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Vaginal *Lactobacillus* microbiota of healthy women in the late first trimester of pregnancy

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Objective This study was undertaken to characterise the dominant species of *Lactobacillus* colonising the vagina of healthy pregnant women, to examine some of their phenotypic and genotypic properties, and to gain a better understanding of the potential role of species, which might be associated with infection-free status.

Design A prospective descriptive cohort study.

Setting Department of Obstetrics and Gynaecology, Medical University of Vienna and Medical School, Vienna, Austria.

Sample A total of 200 women in the late first trimester of pregnancy without clinical signs of vaginal infection were included in the study. Of these, 126 women were found to have a normal vaginal flora based on Gram stain.

Methods Culture probes from those 126 women were further processed for identification of *Lactobacillus* species. Overall, 168 colonies from 84 women were identified as belonging to the *Lactobacillus* genus. Based on the combined results of microbiological methods and genus-specific, multiplex, and species-specific polymerase chain reaction, lactobacilli were recovered from 72 women.

Main outcome measures Identification of *Lactobacillus* species of the vaginal flora of healthy pregnant women.

Results The most frequently occurring species were *Lactobacillus crispatus* and *Lactobacillus gasseri*, followed by *Lactobacillus jensenii* and *Lactobacillus rhamnosus*.

Conclusions Our results may have implications on the composition and on the use of *Lactobacillus* preparations for the prevention of recurrent vaginal infection.

Keywords Indentification, *Lactobacillus*, pregnancy, vagina.

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Introduction

The healthy human vagina is dominated by a variety of *Lactobacillus* species, which play an essential role in protecting women from genital infection. Because vaginal infection is an important mechanism of disease responsible for preterm birth,[1] maintaining the natural, healthy balance of the *Lactobacillus* flora in the vagina is particularly important during pregnancy. A deficiency in lactobacilli can upset the microbial balance in the vagina, frequently resulting in the syndrome of bacterial vaginosis,[2,3] which may be associated with a quantitative and qualitative shift from normally occurring lactobacilli to a mixed flora dominated by anaerobic bacteria.[4] According to Nugent *et al.*,[5] bacterial vaginosis is characterised by a complete loss of lactobacilli and by a concomitant increase in Gram-variable and Gram-negative rods, primary among them is *Gardnerella vaginalis*, as well as *Bacteroides*, *Prevotella*, and *Mobiluncus* species.[2,3] However, loss of vaginal lactobacilli also leaves nonpregnant women susceptible to infection which may result in endometritis or even pelvic inflammatory disease.[6,7]

Several species of *Lactobacillus* have been described to populate the vagina to varying degrees. They differ in their ability to grow and to produce either lactic acid or hydrogen peroxide.[8] Some of the species identified so far include *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus jensenii*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus vaginalis*, and *Lactobacillus salivarius*. In the most recent reported study on the vaginal *Lactobacillus* flora, Vásquez *et al.*[9] found that the vaginal flora of most participants was dominated by a single *Lactobacillus* species, with the presence of other species

showing wide interindividual variability. The most frequently occurring species were *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *L. jensenii*. In another study by Reid *et al.*,[10] the *Lactobacillus* species recovered most commonly were *L. jensenii*, *L. acidophilus*, *L. casei*, and *L. gasseri*. Evidence suggests that there is considerable geographical variation in the composition of the normal *Lactobacillus* microbiota in the vagina.

The present study was undertaken to characterise the dominant species of *Lactobacillus* colonising the vagina of healthy pregnant women, to examine some of their phenotypic and genotypic properties, and to gain a better understanding of the potential role of certain species, which might be associated with infection-free status.

Materials and methods

This study was performed with the approval of the Ethics Committee of the Medical University of Vienna. Pregnant women between 11 + 0 and 14 + 0 weeks of gestation scheduled to give birth at the Department of Obstetrics and Gynecology, Medical University of Vienna, were included in the study after informed consent had been obtained. To be eligible, women had to be free of subjective complaints, vaginal bleeding, and clinical signs of vaginal infection, and they had to have a microscopically diagnosed normal vaginal flora. In addition to the routine antenatal examination, which includes infection screening with a simple vaginal smear, vaginal secretions for cultures of lactobacilli were obtained with a sterile swab. Smears for infection screening were transferred to the Microbiology Laboratory of the Department of Obstetrics and Gynecology. All preparations were Gram stained and evaluated by the Nugent scoring system.[5]

Culture probes from women with a vaginal flora classified as normal (Nugent score 0–3) and without any evidence of *Candida* colonisation (spores and hyphae) were transferred to transport medium and sent to the University of Natural Resources and Applied Life Sciences, Vienna, for further processing and identification of *Lactobacillus* species by phenotypic and genotypic methods. An overview of analytical methods is shown in Figure 1.

Vaginal swabs were removed from the transport medium and streaked onto MRS agar (Merck, Darmstadt, Germany) for identification of lactobacilli. For characterisation of the accompanying microflora, the same swab samples were used to inoculate *Gardnerella* agar (bioMérieux, Marcy I'Etoile, France), Chromocult Coliform agar (Merck), MacConkey agar (Merck), and Schaedler KV agar with 5% (v/v) sheep blood (BD Diagnostics, Sparks, MD, USA) for total *Enterobacteriaceae*, BBL CHROMagar™ Candida (BD Diagnostics) and YGC agar (Merck) for the isolation of yeasts, and Columbia agar with 5% sheep blood (BD Diagnostics) for the cultivation of fastidious microorganisms. MRS and Schaedler KV agar plates were incubated under anaerobic conditions for 48 hours at 37°C. YGC and CHROMagar Candida plates were cultured for a minimum of 5 days at

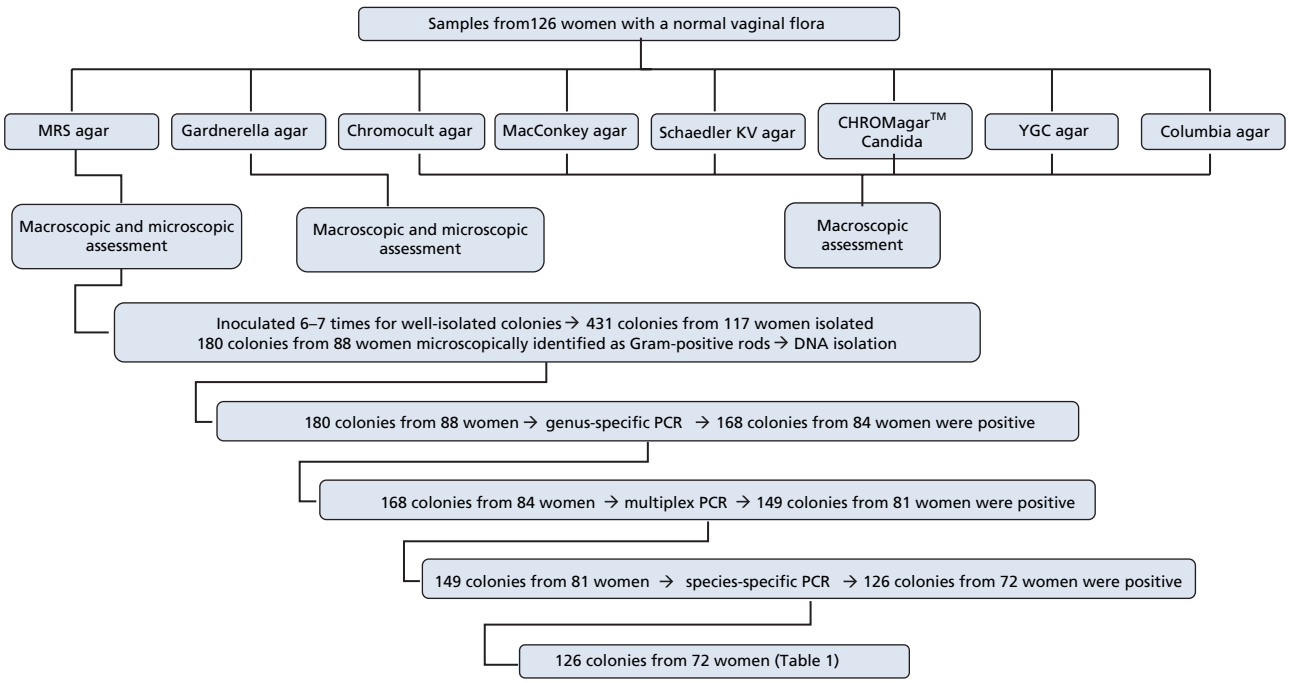

Figure 1. Flow chart of the isolation of preparations.

25°C. The remaining plates were incubated aerobically for 24–48 hours at 37°C, except for *Gardnerella* agar, which was incubated in an anaerobic jar under microaerophilic atmosphere.

All colonies produced on agar plates underwent macroscopic examination and were then subjected to decontamination, with the exception of bacterial isolates harvested from MRS agar. These isolates were further identified based on Gram stains and assessment of colonic morphology. Isolates were excluded if presumptive lactobacilli were not culturable on MRS agar or not verifiable by Gram stain. Identified lactobacilli colonies were further characterised to the genus level by polymerase chain reaction (PCR).

For the DNA studies, each *Lactobacillus* isolate was pregrown in MRS broth incubated under anaerobic conditions for 24 hours at 37°C; 1.5 ml of this bacterial culture were centrifuged (5415 R; Eppendorf, Hamburg, Germany) for 7 minutes at 4°C (5900 *g*) . The pellet was resuspended in 900 μl sterile sodium chloride solution (0.9%), blended, and again centrifuged. The supernatant was discarded, and the washing step was repeated. The pellet was washed using 900 μl of sterile ethylenediamine tetraacetic acid (50 mM, pH 8) and then stored at –20°C. The Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) was used to obtain DNA samples according to the manufacturer's protocol.

In the first PCR assay, isolates were identified to the genus level using the primers LbLMA1-rev and R16-1 according to Dubernet *et al.*[11] The reaction mixture (total volume 25 μl) contained 1 μl of each primer (10 mM), 10× PCR buffer (Finnzymes, Espoo, Finland), deoxyribonucleotide triphosphate (dNTP)-Mix (10 mM) (Roth, Karlsruhe, Germany), DynaZyme (2 U/μl) (Finnzymes), and 0.5 μl bacterial DNA. Amplification was carried out in a Mastercycler gradient (Eppendorf) with the following temperature programme: initial denaturation at 94°C for 4 minutes; 35 cycles consisting of denaturation at 94°C for 1 minutes, annealing temperature at 55°C for 1 minutes including time increment of 2 seconds, extension at 72°C for 1 minutes, and a final extension step at 72°C for 8 minutes. The PCR products were visualised by agarose gel electrophoresis and photographed under ultraviolet exposure.

Colonies identified as belonging to the genus *Lactobacillus* by means of PCR and all type strains were then assessed by multiplex PCR as described by Song *et al.*[12] PCR-G was used for grouping the lactobacilli with the group-specific primers Lac-2, Ldel-7, LU-5, LU-3, and LU-1, followed by PCR using species-specific primers for identification at the species level. Primers Ljen-3, Laci-1, and 23-10C were used for the identification of *L. jensenii* and *L. acidophilus*. Primers Lcri-3, Lcri-2, Lgas-3, and Lgas-2 were used for the identification of *L. crispatus* and *L. gasseri*. Primers LU-5', Lpar-4, and Rha II were applied for the detection of *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. PCR with the primers Lsal-1, Lsal-2, Reu-1, Reu-4, Lpla-2, Lpla-3, Lfer-3, and Lfer-4 was performed to identify *L. salivarius*, *Lactobacillus reuteri*, *L. plantarum*, and *L. fermentum*. Additionally, the primer pair 'casei' and 'Y2' were used for identifying *L. casei* according to Ward *et al.*[13]

Randomly amplified polymorphic DNA (RAPD) analysis was performed with the predominant *Lactobacillus* species found in this study. DNA samples were used from previous PCR amplifications. Seven different RAPD primers as described by Daffonchio *et al.*,[14] Andersson *et al.*,[15] Klein *et al.*,[16] Cocconcelli *et al.*,[17] and Johansson *et al.*,[18] as well as the Pharmacia Primer-Set (Pharmacia Biotech, Uppsala, Sweden) were used. The PCR mixture contained 2 μl of RAPD primer (12.5 pmol/μl), 10× PCR buffer (Finnzymes), dNTP-Mix (10 mM) (Roth), DynaZyme (2 U/μl) (Finnzymes), and 0.5 μl of DNA solution. RAPD-PCR was performed in a Mastercycler gradient (Eppendorf) using the following temperature profile: an initial denaturation step at 95°C for 5 minutes, followed by 45 cycles (1 minutes at 95°C, 1 minutes at 36°C, and 2 minutes at 72°C) and the final step at 72°C for 10 minutes. The PCR products were analysed on a 2% agarose gel and visualised by ultraviolet transillumination.

Results

Between January and April 2005, a total of 200 pregnant women were included in this study. The study population was between 18 and 35 years of age, with 98% of women of Caucasian origin. A total of 126 women were found to have a normal vaginal flora according to Nugent *et al.*[5] and to be free of *Candida* colonisation. Isolates from 38 women were excluded because presumptive lactobacilli were not culturable on MRS agar or not verifiable by Gram stain, so that a total of 180 colonies from 88 women remained for further characterisation by genus-specific PCR.

In the first PCR assay, 168 colonies from 84 women were identified as belonging to the genus *Lactobacillus*. The next

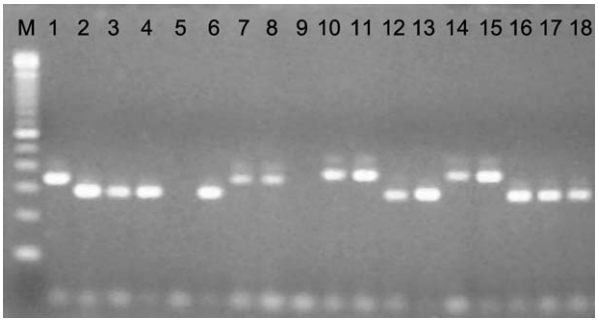

Figure 2. Agarose gel electrophoresis of PCR products from multiplex PCR for grouping of lactobacilli. Lane M, molecular marker (in bp); lanes 1 and 2, positive controls *L. fermentum* with an amplicon of 350 bp and *L. crispatus* with an amplicon of 300 bp; lanes 3 to 18 show lactobacilli identified at the species level by using multiplex PCR.

step, applying combined results of microbiological methods, genus-specific PCR, and multiplex PCR Figure 2, showed that lactobacilli were recovered from 72/126 (57%) women. A flow chart of the isolation of preparations is shown in Figure 1.

Of the 72 women with lactobacilli identified by species-specific PCR, 19 (26.4%) women harboured only *L. gasseri* and 17 (23.6%) women were colonised with only *L. crispatus*. In three (4.2%) women, both of these species were found simultaneously. Overall, 10 of the 72 women harboured at least 2 different *Lactobacillus* species simultaneously. Detailed results are presented in Table 1.

In the 126 *Lactobacillus* colonies from 72 women, 8 different species of lactobacilli were detected and found to dominate the vaginal flora. Of these, *L. crispatus* and *L. gasseri* were the most frequent, identified in 20% and 18% of the 126 *Lactobacillus* colonies. *L. acidophilus*, *L. plantarum*, and *L. salivarius* were not recovered from any of the colonies. Detailed results are summarised in Table 2. Samples of results are shown in Figure 3.

Although a thorough documentation of all species detected in the vaginal swabs was not within the scope of this study, bacterial species other than lactic acid bacteria were detected by selective culture methods, macroscopic and microscopic identification, and Gram staining. The results of the vaginal flora isolated from 126 pregnant women are summarised in Table 2. *Lactobacillus* constituted the predominant flora. There were 40/126 women (31%) mainly colonised by species other than lactobacilli, and 23/126 women (18%) hosted both lactobacilli and Gram-negative rods. Other microorganisms, such as coliforms, *Gardnerella* species, and yeasts, exhibited diminished population densities when lactobacilli were found to be predominant. *Escherichia coli* was even less likely to be recovered from women harbouring *L. crispatus* or *L. gasseri*.

Table 1. Distribution of *Lactobacillus* species in the 72 women with lactobacilli identified by species-specific PCR

***Lactobacillus* species or combinations of species**	**Women with lactobacilli, *n* (%)**
L. gasseri	19 (26.4)
L. crispatus	17 (23.6)
L. jensenii	14 (19.4)
L. rhamnosus	7 (9.7)
L. crispatus and *L. gasseri*	3 (4.2)
L. crispatus and *L. rhamnosus*	2 (2.7)
L. rhamnosus and *L. jensenii*	1 (1.4)
L. rhamnosus and *L. gasseri*	1 (1.4)
Other lactobacilli or lactobacilli combinations	8 (11.2)
L. crispatus and *L. fermentum*	1 (1.4)
L. crispatus and *L. reuteri*	1 (1.4)
L. crispatus and *L. casei*	1 (1.4)

Table 2. Overview of vaginal isolates derived from culture samples

	Women (*n* = 126) colonised with *Lactobacillus* species,[a] *n* (%)
***Lactobacillus* species**	72 (57.1)
L. crispatus	25 (19.8)
L. gasseri	23 (18.2)
L. jensenii	15 (11.9)
L. rhamnosus	10 (7.9)
L. reuteri	4 (3.2)
L. fermentum	2 (1.6)
L. casei	1 (0.8)
L. paracasei	1 (0.8)
	Women (*n* = 126) colonised with other vaginal microbes,[a] *n* (%)
Coliforms	23 (18.2)
E. coli	22 (17.4)
Gardnerella species	13 (10.3)
Yeast (not further specified)	40 (31.7)
Candida albicans	12 (9.5)
Candida krusei	22 (17.4)

[a]Some women were simultaneously colonised with more than one species.

Candida albicans was isolated from 12 women. There was no significant correlation between the presence or absence of lactobacilli and the growth of *C. albicans*.

The predominant *Lactobacillus* species identified by species-specific PCR, namely *L. crispatus*, *L. gasseri*, and *L. jensenii*, were used to generate DNA fingerprints. Samples of women colonised with more than one colony of the same *Lactobacillus* species were further investigated by RAPD-PCR. For *L. crispatus*, 20 samples from 9 women were investigated. The RAPD-PCR results showed that most of the samples within one woman were closely related. Identical bands were found in only one woman. The 20 samples generated 3 different RAPD types Figure 4. For *L. gasseri*, 23 colonies from 9

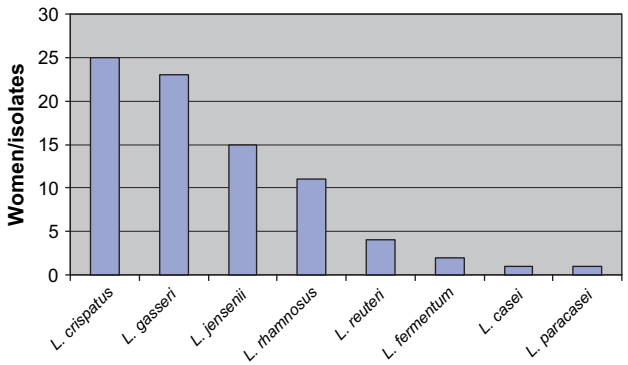

Figure 3. Frequency of detected lactobacilli.

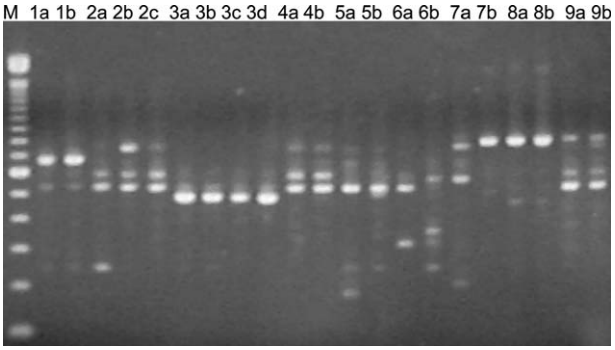

Figure 4. Agarose gel of RAPD products from women who were colonised with more than one colony of *L. crispatus*. Lane M, molecular marker (in bp); lanes 1–9 show groups of *L. crispatus* strains, which differ slightly but indicate close correlation. Different RAPD types are identified.

women were investigated. The majority of the RAPD-PCR patterns within a single subject were different but had high similarity. Only two women showed identical bands. In the 23 colonies, three different RAPD types of this species were found. Similar results were obtained by the RAPD analysis of *L. jensenii* (19 samples from 8 women). The RAPD-PCR fingerprints within one woman differed slightly but indicated a close correlation. In the 19 samples, a maximum of 3 RAPD types was detected.

Discussion

For sometime, the flora of healthy women of childbearing age was believed to be dominated by *L. acidophilus* and *L. fermentum*, followed by *L. brevis*, *L. jensenii*, *L. casei* with all subgroups,[10] and other species. More recently, molecular methods have shown *L. crispatus*, *L. jensenii*, and *L. iners* to be the most common isolates.[10,19] In our study, *L. crispatus* and *L. gasseri* were the most frequently occurring species in the vaginas of pregnant women, followed by *L. jensenii* and *L. rhamnosus*. More specifically, the vaginal flora of all 72 participants in whom lactobacilli were culturable was either dominated by a single or by a combination of two *Lactobacillus* species. In contrast to results reported previously,[10] *L. acidophilus* was underrepresented in our study. However, this is in agreement with results by Vásquez *et al.*[9] and Antonio *et al.*,[20] who found *L. jensenii*, *L. crispatus*, and *L. gasseri* to be the predominant *Lactobacillus* species in the vagina.

There are several reasons that may have led to the contradictory data available on the vaginal *Lactobacillus* flora as determined in different studies. Factors such as the host's state of health, pregnancy, age, or geographical variations may all influence the vaginal flora. Most importantly, however, differences in the methodology used and the lack of reliable identification methods obviously constitute a major problem in characterising the vaginal *Lactobacillus* microbiota. The identification of vaginal lactobacilli based on phenotypic methods comprises sugar fermentation patterns and other biochemical tests and is therefore of limited accuracy and reliability. These tests sometimes cannot differentiate between closely related species. In this study, multiplex and species-specific PCR were performed for an in-depth identification of the strains. Additionally, RAPD analysis was used to generate DNA fingerprints for the three predominant *Lactobacillus* species. Results obtained with species-specific PCR are in accordance with those of Vásquez *et al.*,[9] indicating that *L. crispatus*, *L. gasseri*, and *L. jensenii* can be regarded as the predominant species in the vagina. The RAPD fingerprints of the selected *Lactobacillus* isolates clearly indicated the high homology and close relatedness within one individual, as well as within the species. The majority of the RAPD patterns were practically identical. No more than three different RAPD types per species were detected. This is in general agreement with former observations by Vásquez *et al.*,[9] who applied RAPD analysis, temporal temperature gradient gel electrophoresis, and multiplex PCR for the identification of the vaginal *Lactobacillus* flora. Antonio *et al.*[20] also identified these species based on whole-chromosomal DNA probes. Thus, the implementation of genotypic methods constitutes a convenient way for the classification and species differentiation of vaginal lactobacilli.

Lactobacilli were detected in merely 84 (67%) of the 126 women whose samples were submitted for characterisation of the vaginal flora (Figure 1). This observation led us to assume that a certain proportion of lactobacilli may be viable but not culturable under certain conditions. It may well be that the samples in which *Lactobacillus* cultivation failed exhibit a similar distribution pattern of *Lactobacillus* species, which may be detectable by indirect methods. This particular microbial population will be the subject of another study.

Our results document the frequency distribution of different *Lactobacillus* species and strains, as well as combinations thereof. Because our population consisted of healthy pregnant women in the first trimester of pregnancy, it is likely that the distribution of lactobacilli we found corresponds to that of the general nonpregnant population. In terms of clinical practice, our findings may have an impact both on the substitution therapy with lactobacilli and on the diagnosis of bacterial vaginosis.

A particular feature of bacterial vaginosis is the reduction or absence of lactobacilli in the vaginal flora and an overgrowth of anaerobic and facultative anaerobic organisms. Antibiotic therapy, even though reducing the concentration of the bacteria that cause the symptoms of bacterial vaginosis, also targets the beneficial lactobacilli, further reducing the already depleted *Lactobacillus* flora. Our findings imply that substitution of either *L. crispatus* and *L. gasseri* or *L. jensenii* and *L. rhamnosus* may be more effective at restoring the normal vaginal *Lactobacillus* flora and avoiding relapse of infection, calling into question

the value of vaginal therapeutics containing other species. Probiotic supplements have been proposed as alternative modalities for the maintenance and restoration of a healthy vaginal flora.[21] In this context, our findings may well provide pointers to the composition of future probiotic products specifically directed to women's health. ■

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