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Original article

Preliminary assays for the development of a probiotic for goats

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Abstract – In order to determine probiotic properties, 137 strains of lactic acid bacteria from the feces of Creole goats were screened, only six were resistant to pH 2.0 and bile salts (0.3%). Three strains identified as *Lactobacillus* and two as *Enterococcus* showed agglutination with the treated yeast. Between them, *Lactobacillus* DDL17, DDL19, DDL48 and *Enterococcus* DDE39 demonstrated high specificity in this test because the correspondent agglutination was inhibited by one sugar, suggesting the presence of a lectin-like structure in their cell walls, which could be due to adhesion ability. Another *Enterococcus* strain (DDE55) showed low affinity because five sugars inhibited the agglutination of the treated yeasts. The results of hydrophobic properties showed that the strains who were able to agglutinate yeasts presented similar hydrophobic characteristics as hexadecane, xylene and toluene, but high specificity was not related to a high hydrophobicity. Only two strains (*Lactobacillus* DDL19 and DDL48) showed aggregation with the lowest concentration of ammonium sulfate, complementing the hydrophobicity assay. Only one strain, *Lactobacillus* DDL48, showed an inhibition against an enteric indicator strain (*Salmonella* Typhimurium and *Escherichia coli* O111). This inhibitory action was not affected by the addition of catalase and no inhibition was detected after neutralizing the supernatant culture fluid. These strains could be preselected in order to complete studies focused on designing a probiotic for use in goat feed.

probiotic / lactic acid bacteria / goats

1. INTRODUCTION

Antibiotics have been widely used in most domestic animals not only to prevent infections caused by pathogenic bacteria, but also for their positive incidence on animal performance (liveweight gain and feed efficiency). The essential role of antibiotics in maintaining a good sanitary status in herds has been recognized, but at present their utilization as growth stimulants are more questionable and there are few antimicrobials permitted [1]. The appearance of resistant bacterial populations, the presence of residual antibiotics in foods of animal origin (unacceptable by the Alimentary Code) and the increasing interest for organic production (where the use of chemical substances are reduced or eliminated) has led to the search for other kinds of preventive methods. The stress that animals could suffer for nutritious and/or environmental reasons, affects the intestinal microflora negatively, causing the establishment of pathogens which

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cause diarrhea and/or bad absorption of nutrients [2, 3]. At the moment it is accepted, in a general way, that productive indexes from the herd are more significant and susceptible as indicators of illness, especially if they are of the subclinical type. The good sanitary status of livestock should include a program of preventive medicine. To reestablish the normal intestinal conditions, in recent years, the industry has begun to adopt probiotics, feed supplements composed of pure or mixed cultures of live microorganisms which affect human or animal consumers beneficently, achieving an intestinal microbial balance [4]. The administration of probiotic foods to animals represents an excellent alternative, avoiding the introduction of illnesses on the farm. Satisfactory results have already been obtained in calves, pigs, chickens and lambs, achieving a good general health and a better animal productive performance, translated by a higher live-weight gain, better feed efficiency and digestion and increments in milk and egg productions [5, 6]. Probiotics include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and yeasts like *Saccharomyces cerevisiae* and *S. boulardii* [5], and in particular *Lactobacillus* [7–9], *Bifidobacterium* [10, 11] and *Enterococcus* [12–15]. A probiotic microorganism that will be used as a microbial supplement in animal feeding, should survive and grow under rumen-like conditions, resist to gastric acidity and the presence of lisozyme, bile salts and pancreatic enzymes [4, 16]. These characteristics can be evaluated in vitro and can be used for strain selection [17]. The abomasum and duodenal acidities and the high concentration of bile in the intestine are the first factors to consider in probiotic selection. Probiotic microorganisms must be selected from specific host because particular gastrointestinal conditions of ruminants [16].

This study was designed to isolate, characterize and further select beneficial lactobacilli and bifidobacteria strains in order to elaborate a probiotic for the use in caprine feed.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

Lactic Acid Bacteria (LAB) were isolated from the feces of healthy Creole goats. The animals grazed on alfalfa, oats and Rhodes grass, and once a day they was received a ration of corn grain or wheat pellet.

The strains were isolated at the Technological Ecophysiology Laboratory of CERELA (Tucumán, Argentina) and were identified with API 50 CH and other complementary tests according to the criteria of Bergey's Manual of determinative bacteriology, 9th edition [18].

The nine strains used belonged to *Lactobacillus*: DDL17, DDL19, DDL48 and DDL51; *Bifidobacterium*: DDBA and DDB23 and *Enterococcus*: DDE39, DDE49 and DDE55.

Pathogenic strain *Escherichia coli* O111 was isolated from a goat kid suffering from enteropathology, and was characterized in the Bacteriology Service at the Universidad Nacional de Tucumán, Argentina. *Salmonella* Typhimurium and *Listeria monocytogenes* were provided for this Bacteriological Service.

All strains were activated by successive subculture in LAPTg broth [19] (lactic acid bacteria) or Brain Heart Infusion broth (Merck) (enterobacteria) and incubated at 37 °C for 12 h before use.

All strains were kept at –20 °C in LAPTg broth with 30% glycerol.

Saccharomyces cerevisiae was provided by the Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), Tucumán, Argentina.

2.2. Resistance to low pH and growth under acidity conditions

The resistance of the isolates to pH 3.0 was tested as follows: overnight cultures of the isolates were centrifuged for 10 min at 3000 *g*. After resuspending the pellet in

sterile saline solution –until a concentration of 10^7 cells·mL^{-1} it was diluted 1/19 in sterile saline solution at pH 3.0. After 0, 1 and 2 h of incubation at 37 °C, adequate dilutions were plated in selective agar medium and incubated at 37 °C for 48 h. The control assays were performed using a sterile saline solution at pH 7 as the diluent.

Overnight cultures of resistant isolates (100 µL) were cultured in 5 mL of LAPTg broth adjusted to pH 2 and pH 3 using 4 N HCl. Growth at 37 °C was spectrophotometrically monitored at 560 nm (OD_{560nm}) for 2 h at intervals of 15 min using a Spectronic 20, Bausch & Lomb. The appropriate dilutions obtained from each interval were plated into LAPTg agar medium and incubated at 37 °C for 48 h to determine the number of viable cells.

2.3. Resistance to bile salts

The strains able to grow under acidic conditions were studied for their resistance to bile salts. Overnight cultures of resistant isolates (100 µL) were cultured in 5 mL of LAPTg broth supplemented with Oxgall (Difco) 0.15% or 0.30%. (w/v). The cultures were incubated at 37 °C for 24 h and growth was monitored by measuring the OD_{560nm} from 0 h to 24 h (intervals of 8 h). Appropriate dilutions obtained from each interval were plated into LAPTg agar and incubated at 37 °C for 48 h.

2.4. Salt aggregation test (SAT)

The hydrophobic characteristic of the bacterial surface of strains was determined by the method of Jonsson and Wadström [20]. Lactic acid bacteria were grown in 5 mL of LAPTg broth for 16 h at 37 °C. Bacterial cells were harvested by centrifugation (3 000 *g* for 15 min), washed twice with phosphate buffered saline (PBS) pH 7 and suspended in PBS at a concentration of 10^7 cells·mL^{-1}. Bacterial cell suspensions (25 µL) were mixed with equal volumes of ammonium sulphate of various molarities (0.2–4.0 mol·L^{-1}) on microscopic slides. The lowest concentration of ammonium sulphate giving a visible aggregation was scored as the SAT hydrophobicity value.

2.5. Detection of antibacterial activity

The antibacterial activity was tested by the agar-well diffusion assay [21]. The indicator strains used were *Listeria monocytogenes*, *Salmonella* Typhimurium and *Escherichia coli* O111 (10^5–10^6 CFU·mL^{-1}). Seventy-five microliters of an overnight culture of indicator microorganism were mixed with 12 mL of Brain Heart Infusion (Merck) soft agar (0.7% w/v) at 45 °C and poured into Petri dishes. After solidification, the wells of 5 mm diameter were cut into the agar plates and 30 µL of supernatant of the overnight probiotic culture were added to each well. The supernatants were obtained by growing the potentially inhibitory producer strain overnight in LAPTg broth at 37 °C. The cells were then removed by centrifugation (3 000 *g* for 15 min) and the supernatant was filtered through a 0.22 µm pore-size filter (Millipore), adjusted to pH 6 with sterile NaOH 1 N, and treated with catalase (0.5 mg·mL^{-1}, SIGMA) at 25 °C for 30 min. The treated cell-free supernatant placed in the wells was allowed to diffuse into the agar for 4 h at room temperature. The plates were then incubated at 37 °C and examined after 24 h for inhibition.

2.6. Agglutination assay to study the production of lectin-like substances

Lactic acid bacteria were grown in 5 mL of LAPTg broth for 16 h at 37 °C. Bacterial cells were collected by centrifugation (3 000 *g* for 15 min), washed three times with phosphate buffered saline (PBS) pH 7.4 and suspended in PBS until a concentration of 10^8 bacterial cells·mL^{-1}. Agglutination was monitored visually on microscopic slides by mixing 10 µL of the sample with 5 µL PBS pH 7.4 and 10 µL of a suspension of glutaraldehyde treated *Saccharomyces cerevisiae* (10^8 cells·mL^{-1}). The yeast cells were prepared by incubation in PBS with glutaraldehyde (1 mg·mL^{-1}) for 1 h at 25 °C, washed

twice with PBS, incubated for 30 min at 25 °C with 10 mg·mL^{-1} glycine and washed as above. The treated yeast cells were stored at 4 °C as a suspension in PBS (0.1 g·mL^{-1}) containing 0.02% sodium azide [22].

Inhibition agglutination assay

The ability of different sugars to inhibit agglutination was tested by mixing 10 µL of bacterial cell suspension in PBS with 5 µL of the sugar solution (fucose, *N*-acetyl-glucosamine, glucose, galactose, sucrose, lactose, mannose and sialic acid). Five microliters of these carbohydrate solutions were individually added at 0.2 and 1 M, prior to the addition of 10 µL of the treated yeast suspension.

2.7. Cell surface hydrophobicity

Bacterial surface hydrophobicity was determined by the cell adherence to hydrocarbons assay [23]. The assayed bacteria were grown at 37 °C in LAPTg broth. Unless otherwise stated, the bacteria were collected by centrifugation from the early logarithmic growth phase, washed twice and resuspended in a physiological solution (PS) to an optical density (OD_{600}) of 0.5–0.7. Test hydrocarbon (1 mL) (hexadecane, toluene and xylene) was added to test tubes containing 3 mL of washed cells. The mixtures were blended on a vortex mixer for 90 s. The tubes were left to stand for 15 min for separation of the two phases and the OD of the aqueous phase was measured. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the original bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage hydrophobicity was calculated using the following equation:

$$\% \text{ hydrophobicity} = \frac{(OD_{600}\text{before mixing} - OD_{600}\text{ after mixing}) \times 100}{OD_{600}\text{before mixing}}.$$

2.8. Mixed cultures

Mixed cultures (a potentially probiotic strain + pathogen microorganism) were studied.

LAPTg broth (10 mL) were inoculated with 10^6–10^7 CFU·mL^{-1} of individual probiotic strains and 10^6 CFU·mL^{-1} of pathogens. Pure (Control) and mixed cultures were incubated for 24 h at 37 °C and followed by measuring of OD_{560} (only Control cultures). Lactobacilli counts were determined on Rogosa agar (Merck), bifidobacteria on HHD agar [24], *Salmonella* Typhimurium and *Escherichia coli* O111 on Mc Conkey agar (Merck). All plates were incubated at 37 °C for 24–48h.

2.9. Statistical analysis

All the experiments were carried out in triplicate. The results were statistically evaluated with one way ANOVA and statistical significance was considered when $P < 0.05$.

3. RESULTS AND DISCUSSION

The study of the tolerance of lactic acid bacteria, isolated from fecal samples of 30 healthy Creole goats, to acidity (pH 2) and bile salts (0.3%), determined that only six can be considered potentially beneficial microorganisms. From 137 strains, only nine were able to grow in culture media added to 0.15% bile salts (Tab. I). The first host attributes, affecting the strain selection for probiotic use are the high acidity in the stomach or abomasum and bile salt concentration in the small intestine. The incubation of strains at a lower pH for some hours was used to screen potentially probiotic strains for their ability to survive in the stomach [4]. On the contrary, agar medium supplemented with 0.15% of oxgall was used to enumerate resistant bacteria to bile salts [25, 26], but a value of 0.3% was considered like a critical concentration for the selection of resistant strains [9, 27–29]. Gilliland et al. [27] reported a great dispersion among *Lactobacillus acidophilus* strains isolated from calf intestinal contents in their ability to grow in vitro in the presence of bile salts. In addition, when two strains, one exhibiting low and other high tolerance to

Table I. Selection of potentially probiotic strains isolated from the feces of Creole goats.

Microorganisms		*Lactobacillus*				*Bifidobacterium*		*Enterococcus*		
		DDL17	DDL19	DDL48	DDL51	DDBA	DDB23	DDE39	DDE49	DDE55
Resistance	Bile salts 0.15%	+	+	+	+	+	+	+	+	+
	Bile salts 0.30%	+	+	+	–	+	–	+	–	+
	pH 2	+	+	+	–	+	–	+	–	+

+: growth of strain. –: no growth of strain.

bile were administered orally to calves, the more resistant strain caused a greater increase in the number of lactobacilli than the one possessing a low tolerance [30].

One strain, *Lactobacillus* DDL48, showed antimicrobial activity against *Salmonella* Typhimurium and *Escherichia coli* O111, with an inhibition zone of 8–10 and 11–13 mm diameter, respectively. These effects were observed with a non-adjusted pH supernatant. The remainding lactic acid bacteria strains were ineffective against the pathogens above mentioned pathogens and any strain against *Listeria monocytogenes* (with and without adjusted pH supernatants) (Tab. II). However, the supernatants obtained from cultures of *Lactobacillus* DDL48, adjusted to a pH 6 with a NaOH solution or treated with catalase (in order to exclude the acidity and hydrogen peroxide effects, respectively) were unable to inhibit the growth of the pathogens. From these results it can be infered that any strain considered in this report is antimicrobial, and the antagonistic effects observed in *Lactobacillus* DDL48 against *Salmonella* Typhimurium and *Escherichia coli* O111 were due to low pH for the organic acid production.

Previous works related to lectin-like structures in lactobacilli isolated from chickens [8, 29] or from pigs [9] present different carbohydrate specificity; from poultry strains, glucose/mannose mediated the interaction of *Lactobacillus animalis*, fucose/mannose the agglutination of *Lactobacillus fermentum* and N-acetyl-glucosamine the assay of *Lactobacillus fermentum* subsp. *cellobiosus*. However the results reported by Gusils et al. [9] on *Lactobacillus acidophilus* C2, a pig strain, indicate that the lectin-like structure has sucrose as specific sugars of binding yeast. In addition, Morata de Ambrosini et al. [31] reported that the interaction treated yeasts-*Lactobacillus casei* CRL431, a strain of human origin, is mediated by mannose/sucrose. The great variability observed from sugars involved in yeast agglutination, suggest that the binding specificity is a characteristic of strain and independent of their origin.

The aggregation ability of two strains isolated from goats was recorded as positive for the salt aggregation test (SAT), and was positive for five strains for the agglutination of treated yeasts assays (Tab. II). The addition of lactose (1 M) inhibited agglutination from *Lactobacillus* DDL17, and the addition of sialic acid (0.2 M) was able to inhibit the agglutination from the other lactobacilli strains, namely DDL19 and DDL48. This agglutination assay performed with *Enterococcus* DDE39 was inhibited only after the addition of N-acetyl-glucosamine (0.2 M). The results on the high specificity found in the agglutination test from these four strains, could be correlated to the presence of external lectin-like structures with a carbohydrate affinity. On the contrary, *Enterococcus* DDE55 showed an affinity for five sugars because the addition of glucose, lactose, mannose, N-acetyl-glucosamine and sucrose determined the inhibition of the agglutination of treated yeasts by this strain (Tab. II).

It is known that the hydrophobic nature of prokaryotic cell walls is involved in several

Table II. Selection criteria of potentially probiotic strains isolated from the feces of Creole goats.

	Microorganisms	DDL17	DDL19	DDL48	DDBA	DDE39	DDE55
Antimicrobial spectrum	*Escherichia coli* O111	–[a]	–	+[b]	–	–	–
	Salmonella Typhimurium	–	–	+	–	–	–
	Listeria monocytogenes	–	–	–	–	–	–
Potential adhesive properties	Aggregation[c]	–	0.2	0.2	–	–	–
	Agglutination	+[d]	+	+	–[e]	+	+
	Agglutination assays with the addition of a carbohydrate solution						
	Fucose	+[d]	+	+	ND[h]	+	+
	Galactose	+	+	+	ND	+	+
	Glucose	+	+	+	ND	+	–[g]
	Lactose	–[f]	+	+	ND	+	–[g]
	Mannose	+	+	+	ND	+	–[g]
	N-acetyl-glucosamine	+	+	+	ND	–[g]	–[g]
	Sucrose	+	+	+	ND	+	–[g]
	Sialic acid	+	–[g]	–[g]	ND	+	+
	Hydrophobicity (%)						
	Hexadecane	26 ± 10	60 ± 14	47 ± 9	16 ± 3	2 ± 1	2 ± 1
	Xylene	35 ± 3	57 ± 14	68 ± 14	27 ± 10	5 ± 3	–
	Toluene	31 ± 7	63 ± 8	69 ± 15	21 ± 5	4 ± 3	–

[a] No inhibition zone.
[b] Inhibition zone.
[c] The lowest concentration of ammonium sulphate giving visible aggregation.
[d] Agglutination of treated yeasts.
[e] No agglutination of treated yeasts.
[f] 1 M final concentration.
[g] 0.2 M final concentration.
[h] Not determined.

biological processes such as the interaction of bacteria-intestinal epithelial cells or bacteria-phagocytic cells [32]. Rosenberg et al. [23] demonstrated that bacterial strains possessing superficial hydrophobic characteristics added to organic solvents, but not the strains without hydrophobic characteristics. For this reason the bacterial adhesion to organic solvents was proposed as a technique to study superficial cellular hydrophobicity [22]. The use of this assay was limited due to the harmful effects of xylene on the cell walls inducing lysis. However the use of hexadecane does not have negative effects on bacterial cells, and this organic solvent was recommended [33].

Our results on hydrophobic properties showed that the strains able to agglutinate yeasts presented similar hydrophobic characteristics for the three assayed solvents (Tab. II). The highest hydrophobic percentages corresponded to the lactobacilli strains, namely DDL19 and DDL48, which demonstrates that these microorganisms presented important superficial characteristics, besides a high binding specificity from the agglutination assays. The aggregation with low ammonium sulphate (0.2 M) of these two strains confirmed their hydrophobic characteristics. However, *Enterococcus* DDE39, who also presented high specificity in the yeast agglutination test, did not turn out to

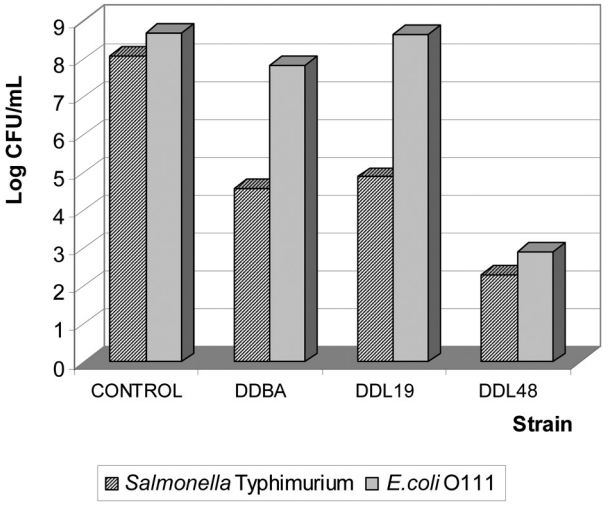

Figure 1. Inhibition of enteropathogenic microorganisms by *Lactobacillus* or *Bifidobacterium* strains in mixed cultures. Control: Pure cultures of pathogens ▨ *Salmonella* Thyphimurium; □ *Escherichia coli* O111. DDBA ▨: Mixed culture between *Bifidobacterium* DDBA and *Salmonella* Thyphimurium. DDBA □: Mixed culture between *Bifidobacterium* DDBA and *Escherichia coli* O111. DDL19 ▨: Mixed culture between *Lactobacillus* DDL19 and *Salmonella* Thyphimurium. DDL19 □: Mixed culture between *Lactobacillus* DDL19 and *Escherichia coli* O111. DDL48 ▨: Mixed culture between *Lactobacillus* DDL48 and *Salmonella* Thyphimurium. DDL48 □: Mixed culture between *Lactobacillus* DDL48 and *Escherichia coli* O111.

be a hydrophobic strain. These results were in agreement with the scientific bibliography regarding the general criteria for selecting probiotic microorganisms, which points out that several tests are complementary but not restricting. The mechanism of adhesion requires the participation of different surface constituents that interact in a sequential manner to overcome repulsive forces [34].

Different double mixed cultures between potentially probiotic strains and host-specific pathogenic microorganisms were carried out using competition assays. After incubation (37 °C, 24 h), the evaluated strain counts did not present significant differences ($P > 0.05$) with respect to pure cultures (control). On the contrary, in the same mixed cultures, partial inhibition of the pathogens was observed. Considering the results of detection of antimicrobial activity it can be inferred that the growth of *Salmonella* Typhimurium was affected by 3 strains, but the greatest inhibition effect was of *Lactobacillus* DDL48, when the pathogen counts diminished almost 6 log units. The same antagonistic effect was observed against *Escherichia coli* O111, while the *Lactobacillus* DDL19 and *Bifidobacterium* DDBA strains did not exert inhibitory action against these pathogenic microorganisms (Fig. 1). The antipathogenic effect observed in the mixed culture between *Lactobacillus* DDL48-*Salmonella* Typhimurium and *Lactobacillus* DDL48-*Escherichia coli* O111 could be explained as a nutritional competition with the addition of an acidity effect, but not as the production of antimicrobial substances like bacteriocins or hydrogen peroxide. In the mixed cultures between *Lactobacillus* DDL19-*Salmonella* Typhimurium and *Bifidobacterium* DDBA-*Salmonella* Typhimurium, the antipathogenic effects was owed to only a nutritional competition.

Among the 137 strains isolates, only six were selected because of their potentially probiotic properties considering adhesion capacity, antipathogenic activity against enteric bacteria, their resistance to bile salts (0.3%) and a pH 3.0. At the present, we are studying the nematicidal activity of the pre-selected strains. The non-translocation concentration of probiotic selected strains must be determined before performing probiotic assays in goats to measure their effect on the animal is performance and sanitary status.

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