Full Paper

***Lactobacillus rhamnosus* L60, a potential probiotic isolated from the human vagina**

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The vagina has been increasingly viewed as an “ecosystem” whose normal microflora help protect it from invading pathogens, including those that cause urinary tract infections and sexually transmitted diseases. We tested new strains of lactobacilli as potential probiotics for maintenance of urogenital tract health, as well as prevention and therapy of urogenital infections. A strain of lactobacilli isolated from the vagina of nonpregnant, healthy, premenopausal women was identified as *Lactobacillus rhamnosus* L60 by 16S rDNA sequence homology. L60 was evaluated for antimicrobial activity, in vitro antibiotic resistance, autoaggregation, surface hydrophobicity, co-aggregation with other bacterial species, hydrogen peroxide (H₂O₂) production, and bacterial adherence. It displayed a wide spectrum of antimicrobial activity against urogenital pathogens, and resistance to antibiotics commonly prescribed for infections caused by these pathogens. L60 produced H₂O₂, adhered to vaginal epithelial cells, co-aggregated with *Escherichia coli* and *Candida albicans*, and displayed self-aggregation. In view of these characteristics, L60 is considered a potential probiotic, and will be further evaluated for preventive and therapeutic application locally in the vaginal tract.

Key Words—antimicrobial activity; bacteriocin; *Lactobacillus rhamnosus*; probiotic

Introduction

The vaginal flora in healthy premenopausal women contain predominantly *Lactobacillus* species (10⁷–10⁸ cfu/g fluid), which prevent urogenital tract infection (UTI). Reduction or elimination of lactobacilli increases the risk of UTI (Lepargneur and Rousseau, 2002; Reid and Burton, 2002; Vásquez et al., 2002).

Some lactobacilli play a protective role by producing compounds such as hydrogen peroxide (H₂O₂),

lactic acid, and bacteriocins, which inhibit the growth of potential pathogens (Reid et al., 2004). Other functions of lactobacilli include competitive exclusion of pathogens from the cell surface, co-aggregation with certain pathogenic bacteria, adherence to epithelial cells, and biofilm formation based on autoaggregation and surface hydrophobicity (Dunne et al., 2001). Previous studies indicate that autoaggregation of probiotic strains is necessary for adherence to vaginal epithelial cells, and that co-aggregation leads to formation of a barrier that prevents colonization by pathogens (Boris et al., 1998; Reid et al., 1990; Zhou et al., 2004). Most bacteriocins from lactic acid bacteria have been isolated from species of the genus *Lactobacillus*, probably because of the diversity of its species and habitats (Eijsink et al., 1998). A majority of the *L. fermentum*

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and *L. acidophilus* strains produce bacteriocins (Tahara and Kanatani, 1997). Lactacin B was the first antimicrobial peptide from *L. acidophilus* that was clearly identified as a bacteriocin (Barefoot and Klaenhammer, 1984).

We report here the beneficial properties of *L. rhamnosus* L60 strain as a potential probiotic for use in therapy of UTI, maintenance of urogenital tract health, and reduction of the risk of infection.

Materials and Methods

Microorganisms and culture media. *L. rhamnosus* strain L60 (hereafter termed "L60" for convenience) was isolated from vaginal swabs from nonpregnant, healthy, premenopausal patients seen at the Dept. of Obstetrics and Gynecology, Río Cuarto Hospital, Córdoba, Argentina. Lactic acid bacteria were grown in de Mann, Rogosa, and Sharpe (MRS) broth or agar for 24 h at 37°C under a 5% CO₂ atmosphere. L60 was identified by standard biochemical tests (Bergey, 1994), the API 50 CHL system (BioMérieux, France) (Nigatu et al., 2000), and 16S rRNA analysis (Cepeda and Santos, 2000). The strain was stored at -80°C in MRS broth containing 30% (v/v) glycerol.

Other bacterial strains were from clinical specimens obtained at Río Cuarto Hospital, and were identified by standard biochemical tests (Bergey, 1994). *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes* were propagated in eosin-methylene blue (E.M.B.; Britania). *Staphylococcus epidermidis*, *S. saprophyticus*, *S. aureus*, *Enterococcus faecalis*, and *Gardnerella vaginalis* were grown in human blood agar, *Candida albicans* and *C. glabrata* in Sabouraud agar (Britania), and *Neisseria gonorrhoeae* in Thayer Martin agar. Optimal incubation atmosphere was determined for each strain.

16S rRNA analysis. For preparation of bacterial DNA templates, colonies were suspended in 500 µl sterile physiological saline solution and centrifuged at 10,000 rpm for 10 min. Supernatant was removed, and the pellet was suspended in 500 µl InstaGene Matrix (Bio-Rad, Hercules, USA) (Cepeda and Santos, 2000). This suspension was incubated for 30 min at 56°C, then heated for 10 min at 100°C. The supernatant was used as a bacterial DNA template for PCR reaction. To amplify 16S ribosomal DNA, direct PCR was performed utilizing 1 µl DNA template in 20 µl PCR reaction mix-

ture containing the universal primers 27F (5'-AGAGTTT-GATCCTGGCTCAG-3') and 1492R (5'-TACGGTTAC-CTTGTTACGACTT-3') (Lane, 1991) (numbering is based on the *Escherichia coli* 16S rRNA gene (Brosius et al., 1978). Amplification was conducted for 35 cycles, at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s. The DNA-amplified fragments were purified of unincorporated PCR primers and dNTPs using a Montage PCR Clean-up kit (Millipore). Purified PCR products of approximately 1,400 bp were sequenced with an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) by Macrogen, Inc. Laboratories (Korea). The 16S rRNA gene sequences were (i) subjected to the BLAST search program (National Center for Biotechnology Information) (Altschul et al., 1997) to find similar sequences, and (ii) aligned using CLUSTAL W software (Thompson et al., 1994) with reference sequences obtained from the RDP Select Sequence function (Maidak et al., 2001) and GenBank.

Antimicrobial activity. Inhibitory activity of L60 was measured by an agar spot test. Indicator microorganisms used for detection were: *E. coli*, *P. mirabilis*, *K. pneumoniae*, *E. aerogenes*, *S. epidermidis*, *S. saprophyticus*, *S. aureus*, *E. faecalis*, *G. vaginalis*, *C. albicans*, *C. glabrata*, and *N. gonorrhoeae*. The surface of a Mueller Hinton agar plate (or Thayer Martin agar plate in the case of *N. gonorrhoeae*) was sown from a standardized suspension of each indicator microorganism. L60 was cultured for 24 h at 37°C in MRS broth, culture supernatant was neutralized, and 100 µl was spotted onto the surface of the agar plate and incubated for 24 h at 37°C. Inhibitory effect of L60 on bacterial growth was measured as described previously (Barberis et al., 2002).

Preliminary characterization of antimicrobial substances. Effects of trypsin, protease type VI, and urease on antimicrobial activity were tested. Each enzyme was prepared at a concentration of 1 mg/ml, added to the crude supernatant sample (final concentration 0.5–0.25 mg/ml), incubated for 1 h at 37°C, and the inhibitory activity was tested. Next, the crude supernatant sample was treated with catalase (final concentration 5 mg/ml) and dissolved in 50 mmol⁻¹ KH₂PO₄ buffer (pH 7.0) at 25°C to completely eliminate possible inhibitory effect of H₂O₂. Finally, heat sensitivity and pH stability were tested. The crude bacteriocin sample was heated for 60 min at 100°C and for 10 min at 121°C before testing the activity. To test the influence of pH,

the sample was adjusted to a pH of 2.0, 4.0, 5.0, 6.0, 7.0, 9.0, or 11.0 with HCl or NaOH, mixed, left standing for 5 h, and inhibitory activity was tested (Ali et al., 1995; Benoit et al., 1994). For testing temperature and enzyme sensitivity, and pH stability, the remaining activity was assayed by the agar well diffusion method and compared with the activity of a corresponding control. *E. coli* was used as an indicator strain. To establish the inhibitory spectrum of a cell-free culture, supernatant fluid derived from L60 incubated for 24 h at 37°C in MRS broth, adjusted to pH 5.5, was spotted onto indicator lawns of various strains of lactic acid bacteria, or other Gram-positive and Gram-negative bacteria. Lawns were prepared by propagating fresh bacterial cultures to an optical density of 0.45 (at 600 nm), adding 200 µl of cell suspension to 3.5 ml of overlay agar, and incubating plates for 24 h at 37°C. For molecular size estimation, dialysis membranes of MWCO 10,000 and 7,000 Da were placed in agar MRS plates, and the producer streak was separated from the sensitive strain when an inhibitory halo was observed (Barberis et al., 1994).

In vitro antibiotic resistance. The antimicrobial resistance of L60 was determined by the disk diffusion test (Britania) described for Kirby-Bauer (Boyle et al., 1973). The antibiotic agents studied were penicillin (10 U disk), clindamycin (2 µg disk), erythromycin (15 µg disk), rifampicin (5 µg disk), nitrofurantoin (300 µg disk), cefotaxime (30 µg disk), ampicillin (10 µg disk), cephalotine (30 µg disk), trimethoprim-sulfamethoxazole (1.25/23.75 µg disk), piperacillin-tazobactam (100/10 µg disk), gentamicin (10 µg disk), amikacin (30 µg disk), pipemidic acid (20 µg disk), ofloxacin (5 µg disk), ciprofloxacin (5 µg disk), ceftacidim (30 µg disk), norfloxacin (10 µg disk), nalidixic acid (30 µg disk), vancomycin (30 µg disk), and metronidazole (5 µg disk).

Autoaggregation test. L60 was grown for 18 h at 37°C in MRS broth. Cells were resuspended in phosphate-buffered saline (PBS) (pH 6.2) to a final concentration of 5×10^9 cfu/ml. A drop was placed on a glass microscope slide and examined macroscopically and microscopically. Autoaggregation was determined as the ability to form aggregates within 2 min (Andreu et al., 1995).

Surface hydrophobicity. Surface hydrophobicity of lactobacilli was studied by the salt aggregation test (SAT). Bacteria were resuspended in 0.02 mol/L sodium phosphate (pH 6.8) to a final concentration of

5×10^9 cfu/ml. Solutions of ammonium sulfate (4.0, 2.0, 1.5, 0.5 mol/L) were mixed with an equal volume of cell suspension on a glass slide. The lowest final concentration of ammonium sulfate causing the bacteria to aggregate was defined as the SAT value. Strains were classified into three groups: high surface hydrophobicity (SAT <0.9 mol/L), intermediate hydrophobicity (SAT 0.9–1.5 mol/L), and hydrophilic (SAT >1.5 mol/L) (Andreu et al., 1995).

Co-aggregation assays. Suspensions of lactobacilli in PBS were adjusted to optical density (at 600 nm)=0.6. Aliquots (500 µl) of L60 were mixed with an equal volume of *E. coli*, *G. vaginalis*, *C. albicans*, or *C. glabrata* strain by vortexing at 100 rpm for 4 h, and incubated at 37°C. Suspensions were Gram-stained and observed under a microscope. A co-aggregation assay was positive if L60 formed aggregates with the other strain (Reid et al., 1990).

H₂O₂ production. H₂O₂ production was studied by the qualitative method of Eschenbach et al. (1989). L60 was plated onto 20 ml MRS agar containing 5 mg 3,3',5,5'-tetramethylbenzidine (TMB; 87748; Fluka Chemie AG) and 0.20 mg horseradish peroxidase (ICN Biomedicals, Inc.). Peroxidase generates O₂ from any H₂O₂ produced by the lactobacilli, and TMB stains colonies blue in the presence of O₂. After 48 h incubation under 5% CO₂ in air, colonies that produce H₂O₂ on MRS agar appear dark blue, whereas nonproducers are colorless (Felten et al., 1999).

Bacterial adherence assay. Vaginal epithelial cells were collected on menstrual cycle days 17 and 18 from a healthy volunteer who was not using a spermicidal product or oral contraceptive, was not receiving antibiotics, and had no known vaginal pathology. Cells were washed with and suspended in $1 \times$ Earle's medium. Overnight cultures of lactobacilli to be tested were suspended (10^8 cells/ml) in Earle's medium. Equal volumes of bacterial suspension and vaginal cells were mixed and incubated at 37°C with rotary shaking (100 rpm/min) for 30 min. The resulting suspension was passed through 8–10 µm pore size Millipore filters and washed with 1 volume Earle's medium. Cells retained on the filter were placed on albumin-coated and non-coated microscope slides, fixed with ethanol, and Gram-stained (Bonetti et al., 2003). Adherence was assayed by counting the number of bacteria adhered to the first 25 intact epithelial cells seen. Two controls were used for each trial: (i) assay without lactobacilli added, to measure indigenous lactobacilli

remaining on vaginal cells after repeated washes; (ii) assay including L60, to measure reproducibility (Reid and Burton, 2002).

Statistical analysis. All experiments were performed in duplicate. Counts of adherent bacteria on epithelial cells were expressed as mean $\pm$ standard deviation. Data were analyzed by Student's *t* test (Microsoft Excel software).

Results

Selection and identification of *Lactobacillus*

The isolated strain was identified as *L. rhamnosus* L60 by biochemical tests and the API 50 CHL system (BioMérieux). The identification was confirmed by 16S rDNA sequence analysis and registered in the GenBank database system (<http://www.ncbi.nlm.nih.gov/sites/entrez>) under accession number EF495247 (1,402 bp). 16S rDNA of the isolate was 99% identical with that of other *L. rhamnosus* strains registered in the GenBank database system (Table 1).

Antimicrobial activity and antibiotic resistance of L60

The L60 strain was selected based on a wide spectrum of antimicrobial activity against urogenital pathogens (Table 2), and resistance to antibiotics commonly applied for infections caused by these pathogens. The

strain was resistant to ofloxacin, ciprofloxacin, ceftacide, norfloxacin, nalidixic acid, vancomycin, and metronidazole.

Characterization of antimicrobial substance

The inhibitory effect of L60 on bacterial growth was not significantly altered by heat treatment, suggesting that the antimicrobial substance produced is strongly resistant to heat. Effect of pH variation on antimicrobial activity was tested after 5 h incubation in MRS agar. All samples showed inhibitory activity in a wide range from pH 4.0 to 7.0, with optimal activity at pH 5. The antimicrobial activity was more stable at acid and neutral pHs. At alkaline pH, the strain became progressively inactive. The antimicrobial substance was a dialyzable molecule smaller than 7,000 Da. Effects of enzymes (trypsin, protease VI) on the antimicrobial activity were also tested. The activity was completely eliminated by trypsin, indicating that the inhibitory material was proteinaceous; it was therefore referred to as a bacteriocin. Catalase and urease had no effect on the activity.

Probiotic properties

L60 showed self-aggregation, producing macroscopic granules; the phenomenon was clearly observable under the light microscope. The strain showed

Table 1. *Lactobacillus* identified on the basis of percent similarity to 16S sequences in GenBank.

Species identification	GenBank accession no. ^a	% Similarity ^b
<i>Lactobacillus rhamnosus</i> L60	EF495247	100
<i>Lactobacillus rhamnosus</i> IDCC 3201	EF533991	99
<i>Lactobacillus rhamnosus</i> LV108	EF534204	99
<i>Lactobacillus rhamnosus</i>	AM491821	99
<i>Lactobacillus rhamnosus</i> MNFLM01	AB288235	99
<i>Lactobacillus rhamnosus</i> LR2	AY675254	99
<i>Lactobacillus rhamnosus</i> F11	AF243146	99
<i>Lactobacillus rhamnosus</i>	AY370680	99
<i>Lactobacillus rhamnosus</i> LR3	AY675253	99
<i>Lactobacillus rhamnosus</i> MCRF-412	AY299488	99
<i>Lactobacillus rhamnosus</i> 11D	AY773958	99
<i>Lactobacillus rhamnosus</i> strain P15	AY852248	99
<i>Lactobacillus</i> sp. BCRC16000	AY773946	99
<i>Lactobacillus</i> sp.	AY363384	99
<i>Lactobacillus casei</i>	AB008205	98

^a GenBank accession no. based on 16S sequences available on the GenBank database system (<http://www.ncbi.nlm.nih.gov/sites/entrez>).

^b % Similarity of 16S sequences available in GenBank, compared to EF495247 sequence.

Table 2. Inhibition of various urogenital pathogens by the bacteriocin produced by *L. rhamnosus* strain L60.

Pathogenic microorganism	Number of strains	Inhibitory halo (mm)	Inhibited strains (%)
<i>Escherichia coli</i>	98	39	100
<i>Proteus mirabilis</i>	12	40	100
<i>Klebsiella pneumoniae</i>	6	36	100
<i>Enterobacter aerogenes</i>	5	49	100
<i>Gardnerella vaginalis</i>	8	32	100
<i>Neisseria gonorrhoeae</i>	3	12	100
<i>Staphylococcus epidermidis</i>	10	21	100
<i>Staphylococcus saprophyticus</i>	4	30	100
<i>Staphylococcus aureus</i>	2	36	100
<i>Enterococcus faecalis</i>	6	31	100
<i>Candida albicans</i>	10	20	100
<i>Candida glabrata</i>	3	0	0

(A)

(B)

Fig. 1. Microscopic observation of co-aggregation between *L. rhamnosus* strain L60 and *E. coli* (A), and lack of co-aggregation between L60 and *C. glabrata* (B).

high hydrophobicity by SAT assay with a minimal ammonium sulfate concentration of 0.5 mol/L. L60 showed co-aggregation with *E. coli*, *G. vaginalis*, and *C. albicans*, but not with *C. glabrata*. Figure 1A shows the microscopic appearance of aggregates formed between *E. coli* and L60, in contrast to isolated *C. glabrata* cells surrounding L60 aggregates (Fig. 1B). This property may constitute an important defense mechanism against infection in the urogenital tract. In addition, L60 produced H_2O_2 , as indicated by the color change of colonies exposed to air following anaerobic growth on TMB agar.

Adherence to vaginal epithelial cells differed greatly between cells treated vs. not treated with L60 ($p < 0.001$). Control (non-treated) epithelial cells showed an adherence of 9.17 ± 3.12 lactobacilli/cell, in contrast to 36.12 ± 3.98 for treated epithelial cells (Table 3). One out of the 25 observed cells for each treatment is shown in Fig. 2A (control) and Fig. 2B (L60-treated).

Table 3. Adherence of *L. rhamnosus* L60 to vaginal epithelial cells.

	Number of L60 adhered ^a
Control	9.17 ± 3.12
Treated with L60	36.12 ± 3.98

^a Number of *L. rhamnosus* L60 adhered to control (non-treated) and treated vaginal epithelial cells (mean $\pm$ standard deviation of 25 repetitions). Difference between the means is significant ($p < 0.01$).

(A)

(B)

Fig. 2. Adherence of *L. rhamnosus* strain L60 to epithelial cells.

A: control. B: cells treated with L60.

Discussion

Lactobacillus rhamnosus strain L60 was isolated, and identified on a biochemical basis (API 50 CHL system) and molecular basis (16S rDNA sequence analysis).

The bacteriocin produced by L60 was partially characterized. It was sensitive to protease, indicating that

the inhibitory material was proteinaceous, and inhibited under conditions which eliminate the effects of organic acids. Its antimicrobial activity was not abolished by treatment with catalase or urease.

Like most of the known bacteriocins produced by *Lactobacillus* strains, the inhibitory material is a heat-stable and low molecular mass (<7,000 Da) peptide. The bacteriocin activity was stable at a wide pH range (4.0–7.0). According to these biochemical characteristics, L60 bacteriocin is a “class II lactic acid bacterium bacteriocin” under the classification of Kemperman et al. (2003).

Tests of antimicrobial activity of L60 showed inhibition of Gram-positive aerobic cocci and Gram-negative rods, similar to the results of Strus et al. (2002). Rates of bacterial resistance to antibiotics used for treatment of urogenital disease were high. Susceptibility to antibiotics is an important consideration in development of probiotic agents. L60 strain was selected based on resistance to seven antibiotics. Our results are partially consistent with those from previous studies (Masco et al., 2006; Zhou et al., 2005).

Necessary characteristics for a *Lactobacillus* strain to serve as an effective prophylactic agent include avid adherence to vaginal epithelial cells, interference with the adherence of other bacteria, production of bacteriocin, and production of H₂O₂ capable of inhibiting the growth of pathogens (Zhou et al., 2004). L60 showed high hydrophobicity, a result similar to that of Juarez Tomas et al. (2005), who concluded that hydrophobicity is an important mechanism in bacterial adherence. Millsap et al. (1996) showed that highly negatively charged, hydrophilic lactobacilli adhered minimally, whereas *Lactobacillus* strains with a slightly negatively charged, hydrophobic cell surface adhered strongly (Felten et al., 1999).

L60 co-aggregated with *E. coli*, *G. vaginalis*, and *C. albicans*, but not with *C. glabrata*, results similar to those of Boris et al. (1998). Such co-aggregation could be an important factor in maintaining vaginal health because it produces an area around the pathogen where the concentration of antimicrobial substances produced by the lactobacilli is increased. L60 also produces H₂O₂, whose toxicity is due to the oxidative effect of the molecule itself or its metabolites (OH, O₂) (Tomas et al., 2004).

L60 showed a high capacity for adherence. Mean numbers of human urogenital lactobacilli adhering to uroepithelial cells ranged from 8–67 in studies by Boris

et al. (1998), and 23–48 in studies by Mastromarino et al. (2002). Numbers of adhering lactobacilli in the present study were comparable. Such adherence may promote colonization of the vaginal epithelium through formation of a bacterial “film” that tends to exclude pathogens from the mucosa (Aroutcheva et al., 2001; Reid, 1999).

In conclusion, the lactobacilli used in this study may protect the vaginal epithelium through a series of barrier mechanisms (adherence), interference mechanisms (co-aggregation with potential pathogens), and production of antimicrobial substances. They appear to be excellent candidates for development as prophylactic agents. Further studies are needed to evaluate their immunomodulatory capabilities. The L60 strain has been selected for further studies of its probiotic properties and possible therapeutic application locally in the vaginal tract.

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