Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates

Rebeca Martín, Nora Soberon, Mario Vaneechoutte, Ana B. Florez, Fernando Vazquez, Juan E. Suárez

RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2008) 11:261-266
DOI: 10.2436/20.1501.01.70 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates

Rebeca Martín,[1] Nora Soberón,[1] Mario Vaneechoutte,[2] Ana B. Flórez,[1] Fernando Vázquez,[1,3] Juan E. Suárez[1]*

[1]Laboratory of Microbiology, University Institute of Biotechnology, University of Oviedo, Oviedo, Spain. [2]Laboratory of Bacteriology and Virology, University Hospital Ghent, Ghent, Belgium. [3]Hospital Monte Naranco, Oviedo, Spain

Received 19 September 2008 · Accepted 15 November 2008

Summary. The probiotic relevant characteristics of 45 strains of vaginal *Lactobacillus* isolated from healthy women were analyzed. Of these, 21 strains were classified as *L. crispatus*, 17 as *L. jensenii*, six as *L. gasseri*, and one as *L. plantarum*. The rate of acidification varied significantly between the strains as did their ability to form biofilms. None used glycogen as a fermentable carbohydrate. H_2O_2 generation was common, especially among *L. jensenii* isolates (88%). No bacteriocinogenic strains were detected. Most strains harbored plasmids (from 1 to 7) of various sizes, those in excess of 50 kb being frequent. One of these plasmids was found to be promiscuous since it hybridized with extrachromosomal bands of 15 isolates. All strains were resistant to metronidazole, ciprofloxacin, gentamicin, clindamycin, trimethoprim, and sulfametoxazole and susceptible to a series of β-lactams, erythromycin, tetracycline, and benzalkonium chloride. Almost half of the strains were highly resistant to nonoxinol 9, which is commonly used as a spermicide. Based on these analyses, strains of all three common species are proposed as new probiotic candidates. [**Int Microbiol** 2008; 11(4):261-266]

Key words: *Lactobacillus* spp. · vaginal lactobacilli · probiotics · plasmids · antibiotic susceptibility/resistance · spermicides

Introduction

The prominence of lactobacilli in the human vaginal microbiota, was noted as early as 1892, in a study published by Döderlein [19]. In most papers published since then, vaginal lactobacilli recovered from healthy women comprise at least 70% of the total bacteria isolated [27,36]. These gram-positive rods make use of two mechanisms to protect the mucosa from the settlement of undesirable microorganisms: (i) exclusion, driven by the formation of a biofilm that masks the epithelial cell receptors [13,33], and (ii) inhibition of growth, due to generation of lactic acid from the fermentative catabolism of sugars, especially glucose, that result from glycogen secretion and hydrolysis [9,10]. As a consequence, the physiological pH of the vagina is around 4, which is inhibitory to most microorganisms associated with vaginal pathology [27]. Microbial antagonism is also exerted by the production of hydrogen peroxide [23], with *Lactobacillus crispatus* and *L. jensenii* being the most protective although not all H_2O_2 producing lactobacilli are equally beneficial [3]. Other antimicrobial agents, such as bacteriocins, are much less common among vaginal than among environmental lactobacilli, and in their effect has only been demonstrated in vitro [5,31]. The protective role of lactobacilli becomes evident when their concentrations drop, in which case the pH of their environment tends to increase. This, in turn, favors colonization by intestinal bacteria and the overgrowth of microorganisms indigenous to the vagina but usually present in low numbers, all of which may behave as opportunistic pathogens. These include the etiological agents of bacterial

***Corresponding author:** J.E. Suárez
Area de Microbiología, Facultad de Medicina
Universidad de Oviedo
Julián Clavería 6
33006 Oviedo, Spain
Tel. +34-985103559. Fax +34-985103148
E-mail: evaristo@uniovi.es

This article contains supporting information [SI] online, consisting of one table (T1-SI), at the Int. Microbiol. website [www.im.microbios.org].

vaginosis (mainly *Gardnerella vaginalis*, *Mycoplasma hominis*, *Atopobium vaginae*, *Prevotella* spp. and *Peptostreptococcus* spp.), yeast vaginosis (several *Candida* species), trichomoniasis [1,24,43], and the potential pathogen *Streptococcus agalactiae* [8].

The term "probiotic" refers to "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4]. The microbial antagonism exerted by lactobacilli and their extremely rare association with pathology [11,20] have promoted their use for protection of the intestinal and vaginal mucosae [2,18,21,32]. However, the success of oral administration on intestinal colonization has been hindered by the complexity and homeostasis of the autochthonous bacterial communities [45] and, possibly, by the genetic characteristics of the hosts [50]. In contrast, lactobacilli are dominant in the vagina and their administration is envisaged mainly in response to a prior drop in the concentration of the indigenous microbiota due to a pathological process or to antibiotic treatment, which should facilitate the establishment of the instilled probiotic lactobacilli. The success obtained so far with the use of a cocktail containing the spermicide-resistant *L. rhamnosus* GR1 and H_2O_2-producing *L. reuteri* RC-14 strains either by themselves [37] or associated with antimicrobial chemotherapy [4] has promoted the search for other, equally beneficial, bacterial isolates.

The aim of this work was to select newly isolated vaginal lactobacilli to be used as probiotic candidates on the basis of their adherence characteristics, production of pathogen-antagonistic compounds, compatibility with other vaginal lactobacilli, and lack of plasmid-encoded antibiotic resistance determinants.

Materials and methods

Microorganisms, culture conditions, and taxonomic classification. Lactobacilli were collected from vaginal swabs of 50 healthy, reproductive-aged women with no history of vaginal problems. The bacteria were initially isolated on chocolate agar plates (Oxoid, Cambridge, England) incubated at 37°C under a 10% CO_2 atmosphere. White colonies composed of gram-positive, rod-shaped, and catalase-negative bacteria were re-isolated on MRS agar (Oxoid) supplemented with either 1% hemoglobin (Sigma, St. Louis, USA) or 20 µg hemin/ml (Sigma) (MRS-H) and incubated under the same conditions. Liquid cultures in MRS broth were incubated at 37°C without agitation. Glycogen utilization was tested in MRS fermentation broth (ADSAMicro, Madrid, Spain) supplemented with 0.5% glycogen (Sigma). The isolates were identified by tDNA-PCR of alkalinized DNA extracts obtained from single colonies as described previously [6].

Acid, hydrogen peroxide, and bacteriocin production. The pH values of the cultures were monitored at 8, 16, and 24 h of incubation and were plotted against a standard curve made with MRS medium containing increasing concentrations of DL-lactic acid (Sigma). H_2O_2 generation was tested by placing the newly isolated lactobacilli on plates of TMB-plus medium [35], which contained 100 µM 3,3′, 5,5′-tetramethylbenzidine (Sigma) and 10 µg horseradish peroxidase (Sigma)/ml. The plates were incubated anoxically at 37°C for 48 h and then exposed to air for 30 min. The development of a pale or an intense blue color was considered as an indication of weak or strong H_2O_2 production, respectively.

Bacteriocin production was tested on MRS-H medium by placing drops (3 µl) of culture from every strain on lawns of each of them and then determining the development of inhibition halos. In addition, filtered culture supernatants were obtained at different incubation times and 3-µl aliquots were placed on the lawns.

Quantification of biofilm formation. Cultures (200 µl) in MRS were set in the wells of polystyrene microtiter dishes (Nunc-Maxisorp, Roskilde, Denmark) and incubated overnight. Afterwards, 50 µl of a 1% solution of crystal violet was added to each well, which was then left to stand for 10 min at room temperature and then rinsed thoroughly with distilled water. After the addition of 200 µl of 95% ethanol, color extraction was allowed to proceed for 10 min and the absorbance was then determined with a plate reader (Tekan, Barcelona, Spain) at 620 nm [34].

Determination of antimicrobial susceptibilities. The minimum inhibitory concentration (MIC) for 13 antibiotics (including sulfametoxazole and amoxicillin/clavulanic acid) and two spermicidal compounds were determined on MRS-H agar according to the standardized agar dilution technique of the Clinical and Laboratory Standards Institute (CLSI) [17]. For erythromycin and doxycycline, the E-test procedure was used as well, in accordance with the conditions recommended by the supplier (AB Biodisk, Solna, Sweden). The results were recorded after 48 h of incubation at 37°C under 10% CO_2.

DNA-related techniques. The plasmid profiles of the strains were determined by two procedures; alkaline lysis [26] and in situ lysis [48] with the following modifications to adapt it for use with lactobacilli: liquid cultures were grown to an OD_{600} of 0.5, and cells were lysed in the wells of the agarose gels using a mix of lysozyme and mutanolysin (Sigma) (at 1.5 mg/ml and 1 unit/µl, respectively, final concentrations). Southern blot analysis was performed as described [40]. The presence of tetracycline and erythromycin resistance determinants was assessed by PCR of total DNA extracted from overnight cultures using the GeneElute Bacterial Genome DNA kit (Sigma). For tetracycline, the degenerated universal primers for genes encoding ribosomal protection proteins D1–D2 [16] and tet1–tet2 [7] as well as gene-specific primers for *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, and *tetW* [21,42] were used. Primers specific for the erythromycin resistance genes *ermA*, *ermB*, *ermC*, *ermF* [14], and *mefA* [29] were also tested. The PCR conditions used in each case were those described by the respective authors.

Complementarily, reactions were run in which the annealing temperatures were lowered by 5°C. Positive amplification control strains included *Lactococcus lactis* K214 (*tetS*), *L. lactis* IPLA31008 (*tetM*), *Bifidobacterium longum* H66 (*tetW*), *Lactobacillus johnsonii* G41 (*ermB*), and *Staphylococcus aureus* IPLA100 (*ermA* and *ermC*). Suitability of the DNA extracted from the *Lactobacillus* strains for PCR was tested through amplification of a 233-bp fragment corresponding to a universal bacterial 16S ribosomal DNA segment [38].

Results

Isolation and taxonomy of vaginal lactobacilli. To minimize multiple isolations of the same strain, only one colony was retained among those growing on the chocolate agar plates for each vaginal exudate. Fifty colonies showing

a phenotype compatible with lactobacilli (see Materials and methods) were selected and classified through analysis of their tDNA-PCR profiles. Of these, 23 turned out to be *L. crispatus*, eight were identified as *L. gasseri*, 18 as *L. jensenii*, and one as *L. plantarum*. After characterization of the strains, some pairs of isolates appeared to be identical, in which case only one representative was retained. The total number of strains was thus reduced to 45 (Table 1-SI).

Acid generation. A wide range of pH values (from about 6 down to 3.9, which corresponded to 0.03–0.67% lactic acid) were obtained after the strains had been grown for 8 h (exponential phase) in MRS with glucose as the fermentable sugar (Table 1-SI). Considered as a whole, the *L. gasseri* isolates were the most acidifying. At 16 h of incubation (transition from the exponential to the stationary phase for most strains), the pH values ranged from 3.6 to 4.4 (i.e., from 1.04% to 0.35% lactic acid equivalents) for all but two strains. The span of pH values became reduced to 0.6 units (from 3.4 to 3.9, i.e., between 1.41% and 0.67% lactic acid) in 24 h-old cultures (stationary phase). The lack of growth and acidification in MRS containing glycogen as the sole fermentable carbohydrate suggested that none of the 45 vaginal lactobacilli used glycogen as a carbon source.

Hydrogen peroxide production. A substantial proportion of the isolates (71%) generated H_2O_2 (Table 1-SI), as revealed by the blue color that appeared on plates of TMB-plus upon exposure to air. The highest proportion was found among *L. jensenii* isolates, 15 out of 17 (88%) being positive, including ten strong producers. About two-thirds of the *L. crispatus* and the *L. gasseri* strains generated H_2O_2 as well, although the frequency of high producers was higher among those belonging to the former species (77 vs. 50%). The single *L. plantarum* isolate produced no detectable amounts of hydrogen peroxide.

Bacteriocin production. None of the vaginal lactobacilli exerted any inhibitory effects on any of the others, neither when pairs of strains were grown one on top of the other on solid media nor when supernatants of the culture medium (taken at different times of incubation) were placed on lawns of the test microorganisms.

Biofilm formation. Some of the strains showed only a weak ability to form biofilms when incubated on polystyrene microtiter plates, as judged by their capacity to retain crystal violet (OD_{600} < 0.2). Most of the other strains generated well-structured biofilms (OD_{600} between 0.2 and 0.35). Finally, three *L. jensenii* strains adhered strongly to the plastic substrate (OD_{600} > 0.65) (Table 1-SI).

Plasmid content. An in situ lysis procedure was used to detect large plasmids that might have passed unnoticed upon extraction by the standard alkaline lysis method. The combination of the data obtained revealed extrachromosomal DNA bands in 41 strains, i.e., in more than 90%, ranging in number from one to seven (Fig. 1 and Table 1-SI). At least 13 strains harbored megaplasmids, with apparent sizes in excess of 50 kb and up to 150 kb; in some cases, these were present together with very small replicons (Fig. 1).

One of the bands, with an apparent size of 15 kb, migrated to a similar position in many isolates, suggesting the presence of a promiscuous plasmid. This possibility was tested in a Southern blot hybridization experiment between the entire plasmid complement of the 45 strains and the purified DNA of the band extracted from one of the *L. gasseri* isolates. Fifteen strains, belonging to the three common species, showed positive hybridization, although the highest proportion was found among *L. jensenii* strains (almost 50%) (Table 1-SI).

Antimicrobial susceptibility. All the strains tested were resistant to metronidazole (MICs: >128 mg/l), ciprofloxacin (MICs: 32–128 mg/l), gentamicin (MICs: 32–64 mg/l), clindamycin (MICs: >128 mg/l), trimethoprim and sulfametoxazole (MICs: ≥32 mg/l). Conversely, all

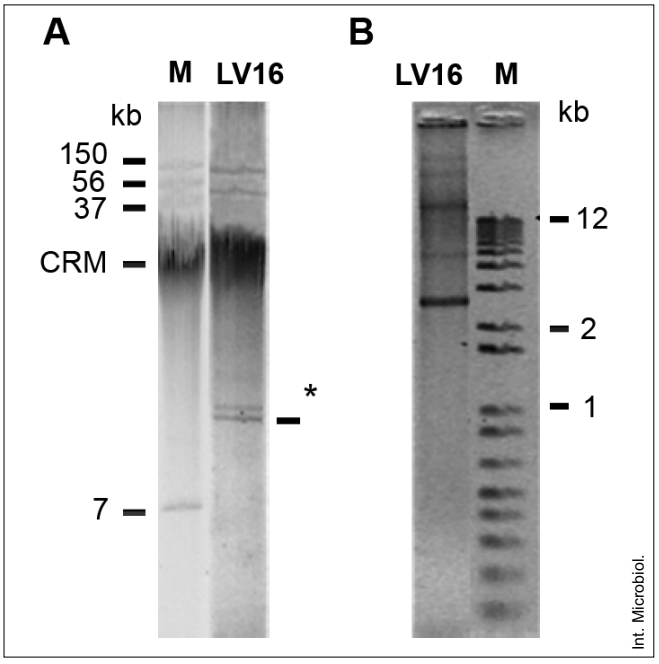

Fig. 1. Plasmid profiles of *Lactobacillus gasseri* LV16 as observed after in situ lysis (**A**), and alkaline lysis (**B**). The size standards (lanes M) were the plasmid complement of *Escherichia coli* V517 (NCTC 50193) (in A) and the 1 kb Plus DNA Ladder (Invitrogen) (in B). The arrow with an asterisk points towards the common plasmid band that hybridized with the 15-kb plasmid extracted from *L. gasseri* LV12. CRM: chromosomal DNA.

strains were extremely susceptible to a series of β-lactam antibiotics, including penicillin G (MICs: ≤1 mg/l), cloxacillin (MICs: 1–4 mg/l), ampicillin and ampicillin plus clavulanic acid (MICs: <1 mg/l, but some strains resisted up to 4 mg/l, irrespective of the presence of the β-lactamase inhibitor). Moderate resistance to cefalotin (MICs: 8–16 mg/l) was observed for some *L. crispatus* strains, while the the MICs of the remaining strains were <1 mg/l.

The use of the microdilution method for testing erythromycin susceptibility rendered unreliable data, which is why the E-test was subsequently used. According to the results of this procedure, most strains were susceptible to erythromycin (MICs: ≤1 mg/l). The exceptions were two *L. crispatus* strains and one *L. jensenii* (MICs: 2 mg/l) and one *L. gasseri* strain (MIC: 4 mg/l). The CLSI standard dilution method rendered a greater dispersion of the MIC results for tetracycline (MICs: 2–16 mg/l) with about one-third of the strains grouped at each extreme. All strains that were highly susceptible to tetracycline were even more so to doxycycline, as were all the *L. gasseri* and *L. jensenii* strains resistant to the former antibiotic (with decreases in MIC from 16 to ≤4 mg/l). However, the tetracycline-resistant *L. crispatus* strains showed comparable levels of resistance to doxycycline. These data were confirmed by E-tests.

The dispersion of MICs obtained for tetracycline-doxycycline and erythromycin, together with the common occurrence of plasmids among the strains under study (see above) suggested that some of the plasmids might harbor transferable antibiotic resistance genes. To test this possibility, total DNA of all strains was subjected to PCR to search for genes encoding tetracycline efflux membrane proteins (*tetK* and *tetL*) and ribosomal protection determinants. The reactions included two degenerate universal primer pairs and those specific for *tetM*, *tetO*, *tetS* and *tetW*. The following erythromycin resistance determinants were checked: *ermA*, *ermB*, *ermC* and *ermF*, all encoding 23S RNA methyltransferases, and *mefA*, whose gene product mediates export of erythromycin out of the cell. Positive amplification was not obtained from any of the reactions, not even when the annealing temperature was lowered by 5°C, while the positive controls yielded amplicons of the predicted size. As a further control, the integrity of the DNAs extracted from the *Lactobacillus* strains and their suitability for PCR were confirmed by amplification of a 233-bp fragment corresponding to a universal eubacterial 16S ribosomal DNA segment.

All strains were extremely susceptible to benzalkonium chloride, formerly used as a spermicide; at a concentration of 0.01%, only two *L. gasseri* isolates were able to grow, their MIC being 0.05%. In contrast, 20 strains, evenly distributed among all species, grew in the presence of 4% nonoxinol-9, while the rest were still quite resistant to this spermicide, with MICs higher or equal to 0.1% of the compound (Table 1-SI).

Discussion

The goal of this work was to select strains of vaginal lactobacilli with a probiotic potential, which is why the exudates were taken from healthy fertile women. The search was specifically addressed towards organisms able to grow under a 10% CO_2 atmosphere or aerobically, to facilitate their propagation and survival. The media were supplemented with a heme source to provide the cells with an essential substrate to generate peroxidases, which allow cells to cope with the oxidative stress produced under (partial) aerobiosis [12]. The convenience of this procedure was proven since the growth of most of the isolates improved greatly after addition of hemin or hemoglobin to the solid medium.

The taxonomic classification of the isolated lactobacilli confirms the reported dominance of *L. crispatus*, *L. jensenii*, and, to a lesser extent, *L. gasseri* in the vagina [3,46]. The single *L. plantarum* isolate recovered was interpreted as an intestinal contaminant that had transiently colonized the vaginal environment (apart from its exotic location it did not generate any biofilm nor did it produce H_2O_2). The relatively higher frequencies of isolation of *L. crispatus* and *L. jensenii* strains correlated with the higher proportion of strong H_2O_2 producers among them when compared to *L. gasseri*, reflecting previous observations of the dominance of H_2O_2-producing strains of these two species in the microbiota of healthy women [3].

The kinetics of acid generation from glucose and consequently the growth rate of the strains varied significantly, indicating that some adapted more easily than others to the microaerophilic conditions used. These strains are considered to be the best probiotic candidates, since the vaginal environment is presumed to be microaerophilic as well. However, the percentages of lactic acid equivalents accumulated by old cultures became quite similar, indicating that the problem for the slow acid producers was a consequence of lack of adaptation to the particular conditions of growth used rather than to an intrinsic inability to ferment the sugar efficiently.

None of our strains were able to degrade glycogen. This is not a new observation, but it is pertinent to mention it here because it is frequently assumed, even in specialized literature, that lactobacilli degrade the glycogen secreted by the parietal vaginal cells, as a preliminary step towards fermentation of the resulting glucose. This assumption persists in spite of the opposite conclusions reached in the 1960s [39,44,49].

The ability to generate a biofilm, which was observed in many of the strains and especially in three *L. jensenii* isolates, may be considered as a positive trait because it might result in the formation of a barrier to pathogen attachment to the vaginal mucosa [13,33]. A substantial proportion of lactic acid bacteria isolated from environmental sources, including those from home-made fermented foods, produce bacteriocins [25,30]. However, none of the vaginal lactobacilli used in this work showed antimicrobial activities that could be ascribed to bacteriocins on any other of the isolates. This would ensure their compatibility in the hypothetical case that mixed-culture formulations were considered to be convenient for vaginal instillation.

More than 90% of the vaginal strains isolated as part of this work harbored plasmids, frequently >50 kb in size. Megaplasmids were previously found to be abundant in a variety of *Lactobacillus salivarius* strains, most of which had originated from the digestive tract of humans and other animals [28]. They were scarce in a series of other lactobacilli of different origins, although that work did not include vaginal isolates. Analysis of one of those megaplasmids in [28] revealed that it was promiscuous and that it encoded a bile salt hydrolase able to confer a selective advantage in the intestinal habitat. The finding among our isolates of another promiscuous plasmid (harbored by one-third of the strains of all three main species) suggested that it likewise encodes traits useful for vaginal colonization.

Apparently, none of the strains under scrutiny encoded transmissible antibiotic resistance determinants, in spite of their abundant plasmid complement, according to the quite uniform picture obtained. Indeed, most isolates were either very resistant (metronidazole, ciprofloxacin, gentamicin, clindamycin, trimethoprim, and sulfametoxazole) or susceptible (β-lactams, erythromycin, tetracycline, and benzalkonium chloride) to the antimicrobials tested. This suggests that impermeability lies behind the observed resistance phenotypes, although it was previously reported that resistance to metronidazole might be due to a lack of hydrogenase activity [15]. Besides, the data also indicated that no resistance genes coding for β-lactamases, ribosomal protection, or antibiotic-efflux proteins are present in these bacteria. This was further confirmed by the lack of amplification obtained after PCR during a search for the most conspicuous erythromycin and tetracycline resistance determinants described for gram-positive bacteria. This also indicates that the moderate resistance of some strains towards these last two antibiotics is due to restriction of their access to the cytoplasm of the cells. As a corollary, it might be concluded that the use of these vaginal lactobacilli as probiotic bacteria would not represent any risk of transmission of antibiotic resistance genes to the microbiota present in the cavity.

Complementarrily, the high level of resistance to metronidazole, clindamycin, and nonoxinol 9 may be considered another positive trait supporting the use of these strains as vaginal probiotics. Metronidazole and clindamycin are the agents of choice for the treatment of bacterial vaginosis and, in the case of metronidazole, for trichomoniasis as well. Consequently, lactobacilli could be administered concomitantly with the antibiotics and not only after the end of treatment. Nonoxinol 9 is the most commonly used spermicide, included both in creams and in condom lubricants and has been associated with relapses of bacterial vaginosis [47]. Accordingly, bacterial strains that are resistant to this antiseptic would be convenient to use as probiotics.

Finally, the data obtained and summarized in Table 1-SI were used to select the best probiotic candidates. Thresholds were set at 0.35% lactic acid equivalents (pH = 4.4 in MRS medium) after 8 h of incubation, positive generation of H_2O_2, absorbance ≥ 0.2 for biofilm formation, and MIC ≥ 2 mg/l for nonoxinol 9. One strain of each species, namely *L. jensenii* LV18, *L. crispatus* LV26, and *L. gasseri* LV36, fulfilled all four restrictions, while almost half (47%) passed more than three of them, with proportions of 66, 59, and 33% among the *L. gasseri*, *L. jensenii* and *L. crispatus* isolates, respectively.

Acknowledgements. This work was supported by CICYT grants SAF2004-0033 and BFU2007-65781from the Spanish Ministry of Science and Technology and the FEDER Plan. We warmly thank Laboratorios Normón for the kind gift of the antimicrobials used in this work. N.S. and R.M. are holders, respectively, of a fellowship associated with this grant and a scholarship from FICYT (Principado de Asturias). The support of Mercadona S.A. is also acknowledged. We thank Prof. K.F. Chater for critical reading of the manuscript.

References

1. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK (1983) Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am J Med 74:14-22
2. Andreu A (2004) *Lactobacillus* as a probiotic for preventing urogenital infections. Rev Med Microbiol 15:1-6
3. Antonio MA, Rabe LK, Hillier SL (2005) Colonization of the rectum by *Lactobacillus* species and decreased risk of bacterial vaginosis. J Infect Dis 192:394-398
4. Anukam K, Osazuwa E, Ahonkhai I, Ngwu M, Osemene G, Bruce AW, Reid G (2006) Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. Microbes Infect 8:1450-1454
5. Aroutcheva AA, Simoes JA, Faro S (2001) Antimicrobial protein produced by vaginal *Lactobacillus acidophilus* that inhibits *Gardnerella vaginalis*. Infect Dis Obstet Gynecol 9:33-39
6. Baele M, Vaneechoutte M, Verhelst R, Vancanneyt M, Devriese LA, Haesebrouck F (2002) Identification of *Lactobacillus* species using tDNA-PCR. J Microbiol Methods 50:263-271

7. Barbosa TM, Scott KP, Flint HJ (1999) Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environ Microbiol 1:53-64
8. Bayó M, Berlanga M, Agut M (2002) Vaginal microbiota in healthy pregnant women and prenatal screening of group B streptococci (GBS). Int Microbiol 5:87-90
9. Boris S, Suárez JE, Vazquez F, Barbés C (1998) Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun 66:1985-1989
10. Boskey ER, Cone RA, Whaley KJ, Moench TR (2001) Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Hum Reprod 16:1809-1813
11. Cannon JP, Lee TA, Bolanos JT, Danziger LH (2005) Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. Eur J Clin Microbiol Infect Dis 24:31-40
12. Chaillou S, Champomier-Verges MC, Cornet M, et al. (2005) The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat Biotechnol 23: 1527-1533
13. Chan RC, Bruce AW, Reid G (1984) Adherence of cervical, vaginal and distal urethral normal microbial flora to human uroepithelial cells and the inhibition of adherence of gram-negative uropathogens by competitive exclusion. J Urol 131:596-601
14. Chung WO, Werckenthin C, Schwarz S, Roberts MC (1999) Host range of the *ermF* rRNA methylase gene in bacteria of human and animal origin. J Ant Chemother 43:5-14
15. Church, DL, Bryant RD, Sim V, Laishley EJ (1996) Metronidazole susceptibility and the presence of hydrogenase in pathogenic bacteria. Anaerobe 2:147-153
16. Clermont D, Chesneau O, de Cespedes G, Horaud T (1997) New tetracycline resistance determinants coding for ribosomal protection in streptococci and nucleotide sequence of *tet(T)* isolated from *Streptococcus pyogenes* A498. Antimicrob Agents Chemother 41:112-116
17. CLSI (2004) Methods for antimicrobial susceptibility testing of anaerobic bacteria: approved standard, M11-A6, 6th edn. Vol. 24, No. 2. Paikka, USA
18. Delgado S, Suárez A, Mayo B (2007) Dominant cultivable *Lactobacillus* species from the feces of helthy adults in northern Spain. Int Microbiol 10:141-145
19. Döderlein ASG (1892) Das Scheidensekret und seine Bedeutung für das Puerperalfieber. O. Durr. Leipzig
20. Gasser F (1994) Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull Inst Pasteur 92:45-67
21. Gevers D, Danielsen M, Huys G, Swings J (2003) Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol 69:1270-1275
22. Guarner F (2006) Enteric flora in health and disease. Digestion 73, Suppl 1:5-12
23. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wolner-Hanssen P, Holmes KK (1996) Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J Infect Dis 174:1058-1063
24. Hay P (2005) Life in the littoral zone: lactobacilli losing the plot. Sex Transm Infec 81:100-102
25. Herrero M, Mayo B, González B, Suárez JE (1996) Evaluation of technologically important traits in lactic acid bacteria isolated from spontaneous fermentations. J Appl Bacteriol 81:565-570
26. Leenhouts KJ, Kok J, Venema G (1989) Campbell-like integration of heterologous plasmids into the chromosome of *Lactococcus lactis* subs. *lactis*. Appl Environ Microbiol 55:394-400
27. Lepargneur JP, Rousseau V (2002) Rôle portecteur de la flore de Doderleïn. J Gynecol Obstet Biol Reprod 31:485-494
28. Li Y, Canchaya C, Fang F, Raftis E, Ryan KA, van Pijkeren JP, van Sinderen D, O'Toole PW (2007) Distribution of megaplasmids in *Lactobacillus salivarius* and other lactobacilli. J Bacteriol 189:6128-6139
29. Luna VA, Sydney Cousin JR, Whittington WLH, Roberts MC (2000) Identification of the conjugative *mef* gene in clinical *Acinetobacter jejunii* and *Neisseria gonorrhoeae* isolates. Antimicrob Agents Chemother 44:2503-2506
30. Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H (1996) Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70:113-128
31. Ocaña VS, Pesce de Ruiz Holgado AA, Nader-Macías ME (1999) Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. Appl Environ Microbiol 65:5631-5635
32. Olivares M, Díaz-Ropero MP, Gómez N, et al. (2006) The comsumption of two new probiotic strains, *Lactobacillus gassei* CECT 5714 and *Lactobacillus corynifirmis* CECT 5711, boots the immune system of healthy humans. Int Microbiol 9:47-52
33. Osset J, Bartolomé RM, García E, Andreu A (2001) Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. J Infect Dis 183:485-491
34. O'Toole GA, Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449-461
35. Rabe LK, Hillier SL (2003) Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. J Clin Microbiol 41:3260-3264
36. Redondo-López V, Cook RL, Sobel JD (1990) Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev Infect Dis 12:856-872
37. Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, Bruce AW (2003) Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol Med Microbiol 35:131-134
38. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS (1990) The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 323:1573-1580
39. Rogosa M, Sharpe ME (1960) Species differentiation of human vaginal lactobacilli. J Gen Microbiol 23:197-201
40. Sambrook JE, Fritsch F, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
41. Sanders ME (2003) Probiotics: considerations for human health. Nutr Rev 61:91-99
42. Scott KP, Melville CM, Barbosa TM, Flint HJ (2000) Occurrence of the new tetracycline resistance gene *tet(W)* in bacteria from the human gut. Antimicrob Agents Chemother 44:775-777
43. Sobel JD (2000) Bacterial vaginosis. Annu Rev Med 51:349-356
44. Steward-Tull BA (1964) Evidence that vaginal lactobacilli do not ferment glycogen. Am J Obstetr Gynecol 88:676-679
45. Tannock GW (2004) A special fondness for lactobacilli. Appl Environ Microbiol 70:3189-3194
46. Vallor AC, Antonio MA, Hawes SE, Hillier SL (2001) Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. J Infect Dis 184:1431-1436
47. Watts DH, Rabe L, Krohn MA, Aura J, Hillier SL (1999) The effects of three nonoxynol-9 preparations on vaginal flora and epithelium. J Infect Dis 180:426-437
48. Wheatcroft R, McRae GD, Miller WR (1990) Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development. Mol Plant-Microb Interact 3:9-17
49. Zoetendal EG, Vaughan EE, de Vos WM (2006) A microbial world within us. Mol Microbiol 59:1639-1650

Table 1-SI. General characteristics of the *Lactobacillus* spp. strains described in this work[a]

Species	Strain	% lactic acid[b]	H_2O_2[c]	Biofilm[d]	Number of plasmid bands	Hybridization[e]	MIC to nonoxinol 9 (%)
L. crispatus	LV3	0.57	–	0.19	2	–	>4
	LV6	0.11	++	0.21	2	–	1
	LV8	0.59	–	0.19	2	–	2
	LV25	0.48	+	0.16	1	–	4
	LV26	0.37	+	0.25	1	–	2
	LV27	0.06	++	0.20	4	+	1
	LV28	0.05	++	0.20	4	–	>4
	LV33	0.21	–	0.26	1	–	1
	LV35	0.63	–	0.21	3	–	>4
	LV37	0.20	++	0.23	0	–	0.1
	LV41	0.03	++	0.26	1	–	0.1
	LV42	0.04	++	0.23	4	–	0.1
	LV43	0.04	++	0.22	0	–	0.1
	LV45	0.07	++	0.24	2	+	2
	LV48	0.40	–	0.20	1	–	>4
	LV50	0.30	++	0.19	3	–	>4
	LV53	0.60	–	0.19	1	+	>4
	LV54	0.61	–	0.15	4	–	>4
	LV55	0.23	++	0.21	2	+	2
	LV65	0.09	–	0.30	2	+	>4
	LV66	0.07	++	0.32	2	+	1
L. gasseri	LV12	0.18	+	0.16	1	+	>4
	LV16	0.39	–	0.20	7	+	2
	LV19	0.53	++	0.26	4	–	1
	LV21	0.37	++	0.19	2	–	>4
	LV36	0.55	++	0.21	2	–	> 4
	LV61	0.67	–	0.13	0	–	0.1
L. jensenii	LV15	0.21	++	0.30	5	+	>4
	LV18	0.38	++	0.34	5	–	>4
	LV22	0.32	++	0.24	1	+	>4
	LV30	0.42	++	0.19	1	+	1
	LV31	0.61	++	0.16	2	+	1
	LV38	0.10	++	0.20	1	+	>4
	LV39	0.32	++	0.21	2	+	1
	LV40	0.33	++	0.21	0	–	1
	LV47	0.24	–	0.20	2	–	>4
	LV49	0.32	++	0.21	5	–	>4
	LV57	0.22	++	0.20	2	–	2
	LV58	0.44	++	0.26	3	+	1
	LV59	0.10	++	0.21	1	–	1
	LV60	0.58	++	0.17	1	–	>4
	LV62	0.16	–	0.67	3	+	>4
	LV63	0.23	++	0.88	3	–	2
	LV64	0.23	++	0.67	1	–	2
L. plantarum	LV2	0.15	–	0.19	5	–	>4

[a]Data reported in this table are means of at least three independent experiments.
[b]Data taken at 8 h of incubation.
[c]Symbols: ++ strong H_2O_2 producers; + weak producers.
[d]OD_{600} of ethanol-solubilized crystal-violet-stained cultures.
[e]Strains that hybridized with the plasmid extracted from *L. gasseri* LV12.