Characterisation of potentially probiotic vaginal lactobacilli isolated from Argentinean women

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ABSTRACT

This study aims to evaluate potentially beneficial properties of 20 strains of vaginal lactobacilli isolated from women in Tucumán, Argentina, by determining acid and hydrogen peroxide production and auto-aggregation ability. The microorganisms were characterised genetically by amplified ribosomal 16S-DNA restriction analysis (ARDRA). *Lactobacillus gasseri* and *L. rhamnosus* were the predominant species identified among the 20 vaginal lactobacilli strains. Most achieved low pH values after 12 h incubation at 37°C and produced hydrogen peroxide in static culture. However, pH decrease and semi-quantitative hydrogen peroxide production of most homofermentative lactobacilli were significantly higher than those of heterofermentative lactobacilli. Of the 20 strains studied, only three demonstrated remarkable auto-aggregation patterns. Four strains were selected for possible use in a probiotic product for vaginal application; however, further *in vitro* study of other potentially probiotic characteristics is required before attempting clinical trials.

KEY WORDS: Lactobacillus.
Probiotics.
Genetic identification.

Introduction

Lactobacilli are the predominant microorganisms in the human vagina, and are widely proposed to have a probiotic effect in restoring the ecological balance of the urogenital tract.[1] The first step in designing a probiotic product is to isolate and identify strains from host sites where it will be applied. This is followed by the selection of probiotic strains by evaluation of potentially beneficial characteristics and clinical efficacy.[2]

Characteristics such as adhesion, production of inhibitory substances (organic acids, hydrogen peroxide, bacteriocins), and the ability to modulate the immune response are proposed mechanisms that explain the protective role of lactobacilli in the vaginal tract.[1] Lactic acid (and other organic acids) produced by lactobacilli contribute to the maintenance of an acidic environment (pH<4.5) and to a healthy and balanced ecosystem.[3] However, epidemiological study demonstrates that the presence of lactobacilli (mainly hydrogen peroxide-producing lactobacilli) in the human vagina correlates with a lower risk of urogenital infection.[4]

The main objective of current research is to select potentially beneficial lactobacilli to be included in a vaginal probiotic product used to reconstitute the indigenous microbiota. In a previous work, 134 human vaginal lactobacilli isolated from 200 women in Tucumán, Argentina, were classified taxonomically by phenotypic tests.[5] However, the identification of isolates by classical tests is sometimes difficult and unreliable.

Correct taxonomic classification using molecular biology tools is a fundamental requirement in order to identify potentially probiotic microorganisms.[6] Different techniques have been applied to identify vaginal lactobacilli and these include DNA-DNA hybridisation,[7–9] denaturing gradient gel electrophoresis (DGGE),[10] randomly amplified polymorphic DNA (RAPD) analysis[11] and ribosomal 16S-DNA (16S-rDNA)-based methods.[10,12–17] With the exception of DNA-DNA hybridisation, which gives a result as either a positive or a negative signal, all generate specific DNA band profiles (DNA fingerprints) that can be used to distinguish microorganisms to the strain level.[11]

Previous studies have shown that culture conditions can influence production of inhibitory substances.[18,19] For example, hydrogen peroxide production by some vaginal lactobacilli is detected only after vigorous shaking of the culture.[18]

The present study aims to identify vaginal lactobacilli strains using amplified ribosomal 16S-DNA restriction analysis (ARDRA)[12] and evaluate potentially beneficial characteristics (acidity, hydrogen peroxide production under static culture conditions, and auto-aggregation ability).

Materials and methods

Microorganisms and growth conditions

Twenty strains of previously isolated vaginal lactobacilli that could be potentially included in a vaginal probiotic product were studied.[18,19] Prior to experimentation, each strain was frozen in milk-yeast extract at –70°C, cultivated in LAPTg broth (peptone: 15 g/L; tryptone: 10 g/L; glucose: 10 g/L; yeast extract: 10 g/L; Tween 80: 1 mL [pH 6.5])[20] at 37°C for 24 h and then subcultured (x2) at 37°C for 12 h.

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Table 1. Genetic classification and vancomycin resistance of vaginal lactobacilli.

Metabolic group	Species*	No†	Vancomycin resistant
Obligate homofermentative	*L. acidophilus*	2	0
	L. gasseri	7	0
	L. johnsonii	2	0
	L. salivarius	2	2
Facultative heterofermentative	*L. rhamnosus*	4	4
	L. paracasei	2	2
Obligate heterofermentative	*L. reuteri*	1	1

*Genetic identification by amplified ribosomal 16S-DNA restriction analysis.
†Number of strains for each *Lactobacillus* species.

Table 2. Characteristics of potentially probiotic vaginal lactobacilli.

Genetic identification	A_{540}*	pH†	H_2O_2‡	Auto-aggregation (%)
L. gasseri CRL 1252	0.92	3.80	++	2
L. gasseri CRL 1259§	1.40	3.69	+	7
L. gasseri CRL 1264	1.45	3.66	+	6
***L. gasseri* CRL 1311**	**1.45**	**3.63**	++	**15**
***L. gasseri* CRL 1320§**	**1.30**	**3.61**	+	**16**
***L. gasseri* CRL 1509**	**1.30**	**3.68**	+	**72**
L. gasseri CRL 1307§	1.50	3.70	w	28
L. acidophilus CRL 1251	1.40	3.62	++	35
L. acidophilus CRL 1266	1.25	3.78	++	81
L. johnsonii CRL 1292	0.92	3.93	–	7
L. johnsonii CRL 1294	1.40	3.67	w	76
L. salivarius CRL 1296	0.54	4.38	–	9
L. salivarius CRL 1328§	0.70	4.02	–	29
L. rhamnosus CRL 1261	0.85	4.01	w	7
L. rhamnosus CRL 1332	0.70	4.00	+	11
L. rhamnosus CRL 1508	0.90	3.96	+	3
L. rhamnosus CRL 1511	1.00	3.98	w	10
L. paracasei CRL 1322	0.90	4.12	w	5
L. paracasei CRL 1512	1.05	4.01	w	4
***L. reuteri* CRL 1324§**	**0.77**	**4.28**	++	**7**

*Absorbance at 540 nm after 12 h incubation at 37°C in LAPTg broth.
†pH reached after 12 h incubation at 37°C in LAPTg broth.
‡Qualitative H_2O_2 determination scored by colour intensity (blue) of lactobacilli colonies on TMB-MRS plates: ++ (strongly positive), + (moderately positive), w (weakly positive), – (negative).
§Strains produced uropathogen inhibition by lactic acid in a previous study.[19]
Microorganisms selected for further study are in bold.

Evaluation of properties

A third subculture of each strain, obtained as described above, was used to determine absorbance (*A*) measurements at 540 nm (Spectronic 20, Bausch and Lomb, Rochester NY) as a measure of bacterial growth, final pH as an indirect measure of acid production during growth, auto-aggregation ability, and qualitative estimation of hydrogen peroxide production.

Extent of auto-aggregation was assessed using a technique described by Ocaña and Nader-Macías.[21] Briefly, A_{600} variation of cellular suspensions in phosphate-buffered saline (PBS. NaCl: 8 g/L; KH_2PO_4: 0.34 g/L; K_2HPO_4: 1.21 g/L [pH 7]) was monitored every hour for four hours, and the percentage auto-aggregation calculated by the formula: auto-aggregation (%) = ([*A* final – *A* initial]/ *A* final) x 100.

Hydrogen peroxide production in lactobacilli culture without agitation was determined qualitatively by a plating method using horseradish peroxidase (HRP) incorporated in the tetramethyl-benzidine (TMB) agar medium.[22] The HRP incorporated in the agar medium catalyses oxidation of TMB (chromogenic substrate) to a purple-blue pigment in those colonies that produce hydrogen peroxide.

Lactobacilli strains were inoculated on TMB-MRS plates (peptone: 10 g/L; meat extract: 10 g/L; yeast extract: 5 g/L; glucose: 20 g/L; K_2HPO_4: 2 g/L; sodium acetate: 5 g/L; ammonium citrate: 2 g/L; $MgSO_4$: 0.1 g/L; $MnSO_4$: 0.05 g/L; agar: 15 g/L; Tween 80: 1 mL [pH 6.5])[23] containing 1 mmol/L TMB (3,3´,5,5´-tetramethyl-benzidine [Sigma] dissolved in methanol) and 2 U/mL peroxidase (EC 1.11.1.7, type II; Sigma). Plates were incubated under anaerobic conditions at 37°C for 48 h. A score was assigned according to the intensity of blue colour of lactobacilli colonies on TMB-MRS plates, as follows: strongly positive (++), moderately positive (+), weakly positive (w) and negative (–).

Phenotypic tests

In order to confirm preliminary classification at the metabolic level,[5] gas production from glucose or from gluconate was evaluated. This phenotypic assessment is a necessary step in the ARDRA methodology.[12] Gas production from glucose for each *Lactobacillus* strain was determined in Gibson medium (yeast extract: 2.5 g; glucose: 5 g; non-fat milk: 800 mL; nutritious agar: 200 mL; $MnSO_4$: 0.04 g [pH 6.5]).[24] Gas production from gluconate was carried out in gluconate medium (tryptone: 1.5 g/L; yeast extract: 1 g/L; K_2HPO_4: 1 g/L; gluconate: 40 g/L [pH 7]), sealed with a layer of Vaseline-paraffin (1:1). Both media were inoculated with lactobacilli and incubated at 37°C for 48 h.

In order to establish vancomycin resistance, lactobacilli were inoculated on MRS agar with vancomycin (15 mg/L) and incubated under anaerobic conditions at 37°C for 48 h. Growth of lactobacilli in the presence of vancomycin was used as an identification key.[25]

Amplification and restriction analysis of 16S-rDNA

The ARDRA methodology described by Ventura *et al*.[12] consists of 16S-rDNA amplification, digestion with four restriction enzymes, and comparison of restriction profiles obtained with theoretical digestion profiles. Theoretical restriction profiles were obtained previously by computer analysis of the 16S-rDNA sequences available from the database, and were confirmed using ARDRA analysis of type strains.[12]

Extraction of DNA was performed as described by Azcárate-Peril and Raya.[26] Briefly, cells were lysed with lysozyme and sodium dodecyl sulphate (SDS), RNA was eliminated with RNase, deproteinisation was achieved with

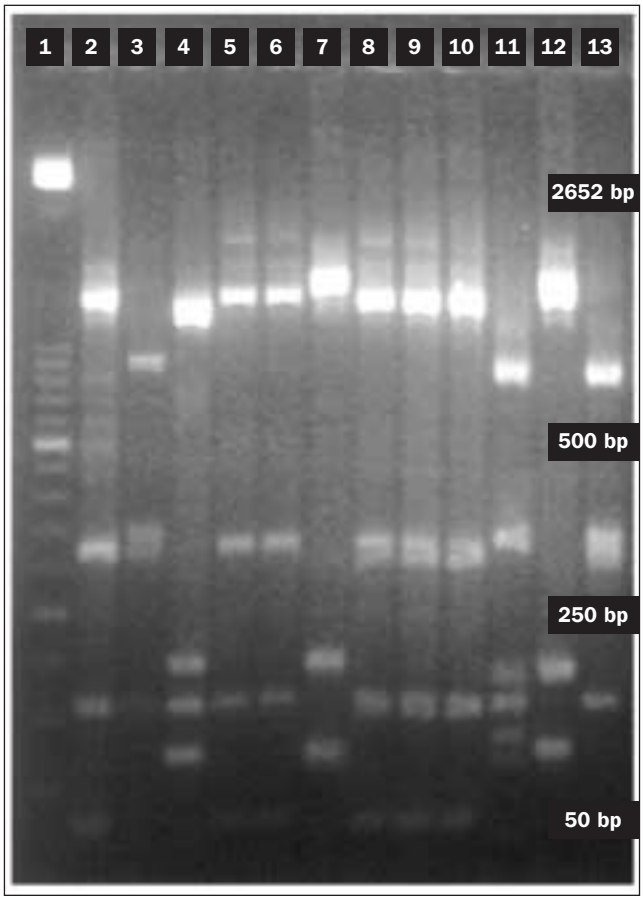

Fig. 1. Agarose gel electrophoresis of 16S-rDNA digested with Sau3AI. Lane 1: 50-bp DNA ladder molecular marker MXIII;lane 2: CRL 1259, profile corresponding to *L. gasseri* or *L. johnsonii*; lane 3: CRL 1261, *L. casei* or *L. rhamnosus*; lane 4: CRL 1266, profile corresponding to *L. acidophilus* group; lanes 5 and 6: CRL 1292 and 1294, *L. gasseri* or *L. johnsonii*; lane 7: CRL 1296, *L. salivarius*; lanes 8, 9 and 10: CRL 1307, 1311 and 1320, *L. gasseri* or *L. johnsonii*; lane 11: CRL 1322, *L. paracasei*; lane 12: CRL 1328, *L. salivarius*; lane 13: CRL 1332, *L. casei* or *L. rhamnosus*.

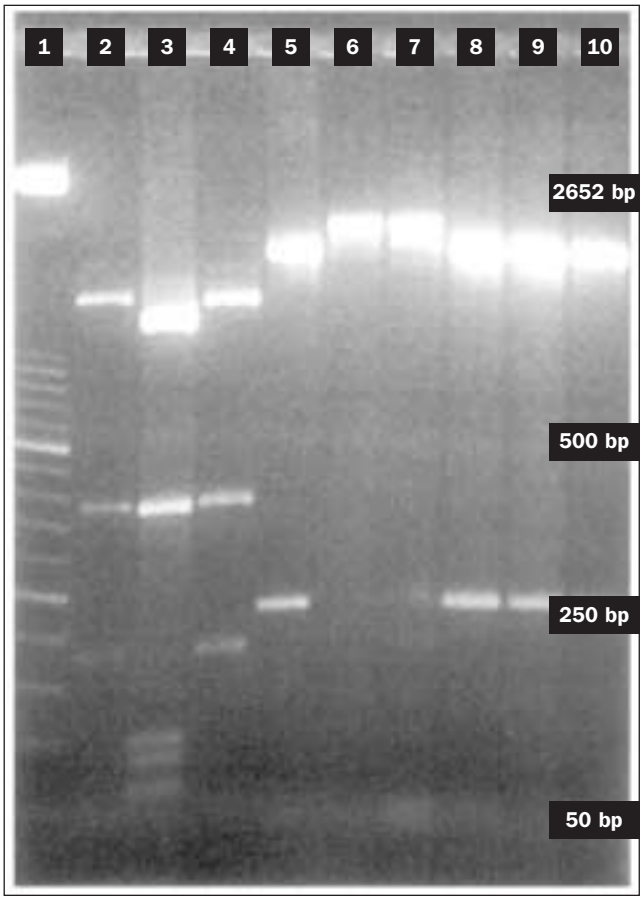

Fig. 2. Agarose gel electrophoresis of 16S-rDNA digested with HinfI (lanes 2 to 4) and DraI (lanes 5 to 12). Lane 1: 50-bp DNA ladder molecular marker MXIII; lane 2: CRL 1261, *L. rhamnosus*; lane 3: CRL 1266, *L. acidophilus*; lane 4: CRL 1332, *L. rhamnosus*; lane 5: CRL 1259, *L. gasseri*; lanes 6 and 7: CRL 1292 and 1294, *L. johnsonii*; lanes 8, 9 and 10: CRL 1307, 1311 and 1320, *L. gasseri*.

proteinase K and chloroform-isoamyl alcohol mixture, and nucleic acids were precipitated with isopropyl alcohol.

To the aqueous DNA solution (1 µL), a reaction mixture (20 mol/L Tris-HCl; 50 mol/L KCl; 200 µmol/L each deoxynucleoside triphosphate; 10 ng/mL primers [P0 and P6];[27] 1.5 mol/L $MgCl_2$; 1.5 units *Thermus aquaticus (Taq)* polymerase and water to a final volume of 24 µL) were added. Reactions were performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). The amplification cycle comprised one cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, annealing at 58°C for 30 sec, an extension at 72°C for 2 min, and cooling at 4°C. The amplification products were subjected to gel electrophoresis in 0.8 % agarose at 100 V for 30 min, followed by ethidium bromide staining.

The amplified 16S-rDNA of each strain was first digested with the enzyme Sau3AI (Roche Diagnostics), and where necessary the amplified product was digested also with HinfI, DraI or HincII. Digested 16S-rDNA was subjected to electrophoresis in 2.5 % agarose gel at 120 V for 2–3 h, followed by ethidium bromide staining.

Results

Genetic identification by ARDRA

Figures 1 and 2 show the restriction profiles obtained from the amplified 16S-rDNA in 12 of the 20 vaginal lactobacilli strains evaluated, and a genetic classification was obtained by comparing them with the standardised digestion profiles previously reported by Ventura *et al.*[12] Among the 20 strains studied, the majority (65%) was assigned to the homofermentative group (Table 1).

Seven different species – *L. gasseri* (seven strains) and *L. rhamnosus* (four strains) – were identified by ARDRA. All the strains belonging to the heterofermentative group were resistant to vancomycin. Among species in the homofermentative group, only those classified as *L. salivarius* were resistant to the assayed antibiotic.

Evaluation of potentially beneficial properties

Results of biomass, pH, auto-aggregation assessment and hydrogen peroxide production for each strain are summarised in Table 2. The pH of the growth medium inoculated with lactobacilli decreased to values close to pH 4.0, and showed an inverse relationship to biomass. Average

values of final pH after 12 h incubation at 37°C in LAPTg were significantly lower for homofermentative lactobacilli (3.80±0.22) than for heterofermentative lactobacilli (4.05±0.11).

Most strains (17/20) produced hydrogen peroxide in TMB-MRS agar plates (Table 2). Eight of the 13 strains in the homofermentative group were moderate or strong hydrogen peroxide producers, and mainly were of the *L. acidophilus* group. In contrast, four out of seven heterofermentative strains were weak hydrogen peroxide producers.

Among the 20 strains evaluated, only three (*L. acidophilus* CRL 1266, *L. gasseri* CRL 1509 and *L. johnsonii* CRL 1294) demonstrated a remarkable auto-aggregating pattern (auto-aggregation >70%; Table 2). Four lactobacilli strains showed strong or moderate hydrogen peroxide production in static culture, low pH values after 12 h incubation at 37°C and/or remarkable auto-aggregation pattern, and these were regarded as worthy of further study.

Discussion

In this study several potentially beneficial characteristics of vaginal lactobacilli are evaluated. However, different *in vitro* techniques have been used to characterise strains isolated from the human vagina.[4,7,8,28–30] These tests provide an approach by which adhesion or the production of inhibitory substances can be determined,[2] and these tests must be performed before attempting expensive clinical trials.[28]

A recent study supports the hypothesis that vaginal lactobacilli are the main producers of lactic acid in the human vagina.[3] Elsewhere, high levels of hydrogen peroxide produced *in vitro* by vaginal lactobacilli, isolated from pregnant women at high risk of preterm birth, was associated with a reduced incidence of bacterial vaginosis at 20 weeks' gestation and of subsequent chorioamnionitis.[13]

Detection of hydrogen peroxide can be achieved by several qualitative and quantitative tests.[22] In a previous study, the level of hydrogen peroxide produced by vaginal lactobacilli was not detected in static cultures by the quantitative *o*-dianisidine-peroxidase spectrophotometric method;[18] however, it is possible that the microorganisms generated levels below the detection threshold of the quantitative method used.

The qualitative TMB-MRS method used in the present study provided evidence of hydrogen peroxide production in static culture. These results support those of other workers,[7,8,13] and demonstrate the species specificity of hydrogen peroxide production, as almost all strains in the *L. acidophilus* group (mainly *L. gasseri* and *L. acidophilus* strains) produced the oxidative metabolite.

Experimental evidence suggests that aggregation influences the development of biofilms.[31] In the vaginal tract, auto-aggregation could allow lactobacilli to form a mucosal biofilm and thus prevent the entrance of pathogens.[1] Boris *et al*.[32] reported the selection of three vaginal auto-aggregating lactobacilli (*L. gasseri, L. acidophilus* and *L. jensenii*) among 70 isolates. Similarly, in the present study, all strains demonstrating a marked auto-aggregating pattern belonged to the *L. acidophilus* group.

In this study, genetic identification of 20 potentially probiotic vaginal lactobacilli was performed by ARDRA.[12] This was chosen because it is a reliable and rapid identification method for vaginal and intestinal lactobacilli to the species and subspecies level, is easy to perform and could be applied quickly to a large number of strains. Furthermore, the methodology permits differentiation of closely related species (e.g., those belonging to the *L. acidophilus* and *L. casei* groups) that cannot be distinguished by conventional phenotypic tests.

Most vaginal *Lactobacillus* species identified by ARDRA were classified as belonging to the *L. acidophilus* group, with *L. gasseri* being the predominant species. Prevalence of *L. gasseri* among *Lactobacillus* species in the human vagina has been reported by other authors.[7,8,11–13,15,16] However, different results have been obtained in some studies, where none of the isolates were identified as *L. acidophilus*.[7–9,15,16]

Strains belonging to other predominant species of vaginal microflora (*L. crispatus*, *L. jensenii*, and *L. iners*)[7–11,13–17] were not identified in this study. However, the strains were evaluated using culture methods, which explains, for example, why *L. iners* was not found. *L. iners* can be detected by non-culture-based techniques such as PCR-DGGE and partial sequencing to 16S-rDNA.[10,17]

In summary, most of the lactobacilli strains used in the present work achieved low pH values after 12 h incubation at 37°C and produced hydrogen peroxide in static culture, but only three strains demonstrated a remarkable auto-aggregation pattern. Expression of these potentially beneficial characteristics suggests that these lactobacilli can create a vaginal environment unfavourable for the growth or adhesion of pathogens. Therefore, use as a vaginal probiotic product could prove to be an effective alternative to prevent or treat genital infection in women.

Some *Lactobacillus* species have been evaluated as candidates for eventual use in a vaginal probiotic product,[33] and others (e.g., *L. reuteri* CRL 1324 and *L. gasseri* CRL 1509, CRL 1311 and CRL 1307) have been selected for further study of their potentially beneficial properties. Genetic identification to the species level is an important step in this process. Techniques such as ARDRA and other molecular biology tools will prove useful in the identification of lactobacilli suitable for inoculation to women[34] or animal models to establish vaginal colonisation of potentially probiotic microorganisms.

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