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Effect of Two Probiotic Strains of *Lactobacillus* on In Vitro Adherence of *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Staphylococcus aureus* to Vaginal Epithelial Cells

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Abstract The lactobacilli probiotics maintain a normal vaginal biota and prevent disease recurrence. This microorganisms form a pellicle on the vaginal epithelium that acts as a biologic barrier against colonization by pathogenic bacteria. In this paper were realized assays of exclusion, competition, and displacement. For these test, vaginal epithelial cells, two strains of lactobacilli and pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus agalactiae* and *Listeria monocytogenes*) were used. The lactobacilli strains showed a great capacity of adherence, with a mean of 83.5 ± 26.67 *Lactobacillus fermentum* cells and 56.2 ± 20.87 *Lactobacillus rhamnosus* cells per vaginal epithelial cells. *L. fermentum* and *L. rhamnosus* were able to reduce the adherence of *S. aureus*, *S. agalactiae* and *L. monocytogenes* in a significant level in this assay ($P < 0.01$). The lactobacilli used in this study protect the vaginal epithelium through a series of barriers and interference mechanisms. The aim of present study was to assess the ability of vaginal *Lactobacillus* strains, selected for their probiotic properties, to block the adherence of pathogenic microorganisms in vitro by displacement, competition, and exclusion mechanisms.

Introduction

Most studied probiotic bacterial strains belong to the genus *Lactobacillus*. Lactobacilli naturally inhabit both the gastrointestinal tract and the female reproductive tract of healthy humans. In the female reproductive tract, lactobacilli make up the majority of the vaginal microbiota [25, 26]. Lactobacilli have been shown to play a role in protecting women from infection by incoming pathogens, including HIV [22], and epidemiological evidence suggests that women with high numbers of vaginal lactobacilli have reduced susceptibility to gonorrhea and chlamydia following exposure [24]. Clearly, lactobacilli are a key component of the human defense against colonization by sexually transmitted pathogens. It is generally accepted that lactobacilli play a major role in maintaining the urogenital health by preventing the overgrowth and invasion of pathogenic bacteria [12, 15] by a combination of competitive exclusion, competition for nutrients, and production of antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants [3, 12, 20, 23].

The use of human lactobacilli as probiotics which restore and maintain a normal vaginal biota and prevent disease recurrence is very important. Lactobacilli form a pellicle on the vaginal epithelium that acts as a biologic barrier against colonization by pathogenic bacteria. In this sense, previous studies have reported that adhesive lactobacilli can inhibit the attachment of pathogens to urogenital epithelial cells in vitro [8, 10]. After inhibition of adherence to epithelial cells, the pathogen could be killed by the antimicrobial agents found in the vaginal mucus. Thus, the pathogen could not cause an infection [25].

The uropathogenic strains *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Staphylococcus aureus* cause several infectious diseases in humans. These pathogens may also cause neonatal sepsis [21].

The aim of present study was to assess the ability of vaginal *Lactobacillus* strains, selected for their probiotic

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properties, to block the adherence of pathogenic microorganisms in vitro by displacement, competition, and exclusion mechanisms.

Materials and Methods

Bacterial Strains and Culture Conditions

Lactobacillus fermentum strain L23 and *L. rhamnosus* strain L60 have been well identified by standard biochemical tests [1], by the API 50 CHL system (BioMérieux, Inc., France) [11], and by 16S rRNA analyses [4, 9, 17].

The 16S rRNA sequences of both lactobacilli were deposited in the GenBank according to the last recommendation of FAO/WHO during 2001 [19]. The GenBank accession numbers GQ 455406 and EF 495247 have been assigned to *L. fermentum* L23 and *L. rhamnosus* L60, respectively.

Streptococcus agalactiae or group B streptococci (GBS) isolates were recovered from pregnant patients of the Gynecology Service at the New Hospital of Río Cuarto, Córdoba, Argentina. These isolates were identified by Gram staining followed by standard biochemical tests and, in addition, by Group B-specific latex agglutination (Slidex Strepto-Kit, BioMérieux, Marcy l'Etoile, France) [1]. They were inoculated on 5 % sheep blood agar plates (BioMerieux, Inc., France), and incubated at 37 °C with 5 % CO_2 for 24–48 h. Isolates were stored at −80 °C in tryptic soy broth (TSB) (Merck, Germany) containing 30 % (v/v) glycerol. Each isolate was reactivated by re-culturing once in TSB broth prior to experiments and then was directly inoculated on the corresponding agar medium.

The samples for isolation of *L. monocytogenes* were directly inoculated on selective agar (*Listeria* Agar; Oxoid). Typical *L. monocytogenes* colonies (which are blue-green with a surrounding precipitate) were isolated and purified by re-streaking on 3 % blood agar, where bacterial growth appeared surrounded by a β-hemolysis zone after 24 h of incubation. The purified colonies were then inoculated on tryptone soy agar (TSA). Single pure isolated colonies were grown overnight in TSB. After being identified by standard biochemical tests, the isolates were frozen in cryovials in a glycerol/TSB mixture at −20 °C [1].

Staphylococcus aureus isolates were obtained from several clinical samples and then tested. Isolates were inoculated on blood agar and incubated aerobically at 35 °C. After 24 h of incubation, the isolates formed white, entire, convex, glistening colonies that were surrounded by a zone of β-hemolysis and had between 5 and 6 mm of diameter. The isolates were first identified as *S. aureus* by standard biochemical techniques [7]. Isolates were plated onto sterile manitol salt agar (MSA) and incubated at 37 °C for 24 h. Characteristic colonies were subcultured once on MSA and twice in blood agar for purification. Purified colonies were maintained on nutrient agar slants at 4 °C until used. Isolates were identified using sugar fermentation tests and the API Staph (BioMerieux, France), as described above [5, 7, 13].

Collection and Washing of Vaginal Epithelial Cells

Vaginal epithelial cells (VEC) were collected from a healthy volunteer during days 17 and 18 of her menstrual cycle. The patient was not using a spermicidal product nor an oral contraceptive, was not receiving antibiotics, and had no known vaginal pathology. Cells were washed three times and suspended in 1× Earle medium.

Bacterial Adherence Assay

Overnight cultures of the lactobacilli to be tested were suspended (10^8 cells/ml) in Earle medium. Equal volumes of bacterial suspension and VEC were mixed and incubated at 37 °C with orbital shaking (100 rpm) for 30 min. The resulting suspension was filtered through 8–10 μm pore size Millipore filters and washed with Earle medium. Cells retained on the filter were placed on albumin-coated and non-coated microscope slides, fixed with ethanol, and Gram stained [2]. Adherence was assessed by counting the number of bacteria adhered to the first 25 intact epithelial cells observed. Two controls were used: (i) assay without addition of lactobacilli to measure the indigenous lactobacilli remaining on vaginal cells after three washes and (ii) assay including L23 and L60 to measure reproducibility [20].

Overnight cultures of *S. aureus* and *S. agalactiae* were suspended (10^8 cells/ml) in Earle medium. Equal volumes of each bacterial suspension and vaginal cells were mixed and incubated at 37 °C with orbital shaking (100 rpm) for 30 min. The resulting suspension was processed as described above.

In the assay with *L. monocytogenes*, a modification of the counting technique was used due to the difficulty in microscopically differentiating lactobacilli from listeria cells. To differentiate *Listeria* cells from LAB, a turbidimetric test was used. In exclusion, competition and displacement tests with listeria strains, after incubation and washing the mixture (cells, lactobacilli and listeria), the surface of Muller Hinton agar plates, which the lactobacilli growth is impossible, were seeded. After incubation the listeria growth was removed and the suspensions in PBS were made and measured their absorbances, which were compared with the control curve.

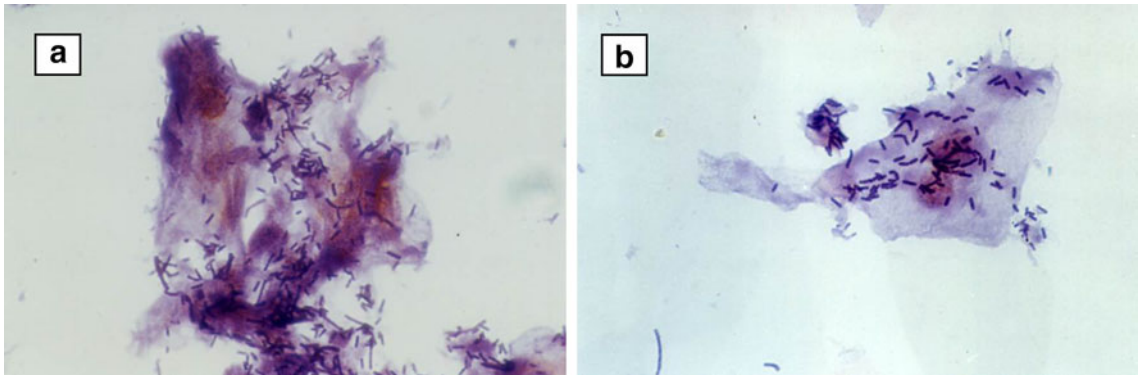

Fig. 1 Lactobacilli probiotic strains adhered to vaginal epithelium cells as observed by light microscope after Gram staining (×100). **a** *L. fermentum*; **b** *L. rhamnosus*

Table 1 Bacteria adhered to VEC

Microorganisms	Average of adhered bacteria ± SD
L. fermentum (L23)	83.5 ± 26.67
L. rhamnosus (L60)	56.2 ± 20.87
S. aureus 1	113.4 ± 45.5
S. aureus 2	114 ± 43.51
S. aureus 3	112 ± 21
S. agalactiae (GBS) 1	132.3 ± 60
S. agalactiae (GBS) 2	134.5 ± 61.35
S. agalactiae (GBS) 3	134.5 ± 60.62

Table 2 Absorbance values of *Listeria* strains adhering to vaginal cells

Microorganisms	Absorbance average of adhered bacteria ± SD
L. monocytogenes 1	1.260 ± 0.0015
L. monocytogenes 2	1.116 ± 0.0017
L. monocytogenes 3	1.301 ± 0.0015

Assay of Exclusion, Competition, and Displacement

For the exclusion assay, VEC and lactobacilli (1×10^8 CFU/ml) were mixed in a 1:1 ratio and incubated with agitation at 37 °C for 1 h. Pathogens (1.5×10^8 CFU/ml) were added later, and the resulting suspension was incubated for an additional hour.

For the competition assay, VEC, lactobacilli, and pathogenic bacteria (the same amounts as for the exclusion assay) were incubated together with orbital shaking (100 rpm) for 60 min.

For the displacement assay, equal volumes of pathogenic bacterial suspensions and vaginal cells were mixed and incubated at 37 °C with orbital shaking (100 rpm) for 60 min. Then, a suspension of lactobacilli (3×10^8 CFU/ml) was added to determine if lactobacilli were able to displace initially adhered pathogens.

Statistical Analyses

All bacterial counts were expressed as mean ± SD and were log transformed for each experiment. An analysis of variance (Sigma Stat Statistical Software V 2.0, for Windows NT and 3.1; SPSS) was used for differences in numbers of viable microorganisms from several treatment groups. A P value of 0.05 was considered statistically significant.

Results

The lactobacilli strains showed a great capacity of adherence, with a mean of 83.5 ± 26.67 *L. fermentum* cells and 56.2 ± 20.87 *L. rhamnosus* cells per VEC. Thus, *L. fermentum* showed the highest capacity of adherence ($P < 0.05$). The adherence of lactobacilli to vaginal epithelium cells is shown in Fig. 1a, b.

The pathogenic bacterial species studied were able to adhere to the vaginal epithelium cells (Table 1). Absorbance values of the tested *Listeria* strains are shown in Table 2. They represent the degree of adherence of these strains to VEC. The adherence means of pathogenic microorganisms to 30 vaginal epithelium cells were 113.13 for *S. aureus* and 133.76 for *S. agalactiae*. On the other hand, the average value of *L. monocytogenes* absorbance was 1.22.

The results of the exclusion assay are detailed in Tables 3 and 4. The blocking effect against the pathogenic microorganisms did not vary among the studied lactobacilli strains ($P < 0.05$). The percentages of adhesion inhibition for all the pathogenic strains tested were high (Table 5). After evaluating the competitive exclusion of pathogenic microorganisms by lactobacilli, an analysis of variance was

Table 3 Counts of *S. aureus* and *S. agalactiae* adhered to VEC in the presence of *L. fermentum* or *L. rhamnosus* in the different assays: exclusion, competition and displacement

	Bacterial count (bacteria/VEC) (mean ± SD)					
	L23			L60		
	Exclusion	Competition	Displacement	Exclusion	Competition	Displacement
Strains S. a.						
1	18.6 ± 13.97	8.5 ± 6.50	41.7 ± 14.29	13.2 ± 8.61	6.8 ± 3.91	32.8 ± 18.49
2	18.7 ± 14.80	9.2 ± 7.37	37.9 ± 17.37	13.3 ± 9.05	6.3 ± 4.32	34.8 ± 21.02
3	19.6 ± 12.57	9.4 ± 7.34	43.5 ± 16.43	14.5 ± 7.47	6.4 ± 3.75	32.6 ± 17.63
Average	19 ± 13.78	9 ± 7.07	41 ± 16.03	13.7 ± 8.38	6.5 ± 4	33.4 ± 19.05
Strains GBS						
1	55.9 ± 24.02	9.9 ± 7.55	42.6 ± 17.15	53.3 ± 25.12	12.1 ± 4.89	52.3 ± 25.01
2	59.3 ± 27.46	9.1 ± 8.36	45 ± 19.70	49.8 ± 24.92	11.3 ± 6.81	52.6 ± 26.81
3	56.6 ± 25.97	10.2 ± 7.84	43 ± 18.46	49.4 ± 25.06	11.7 ± 6.31	50.8 ± 25.68
Average	57.3 ± 25.82	9.7 ± 7.92	43.5 ± 18.44	50.8 ± 25.03	11.7 ± 6	51.9 ± 25.83

S. a. Staphylococcus aureus, *GBS* group B streptococci (*Streptococcus agalactiae*)

Table 4 Absorbance of *L. monocytogenes* strains adhered to VEC in the presence of *L. fermentum* or *L. rhamnosus* in the different assays: exclusion, competition and displacement

Strains L. m.	Bacterial absorbance (mean ± SD)					
	L23			L60		
	Exclusion	Competition	Displacement	Exclusion	Competition	Displacement
1	0.048 ± 0.0011	0.529 ± 0.0015	0.426 ± 0.0011	0.056 ± 0.0015	0.350 ± 0.001	0.710 ± 0.0015
2	0.051 ± 0.001	0.528 ± 0.0015	0.422 ± 0.0017	0.049 ± 0.001	0.336 ± 0.002	0.723 ± 0.0015
3	0.044 ± 0.0005	0.529 ± 0.0017	0.426 ± 0.0010	0.059 ± 0.0011	0.370 ± 0.0011	0.700 ± 0.0015
Average	0.048 ± 0.0009	0.529 ± 0.0016	0.425 ± 0.0013	0.055 ± 0.0012	0.352 ± 0.0014	0.711 ± 0.0015

L. m. Listeria monocytogenes

performed. This inhibition was statistically significant compared to control ($P < 0.05$). In the exclusion test, *L. fermentum* inhibited the adhesion of *L. monocytogenes* with an average percentage of 96.1.

The percentages of reduction of *S. aureus* and GBS adherence observed when these pathogenic microorganisms competed with both probiotic lactobacilli for binding sites ranged between 91.2 and 94.3 ($P < 0.05$).

When the lactobacilli were added after the pathogenic microorganisms (displacement assay), the reduction percentages of adherence to VEC were lower than in the exclusion and competition assays. However, *L. fermentum* and *L. rhamnosus* were able to reduce the adherence of *S. aureus*, GBS and *L. monocytogenes* in a significant level in this assay ($P < 0.01$).

Discussion

Lactobacilli are believed to interfere with pathogenic microorganisms by different mechanisms. The first is competitive exclusion of genitourinary pathogens from receptors present on the surface of the genitourinary epithelium. The competition for space to adhere between indigenous bacteria and exogenous pathogens results in the competitive exclusion of pathogenic bacteria [6, 14]. Second, lactobacilli coaggregate with some pathogenic bacteria, a process that, when linked to the production of antimicrobial compounds, such as lactic acid, hydrogen peroxide, bacteriocin substances, and possibly biosurfactants, causes an inhibition of the growth of the pathogen [18].

The vaginal *Lactobacillus* strains used in this study have previously shown adhesive properties and antagonistic effects against urogenital pathogens by antimicrobial compounds and resistance to spermicides in vitro [16].

Adherence of bacteria to epithelial cells has been shown to be an important factor in the colonization of mucous membranes. In this study, the average values of adhesion of lactobacilli and pathogenic microorganisms to VEC match with those reported by Pascual et al. [16] for *L. fermentum*. The average adhesion of *L. fermentum* was greater than

Table 5 *L. fermentum* and *L. rhamnosus* inhibition of *S. aureus*, *S. agalactiae* and *L. monocytogenes* adherence to VEC

	Inhibition percentage (%)					
	L23			L60		
	Exclusion	Competition	Displacement	Exclusion	Competition	Displacement
Strains S. a.						
1	83.6	92.5	63.2	88.4	94	71
2	83.6	91.9	66.8	88.3	94.5	69.5
3	82.5	91.6	61.2	87	94.3	70.9
Average	83.2	92	63.7	87.9	94.3	70.5
Strains GBS						
1	77.7	92.5	67.8	79.7	90.8	60.5
2	75.9	93.3	66.5	73	91.6	60.9
3	77.9	92.4	68	72.9	91.3	62.2
Average	77.1	92.7	67.4	75.2	91.2	61.2
Strains L. m.						
1	96.2	58	66.2	95.5	72.2	43.7
2	95.4	52.7	62.2	95.6	69.9	35.2
3	96.6	59.3	67.3	95.5	71.6	46.2
Average	96.1	56.7	65.2	95.5	71.2	41.7

S. a. Staphylococcus aureus; *GBS* group B streptococci (*Streptococcus agalactiae*); *L. m. Listeria monocytogenes*

L. rhamnosus. Kwok et al. [8], working with *L. crispatus* CTV-05, observed similar adhesion values.

In our in vitro model, the exclusion, competition, and displacement assays showed that treatment with *L. fermentum* or *L. rhamnosus* caused a decrease in the number of all of the pathogenic strains adhering to epithelial cells. These results agree with Osset et al. [15], who found that *Lactobacillus* LB35 was able to block the adhesion of *E. coli* EC26 to VEC through these mechanisms.

Competition profiles for *S. aureus* and GBS by lactobacilli were, however, very different from those of exclusion and displacement. The degree of competition was generally much higher than the degree of inhibition achieved by exclusion and displacement. The degree of competition was strain-dependent and was probably determined by the affinity of adhesins on the respective bacterial surfaces for the stero-specific receptors that they are competing for, or their relative positions in the case of steric hindrance [11].

In conclusion, the lactobacilli used in this study may protect the vaginal epithelium through a series of barriers (self-aggregation, co-aggregation with potential pathogens, and adherence) and interference (receptor binding interference block) mechanisms.

The selection of probiotics that directly compete with pathogenic microorganisms, which produce antimicrobial compounds such as bacteriocins and that have the ability to colonize the vaginal mucous membranes is a logical approach for the development and use of such probiotics for the therapeutic treatment of infectious diseases caused by *L. monocytogenes*, *S. agalactiae*, and *S. aureus*.

Consequently, these two probiotic strains of *Lactobacillus* may be excellent candidates for eventual use as prophylactic agents. Studies to further evaluate their feasibility as such are underway.

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