BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 43 (04) 325-408 April 2010

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, March 2010, Volume 43(4) 338-344

Identification and *in vitro* production of *Lactobacillus* antagonists from women with or without bacterial vaginosis

K.M.G.R. Branco, R.M.D. Nardi, J.L.S. Moreira, A.C. Nunes, L.M. Farias, J.R. Nicoli and M.A.R. Carvalho

The Brazilian Journal of Medical and Biological Research is partially financed by

CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico
Ministério da Ciência e Tecnologia
CAPES
Ministério da Educação
BRASIL UM PAÍS DE TODOS GOVERNO FEDERAL
FAPESP

Institutional Sponsors

SciELO
USP UNIVERSIDADE DE SÃO PAULO
USP Campus Ribeirão Preto
Faculdade de Medicina de Ribeirão Preto
FAEPA HCFMRP
AFIP
UNICAMP
SHIMADZU
GE Healthcare

Brazilian Journal of Medical and Biological Research (2010) 43: 338-344
ISSN 0100-879X

Identification and *in vitro* production of *Lactobacillus* antagonists from women with or without bacterial vaginosis

K.M.G.R. Branco[1], R.M.D. Nardi[1], J.L.S. Moreira[2], A.C. Nunes[2], L.M. Farias[1]*, J.R. Nicoli[1]* and M.A.R. Carvalho[1]*

[1]Departamento de Microbiologia, [2]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Abstract

Lactobacilli isolated from the vaginal tract of women with and without bacterial vaginosis (BV) were identified and characterized for the production of antagonists. Bacterial samples were isolated from healthy women (N = 16), from patients with clinical complaints but without BV (N = 30), and from patients with BV (N = 32). Identification was performed using amplified ribosomal DNA restriction analysis. Production of antagonistic compounds was evaluated by the double-layer diffusion technique using Gram-positive (N = 9) and Gram-negative bacteria (N = 6) as well as yeast (N = 5) as indicator strains. Of a total of 147 isolates, 133 were identified as pertaining to the genus *Lactobacillus*. *Lactobacillus crispatus* was the species most frequently recovered, followed by *L. johnsonii* and *L. jensenii*. Statistical analysis showed that *L. crispatus* was more frequent in individuals without BV ($P < 0.05$). A higher production of antagonists was noted in *L. crispatus* isolates from healthy women ($P < 0.05$). More acidic local pH and higher H_2O_2 production by isolated lactobacilli from healthy women suggest these mechanisms as the possible cause of this antagonism. In conclusion, a significant correlation was detected between the presence and antagonistic properties of certain species of *Lactobacillus* and the clinical status of the patients.

Key words: *Lactobacillus*; Bacterial vaginosis; Antagonism; Hydrogen peroxide; Identification

Introduction

A healthy vaginal ecosystem is generally dominated by certain species of *Lactobacillus*, which exert a significant influence on the microbiology of the vagina (1). They play a protective role mainly by a combination of steric exclusion and the production of inhibitory substances (2). The lactobacilli metabolize glucose essentially to lactic acid, which contributes to the maintenance of a low vaginal pH (4.0-4.5) and reduces the growth of most pathogenic microorganisms. Many isolates of vaginal lactobacilli also produce H_2O_2, a compound having broad antimicrobial activity. Women colonized by H_2O_2-producing lactobacilli have been associated with decreased susceptibility to human immunodeficiency virus (HIV) infection (3) and bacterial vaginosis (BV) (4,5). However, *in situ* H_2O_2 production by these lactobacilli has never been demonstrated.

Bacterial vaginosis affects millions of women and is associated with several serious health problems. The cause of BV remains poorly understood despite numerous studies based on cell cultures (6). Bacterial vaginosis is characterized by a depletion of *Lactobacillus* spp and an overgrowth of diverse aerobic, anaerobic and micro-aerophilic microorganisms such as *Gardnerella vaginalis*, *Prevotella* spp, *Peptostreptococcus* spp, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Mobiluncus* spp (7). A recent study using ribosomal DNA sequences showed that women with BV had higher bacterial diversity with many newly recognized species. Data obtained from the vaginal ecosystem of healthy women showed a lower bacterial diversity and confirmed the predominance of *Lactobacillus* species (8). These data suggest that, in contrast to the gastrointestinal microbial ecosystem, where potent beneficial functions of the microbiota are related to a rich biodiversity, a healthy vaginal ecosystem is characterized by a low microbial diversity consisting almost exclusively of predominant lactobacilli.

Correspondence: J.R. Nicoli, Departamento de Microbiologia, Instituto de Ciências Biológicas, UFMG, Caixa Postal 486, 30161-970 Belo Horizonte, MG, Brasil. Fax: +55-31-3409-2730. E-mail: jnicoli@icb.ufmg.br

*These authors contributed equally to this study.

Received July 3, 2009. Accepted February 11, 2010. Available online March 5, 2010. Published April 12, 2010.

Additionally, recent data indicate that female genital-tract HIV load correlates inversely with *Lactobacillus* species and positively with BV (9).

There is increasing interest in the selection of particular species of *Lactobacillus* to serve as vaginal probiotics for the prevention of sexually transmitted infections and BV. The strains are generally selected on the basis of their dominance in the healthy vaginal ecosystem, their ability to inhibit BV pathogens *in vitro*, their high acid production, their production of H_2O_2, and their ability to bind exfoliated vaginal epithelial cells *in vitro*.

In the present study, *Lactobacillus* isolated in a previous study from the vaginal tract of patients with and without BV was identified by amplified ribosomal DNA restriction analysis (ARDRA) and characterized for the *in vitro* production of antagonistic substances to evaluate if some correlation exists between the presence and antagonistic properties of certain species of *Lactobacillus* and the clinical status of the patients.

Material and Methods

Ethical aspects

This project was approved by the Ethics Committee of the Federal University of Minas Gerais (ETIC #062/03) and written informed consent was obtained from all subjects before inclusion in the study.

Patients

The bacterial samples were isolated from healthy women (N = 16), patients with clinical complaints (human papilloma virus, herpes, candidiasis), but without BV (N = 30), and patients with BV (N = 32). Patients were screened at the Gynecology Service, University Hospital, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

Lactobacillus isolates

A total of 147 bacteria isolated from the subjects were selected as presumptive *Lactobacillus* based on the following characteristics: Gram-positive micro-aerophilic and catalase-negative rods isolated on de Man, Rogosa and Sharp (MRS) agar (Merck, Germany) at 37°C. The bacterial samples were maintained at -80°C in MRS broth medium (Difco, USA) supplemented with 10% glycerol.

DNA extraction

Chromosomal DNA was isolated from overnight cultures of all isolates in 10 mL MRS broth. After washing the cells with deionised water, the pellet was obtained by centrifugation at 14,000 *g* for 5 min at 4°C, suspended in 1 mL 5 M LiCl, and incubated for 1 h with constant shaking. After a second washing with 1 mL deionized water, the pellet was suspended in 1 mL protoplasting buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mg/mL lysozyme, 100 µg/mL RNAse). After incubation for 1 h at 37°C and centrifugation at 14,000 *g* for 5 min at 4°C, the pellet was suspended in 500 µL protoplasting buffer without lysozyme and 100 µL 10% sodium dodecyl sulfate was added to allow cells to lyse. After lysis, the mixture was extracted once with phenol, phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). After isopropanol precipitation, the DNA was dissolved in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR amplification of the 16S-23S rDNA intergenic spacer

The 16S-23S intergenic spacer region was amplified by the method of Moreira et al. (10) using the primer 16-1A (GAATCGCTAGTAATCG) that anneals to a conserved region of the 16S rRNA genes, and primer 23-1B (GGGTT CCCCCATTCGGA) that anneals to a conserved region of the 23S rRNA genes using a PTC-100® Thermal cycler (MJ Research Inc., USA). The reaction mixture (50 µL) contained 10 pM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, reaction buffer containing 1.5 mM $MgCl_2$, 5 U Taq DNA polymerase (Phoneutria Biotecnologia & Serviços, Brazil), and 5 µL of template DNA solution. The amplification program was 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and finally 72°C for 10 min. PCR products were electrophoresed on 1.4% agarose gel and visualized by UV transillumination after staining with an ethidium bromide solution (5 µg/mL).

Amplified ribosomal DNA restriction analysis

ARDRA of 16S-23S rRNA intergenic regions was performed by the method of Moreira et al. (10). Briefly, the 16S-23S rRNA intergenic spacer regions of lactic acid bacteria were amplified by PCR and digested with a set of 12 enzymes chosen after compilation of nucleotide sequences already deposited at GenBank and *in silico* restriction digestion using the Webcutter 2.0 tool (Max Heiman 1997; http://rna.lundberg.gu.se/cutter2/). *Sph*I, *Nco*I and *Nhe*I enzymes hydrolyzed inside the 16S gene; *Ssp*I, *Sfu*I, *Dra*I, *Vsp*I, *Hin*cII, and *Eco*RI enzymes hydrolyzed inside the intergenic region, and *Avr*II and *Hin*dIII enzymes hydrolyzed inside the 23S gene. *Eco*RV enzyme hydrolyzed inside the spacer region for the *L. casei* group and in the 23S gene for *L. acidophilus* group. For several lactobacillus species no spacer nucleotide sequences have been reported and only fragments of the 16S and/or 23S genes were found. All restriction enzymes were purchased from Promega Corporation (USA).

In vitro antagonism assay

The isolated bacteria were cultured in MRS broth for 24 h at 37°C in an anaerobic chamber (Forma Scientific Company, USA) containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. After growth, an aliquot (5 µL) of the culture was spotted onto MRS agar (Difco). After incubation at 37°C for 48 h under anaerobic conditions, the cells were killed

by exposure to chloroform for 20 min. Residual chloroform was allowed to evaporate and Petri dishes were overlaid with 3.5 mL brain heart infusion, MRS or Sabouraud soft agar (0.7%) (Difco), which had been inoculated with 0.2 mL of a 24-h culture of *L. casei* ATCC 334, *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4356, *L. delbrueckii* subsp lactis ATCC 7830, *L. brevis* ATCC 367, *L. fermentum* ATCC 9338, *Staphylococcus aureus* ATCC 33591, *Enterococcus faecalis* ATCC 4083, *Prevotella intermedia* ATCC 25611, *Bacteroides fragilis* ATCC 25285, *Bifidobacterium bifidum* ATCC 29521, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 27853, *Gardnerella vaginalis* ATCC 14018, *Mobiluncus mulieris* ATCC 35239, *Candida albicans* ATCC 18804, *C. glabrata* ATCC 2001, *C. krusei* ATCC 20298, *C. parapsilosis* ATCC 22019, or *C. tropicalis* ATCC 750. After 24 h of incubation at 37°C under aerobic or anaerobic conditions depending on the indicator strain, the antagonistic activity was determined as the presence of a growth inhibition zone using the method of Tagg as modified by Gomes et al. (11).

Determination of hydrogen peroxide production

To test for the production of H_2O_2 by lactobacillus isolates, a qualitative assay on tetramethylbenzidine (TMB; Sigma, USA) agar plates was done as described by Rabe and Hillier (12). Lactobacilli were inoculated onto TMB agar plates and incubated in the anaerobic chamber at 37°C. After 40 h, the plates were exposed to ambient air. If the lactobacilli produce H_2O_2, a reaction occurred with horseradish peroxidase (Sigma) present in the medium, which oxidizes TMB, causing the colonies to turn blue. *L. acidophilus* ATCC 4356 was used as control.

Statistical analysis

Data were analyzed using the Minitab Release 14.20 software (Minitab Inc., Copyright© 2005). The Mann-Whitney rank sum test, the Fisher exact test and Kruskal-Wallis one-way analysis of variance followed by pair-wise multiple comparison using the Student-Newman-Keuls method were used. $P < 0.05$ was considered to be statistically significant.

Results

A total of 147 presumptive *Lactobacillus* samples were isolated in a previous study (Soares-Brandão KLK, unpublished results) from healthy women, patients with clinical complaints but without BV, and patients with BV.

Among these isolates, 133 were confirmed as pertaining to the genus *Lactobacillus* by ARDRA and then characterized for *in vitro* production of antagonistic substances. Table 1 shows that the frequency of subjects presenting at least one *Lactobacillus* isolate was apparently higher in healthy women (87.5%) than in the two other groups (66.7 and 65.6% for patients with clinical complaints but without BV and patients with BV, respectively). However, statistical

Table 1. Total number and percent of women harboring the *Lactobacillus* species in dominant levels isolated from healthy women, from patients with complaints but without bacterial vaginosis (BV), and from patients with BV.

	Healthy (N = 16)	With complaints (N = 30)	With BV (N = 32)
*Lactobacillus**	14 (87.5)[a]	20 (66.7)[a]	21 (65.6)[a]
L. crispatus	15 (57.1)[a]	24 (60.0)[a]	5 (13.6)[b]
L. johnsonii	6 (21.4)[a,b]	9 (15.0)[a]	15 (45.5)[b]
L. jensenii	2 (21.4)[a]	8 (20.0)[a]	13 (36.4)[a]
L. reuteri	1 (7.1)	1 (5.0)	5 (18.2)
L. rhamnosus	1 (7.1)	1 (5.0)	4 (13.6)
L. agilis	1 (7.1)	3 (15.0)	1 (4.6)
L. gasseri	1 (7.1)	1 (5.0)	2 (9.1)
L. acidophilus	1 (7.1)	2 (5.0)	0 (0)
L. delbrueckii	2 (14.3)	0 (0)	0 (0)
L. vaginalis	0 (0)	0 (0)	2 (9.1)
L. fermentum	0 (0)	0 (0)	2 (3.6)
L. brevis	1 (7.1)	0 (0)	0 (0)
Lactobacillus spp	0 (0)	0 (0)	4 (9.1)
Total	31	49	53

*Subjects with at least one *Lactobacillus* isolate. Isolates were identified by amplified ribosomal DNA restriction analysis using the 16S-23S intergenic regions. Data are reported as total number with percent in parentheses. [a,b]Different letters on the same line indicate significant differences by the Fisher exact test ($P < 0.05$).

Table 2. Mean antagonism frequency (% ± SD) against all indicator strains produced by *Lactobacillus crispatus*, *L. johnsonii* and *L. jensenii* isolated from healthy women, from patients with complaints but without bacterial vaginosis (BV), and from patients with BV.

	L. crispatus	*L. johnsonii*	*L. jensenii*
Healthy	63.7 ± 30.9^{a1} (15)	45.8 ± 38.5^{a2} (6)	47.5 ± 37.9^{a2} (2)
With complaints	35.5 ± 23.8^{b1} (24)	42.7 ± 31.6^{a1} (9)	15.0 ± 19.4^{b2} (8)
With BV	18.0 ± 22.4^{c1} (5)	25.3 ± 22.3^{b1} (15)	21.8 ± 19.9^{b1} (13)

The total number of *Lactobacillus* isolates of each species tested against 20 indicator strains is reported in parentheses. ^{a,b,c}Different letters in the same column indicate significant differences by Kruskal-Wallis one-way analysis of variance followed by pairwise multiple comparison using the Student-Newman-Keuls test ($P < 0.05$). ^{1,2}Different numbers on the same line indicate significant differences by Kruskal-Wallis one-way analysis of variance followed by pairwise multiple comparison using the Student-Newman-Keuls test ($P < 0.05$).

Table 3. Susceptibility (%) of each indicator strain to antagonism produced by all *Lactobacillus* species isolated from healthy women, from patients with complaints but without bacterial vaginosis (BV), or from patients with BV.

	Healthy (N = 16)	With complaints (N = 30)	With BV (N = 32)
L. acidophilus	90.2* (92.9)**	51.0 (85.0)	22.6 (36.4)
L. brevis	22.6 (28.6)	6.1 (5.0)	15.1 (22.7)
L. plantarum	61.3 (64.3)	20.4 (35.0)	15.1 (18.2)
L. delbrueckii	71.0 (64.3)	34.7 (70)	18.9 (27.3)
L. fermentum	83.9 (76.6)	34.7 (75.0)	28.2 (36.4)
L. casei	35.5 (35.7)	24.5 (45.0)	20.8 (31.8)
S. aureus	51.6 (85.7)	26.5 (75.0)	22.6 (31.8)
B. bifidum	38.7 (64.3)	32.7 (55.0)	15.1 (22.7)
E. faecalis	25.8 (28.6)	8.2 (15.0)	11.3 (4.6)
G. vaginalis	77.4 (64.3)	59.2 (95.0)	20.8 (40.9)
M. mulieris	74.2 (78.6)	32.7 (90.0)	11.3 (31.8)
B. fragilis	93.6 (85.7)	69.4 (90.0)	75.4 (95.5)
P. intermedia	77.4 (92.9)	65.3 (100)	75.4 (81.8)
P. aeruginosa	38.7 (28.6)	6.1 (10.0)	17.0 (4.6)
P. vulgaris	58.1 (64.3)	28.6 (35.0)	22.6 (27.3)
C. krusei	0 (0)	0 (0)	1.9 (4.6)
C. parapsilosis	25.8 (24.4)	8.2 (20.0)	3.8 (9.1)
C. albicans	48.4 (57.1)	26.5 (50.0)	13.2 (27.3)
C. glabrata	54.8 (71.4)	22.5 (30.0)	7.6 (18.2)
C. tropicalis	77.4 (64.3)	55.1 (80.0)	22.6 (36.4)
Mean	55.3^{a} (58.6)^{a}	30.6^{b} (53.0)^{a}	22.1^{b} (30.5)^{b}

*Number of *Lactobacillus* positive for antagonism/total number of *Lactobacillus* tested. Positive/negative tests were determined as presence/absence of inhibitory zone. **Number of patients with *Lactobacillus* positive for antagonism/total number of patients. ^{a,b}Different letters indicate significant differences by the Mann-Whitney rank sum test ($P < 0.05$).

analysis of these data showed a tendency but not a significant difference ($P = 0.10$). Considering all groups, *L. crispatus* was the species most frequently recovered (33.1% of the 133 isolates), followed by *L. johnsonii* (22.6%) and *L. jensenii* (17.3%). In healthy women, a mean value of 1.94 *Lactobacillus* species/individual was observed. Statistical analysis showed that *L. crispatus* was more frequent in individuals without BV ($P < 0.05$), whereas *L. johnsonii* and *L. jensenii* tended to be most frequently found in patients with BV.

Table 2 shows the overall antagonistic capacity as the mean growth inhibition frequencies against all the indicator strains of the three more frequently isolated *Lactobacillus* species (*L. crispatus*, *L. johnsonii*, and *L. jensenii*). *L. crispatus* isolates from healthy women showed the highest antagonistic or inhibitory ability ($P < 0.05$), either when compared with the two other *Lactobacillus* species from the same group of women or when compared with *L. crispatus* isolates from the other two patient groups. In patients with BV, all the *Lactobacillus* isolates, independently of the species, showed similar low antagonistic or inhibitory capacity.

Table 3 shows that, independently of indicator strains, a higher frequency ($P < 0.05$) of antagonism was noted for *Lactobacillus* species isolated from healthy women (55.3%) when compared to those from both patients with complaints but without BV (30.6%) and with BV (22.1%). Among indicator strains, *L. acidophilus*, *L. fermentum* and *B. fragilis* were the most sensitive, followed by important pathogens such as *G. vaginalis* and *M. mulieris*.

Table 4 shows a significantly higher frequency ($P < 0.05$) of cells producing H_2O_2 by all *Lactobacillus* species isolated from healthy women (all of them, except one *L. brevis* isolate, 96.8%) when compared to those from both patients with complaints but without BV (65.3%) or with BV (35.8%). This difference is due to a progressive decrease of antagonistic activity for practically all *Lactobacillus* species, but with a higher reduction for *L. jensenii* and *L. johnsonii* isolates. For example, isolates of the three predominant species produced more frequently H_2O_2

Table 4. Number and percent of *Lactobacillus* species producing hydrogen peroxide isolated from healthy women, from patients with complaints but without bacterial vaginosis (BV) and from patients with BV.

	Healthy (N = 16)	With complaints (N = 30)	With BV (N = 32)	Total (N = 78)
L. crispatus	15/15 (100)[a]	20/24 (83.3)[ab]	2/5 (40.0)[a]	37/44 (84.1)
L. johnsonii	6/6 (100)[a]	1/9 (11.1)[b]	4/15 (26.7)[b]	11/30 (36.7)
L. jensenii	2/2 (100)[a]	5/8 (62.5)[a]	2/13 (15.4)[b]	9/23 (39.1)
L. reuteri	1/1 (100)	1/1 (100)	1/5 (20.0)	3/7 (42.9)
L. rhamnosus	1/1 (100)	1/1 (100)	3/4 (75.0)	5/6 (83.3)
L. agilis	1/1 (100)	2/3 (66.7)	0/1 (0)	3/5 (60.0)
L. gasseri	1/1 (100)	0/1 (0)	1/2 (50.0)	2/4 (50.0)
L. acidophilus	1/1 (100)	2/2 (100)	-	3/3 (100)
L. delbrueckii	2/2 (100)	-	-	2/2 (100)
L. vaginalis	-	-	2/2 (100)	2/2 (100)
L. fermentum	-	-	1/2 (50.0)	1/2 (50.0)
L. brevis	0/1 (0)	-	-	0/1 (0)
Lactobacillus spp	-	-	3/4 (75.0)	3/4 (75.0)
Total (%)	30/31 (96.8)[a]	32/49 (65.3)[b]	19/53 (35.8)[c]	81/133 (60.9)

Hydrogen peroxide was measured as described originally by Rabe and Hillier (12). [a,b,c]Different letters on the same line indicate significant differences by the Fisher exact test ($P < 0.05$).

($P < 0.05$) when recovered from healthy women (100% of *L. crispatus*, *L. johnsonii* and *L. jensenii*) than when isolated from patients with complaints but without BV (83.3, 11.1 and 62.5% of *L. crispatus*, *L. johnsonii*, and *L. jensenii*, respectively) or with BV (40.0, 26.7, and 15.4% of *L. crispatus*, *L. johnsonii*, and *L. jensenii*, respectively).

Determination of vaginal pH suggested a relationship between local acidic pH and BV symptoms. Most of the healthy women (93.3%) and patients with complaints but without BV (89.3%) had a vaginal pH value lower than 4.5, whereas this value was observed only in 50% of patients with BV ($P < 0.05$). Additionally, *L. crispatus* tended to be more frequently (85.7%) isolated from women with lower vaginal pH values when compared to *L. johnsonii* (64.3%, $P = 0.14$) and *L. jensenii* (61.5%, $P = 0.11$).

Discussion

A recent USA study showed that *L. acidophilus* and *L. fermentum* were the primary species colonizing the vagina of adolescent women, but the results were obtained using phenotypic identification methods such as biochemical assays (13). However, the group of organisms previously known as *L. acidophilus* was shown to be highly heterogeneous, and was divided subsequently into DNA homology groups A and B to form the six separate species: *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1), and *L. johnsonii* (B2) (14). *L. jensenii* and *L. iners* are other common lactobacilli found in the human vagina (15,16). The closely related species within the *L. acidophilus* complex are quite difficult and sometimes impossible to differentiate by phenotypic methods. Consequently, accurate genomic methods, such as ARDRA (10), are needed in order to define the *Lactobacillus* microbiota in the vagina. Additionally, some *Lactobacillus* species, such as *L. iners*, do not grow on Rogosa or MRS media traditionally used for lactobacillus recovery (16). This species seems to be dominant both among white (17) and black women (18). However, due to its culture characteristic (growth only on blood agar), its antagonistic ability is not known.

Our results are in general agreement with other studies (17,19,20) showing that only one or a few *Lactobacillus* species colonize the healthy human vagina (1.94 species/individual) and that species within the *L. acidophilus* complex predominate (*L. crispatus*). Antonio et al. (21) applying whole-chromosome DNA probes and Vásquez et al. (17) using randomly amplified polymorphic DNA analysis, TTGE multiplex PCR and 16S ribosomal DNA sequencing showed a dominance of *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii*. In a comparative study between Gram stain of vaginal smears (Nugent's criteria) and tDNA-PCR identification of vaginal cultivable species, Verhelst et al. (20) found that *L. crispatus* was the only *Lactobacillus* species linked to a single grade (as determined by Gram stain-based grading of vaginal smears), namely grade Ia, while the other lactobacilli were more evenly distributed over all specimens. In this same study, the number of *Lactobacillus* species per individual presenting grade I microbiota ranged from 1.5 to 2.0. Recent results obtained by Antonio et al. (19) also suggest that rectal colonization by *L. crispatus* may contribute to the maintenance of a healthy vaginal microbiota.

Lactic acid bacteria play a significant physiological role in the maintenance of the ecological balance mainly because their lactic acid production is responsible for a low pH level

in the tracts. In addition, they also produce many other growth inhibitory substances such as hydrogen peroxide, bacteriocins or lactocins, and some organic acids. Other mechanisms proposed for their microbial antagonism are competition for nutrients, inhibition of pathogen adhesion to surfaces, and stimulation of the immune system. The results obtained here in the *in vitro* antagonism assays (Tables 2 and 3) showed that the *Lactobacillus* isolated from the healthy women presented a higher ability to produce diffusible compounds against potential pathogenic bacteria (*G. vaginalis*, *M. mulieris*), as well as against other *Lactobacillus* species than those isolated from patients with complaints and without BV or with BV. Interestingly, *L. acidophilus* and *L. fermentum* were among the most sensitive indicator strains, and this fact might be an explanation for their low isolation frequencies (Table 1). Among the most frequently recovered *Lactobacillus* species, *L. crispatus* showed the highest antagonistic capacity, but only when isolated from healthy women (Table 2). However, the *in vitro* antagonism assay used in the present study is not specific and only demonstrates the production of one or more diffusible inhibitory substance(s), which could be H_2O_2, organic acids and/or bacteriocins.

L. crispatus and *L. jensenii* are considered to be the main H_2O_2 producers, and the presence of these bacteria has been positively associated with being white, older than 20 years, using barrier contraception, and having a low frequency of BV and gonorrhea (21). In a screening study, Ocanã et al. (22) also observed that among *Lactobacillus* strains isolated from healthy women, an *L. crispatus* strain produced the highest level of H_2O_2. A recent study indicated that douching frequently and having multiple sex partners increase the risk of the absence of protective H_2O_2-producing lactobacilli among women with BV (23). On the other hand, the same report showed that the use of metronidazole might favor the presence of H_2O_2-producing lactobacilli in the vaginal microenvironment. In the present study, *L. crispatus* was also observed to be the most frequent H_2O_2 producer among the *Lactobacillus* species isolated from healthy women. However, the same species did not exhibit this ability when recovered from patients with BV. During an 8-month study, Vallor et al. (2) showed that, in contrast to *L. crispatus* and *L. jensenii* H_2O_2-producing isolates, strains that did not produce the antimicrobial substance were rapidly lost, a fact that supports the role of this production in sustained colonization. However, as demonstrated by diffusion assays, there is not always a direct correlation between the ability for H_2O_2 production and the antagonistic activity of lactobacilli (24). In fact, the potential of H_2O_2 production by *Lactobacillus* described here and in the literature has been demonstrated by *in vitro* assays and one must be cautious when extrapolating it to the *in vivo* environment.

Many investigators believe that lactic acid production is a primary mechanism for maintaining the equilibrium of a healthy vaginal ecosystem, creating an inhospitable environment for the growth of pathogenic microorganisms. A low pH is not favorable for the growth of *G. vaginalis* (optimal pH 6.0 to 6.5) or of various obligate anaerobes, such as *P. bivia* and *Peptostreptococcus* spp (optimal pH 5.5 to 6.0) (25). The presence of lactobacilli and the number of their bacterial cells in the vaginal environment are responsible for pH changes. The results of the present study suggest that the *Lactobacillus* species is also an important factor since women with vaginal pH values lower than 4.5 are preferentially associated with *L. crispatus*. Aroutcheva et al. (1) described very different *in vitro* growth and acid production rates depending on the species of *Lactobacillus* isolated from the human vagina and these characteristics could explain the specific presence of *L. crispatus* in healthy women. Additionally, the activity of H_2O_2 is influenced by the hydrogen ion concentration in the environment. Hydrogen peroxide is stable under acidic conditions and is degraded as the hydrogen ion concentration decreases (26).

Among cultivable lactobacilli, *L. crispatus* seems to be an important species apparently associated with a healthy vaginal ecosystem. However, this bacterium is important for a healthy vaginal ecosystem not only in quantitative terms, but also for its metabolic properties (antagonism). Presumably, hydrogen peroxide and acid production is a mechanism responsible for the predominance and protective characteristics of this *Lactobacillus* species. However, other antagonistic abilities cannot be excluded, such as production of bacteriocin or competition for adhesion sites and nutrients.

Acknowledgments

The authors are grateful to Maria Gorete Barbosa Ribas, Luzia Rosa Resende and José Sérgio Barros de Souza, for valuable technical help.

References

1. Aroutcheva A, Gariti D, Simon M, Shott S, Faro J, Simoes JA, et al. Defense factors of vaginal lactobacilli. *Am J Obstet Gynecol* 2001; 185: 375-379.
2. Vallor AC, Antonio MA, Hawes SE, Hillier SL. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. *J Infect Dis* 2001; 184: 1431-1436.
3. Bowers R. 'Good bacteria' may aid in slowing HIV spread. *AIDS Alert* 2008; 23: 67-68.
4. Strus M, Brzychczy-Wloch M, Gosiewski T, Kochan P, Heczko PB. The *in vitro* effect of hydrogen peroxide on vaginal microbial communities. *FEMS Immunol Med Microbiol* 2006;

48: 56-63.
5. Cherpes TL, Hillier SL, Meyn LA, Busch JL, Krohn MA. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive *Herpes simplex* virus type 2 serology. *Sex Transm Dis* 2008; 35: 78-83.
6. Forsum U, Holst E, Larsson PG, Vasquez A, Jakobsson T, Mattsby-Baltzer I. Bacterial vaginosis - a microbiological and immunological enigma. *APMIS* 2005; 113: 81-90.
7. Srinivasan S, Fredricks DN. The human vaginal bacterial biota and bacterial vaginosis. *Interdiscip Perspect Infect Dis* 2008; 2008: 750479.
8. Dumonceaux TJ, Schellenberg J, Goleski V, Hill JE, Jaoko W, Kimani J, et al. Multiplex detection of bacteria associated with normal microbiota and with bacterial vaginosis in vaginal swabs by use of oligonucleotide-coupled fluorescent microspheres. *J Clin Microbiol* 2009; 47: 4067-4077.
9. Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, et al. Female genital-tract HIV load correlates inversely with *Lactobacillus* species but positively with bacterial vaginosis and *Mycoplasma hominis*. *J Infect Dis* 2005; 191: 25-32.
10. Moreira JL, Mota RM, Horta MF, Teixeira SM, Neumann E, Nicoli JR, et al. Identification to the species level of *Lactobacillus* isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. *BMC Microbiol* 2005; 5: 15.
11. Gomes DA, Souza AM, Lopes RV, Nunes AC, Nicoli JR. Comparison of antagonistic ability against enteropathogens by G+ and G- anaerobic dominant components of human fecal microbiota. *Folia Microbiol* 2006; 51: 141-145.
12. Rabe LK, Hillier SL. Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. *J Clin Microbiol* 2003; 41: 3260-3264.
13. Alvarez-Olmos MI, Barousse MM, Rajan L, Van Der Pol BJ, Fortenberry D, Orr D, et al. Vaginal lactobacilli in adolescents: presence and relationship to local and systemic immunity, and to bacterial vaginosis. *Sex Transm Dis* 2004; 31: 393-400.
14. Fujisawa T, Benno Y, Yaeshima T, Mitsuoka T. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). *Int J Syst Bacteriol* 1992; 42: 487-491.
15. Martin R, Soberon N, Vaneechoutte M, Florez AB, Vazquez F, Suarez JE. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol* 2008; 11: 261-266.
16. Falsen E, Pascual C, Sjoden B, Ohlen M, Collins MD. Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp. nov. *Int J Syst Bacteriol* 1999; 49 (Part 1): 217-221.
17. Vásquez A, Jakobsson T, Ahrne S, Forsum U, Molin G. Vaginal *Lactobacillus* flora of healthy Swedish women. *J Clin Microbiol* 2002; 40: 2746-2749.
18. Anukam KC, Osazuwa EO, Ahonkhai I, Reid G. *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin city, Nigeria. *Sex Transm Dis* 2006; 33: 59-62.
19. Antonio MA, Rabe LK, Hillier SL. Colonization of the rectum by *Lactobacillus* species and decreased risk of bacterial vaginosis. *J Infect Dis* 2005; 192: 394-398.
20. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, et al. Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiol* 2005; 5: 61.
21. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis* 1999; 180: 1950-1956.
22. Ocanã VS, Pesce De Ruiz Holgado AA, Nader-Macias ME. Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. *Curr Microbiol* 1999; 38: 279-284.
23. Beigi RH, Wiesenfeld HC, Hillier SL, Straw T, Krohn MA. Factors associated with absence of H_2O_2-producing *Lactobacillus* among women with bacterial vaginosis. *J Infect Dis* 2005; 191: 924-929.
24. Mastromarino P, Brigidi P, Macchia S, Maggi L, Pirovano F, Trinchieri V, et al. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. *J Appl Microbiol* 2002; 93: 884-893.
25. Biswas MK. Bacterial vaginosis. *Clin Obstet Gynecol* 1993; 36: 166-176.
26. Fontaine EA, Taylor-Robinson D. Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli. *J Appl Bacteriol* 1990; 69: 326-331.