Factors Associated with Acquisition of, or Persistent Colonization by, Vaginal Lactobacilli: Role of Hydrogen Peroxide Production

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To identify factors that predict sustained colonization by vaginal lactobacilli, microbiologic, behavioral, and demographic data were obtained from 101 nonpregnant women at baseline and at 4 and 8 months. A total of 272 isolates of lactobacilli were identified to the species level by use of whole chromosomal DNA homology to type strains. The predominant lactobacilli were the species Lactobacillus crispatus (38%) and L. jensenii (41%). Of 57 women initially colonized by H2O2-producing L. crispatus or L. jensenii, 23 (40%) remained colonized over 8 months, compared with 1 (5%) of 21 women colonized by other H2O2-producing species or by H2O2-negative strains (P = .01). Frequency of sexual intercourse (≥1 sex act per week) was associated with loss of colonization with H2O2-producing lactobacilli (P = .018), as was antibiotic use (P ≤ .0001). Other behavioral and demographic characteristics did not predict sustained colonization. The production of H2O2 is closely linked with species and is a predictor for sustained long-term colonization of the vagina.

First identified in 1894 by the German physician A. Doderlein [1], Lactobacillus has been shown to be the predominant bacterium in the normal vaginal microbial flora found in women of reproductive age [2]. Lactobacilli are facultative anaerobes that colonize the moist surface of the vaginal epithelium, intestinal tract, and oral cavity of humans and nonhuman animals [2, 3]. In reproductive-age women, glycogen is deposited, under estrogenic control, onto the mature vaginal epithelium, where it is broken down to glucose by vaginal epithelial cells and bacterial enzymes [2]. The lactobacilli metabolize glucose to a final end product of lactic acid, which contributes to the maintenance of a low vaginal pH (4.0–4.5) [3, 4]. Many isolates of vaginal lactobacilli produce hydrogen peroxide (H2O2), a compound having broad antimicrobial activity [5, 6]. Lactobacillus crispatus and L. jensenii are the most prevalent species in the vagina, and 94%–95% of these strains produce H2O2 [7]. Women colonized by H2O2-producing lactobacilli have decreased acquisition of human immunodeficiency virus (HIV) infection [8], gonorrhea [8], and bacterial vaginosis [9].

Bacterial vaginosis is the most frequent cause of vaginal discharge in the United States [10]. Symptoms of bacterial vaginosis include vaginal discharge and malodor, although some women are asymptomatic [11]. Clinical signs of bacterial vaginosis include elevated pH (>4.5), homogenous vaginal discharge, a positive whiff test (ammonia odor produced when 10% potassium hydroxide is added to vaginal fluid), and the presence of clue cells (sloughed vaginal epithelial cells coated with bacteria) [10, 11]. Acquisition of bacterial vaginosis is linked with increased number of sex partners [9, 12, 13], use of intrauterine devices [13], and douching [9]. Bacterial vaginosis is associated with adverse pregnancy outcomes [14, 15], pelvic inflammatory disease [16], and increased postoperative infections [17]. In 4 cross-sectional studies, women with bacterial vaginosis had an increased prevalence of HIV seropositivity [18–21], and, in a longitudinal study of pregnant women, women with bacterial vaginosis had an increased incidence of HIV infection [22].

Because colonization by lactobacilli protects against acquisition of bacterial vaginosis and because the risk factors for loss of vaginal lactobacilli are undescribed, we sought to characterize lactobacilli that persistently colonize the vagina and compare them with lactobacilli that colonize transiently. The hypothesis of the present study was that H2O2-producing lactobacilli are more likely to sustain long-term vaginal colonization than are lactobacilli that do not produce H2O2.
Methods

Vaginal samples collected for microbiologic analysis were obtained during 1992–1995 at the sexually transmitted disease clinic and at an adolescent clinic affiliated with the University of Washington (Seattle). After the baseline visit, each woman was asked to return for 2 additional visits ~4 months apart. A total of 263 women were initially enrolled in this study. Of these, 76 failed to return for follow-up, 69 returned for follow-up but not at the specified times, 8 had missing laboratory data, and 9 were not included because the frozen lactobacillus stock samples were irrecoverable. Therefore, a total of 101 women were included in this analysis. The mean interval between baseline and the first follow-up visit was 125 days, and the mean interval to the next follow-up was 150 days.

Demographic and behavioral information was elicited by means of a structured interview. The women ranged in age from 14 to 44 years (mean, 21.8 years). The cohort was 42% white, 39% African American, 8% Asian, and 12% other or mixed race. Sixty-eight percent of the women had had ≥5 lifetime sex partners, 87% used some form of birth control during the study period, and 18% reported douching ≥1 time per month. Antibiotic use during the study was reported by 37% of the women. Thirty-three of the women reported having been diagnosed with bacterial vaginosis and prior sexually transmitted infections, included trichomoniassiasis (3%), gonorrhea (4%), and chlamydiaal infection (10%).

A wet-mount preparation of the vaginal fluid was evaluated by a clinician at each visit. A clinical diagnosis of bacterial vaginosis was made if 3 of the following 4 criteria were met: vaginal pH ≥4.7, presence of clue cells (squamous vaginal epithelial cells covered with vaginal bacteria, giving the cells a granulated appearance and obscured borders), homogenous vaginal discharge, and amine odor when vaginal fluid was mixed with 10% potassium hydroxide [11].

Vaginal specimens, obtained with sterile Dacron swabs and transported in Amies transport medium (MML Diagnostics Packaging), were delivered to the laboratory within 12 h for microbiologic culture. Swabs were used to inoculate 1 Rogosa agar plate (Difco Laboratories) and 2 human blood bilayer Tween plates and 1 Columbia agar with 5% sheep blood plate (Prepared Media Laboratories). The Rogosa plate and 1 human blood bilayer Tween plate were incubated for a minimum of 96 h under anaerobic conditions at 37°C. The remaining plates were incubated for a minimum of 48 h under 6% CO2 at 37°C. Lactobacilli were identified to the genus level by Gram’s stain morphology, negative catalase test, and production of lactic acid, as assessed by gas chromatographic analysis [23]. Those identified as lactobacilli were stored at −70°C in litmus milk (Becton Dickinson Microbiology Systems) for further identification, based on DNA homology.

To test for the production of H2O2 by lactobacilli, a qualitative assay on tetramethylbenzidine agar plates was done [24]. Lactobacilli were inoculated onto tetramethylbenzidine plates and were incubated anaerobically at 37°C. After 48 h, the plates were exposed to ambient air. If the lactobacilli produce H2O2, the H2O2 reacts with the horseradish peroxidase present in the media and oxidizes the tetramethylbenzidine, causing the colonies to turn blue.

For species-level identification, DNA was extracted from Lactobacillus isolates by a method described elsewhere, with some modification [7, 25, 26]. In brief, isolates frozen in litmus milk were thawed and were subcultured onto Columbia 5% sheep blood plates and incubated for 48 h in a 6% CO2 incubator at 37°C. Isolated colonies were grown in PYTSG broth (0.5% [wt/vol] peptone, 1% [wt/vol] yeast, 0.02% Tween 80, 1% [wt/vol] soluble starch, and 1% [wt/vol] glucose) [7]. Sample broths were subjected to centrifugation (2000 g for 20 min), and the pellets were subsequently washed 3 times with TES buffer (30 mM Tris, 5 mM EDTA, and 50 mM NaCl [pH 8.0]). The pellet was resuspended in 1 mL of lysis buffer (25% ultrapure sucrose, 50 mM Tris, and 1 mM EDTA [pH 8.0]) and was incubated for 60 min in a 37°C water bath. The following reagents were added in the prescribed order and were incubated for the following times: 35 U of RNase A (Sigma) for 30 min, 16 U of the nonspecific protease type XIV from Streptomyces griseus (Sigma) for 1 h, and 1 mL of 0.25 M EDTA (pH 8.0) at 25°C for 5 min. After 0.4 mL of 20% SDS was added, the solution was incubated for a minimum of 60 min at 65°C or until the solution was clear. Ten units of protease K (Sigma) was added, and the solutions were incubated for an additional 30 min at the same temperature. The lysate was extracted twice with an equal volume of Tris-buffered phenol and once with an equal volume of chloroform. DNA was precipitated with 95% ethanol and incubation at −20°C. The DNA pellet was washed with 70% ethanol and was allowed to air dry before being suspended in a nominal amount of 10 mM Tris-Cl with 1 mM EDTA (pH 8.0).

DNA from Lactobacillus isolates was extracted and was slot-blotted onto S&S Nytran nylon membranes (Schleicher & Schuell) and probed with whole genomic DNA probes prepared from known American Type Culture Collection (ATCC) strains, as done earlier in the laboratory [7, 26]. In brief, a total of 3.0 μL of purified DNA was suspended in a total volume of 400 μL of 10 mM Tris-HCl with 1 mM EDTA (pH 7.0), to which 0.1 mL of 3.0 N NaOH was added. Samples were incubated for 60 min at 65°C, cooled to room temperature, and neutralized by adding 1.0 mL of 2 M ammonium acetate (pH 7.0). Each sample was evenly aliquoted into 3 adjacent wells and was vacuum-transferred to the membrane. Membranes were heated for 2 h at 80°C. In addition to patient DNA samples, DNA samples from 39 Lactobacillus ATCC strains and Escherichia coli strain 25922 also were blotted, to serve as positive and negative controls for possible cross-hybridization of the species. Membranes were prehybridized in 50% formamide buffer at 42°C for 2 h. By randomly labeling the whole genomic DNA with [α-32P]dATP (NEN Life Science Products), using the Random Primed DNA Labeling Kit (Boehringer Mannheim), whole chromosomal probes were made from ATCC Lactobacillus strains L. crispatus 33197, L. jensenii 25258, L. gasseri 9857, L. ruminis 25644, L. acidophilus 4356, and the unnamed species designated L. 1086V. Radiolabeled whole chromosomal probes were hybridized with DNA-containing membranes for 24 h at 42°C with constant agitation. Blots were washed twice in 1× standard saline citrate (SSC) and 0.1% SDS for 5 min and then were washed once in 0.1× SSC and 0.1% SDS at 42°C and once at 65°C for 5 min. Blots were exposed to film (X-Omat; Kodak) for 2–4 h before the film was developed.

Fisher’s exact χ2, Pearson χ2, and Mantel-Haenszel χ2 tests were used to compare discrete variables with respect to the presence or absence of H2O2-producing lactobacilli, L. crispatus, and/or L. jensenii. P < .05 was considered statistically significant.
Results

In total, 272 isolates of Lactobacillus were obtained from the 101 women and the 303 visits. Overall, 96 (95%) of the 101 women were positive for lactobacillus colonization on ≥1 visit and 60 (59%) were persistently positive at all visits. The colonization patterns of the 101 women are shown in figure 1. The women were divided into 2 groups, those colonized at baseline with lactobacilli (n = 78) and those not colonized at baseline (n = 23). Of the 78 women colonized by lactobacilli at baseline, 60 maintained persistent colonization for all 8 months. Of the 23 women initially lacking vaginal lactobacilli, 18 (78%) had acquired lactobacilli at 4 or 8 months. These data suggest that vaginal colonization by lactobacilli was dynamic in this cohort, with two-thirds of the women either acquiring or losing vaginal lactobacilli over an 8-month period of follow-up.

All different colony types of Lactobacillus were tested for H2O2, with 190 (70%) of the 272 isolates testing positive for H2O2 production. Of the 78 women who were initially positive for lactobacilli, 61 (78%) were colonized at baseline with H2O2-producing lactobacilli, and 17 were colonized with non–H2O2-producing lactobacilli. Among the women initially colonized by lactobacilli at baseline, H2O2-producing strains were isolated from 52 (87%) of the 60 women persistently colonized for 8 months, compared with 9 (50%) of the 18 women who lost colonization after baseline (P = .004).

Among the 23 women with no lactobacillus colonization at baseline, 18 (78%) had acquired lactobacilli at 4 or 8 months (figure 1). Of those 18, 8 (44%) acquired H2O2-producing strains. Thus, only 8 (35%) of the 23 women initially negative for lactobacillus colonization acquired H2O2-producing strains over 8 months of follow-up.

The frequency of each species of Lactobacillus found at the 3 visits is shown in table 1. Of all vaginal lactobacillus isolates obtained from the cohort at each time point (baseline, 4 months, and 8 months), ~70% were identified as L. crispatus or L. jensenii. There was no statistically significant increase or decrease from baseline in the frequency of any of the species after the initial visit. More than 1 species of lactobacilli was found to cocolonize the vagina in 23 (23%) of the 101 women sometime during the study. Cocolonizing species were combinations of L. crispatus, L. jensenii, and the unnamed Lactobacillus species designated 1086V.

To evaluate the role of species and production of H2O2 in predicting sustained colonization by lactobacilli, the women initially colonized by lactobacilli were divided into 3 groups (table 2). The 2 women who were colonized at baseline by H2O2-negative strains of L. crispatus and the 4 women colonized at baseline by H2O2-negative strains of L. jensenii were grouped with the 15 women colonized by L. gasseri or L. 1086V. Of the 24 women persistently colonized for 8 months by the same species of Lactobacillus, 23 (96%) were colonized with H2O2-
producing strains of *L. crispatus* or *L. jensenii*. Only 1 woman who was initially colonized with an H$_2$O$_2$-negative strain remained consistently positive for the same species throughout the 8 months of follow-up. Women initially colonized by either H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* were significantly more likely to remain colonized for 8 months than were women colonized by other lactobacilli (table 2). None of the 6 women initially colonized by H$_2$O$_2$-negative strains remained colonized over 8 months, which suggests that H$_2$O$_2$-negative lactobacilli are less able to sustain colonization. On the other hand, H$_2$O$_2$ production itself was insufficient to sustain colonization, as evidenced by the fact that 4 women initially colonized by H$_2$O$_2$-producing strains of *L. gasseri* lost the colonization after baseline.

Demographic and behavioral factors associated with persistent colonization by H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* were analyzed. Of 57 women initially colonized with H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* at baseline, 23 remained persistently colonized for 8 months, whereas 34 women lost colonization after baseline. There was no statistically significant association between race, age, number of male sex partners, birth control method, or site of enrollment and persistence or loss of colonization by H$_2$O$_2$-producing *L. crispatus* or *L. jensenii*. However, women who reported having $\geq 1$ act of vaginal intercourse per week over the 8-month period were more likely to lose colonization. Among the 23 women who were persistently colonized by H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* for 8 months, only 8 (35%) had sexual intercourse $\geq 1$ time a week, compared with 23 (68%) of 34 women who lost H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* colonization after baseline ($P = .018$, Fisher’s exact $\chi^2$ test). Whether the loss of H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* identified in this study was due to unprotected vaginal intercourse could not be evaluated because of the lack of condom information for each individual act of intercourse. In addition, women initially colonized with H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* who lost colonization after baseline or after the first follow-up visit were more likely to report antibiotic use during the interim than were women who did not lose colonization (38% vs 8%; $P = .0001$, Mantel-Haenszel $\chi^2$ test).

The acquisition of bacterial vaginosis among women persistently colonized for 8 months by H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* was compared with that among women who were initially colonized with these species but lost colonization. Only 6 (26%) of the 23 women persistently colonized with H$_2$O$_2$-producing *L. crispatus* or *L. jensenii* species throughout the 8 months developed bacterial vaginosis. This is in contrast to an acquisition of bacterial vaginosis for 20 (59%) of 34 women initially colonized with H$_2$O$_2$-producing *L. crispatus* and *L. jensenii* species who lost colonization of these species after baseline ($P = .015$, Mantel-Haenszel $\chi^2$ test).

In the present study, the frequency of douching decreased from 14% at baseline to 5% at 4 months and 6% at 8 months, probably because douching was discouraged by the health care provider at each visit. Thus, douching was too infrequent in this population to evaluate the temporal association between douching and the loss of colonization by lactobacilli.

### Table 1. Frequency of isolation of *Lactobacillus* species from 101 women at baseline and at 4- and 8-month follow-ups.

<table>
<thead>
<tr>
<th>Lactobacillus species</th>
<th>Baseline (n = 86)</th>
<th>4-Month follow-up (n = 86)</th>
<th>8-Month follow-up (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. crispatus</em></td>
<td>33 (38)</td>
<td>33 (38)</td>
<td>37 (37)</td>
</tr>
<tr>
<td><em>L. jensenii</em></td>
<td>35 (41)</td>
<td>35 (41)</td>
<td>38 (38)</td>
</tr>
<tr>
<td>L. 1086V</td>
<td>13 (15)</td>
<td>16 (19)</td>
<td>24 (24)</td>
</tr>
<tr>
<td><em>L. gasseri</em></td>
<td>4 (5)</td>
<td>2 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td><em>L. ruminis</em></td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No Lactobacillus isolated</td>
<td>23 (23)</td>
<td>25 (25)</td>
<td>16 (16)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of women in whom species is isolated or no. of women in whom no *Lactobacillus* species is isolated (% of women in group). Some women were colonized with $\geq 1$ species of *Lactobacillus*. 1086V, Designation for unnamed *Lactobacillus* species.

### Table 2. Association of hydrogen peroxide (H$_2$O$_2$) production by *Lactobacillus* and sustained colonization: role of species.

<table>
<thead>
<tr>
<th>Colonization pattern</th>
<th><em>H$_2$O$_2$</em> positive</th>
<th>No <em>H$_2$O$_2$</em>-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. crispatus</em> (n = 29)</td>
<td><em>L. jensenii</em> (n = 28)</td>
</tr>
<tr>
<td>Persistent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 months</td>
<td>13 (45)</td>
<td>10 (36)</td>
</tr>
<tr>
<td>4 months</td>
<td>6 (21)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Lost colonization after baseline visit</td>
<td>10 (34)</td>
<td>14 (50)</td>
</tr>
</tbody>
</table>

*Women colonized by H$_2$O$_2$-positive *L. crispatus* vs. women not colonized by H$_2$O$_2$-positive *L. crispatus* or *L. jensenii*, Fisher’s exact $\chi^2$ test.

*Women colonized by H$_2$O$_2$-positive *L. jensenii* vs. women not colonized by H$_2$O$_2$-positive *L. crispatus* or *L. jensenii*, Fisher’s exact $\chi^2$ test.

*Includes 4 women colonized by other H$_2$O$_2$-positive *Lactobacillus* strains, including *L. gasseri*, and 17 women colonized by H$_2$O$_2$-negative strains, including *L. gasseri*, *L. ruminis*, and undescribed species designated L. 1086V.
Discussion

In the present study of a population of 101 reproductive-age women, H2O2-producing strains of *L. crispatus* and *L. jensenii* were the most likely to maintain persistent vaginal colonization over a period of 8 months. Published studies have assigned a protective role to H2O2-producing lactobacilli, because women colonized by H2O2-producing strains had decreased acquisition of HIV and *Neisseria gonorrhoeae* infection and of bacterial vaginosis [8, 9, 20]. The present study suggests that the capacity of lactobacilli to produce H2O2 is also associated with persistent colonization. The relevance of H2O2 production is supported by the finding that 85% of the women who were persistently colonized by lactobacilli over 8 months were initially colonized by H2O2-producing strains of lactobacilli, whereas only half of those who lost colonization had H2O2-producing strains at baseline. It was interesting to note that the 6 isolates identified as *L. crispatus* or *L. jensenii* that did not produce H2O2 were lost after the first visit, which supports the role of H2O2 production in sustained vaginal colonization.

On the other hand, the species of *Lactobacillus* present may also be important, because none of the 4 H2O2-producing strains of *L. gasseri* sustained colonization after the enrollment visit. *L. crispatus* and *L. jensenii* were the predominant species of vaginal lactobacilli, accounting for more than half of the total lactobacilli identified at each visit. Furthermore, in the group of 23 women cocolonized with ≥2 *Lactobacillus* species in the vagina, 87% were colonized with *L. crispatus* and 74% with *L. jensenii*. Of the 272 isolates that were identified by DNA homology, no isolates were identified as *L. acidophilus*. This is in agreement with previous studies by Antonio et al. [7], Giorgi et al. [27], and Song et al. [28], in which *L. jensenii*, *L. crispatus*, and *L. gasseri*, rather than *L. acidophilus*, were identified to be the predominant vaginal colonizers of women.

Vaginal intercourse at a frequency of ≥1 time weekly was the only behavior associated with loss of H2O2-producing *L. crispatus* or *L. jensenii* (*P = .018*). In published studies, acquisition of bacterial vaginosis has been associated with exposure to a new sex partner [9, 12, 13, 29], douching [9], and absence of H2O2-producing lactobacilli [9]. In a recent prospective study, Schwabek et al. [29] showed that transient alteration in vaginal flora was correlated with behaviors such as a number of sex partners, frequent vaginal intercourse, and frequent episodes of receptive oral sex [29]. The present study extends these findings to show that sexual intercourse and antibiotic use increases the risk of losing colonization by H2O2-producing lactobacilli. There were too few women reporting a new sex partner in the present study to evaluate whether exposure to new sex partners was a risk factor for loss of lactobacilli. Similarly, the low prevalence and incidence of sexually transmitted infections in this population did not permit the evaluation of the effect of these infections on vaginal colonization by lactobacilli. Finally, this study further confirms that loss of H2O2-producing lactobacilli leads to an increased acquisition of bacterial vaginosis.

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 bacterial vaginosis. In a recent study by McLean and Rosenstein [30], 60 vaginal *Lactobacillus* isolates from healthy volunteers were identified to the species level by means of a commercial biochemical system (50 CH; bioMérieux). Two strains of *L. acidophilus* identified to the species level by use of these phenotypic characteristics were selected as possible probiotic candidates for recolonization of the vagina. These strains were selected on the basis of their ability to inhibit bacterial vaginosis pathogens in vitro, their acid production, their production of H2O2, and their ability to bind exfoliated vaginal epithelial cells in vitro.

The results of our study suggest that H2O2-producing strains of *L. crispatus* or *L. jensenii*, identified by DNA homology, should be considered for vaginal probiotics. To our knowledge, this study is the first to demonstrate that sustained colonization is greatest among *L. crispatus* and *L. jensenii* strains that produce H2O2. Furthermore, only 8 of 23 women initially lacking H2O2-producing strains of *L. crispatus* or *L. jensenii* were spontaneously colonized by these species over 8 months of follow-up. This suggests that recolonization of the vagina with an exogenous probiotic may be the only effective means to establish these species in the vagina.

In this study, we have identified the predominant vaginal species that persistently colonize young sexually active women and have concluded that the production of H2O2 by *L. crispatus* and *L. jensenii* predicts sustained colonization. Production of H2O2 may enhance persistence of *Lactobacillus* through direct inhibition of other components of the vaginal flora; alternatively, H2O2-producing strains of lactobacilli may have surface molecules important in adherence that are coregulated with the genes for H2O2 production. In either case, *L. crispatus* and *L. jensenii*, should be regarded as symbionts that can dominate the vaginal microbial consortium in some women, whereas other species are transient colonizers. The transient nature of colonization was suggested by the data showing that two-thirds of the women lost or acquired *Lactobacillus* colonization during the study. Clearly, biologic or microbiologic factors can lead to loss of even those strains of lactobacilli that produce H2O2. Further studies should focus on the interaction of *Lactobacillus* with vaginal cells to clarify these bacterial–host cell interactions.

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