader Macías [39]. The microorganisms were centrifuged and washed three times in saline and the $OD_{600\ nm}$ = 0.60 ± 0.05 adjusted (Spectronic 20, Bausch and Lomb, Rochester, New York, USA). The suspensions were allowed to settle determining the OD_{600nm} at different periods of time (1, 2, 3, 4 and 24 h). The percentage of autoaggregation was calculated applying an expression similar to that used to determine the percentage of hydrophobicity. The strains autoaggregation was classified as low (0-35%), medium (36% -70%) and high (71-100%).

Production of biofilm

To evaluate the ability of the strains to form biofilms, the technique of quantification of biofilm in polystyrene microplates [40-42] modified in the Laboratory was applied [43]. The culture media assayed were: A) standard MRS and LAPTg broths, and B) MRS and LAPTg broths with individual omission of Tween 80. Lactic bacteria were subcultured in MRS and LAPTg broths as described before. The cell pellets were resuspended in 5 ml of the different culture media, adjusted to 1.5 (OD_{540nm}) corresponding to 2–2,5 x 108 colony forming units per milliliter (CFU/ml), and distributed in 200 µl aliquots in polystyrene microplates wells (bottom U). They were incubated at 37 °C under static conditions for 72 h. The quantification of biofilm formation was performed after discarding the culture medium and washing the wells with 200 µl of phosphate buffered saline (PBS: NaCl 8 g/l; KCl 0.0002 g/l; Na_2HPO_4 1.15 g/l; KH_2PO_4 0.2 g/l; pH 7.4). The formed biofilms in the wells were stained with 0.1% (w/v) Crystal Violet for 30 min at room temperature. Each well was washed with 200 µl sterile distilled water to remove excess dye. The microplates were left inverted for 14-16 h at room temperature. The stained biofilm was eluted with 200 µl of 30% glacial acetic acid and incubated 15 min at room temperature, transfering aliquots (135 µl) to a second microplate (flat bottom) in which the OD was determined at 570 nm (OD_{570nm}) in a microplate spectrophotometer reader (VERSAmax, Molecular Devices, Sunnyvale, California, USA).

Production of exopolysaccharides (EPS)

Qualitative determination

The ability of LAB to produce exopolysaccharides was determined by applying the technique described by Van Geel-Schutten et al. [44]. Strains from different bovine origin (cow reproductive tract, bitch's milk and gastrointestinal tract of calves) were seeded by spot (5-6 drops of 10 µl) in de Man, Rogosa and Sharpe (MRS) agar media supplemented with high concentrations (100 g/l) of different sugars: glucose, fructose, maltose, raffinose, sucrose, galactose or lactose. The plates were incubated at 37 °C for 72-96 h. The EPS producing strains were evidenced by observing the appearance of the colonies, collected with a sterile spatula and the phenotype (ropy/mucous) characteristic of the EPS produced.

Quantitative determination of EPS

To determine the amount of EPS produced by LAB in the qualitative method, the technique described by Quesada et al. [45] was applied. The strains were subcultured three times in MRS broth at 37 °C for 24 h, and later three more times in enriched media (MRS glucose and MRS sucrose, sugars at 10%), at 37 °C for 10 h, and the last one at 37 °C for 72 h. The tubes were centrifuged at 15,000 rpm for 30 min at 4 °C, and the supernatant transferred to sterile bottles in which 2 volumes of 96° (v/v) cold ethanol (National Institute of Viticulture, Argentine) were added, allowed to settle for 48 h in cold room (4 °C), and centrifuged at 15,000 rpm 60 min at 4 °C. The supernatants were discarded and the pellets allowed to dry at room temperature and weighed (up to constant weight values) to calculate the amount of EPS (in milligrams) produced per gram of LAB and

liter of culture medium.

Production of inhibitory substances (organic acids, hydrogen peroxide, bacteriocins) of bovine BL against pathogens causing bovine infections Qualitative determination of H_2O_2

To determine the production of hydrogen peroxide, the qualitative agar plate method was applied, as described by Juarez Tomás et al. [46]. In this method, the peroxidase enzyme (incorporated into the agarized medium) catalyzes the oxidation of a chromogenic substrate (TMB) in presence of hydrogen peroxide. The grown colonies of lactobacilli-H_2O_2 producer strains were colored blue or brown in the presence of oxygen from the air. The reagents used were: Solution A: TMB 17.34 mM in methanol and solution B: 100 U/ml peroxidase. The final concentrations in each plate were: TMB 1mM and peroxidase 2 U/ml. Each strain of lactobacilli was seeded on the surface of the TMB-MRS plates and incubated in microaerophylic conditions at 37 °C for 48 h. At the end of the incubation, the plates were exposed to the air and coloration of the colonies recorded. The production of H2O2 was classified according to the color intensity of the colonies, such as: ++, +, + d (some coloured colonies), and - (negative).

Production of inhibitory substances. Agar diffusion method

To evaluate the inhibitory activity of lactic acid bacteria against pathogenic microorganisms, the plate diffusion technique was used according to Jack et al [47], modified by Ocaña et al [48]. The cell-free supernatants were centrifuged and filtered and added to pathogens (different concentrations) inoculated on soft agar plates. Also the chemical nature of the antagonistic substances was determined: the supernatants were neutralized with NaOH 2 mol/l (Cicarrelli, Argentine) to define the organic acid nature. They were later neutralized (pH: 6.5) and then treated with catalase 1000 U/ml (Sigma-Aldrich, St Louis, USA) to decide if they were oxygen peroxide or bacteriocins.

Referred to the pathogen strains, Triptic Soy Broth (TSB), Brain Heart Infusion (BHI) (Britania, Argentine) and Todd Hewitt plates (1% agar) were prepared by inoculating 15 µl of active pathogenic strains (10^7 - 10^9 CFU) in 15 ml of culture medium (specific for each microorganism), melted and cooled to 30 °C. Once solidified the plates, 6 mm wells were performed in which 30 µl of supernatants (pure, neutralized and neutralized-treated with catalase) of LAB under study were seeded. The plates were incubated for 3 h at room temperature and subsequently at 30 °C for Listeria *monocytogenes* and at 37 °C for the other pathogens for 24-48 h. The size of the diameters of inhibition halos was measured in mm.

Enzymatic activities of LAB isolated from the vagina, mammary gland of adult bovine females, and calves faeces.

The screening of production of different type of enzymes, some of them related with a specific beneficial characteristic was performed in all the strains. To determine amylase activity, LAB were subcultured in MRS broth. 50 µl of each bacterial suspension was added in wells of MRS-agar starch plates, incubated at 37 °C for 48 h. The positive enzymatic activity was evidenced by the addition of Lugol, due to the presence of clear zones around the wells, indicating the enzymatic degradation of the starch. Producer *Lb. amylovorus* CRL1949 strain (provided by Laboratory of Plant Technology Ecophysiology, CERELA-CONICET) was used as positive control.

To evaluate the xylanase activity, LAB suspensions (50 µl) were added in wells (6 mm) performed aseptically on MRS-xylan agar plates. They were incubated at 37 °C for 48-72 h. After that time, a Congo Red solution added on the plate was used as developer, and allowed to act for 15 min. The dye turned the xylan to red, then the positive xylanase activity was evidenced by the presence of clear zones (halos) around the well, indicating the enzymatic degradation of the xylan.

To study the production of cellulase activity in LAB, cell suspensions were seeded in wells (50 µl) performed in MRS-carboxymethyl cellulose (CMC) agar plate. The plates were incubated at 37 °C for 48-72 h without inverting. Then they were stained by adding Congo Red solution to the plate surface and allowed to act for 15 min. The dye stains the CMC red, then the positive cellulase activity was evidenced by the presence of clear zones around the well, indicating the enzymatic degradation of carboxymethylcellulose.

Compatibility between BL strains

The compatibility between the LAB strains from the three tracts under study: vaginal tract and mammary gland of adult bovine females, and gastrointestinal tract of calves, was evaluated by the agar plate diffusion method [49]. Each one of the microorganisms was used as an indicator and as a producer of inhibitory substances. Different LAB concentrations (10^7 and 10^9 CFU/ml) were inoculated into semi-soft LAPTg agar plates (1% agar) as indicators microorganism. In the assays of bacteria as producers, the treatment of supernatants (with NaOH and catalase) was applied to determine the nature of the inhibitory substances (organic acids, hydrogen peroxide or bacteriocins) as described before. The microorganisms, as producers, were activated by successive sub-cultures in MRS broth. The third subculture was centrifuged at 6000 rpm for 10 min. Each supernatant was distributed in three tubes (pure, neutralized with NaOH and neutralized-treated with catalase) and pH measured. Aliquots (25 µl) of the pure and treated supernatants were added in the wells of agar plates containing the indicator strains. The plates were incubated at room temperature for 5 h, and then 24 h at 37 °C.

Statistical analysis

Statistical analysis was performed by applying the Minitab (versión 16) software.

Results and discussion

Surface properties

The strains used in this work were selected previously by their beneficial characteristics [27,28,50], planning at that time their use in each specific homologous tract or ecological system and host. But, when planning to go further in the selection of those strains with higher or more outstanding properties to be used as a single formula, some specific techniques and complementary studies were required. In this sense, the beneficial strains used to complement the probiotical characteristics screening are included in **Table 1**, being isolated from three different bovine ecosystems.

The evaluation of the surface properties of the strains, related to their adhesion capability has shown that most of the strains from the vaginal tract were not able to autoaggregate. The strain with higher index was *Lb. gasseri* CRL1461 as shown in **Figure 1**. Similar pattern was observed in the autoaggregation values of the mammary gland strains, most of them with

low indexes, except Lb. *plantarum* CRL1716 with 51% (Table 2). Referred to the hydrophobicity capability, most of the strains from vagina were non-hydrophobic, being *Lb. gasseri* CRL1461 (60%), *Lb. delbrueckii* CRL1460 (58%), *Lb gasseri* CRL1421 (64%) and CRL1412 (72%) the strains with higher indexes.

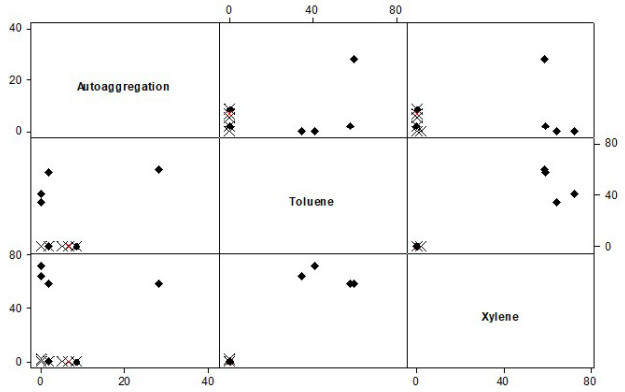


Figure 1: Surface characteristics of selected LAB isolated from vaginal tract. Self-aggregation and hydrophobicity index (in toluene and xylene). Facultative heterofermentative (●), obligate heterofermentative (X) and obligate homofermentative (♦) strains

Table 2: Surface characteristics of LAB strains selected from the bovine mammary gland

Strains		Autoaggregation (%) [a]	Hydrophobicity Hexadecane (%)[b]
Lb. plantarum	CRL 1716	51,95	96,87
Lb. perolens	CRL 1724	5,55	75,09
Lc. lactis subsp lactis	CRL 1655	14,37	13,51
P. pentosaceus	CRL 1831	10,00	0,00
W. cibaria	CRL 1833	8,41	82,91
E. hirae	CRL 1842	4,69	13,33

[a]Autoaggregative capability (Ocaña and Nader-Macias, 2002).
[b]Determined by MATH in hexadecane (Otero et al, 2004).

In the mammary gland strains, three of the strains: *Lb. plantarum* CRL1716, *Lb. perolens* CRL1724 and W. cibaria CRL1833 showed high hydrophobicity indexes. These results indicate that the two characteristics related with the adhesion capability of the strains are not directly correlated, or expressed together in one strain: in some strains only one of them is expressed, while in other strains the other. This type of behavior was also observed in feedlot isolated strains, where some strains showed to be autoagregants, while some other were hydrophobic [26]. In human host isolated strains, this differential activity was also evidenced [51].

Biofilm forming strains

The capability to form biofilm is also a characteristic more frequently and strongly related to the mucosal adhesion and permanence capabilities of the strains under evaluation [43]. From the biofilm formed strains, a different behavior was also obtained. The vaginal strains showed that some of them were able to form biofilm in most of the media assayed. *Lb. fermentum* CRL1573/1574/1575 and 1576 formed biofilm in LAPTg, MRS and MRS without tween 80. *Lb. gasseri* CRL1412/1421, *Lb. delbrueckii* CRL1460, *Lb. fermentum* CRL1556/1559/1560/1563 and 1564 strains formed biofilm only in complete MRS broth, while *Lb. fermentum* CRL1560/1561 and 1564 formed slightly lower biofilm in this media without tween added. In LAPTg with not tween, most of the strains did not form biofilm, as observed in **Figure 2a**.

Referred to the mammary gland isolated strains, *Lb. plantarum* CRL1716 showed to form biofilm only in MRS media (**Figure 2b**).

In the case of calves faeces origin (**Figure 2c**) the strains did not form biofilm in the assayed media.

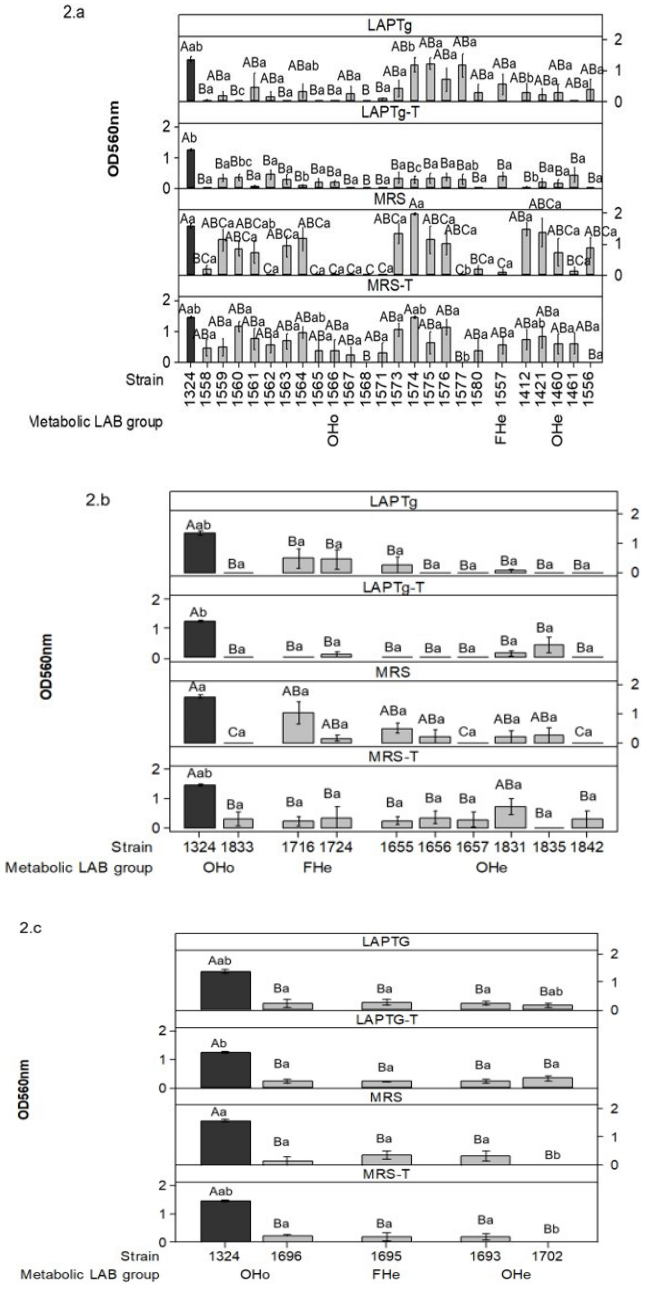


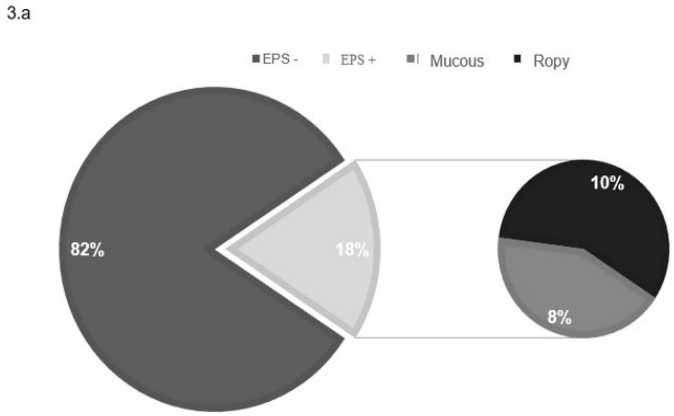
Figure 2: Formation of biofilms of lactic bacteria strains isolated from the reproductive tract (a), mammary gland (b) of cows, and gastrointestinal tract (c) of lactating calves in MRS and LAPTg broths with (0.1%) or without Tween 80. The data are expressed as the mean values of OD_{560nm} ± standard error of the biofilms formed in microplates stained with crystal violet and solubilized with ethanol. The rows with different capital letters indicate significant differences ($p < 0.05$) in the formation of biofilms of the different strains in the same culture medium. The columns with different lowercase letters indicate significant differences ($p < 0.05$) in the formation of biofilms of the same strains in different culture media according to the Tukey test. Methods are described in the text

These results indicate that the capability to form biofilm in different culture media is again a characteristic of each strain, and must be assayed individually in each specific case. These results are similar to the ones described by [43], showing different behavior of the strains isolated from human vagina in different culture media.

Exopolysaccharides production

The production of exopolysaccharides is highly related to the formation of a mucous coverage in a way to promote the approach of the strains to the animal cells or mucosal surfaces. The importance of EPS production in specific strains is also being studied more recently, due to the close relationship between the EPS production and the immune stimulation. Chabot et al. [52] have shown that exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN- in mouse splenocytes, acting as adjuvants or stimulators of the immune system. These immunomodulatory polysaccharides exert their action through cell membrane receptors whose nature, density and cellular distribution contribute to the response. EPS are well known as mitogens for a wide variety of cells [53].

From the 17 strains evaluated, only 6 (18%) were able to produce EPS **(Figure 3a)**, being 2 of them mucous-EPS, and 4 ropy-EPS. The quantification of EPS of the 6 producer strains in sugar-added media have shown that the amount of EPS is different, and depends on the sugar used as main source of production. *Lb. gasseri* CRL1412 and *Lb. fermentum* CRL1576 were the strains showing higher EPS production on MRS glucose **(Figure 3b up)**. When sucrose was the carbon source, *Lb. fermentum* CRL1580/1576 and *W. cibaria* CRL1833 were the higher EPS producer strains, indicating that this characteristic is also related to each specific strain **(Figure 3b low)**. Previous studies indicated that the carbon source added to the culture media plays an important role in the phenotype and total amount of polysaccharide produced. The use of single sugar does not promote a higher EPS production in all the strains, because it depends on the metabolic capability of the strain under assay. Cerning et al. [54] showed that EPS production by *Lb. casei* CG11 in basal medium containing glucose was higher than in the same medium with lactose or galactose. *Lb. casei* CRL87 was able to produce 1.7 times more EPS in galactose than in glucose, according to Mozzi et al. [55].



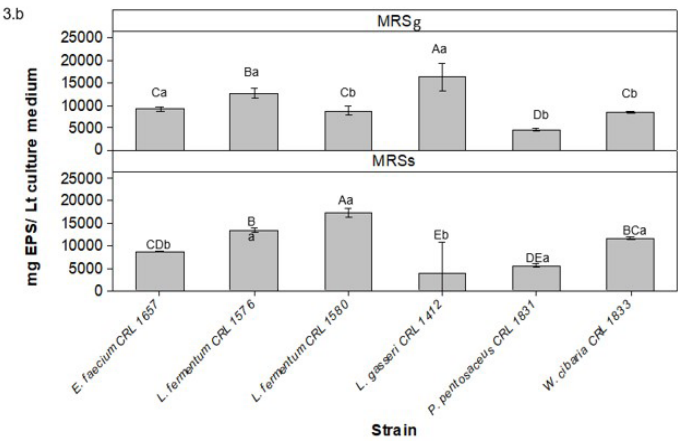


Figure 3: Production of exopolysaccharides (EPS) in lactic acid bacteria strains isolated from different bovine ecosystems. (a) EPS classification according to phenotype, and (b) quantitative determination in MRS broth (10% glucose added) and MRSs (10% sucrose added). The rows with different capital letters indicate significant differences ($p < 0.05$) in EPS production of different strains in the same culture medium tested. The columns with different lowercase letters indicate significant differences ($p < 0.05$) in the EPS production of the same strain in two culture media tested according to the Tukey test

Inhibitory substances production

The assays performed to determine the production of inhibitory substances have shown different degree of H2O2 production in agar media, according to the oxidized colour of the colonies, indicating that all the strains were able to produce H_2O_2. *Lb. gasseri* CRL1412, *Lb. fermentum* CRL1575, *P. pentosaceus* CRL1831, *Lb. johnsonii* CRL 1693 and *Lb. mucosae* CRL1696 strains were the ones showing higher production levels (**Table 3**). When looking at the other inhibitory substances produced from strains isolated from the three ecosystems, the results indicated in **Figure 4** were obtained. The strains isolated from vagina only were able to inhibit *Listeria monocytogenes* (shown as a photo example in **Figure 5**), while those isolated from the mammary gland and calves faeces have shown a wider spectrum of pathogen inhibition. The quantitative determination of the size of haloes is indicated in Tables 4a and 4b. The calves' strains showed to inhibit *L. monocytogenes, E. faecium, S. dysgalactiae, S. uberis, S. aureus, S. dublin, S. Tiphymurium, E. coli* and *S. infantis*. The mammary gland strains inhibited *L. monocytogenes, E. faecium, S. dysgalactiae, S. agalactiae, Staphylococcus* coagulase-negative and *S. bovis*. The inhibitory potential of the LAB strains under evaluation indicates that each strain shows a specific inhibition pattern that must be assayed when studying each particular strain. The ability of various LABs to inhibit the growth of pathogenic microorganisms has been documented, as well as the antimicrobial activities of cell-free supernatants of LAB isolates of different origins. Bacteriocins are a group of potent antimicrobial peptides produced by some microorganisms, including LAB, active against closely related organisms, mainly gram-positive bacteria to obtain a competitive advantage for nutrients in the environment [56]. The LAB strains that produce bacteriocins are protected from their own toxins by the expression of a specific immune protein, encoded in the bacteriocin operon [57]. LABs isolated from bovine ecosystems show various antimicrobial activities, through the production of different metabolites, including lactic acid, hydrogen peroxide and bacteriocins.

Table 3: Qualitative determination of hydrogen peroxide production by lactic bacteria[a]

Strains	Hydrogen peroxide[b]
Lb. gasseri CRL 1412	+++
Lb. gasseri CRL 1421	+
Lb. gasseri CRL 1460	++
Lb. delbrueckki CRL 1461	+
Lb. fermentum CRL 1574	++
Lb. fermentum CRL 1575	+++
Lb. gasseri CRL 1566	++
Lc. lactis subsp lactis CRL 1655	++
Lb. plantarum CRL 1716	+
Lb. perolens CRL 1724	+
P. pentosaceus CRL1831	+++
W. cibaria CRL 1833	++
E. hirae CRL 1842	+
Lb. johnsonii CRL1693	+++
Lb. murinus CRL 1695	++
Lb. mucosae CRL 1696	+++
Lb. salivarius CRL 1702	++

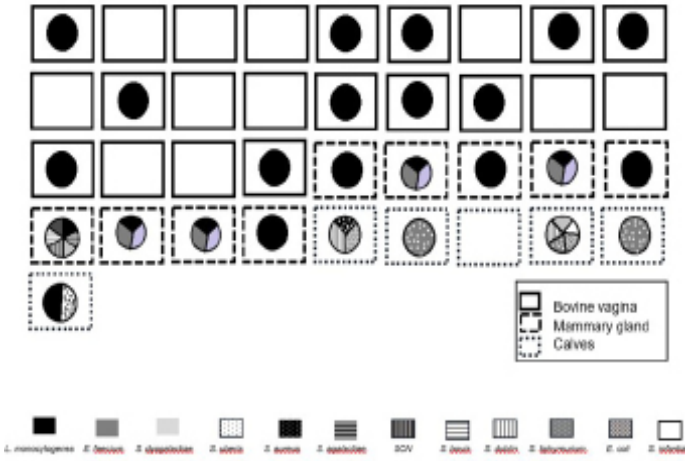


Figure 4: Production of inhibitory substances by lactic bacteria isolated from vagina and mammary gland of adult bovine females and feces of lactating calves. The pathogens tested were: *L. monocytogenes*, *E. faecium*, *S. dysgalactiae*, *S. uberis*, *S. aureus*, *S. agalactiae*, SCN, *S. bovis*, *S. dublin*, *S. tiphymurium*, *E. coli* and *S. infantis*. The quadrants in different trace correspond to the tracts from which the lactic bacteria were isolated. Each rectangle represents one of the selected strain

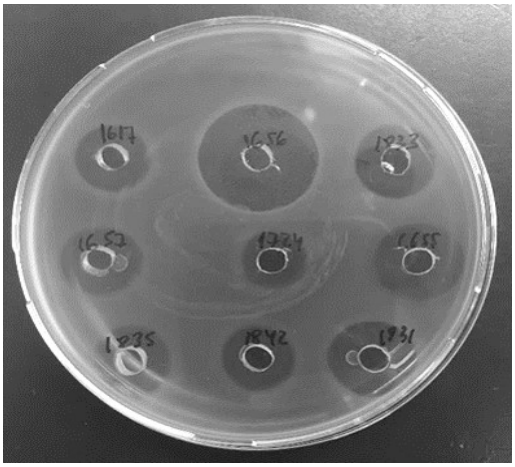


Figure 5: Inhibition of *Listeria monocytogenes* by neutralized supernatants of LAB strains isolated from bovine mammary gland

Table 4: Inhibitory activity of LAB isolates from gastrointestinal tract (4a) of suckling calves and mammary gland (4b) of adult cows against pathogens responsible for infections in cattle, using the plate diffusion technique (Inhibitory Halos in mm)

4a Strain	*L. monocytogenes*		*E. coli*		*S. aureus*		*E. faecium*	
	Neut	N.N	Neut	N.N	Neut	N.N	Neut	N.N
Lb. johnsonii CRL 1693	0	0	2	0	1	0	0	0
Lb. murinus CRL 1695	0	0	0	0	0	0	0	0
Lb. mucosae CRL 1696	0	0	0	0	0	0	0	0
Lb. salivarius CRL 1702	0	0	0	0	0	0	0	0

4b

Strain	*L. monocytogenes*		*E. coli*		*S. aureus*		*E. faecium*		*S. dysgalactiae*		*S. agalactiae*		*S.C.Negat*		*S. bovis*	
	Neut	N.N	Neut	N.N	Neut	N.N	Neut	N.N	Neut.	N.N	Neut	N.N	Neut	N.N	Neut	N.N
Lb. plantarum CRL 1716	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. mundtii CRL 1656	9	11	0	0	0	0	7	8	5	4	0	0	0	0	0	0
W. cibaria CRL 1833	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecium CRL 1657	8	7	0	0	0	0	4	4	1	1	0	0	0	0	0	0
Lb. perolens CRL 1724	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lc. lactis subsp lactis CRL 1655	8	7	0	0	4	5	0	0	2	5	4	4	6	6	2	5
E. hirae CRL 1835	5	6	0	0	0	0	6	6	3	5	0	0	0	0	0	0
E. hirae CRL 1842	4	4	0	0	0	0	1	2	2	0	0	0	0	0	0	0
P. pentosaceus CRL 1831	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Beneficial enzymes

As the strains under evaluation are planned to be included in a multi-strain formula, the evaluation of the beneficial enzymes produced were also of main interest. The enzymes screened were amylase, cellulose and xylanase supported by the possibilities of administration to different aged animals. The adult cows are fed with different type of grains, where the three enzymes evaluated could exert some type of fiber-degradation. Amylase catalyzes the hydrolysis of 1-4 bonds of α-amylase, by digesting glycogen and starch to form simple sugars. When incorporated into the diet of ruminants, starch is degraded mainly by the amylolytic bacteria, which ferment the reserve carbohydrates (starch) present in grains. The digestibility of the starch in the rumen is high and the fraction that manages to pass to the intestine can be degraded by the pancreatic amylase of the animal and thus absorbed as glucose, which favors the ruminant by providing a direct source of glucose. Hence the importance of isolating lactic bacteria with amylolytic properties as potential probiotics for ruminants, since they would favor the digestive process in diets with a high concentration of energy compounds, favoring weight gain. All the strains under study, independently of the isolation origin, were not able to produce xylanase, amylase and celullase enzymes under the conditions assayed.

Compatibility of the strains

The ideal formula to be applied in the three areas of bovines could be designed or formulated with one or two strains isolated from each mucosal tract or ecosystem. When determining the compatibility of the selected strains (with beneficial characteristics), *Lc. lactis subsp lactis* CRL1655 (bacteriocin producer) can be combined only with *Lb. fermentum* CRL1575 and *Lb. gasseri* CRL1566, because inhibits all the other strains. It was observed that *Lb. gasseri* CRL1412 and CRL1421 were the most sensitive strains, since they were inhibited by most of the assayed strains. The other strains show some type of inhibition only to specific strains, and in higher dilutions as shown in **Table 5**. Then, a mixed probiotic formula can de designed or formulated by combining most of the strains, except *Lc. lactis subsp lactis* CRL1655 that must be used individually. Although the use of probiotic products in animals formulated with a combination or multiple-strains is very frequent [58,59], the evaluation of the degree of interactions or compatibility between potentially probiotic LAB strains has not been reported to date, to be applied to different bovine ecosystems. In this assay, the lactobacilli evaluated showed different spectra of inhibition between them. This study, therefore, allows the selection of compatible strains for their potential inclusion in a probiotic / pharmacological product.

Table 5: Compatibility between strains of Lactobacillus isolated from vagina and mammary gland of adult bovine females, and gastrointestinal tract of calves

Indicator Strains[a]	Dilution	Inhibitory substances producer strains[b] (inhibition halos, mm)																
		CRL 1412	CRL 1421	CRL 1460	CRL 1461	CRL 1574	CRL 1575	CRL 1566	CRL 1655	CRL 1716	CRL 1724	CRL 1831	CRL 1833	CRL 1842	CRL 1693	CRL 1695	CRL 1696	CRL 1702
CRL 1412	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	2,0,0	0,0,0	2,3,5	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	1,2,0	2,0,0	3,5,0	5,5,0	2,3,0	3,0,0	3,0,0	5,5,6	2,5,0	0,0,0	5,5,5	5,5,3	5,0,0	5,0,0	5,5,0	0,0,0	5,4,0
CRL 1421	-1	2,3,0	4,4,0	3,5,0	0,2,0	5,4,0	3,0,0	5,5,0	3,4,5	2,0,0	2,0,0	5,5,5	4,5,3	2,0,0	3,4,0	2,0,0	2,3,0	3,4,0
	-2	2,0,0	2,3,2	2,3,0	2,4,0	4,4,0	5,4,0	5,5,0	5,5,6	5,5,0	5,5,5	5,3,6	5,5,6	5,0,0	5,3,3	5,3,2	5,5,3	5,5,5
CRL 1460	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	3,2,0	5,5,6	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1461	-1	0,0,0	0,0,0	0,0,0	0,3,0	0,0,0	0,0,0	0,0,0	8,10,7	0,0,0	0,0,0	3,0,0	4,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	1,1,0	0,0,0	0,0,0	0,0,0	10,8,9	0,0,0	0,0,0	1,1,2	1,1,1	0,0,0	0,0,0	0,1,0	3,2,0	1,2,0
CRL 1574	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	5,0,0	4,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,4,0	2,0,0	2,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

CRL 1575	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	3,0,0	3,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1566	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	5,0,0	3,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	1,4,0	0,0,0	5,0,0	4,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1655	-1	0,0,0	2,0,0	2,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	2,0,0	3,0,0	0,0,0	0,0,0	1,0,0	1,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1716	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,1,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	5,4,7	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1724	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	5,2,1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	8,5,3	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1831	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	4,4,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,3,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1833	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	10,6,3	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,4,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1842	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,2,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,3,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1693	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	7,3,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	2,2,1	2,0,0	2,0,0	10,6,5	3,3,3	3,2,0	4,0,0	3,0,0	2,2,2	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1695	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,3,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	10,4,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1696	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	10,3,1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	1,2,0	5,4,3	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
CRL 1702	-1	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	10,6,4	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	-2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	6,3,2	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

[a]Vaginal and mammary gland lactobacilli from adult bovine female and gastrointestinal tract of calves used as indicator (sensitive) strains of inhibitory substances produced by Lactobacilli.

[b]Vaginal and mammary gland lactobacilli from adult bovine female and gastrointestinal tract of calves used as inhibitory substances producers against Lactobacilli.

The results represent the size of the inhibition halos (in mm) produced by the Lactobacillus supernatant (untreated, neutralized and neutralized and treated with catalase).

Table 6: Homologous beneficial LAB strains selected by their probiotic characteristics for the design of a cattle formula

Strain	Origin	Surface properties								Antimicrobial properties		
		Auto aggregation (%)[a]	Hydro phobicity (%)[b]	Biofilm (OD_{570nm})[c]				EPS (mg/ltmedim)[d]		Inhibition of pathogens[e]	Hydrogenperoxide[f] (Mm de H2O2)	Lactic acid[g] (g/l)
				MRS	MRS-T	LAPTg	LAPTg-T	MRSg	MRSs			
Lb. gasseri CRL 1412	Vaginal Mucosa	0	72	1,48	0,73	0,28	0,01	17,1710	5,500	1	0,27	n.d
***Lb. gasseri* CRL 1421**		0	64	1,37	0,81	0,19	0,16	-		-	0,27	n.d
***Lb. gasseri* CRL 1460**		2	58	0,72	0,59	0,27	0,13	-		-	0	n.d
***Lb. delbrueckii* CRL 1461**		28	58	0,11	0,60	0	0,41	-		-	0,12	n.d
***P. pentosaceus* CRL 1831**	Mammary gland	10	0	0,21	0,73	0,05	0,13	4,500	5,500	-	n.d	0,20
***W. cibaria* CRL 1833**		8	83	0	0,35	0	0	8,450	11,560	-	0,50	n.d
***Lb. johnsonii* CRL 1693**	Calf feces	13	79	0,31	0,18	0,21	0,20	-		2,3,4	0	10.56
***Lb. murinus* CRL1695**		70	18	0,35	0,17	0,25	0,21	-		5	0	6.38
***Lb. mucosae* CRL1696**		16	18	0,14	0,23	0,22	0,22	-		-	0,82	5.7
***Lb. salivarius* CRL1702**		6	35	0	0	0,15	0,31	-		5	0,37	8.12

MRS-T, LAPTg-T: culture medium without Tween 80; MRSg: culture medium enriched with glucose 10%; MRSs: MRS enriched with sucrose 10%; 1: *L. monocytogenes* FBUNT; 2: *E. coli* ATCC12900; 3: *S. dublin* MP/07; 4: *S. aureus* FBUNT; 5: *S. typhimurium* MP/08. N.d: undetermined.

[a] Autoaggregative capability (Ocaña and Nader-Macias, 2002).

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

[c] Determined by technique of quantification of biofilm in polystyrene microplates modified (Leccese Terraf et al., 2016).

[d] Technique described by Quesada et al, 1993 / Gómez, 2005.

[e] Analyzed by the plate diffusion method described by Ocaña et al, 1999 and Juárez Tomás et al, 2004.

[f] Determined by spectrophotometric method of o-dianisidine and peroxidase (Juarez Tomas et al, 2004).

[g] The lactic acid production was obtained by HPLC in a 16 h culture in LAPTg broth (Maldonado et al, 2012).

Conclusions

The results obtained allow to go further in the design of a homologous, host-specific multi-strain probiotic formula to be applied at different tracts in cattle. From 38 LAB strains with potential probiotic properties, only 10 strains showed to share properties or to express different beneficial characteristics were selected, in such a way to complement their effect. **Table 6** shows the compatible strains with beneficial properties, hat will be further evaluated in their technological characteristics.

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References

1. Quigley EM. Basic Definitions and Concepts: Organization of the Gut Microbiome. Gastroenterol Clin North Am. 2017; 46: 1-8.

2. Ma T, Suzuki Y, Guan LL. Dissect the mode of action of probiotics in affecting host-microbial interactions and immunity in food producing animals. Vet Immunol Immunopathol. 2018; 205: 35-48.

3. Bleich A, Fox JG. The Mammalian Microbiome and Its Importance in Laboratory Animal Research. ILAR J. 2015; 56: 153-158.

4. Mendes R, Raaijmakers JM. Cross-kingdom similarities in microbiome functions. ISME J. 2015; 9: 1905-1907.

5. Li K, Bihan M, Methéet BA. Analyses of the stability and core taxonomic memberships of the human microbiome. PLoS One. 2013; 6; 8: e63139.

6. Stumpf RM, Wilson BA, Rivera A, Yildirim S, Yeoman CJ, et al. The primate vaginal microbiome: comparative context and implications for human health and disease. Am J Phys Anthropol. 2013; 152: 119-134.

7. Kleerebezem M, Vaughan EE. Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu Rev Microbiol. 2009; 63: 269-290.

8. Nader Macías MEF, Otero MC, Espeche MC, Maldonado NC. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. J Ind Microbiol Biotechnol. 2008; 35: 1387-1395.

9. Clempson AM, Pollott GE, Brickell JS, Wathes DC. Associations between bovine IGFBP2 polymorphisms with fertility, milk production, and metabolic status in UK dairy cows. Anim Biotechnol. 2012; 23: 101-113.

10. Reverter A, Fortes MR. Breeding and Genetics Symposium: building single nucleotide polymorphism-derived gene regulatory networks: Towards functional genomewide association studies. J Anim Sci. 2013; 91: 530-536.

11. Verbeke J, Van Poucke M, Peelman L, Piepers S, De Vliegher S. Associations between CXCR1 polymorphisms and pathogen-specific incidence rate of clinical mastitis, test-day somatic cell count, and test-day milk yield. J Dairy Sci. 2014; 97: 7927-7939

12. Cheng J, Palva AM, de Vos WM, Satokari R. Contribution of the intestinal microbiota to human health: from birth to 100 years of age. Curr Top Microbiol Immunol. 2013; 358: 323-346.

13. Douillard FP, de Vos W. Functional genomics of lactic acid bacteria: from food to health. Microb Cell Fact. 2014.

14. Eren AM, Sogin ML, Morrison HG, Vineis JH, Fisher JC, et al. A single genus in the gut microbiome reflects host preference and specificity. ISME J. 2004; 9: 90-100.

15. Armas F, Camperino C, Marianelli C. In Vitro Assessment of the Probiotic Potential of Lactococcus lactis LMG 7930 against Ruminant Mastitis-Causing Pathogens. PLoS One. 2017; 9; 12: e0169543.

16. Pellegrino M, Berardo N, Giraudo J, Nader-Macías MEF, Bogni C. Bovine mastitis prevention: humoral and cellular response of dairy cows inoculated with lactic acid bacteria at the dry-off period. Benef Microbes. 2017; 8: 589-596.

17. Shiva C, Arosemena Angulo EL, Calvo MA. Organic acids as a possible alternative to probiotics. Rev Albeitar. 2017; 209: 4-14.

18. FAO/WHO .2001.

19. ISAPP (International Scientific Association for Probiotics and Prebiotics) Prebiotics: A Consumer Guide for Making Smart Choices. 2009.

20. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2015.

21. Genís S, Bach À, Fàbregas F, Arís A. Potential of lactic acid bacteria at regulating Escherichia coli infection and inflammation of bovine endometrium. Theriogenology. 2016; 85: 625-637.

22. Genís S, Bach À, Arís A. Effects of intravaginal lactic acid bacteria on bovine endometrium: Implications in uterine health. Veterinary Microbiology. 2017; 204: 174-179.

23. Genís S, Cerri RLA, Bach À, Silper BF, Baylão M, et al. Pre-calving Intravaginal Administration of Lactic Acid Bacteria Reduces Metritis Prevalence and Regulates Blood Neutrophil Gene Expression After Calving in Dairy Cattle. Front Vet Sci. 2018; 21; 5:135.

24. Pellegrino M, Frola ID, Berardo N, Gobelli D, Nader-Macias MEF, et al. In Vitro Characterization of Lactic Acid Bacteria Isolated from Bovine Milk as Potential Probiotic Strains to Prevent Bovine Mastitis. Probiotics Antimicrob Proteins. 2018.

25. Maldonado NC, Chiaraviglio J, Bru E, De Chazal L, Santos V, et al. Effect of Milk Fermented with Lactic Acid Bacteria on Diarrheal Incidence, Growth Performance and Microbiological and Blood Profiles of Newborn Dairy Calves. Probiotics Antimicrob Proteins. 2017; 10: 668-676.

26. Maldonado NC, Ficoseco Aristimuño C, Mansilla FI, Melián C, Hebert E, et al. Identification, characterization and selection of autochthonous lactic acid bacteria as probiotic for feedlot cattle. Livestock science. 2018; 212: 99-110.

27. Otero MC, Nader Macías MEF. Inhibition of Staphylococcus aureus by H2O2-producing Lactobacillus gasseri isolated from the vaginal tract of cattle. Anim Reprod Sci. 2006; 96: 35-46.

28. Espeche C, Pellegrino M, Frola I, Larriestrac A, Bogni C, et al. Lactic acid bacteria from raw milk as potentially beneficial strains to prevent bovine mastitis. Anaerobe. 2012; 18: 103-109.

29. Aristimuño Ficoseco C, Mansilla I, Maldonado N, Miranda H, Nader Macias F, et al. Safety and Growth Optimization of Lactic Acid Bacteria Isolated from Feedlot Cattle for Probiotic Formula Design. Front Microbiol. 2018; 9: 2220.

30. Frola ID, Pellegrino MS, Espeche MC, Giraudo JA, Nader-Macias ME, et al. Effects of intramammary inoculation of Lactobacillus perolens CRL1724 in lactating cows' udders. J Dairy Res. 2012; 79: 84-92.

31. Frola ID, Pellegrino MS, Magnano G, Giraudo JA, Espeche MC, et al. Histological examination of non-lactating bovine udders inoculated with Lactobacillus perolens CRL 1724. J Dairy Res. 2013; 80: 28-35.

32. Otero MC, Morelli L, Nader Macías M.F. Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. Lett Appl Microbiol. 2006; 43: 91-97.

33. Otero MC, Ocaña VS, Nader-Macías MEF. Bacterial surface characteristics applied to selection of probiotic microorganisms. Public Health Microbiology: Methods and Protocols. Methods in Molecular Biology. 2004; 268: 435-440.

34. Rosenberg M, Doyle RJ. Microbial cell surface hydrophobicity: history, measurement, and significance. Microbial Cell Surface Hydrophobicity. 1990; 1-37.

35. Ocaña V, de Ruiz Holgado AA, Nader-Macías MEF. Characterization of a bacteriocin-like substance produced by a vaginal Lactobacillus salivarius Strain. Appl Environ Microbiol. 1999; 65: 5631-5635.

36. Ocaña VS, de Ruiz Holgado AA, Nader-Macías MEF. Growth inhibition of Staphylococcus aureus by H2O2-producing Lactobacillus paracasei subsp. paracasei isolated from the human vagina. FEMS Immunol Med Microbiol. 1999; 23: 87-92.

37. Ocaña VS, de Ruiz Holgado AA, Nader-Macías MEF. Selection of vaginal H2O2-generating Lactobacillus species for probiotic use. Curr Microbiol. 1999; 38: 279-84.

38. Vandervoorde L, Christiaens H, Verstraete W. Prevalence of coaggregation among chicken lactobacilli. J Appl Bacteriol. 1992; 72: 214-219.

39. Ocaña VS, Nader-Macías MEF. Vaginal lactobacilli: self and coaggregation. Br J Biomed Sci. 2003; 59: 183-190.

40. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol. 1998; 30: 295-304.

41. Lebeer S, Ceuppens J, Geboes K, Van Assche G, Rutgeerts P, et al. Mechanisms of probiotic-host interaction with IBD as a case study: ¿a role for exopolysaccharides? Commun Agric Appl Biol Sci. 2007; 72: 41-45.

42. Lebeer S, Verhoeven LA, Perea Vélez M, Vanderleyden J, De Keersmaecker S. Impact of environmental and genetic factors on biofilm formation by the probiotic strain Lactobacillus rhamnosus GG. Appl Environ Microbiol. 2007; 73: 6768-6775.

43. Leccese Terraf MC, Juárez Tomás MS, Rault L, Le Loir Y, Even S, et al. Biofilms of vaginal Lactobacillus reuteri CRL 1324 and Lactobacillus rhamnosus CRL 1332: kinetics of formation and matrix characterization. Arch Microbiol. 2016; 198: 689-700.

44. Van Geel-Schutten GH, Flesch F, ten Brink B, Smith MR, Dijkhuizen L. Screening and characterization of Lactobacillus strains producing large amounts of exopolysaccharides. Appl Microbiol Biotechnol. 1998.

45. Quesada E, Bejar V, Calvo C. Exoplysaccharide production by Volcaniella eurihalina. Experentia. 1993; 49: 1037-1041.

46. Juárez Tomás MS, Ocaña VS, Wiese B, Nader-Macías ME. Growth and lactic acid production by vaginal Lactobacillus acidophilus CRL 1259, and inhibition of uropathogenic Escherichia coli. J Med Microbiol. 2004; 52: 1117-1124.

47. Jack RW, Tagg JR, Ray B. Bacteriocins of Gram-positive bacteria. Microbiol. Rev. 1995; 59: 171-200.

48. Ocaña VS, Bru E, De Ruiz Holgado AA, Nader-Macias MEF. Surface characteristics of lactobacilli isolated from human vagina. J Appl Microbiol. 1999; 45: 203-212.

49. Juárez Tomás MS, Bru E, Nader-Macías ME. Comparison of the growth and hydrogen peroxide production by vaginal probiotic lactobacilli under different culture conditions. Am J Obstet Gynecol. 2003; 188: 35-44.

50. Maldonado J, Cañabate F, Sempere L, Vela F, Sánchez AR, et al. Human milk probiotic Lactobacillus fermentum CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. J Pediatr Gastroenterol Nutr. 2012; 54: 55-61.

51. De Gregorio E, Rappuoli R. From empiricism to rational design: a personal perspective of the evolution of vaccine development. Nat Rev Immunol. 2014; 14: 505-514.

52. Chabot S, Yu HL, de Léséleuc L, Cloutier D, van Calsteren MR, et al. Exopolysaccharides from Lactobacillus rhamnosus RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-γ in mouse splenocytes. INRA Editions. 2001; 81: 683-697.

53. Ruas-Madiedo P, de los Reyes-Gavilán CG. Invited Review: Methods for the Screening, Isolation, and Characterization of Exopolysaccharides Produced by Lactic Acid Bacteria. J. Dairy Sci. 2015; 88: 843-856.

54. Cerning J, Renard CM, Thibault JF, Bouillanne C, Landon M, et al. Carbon Source Requirements for Exopolysaccharide Production by Lactobacillus casei CG11 and Partial Structure Analysis of the Polymer. Appl Environ Microbiol. 1994; 60: 3914-3919.

55. Mozzi F, Rollán G, de Giori GS, Font de Valdez G. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in Lactobacillus casei CRL 87. J Appl Microbiol. 2001; 91: 160-167.

56. Mokoena MP. Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. Molecules.2017; 22.

57. Parada JL, Ricoy Caron C, Bianchi P, Medeiros A, Soccol CR. Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. Braz. Arch. Biol. Technol. 2007; 50: 521-542.

58. MacPhee RA, Hummelen R, Bisanz JE, Miller WL, Reid G. Probiotic strategies for the treatment and prevention of bacterial vaginosis. Expert Opin Pharmacother. 2010; 11: 2985-2995.

59. Salminen S, Nybom S, Meriluoto J, Collado MC, Vesterlund S, et al. Interaction of probiotics and pathogens-benefits to human health? Curr Opin Biotechnol. 2010; 21: 157-167.

60. Juárez Tomás MS, Nader Macías MEF. Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Microbiology Book Series. Ed.: Méndez-Vilas A. Formatex. 2007; 732-739.