Characterization of vaginal lactobacilli from HIV-negative and HIV-positive Indian women and their association with genital HIV-1 shedding

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Abstract

One of the crucial determinants for successful administration of lactobacilli to the vaginal niche is the use of appropriate Lactobacillus species. In this cross-sectional study 54 human immunodeficiency virus (HIV)-negative and 76 HIV-positive antiretroviral treatment-naïve women were evaluated for culturable vaginal lactobacilli and their association with genital HIV-1 shedding. Lactobacillus species were identified by 16S rDNA sequencing while cervical and plasma HIV-1 viral load was determined by Abbott real-time PCR. Lactobacilli were isolated in 77.8% HIV-negative and 73.7% HIV-positive women. The mean log_{10} plasma and cervical HIV-1 viral loads (RNA copies ml^{-1}) were 3.73±1.02 and 2.85±0.32 respectively. We observed that presence of L. crispatus, L. gasseri or L. jensenii species was associated with undetectable cervical HIV-1 (\(P=0.046\)) and reduced genital HIV-1 shedding (\(P=0.048\)) compared to other species. Our findings endorse using Lactobacillus-based strategies to aid the prevention of HIV-1 transmission among Indian women, however confirmation by future prospective studies is indeed warranted.

It is now very well accepted that lactobacilli are critical components of the microbiota of healthy vagina [1]. Vaginal lactobacilli are reported to offer protection against genital infections, bacterial vaginosis, vaginal candidiasis and recurrent urinary infections [2]. Hydrogen peroxide (H\(_2\)O\(_2\))-producing lactobacilli have been associated with reduced likelihood of genital human immunodeficiency virus (HIV) type 1 (HIV-1) shedding [3]. Thus exogenously applied Lactobacillus probiotics hold promise to restore and/or maintain vaginal health. The use of engineered lactobacilli that can express broadly neutralizing antibodies against HIV-1 and the delivery of microbicide by lactobacilli colonizing the vagina are being researched as strategies for prevention of HIV transmission [4].

One of the crucial determinants for successful administration of lactobacilli to the vaginal niche includes the use of appropriate Lactobacillus species [5]. The currently available vaginal probiotics predominantly contain L. rhamnosus and L. reuteri [6, 7] species. Of the two studies characterizing vaginal lactobacilli among healthy women of reproductive age from India, L. reuteri, L. fermentum and L. salivarius were reported as the predominant species in one [8]; while L. crispatus, L. gasseri, and L. jensenii in the other [9]. To increase the chances of successful colonization, optimal candidates for vaginal probiotics ought to be Lactobacillus species that are normally found in the vagina [10]. Hence understanding of species composition in different geographic regions becomes imperative for the development of better prophylactics.

It is reported that species composition of microorganisms differs in HIV-positive women as compared to HIV-negative women, however studies characterizing the Lactobacillus species among HIV-positive women are sparse, likewise the interactions between Lactobacillus species and genital HIV-1 shedding are not well studied [3, 11]. Better understanding of these dynamics might help inform strategies to improve vaginal health and decrease the risk of HIV transmission.

The present study was thus conducted to identify the culturable vaginal lactobacilli in HIV-negative and HIV-positive women and to determine their association with cervical HIV-1 shedding.

In this cross-sectional study, age-matched 54 HIV-negative and 76 HIV-positive women [55 chronic progressors and 21 long term non-progressors (LTNPs)] attending the National...
AIDS Research Institute Clinics at Pune, India were enrolled from January 2015 to December 2016. LTNPs were defined as individuals with asymptomatic HIV infection for more than 7 years who had stable CD4 counts above 500 cells µl⁻¹ and who had not taken antiretroviral treatment (ART), while chronic progressors were defined as asymptomatic ART-naïve HIV-infected women with CD4 counts <500 cells µl⁻¹. The study was approved by the institutional Ethics Committee of the National AIDS Research Institute (Protocol number: NARI EC/2013–05) and samples were collected after obtaining written informed consent from the participants. Enrollment criteria included women with no vaginal discharge, no visible infection (by speculum examination), and no systemic or vaginal antimicrobial therapy for at least 2 weeks before enrollment.

A pelvic examination was performed by trained physicians for all participants during which vaginal swabs and cervical-vaginal lavage (CVL) samples were collected as described previously [12]. All samples were collected during the proliferative phase (between 7 to 14 days after the first day of the menses) of the menstrual cycle. The vaginal swabs were processed for detection of bacterial vaginosis as per Nugent’s criteria, for Candida colonization by Gram stain and trichomoniasis by wet mount.

For *Lactobacillus* culture the vaginal swabs were plated on Lactobacillus MRS HiVeg (MRS) agar and blood agar (HiMedia Laboratories, Mumbai, India) within 2 h of collection and incubated in anaerobic jars with Anaerogas Pack (HiMedia Laboratories, Mumbai, India) at 37°C for 24 h. Colonies showing Gram-positive bacilli on MRS agar and small transparent colonies from blood agar yielding Gram-positive bacilli but not growing on MRS agar (for identification of *L. iners*) were further evaluated. *Lactobacillus* quantification was done by the calibrated loop method and H₂O₂ production was determined on tetramethyl benzidine agar plates as described previously [12].

Genomic DNA was extracted from *Lactobacillus* isolates using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) as per manufacturer’s instructions and used as template for amplification. The isolates were identified to the species level by sequencing the 16S rDNA using 0.1 mM of forward: 5¢ AGA GTT TGA TCC TGG CTC AG and 3¢ reverse; 5¢ CCC ACT GCT GCC TCG GTG AG 3¢ primers. PCR was performed in 50 µl containing 5 µl 10 x buffer, 2.5 mM MgCl₂, 200 mM each dNTP, 10 pmol of each primer, 0.5 U Taq polymerase and 5 µl of lactobacilli DNA, with the remaining volume made up with sterile distilled water [8]. Amplification was done by initial denaturation at 93°C for 3 min, followed by 30 cycles at 93°C for 1 min, 48°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. The PCR products were purified with the QIA quick PCR purification kit (Qiagen Valencia, CA, USA) and subjected to direct sequencing with the same primers using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on the 3100 Genetic analyser (Applied Biosystems, Foster City, CA, USA). The sequences generated were identified through BLAST search and genus and species were determined by the criteria of 98% sequence identity for species and 95% sequence identity for genus.

HIV serostatus of all participants was determined by commercially available rapid antibody tests; Combaid's RS Advantage HIV 1+2 (Arkray Healthcare Pvt Ltd, Gujarat, India), SD Bioloiobe ½ 3.0 (Alere Medical Pvt Ltd, Gurgoan, India) and Meriscreen HIV 1–2 (Meril Diagnostics Pvt Ltd, Gujrat, India) using the National AIDS Control Organization, India, recommended HIV testing algorithm [13] and CD4 count was estimated using the FACSCalibur flow cytometer (Becton Dickinson, Singapore). Plasma and cervical HIV-1 viral load measurements were done using Abbott m2000rt HIV-1 RealTime PCR (Abbott Molecular, USA). The lower limit of detection of this assay is 40 RNA copies ml⁻¹.

The Stata 10.0 IC software (StataCorp LLC, USA) was used for statistical analysis. Chi-square test was used to determine association of different *Lactobacillus* species with colony formation and H₂O₂ production. Fisher’s exact test is used if expected cell frequency is less than 5. Trends with colony formation were obtained using linear by linear test, while regression analysis was used for controlling for plasma viral load. Results with P value<0.05 were considered as statistically significant.

The median age of women was 36 years (range, 33–39) in the HIV-negative group and 34 years (range, 31–38) in the HIV-positive group, while the mean vaginal pH was 5.0 (range, 3.6–8.0) and 5.0 (range, 3.0–8.0) in HIV-positive and HIV-negative women respectively.

Evaluation of vaginal flora by Nugent’s score revealed that 5/54 (9.3 %) HIV-negative women and 18/76 (23.7 %) HIV-positive women had altered flora (Nugent’s score 4–6), while none of the women in both groups had bacterial vaginosis, trichomoniasis, or *Candida* infection.

Lactobacilli were isolated in 77.8 % (42/54) of HIV-negative and 73.7 % (56/76) of HIV-positive women, with no statistically significant difference in the isolation rates in the two groups (P=0.681). Among the HIV-positive women lactobacilli were isolated in 74.5 % (41/55) of chronic progressors and 71.4 % (15/21) of LTNPs, with no statistically significant difference in the isolation rates in the two groups (P=0.783).

HIV-positive women had significantly reduced quantity of lactobacilli (P=0.009) and H₂O₂-producing isolates, including strong H₂O₂-producers (P=0.015) as compared to the HIV-negative women (Table 1). In the HIV-positive group no statistically significant difference in the quantity of lactobacilli (P=0.860) and H₂O₂-production (P=0.754) was seen among chronic progressors and LTNPs. A trend of increasing H₂O₂ production with increasing quantity of lactobacilli was observed, P=<0.001 (data not shown).
The Lactobacillus species isolated in both groups are presented in Table 2. L. crispatus, L. gasseri and L. jensenii were the major species isolated overall and in both HIV-positive and HIV-negative women. No statistically significant difference in the distribution of Lactobacillus species between the two groups was observed. The Lactobacillus species remained unidentified in 8 isolates, including 4 each from the HIV-negative and HIV-positive group. We observed H₂O₂ production in 35/42 (83.3 %) and 34/56 (60.7 %) Lactobacillus isolates from HIV-negative and HIV-positive women respectively. The species showing H₂O₂ production were L. crispatus, L. gasseri, L. jensenii, L. reuteri, L. vaginalis and L. mucosae.

L. crispatus, L. gasseri and L. jensenii species were present in significantly greater quantity (P=0.001) and were strong H₂O₂-producers (P=0.033) as compared to other species in isolates from both HIV-positive as well as HIV-negative women.

The median CD4 counts in HIV-positive women were 580 cells µl⁻¹ (IQR: 415, 792) overall, 472 cells µl⁻¹ (IQR: 392, 640) in chronic progressors and 770.5 cells µl⁻¹ (IQR: 636, 1051) in LTNPs. No association between isolation rate, species or quantity of lactobacilli and CD4 counts was seen.

The mean log_{10} plasma HIV-1 viral load (RNA copies ml⁻¹) when detectable virus was present, was 3.73 ±1.02 overall, 4.29±0.92 in chronic progressors and 3.11 ±0.74 in LTNPs. The mean log_{10} cervical HIV-1 viral load when detectable virus was present, was 2.85±0.32 overall, 2.85±0.35 in chronic progressors and 2.86±0.31 in LTNPs. The cervical viral load was undetectable in majority of LTNPs (84.2 %) as compared to chronic progressors (57.4 %), (P=0.04).

There was no association between plasma viral load and the quantity of lactobacilli (P=0.170) or presence of either L. crispatus, L. gasseri or L. jensenii species (P=0.777) as compared to other species. After controlling for plasma viral load, undetectable cervical HIV-1 viral loads (P=0.006) and a significantly reduced cervical HIV-1 viral load (P=0.001) with increasing quantity of lactobacilli was seen (Table 3). We observed that presence of either L. crispatus, L. gasseri or L. jensenii species were associated with undetectable cervical HIV-1 viral load (P=0.046) and reduced cervical HIV-1 viral load when detectable virus (P=0.048) was present as compared to other species (Table 3).

Identification and characterization of the Lactobacillus species colonizing women in a particular region becomes imperative, if strategies for restoration of vaginal health through administration of probiotic or engineered lactobacilli are to be planned. The longevity of HIV-positive women with the availability of antiretroviral treatment and their increased risk for acquiring sexually transmitted infections (STIs) warrants the need for understanding the lactobacilli composition in this population. The present report characterizing vaginal Lactobacillus species among HIV-positive women and determining their association with genital HIV-1 shedding addresses an important research gap and will add to the relatively meagre global literature on this topic.

In concordance with reports from different parts of the world L. crispatus, L. gasseri and L. jensenii were the predominant cultivable Lactobacillus species detected in HIV-negative women from India [14–18]. Our results corroborate Madhivanan et al. from South India who reported L. crispatus, L. gasseri and L. jensenii as the predominant cultivable species, however contrast with Garg et al. from North India where L. reuteri, L. fermentum and L. salivarius were reported as the predominant species, indicating that species variation may occur at sub-populations within a country and should be considered while choosing a probiotic [8, 9].
We did not observe any significant difference in the isolation rate and species distribution between the HIV-negative and HIV-positive women narrowing the list of potential probiotic Lactobacillus species to L. crispatus, L. gasseri and L. jensenii that can be applied to both groups. In agreement with other researchers and confirming our previous finding, we found significantly reduced quantity of lactobacilli and H2O2-producing isolates in HIV-positive women [12, 19]. We also report here for the first time a significant positive association between the quantity of lactobacilli and H2O2-production, indicating the importance of optimal dosage in Lactobacillus-based formulations. Furthermore, Hitti et al. in their prospective study demonstrated the effect over time of H2O2-producing lactobacilli on HIV viral load in vaginal secretions and showed that appearance of H2O2-producing lactobacilli between visits was associated with 0.7 log10 decrease in CVL viral load, while their loss resulted in a 0.5 log10 increase in CVL viral load when compared with stable colonization [20]. However, due to the cross-sectional nature of our study we could not show this cause-effect relationship.

It has been reported that in about 30% women with undetectable plasma HIV-1 viral load, detectable virus may be present in the cervicovaginal secretions, suggesting that vaginal bacterial ecosystem might influence genital HIV-1 shedding [3]. In the present study we noted that Lactobacillus species, especially the presence of L. crispatus, L. gasseri and L. jensenii were associated with undetectable genital HIV-1 and reduced genital HIV-1 shedding when the virus was detectable. Our finding corroborates with previous studies which show that Lactobacillus-dominated cervicovaginal microbiota, particularly the L. crispatus-dominated cluster are associated with reduced genital HIV viral load and HIV/STI prevalence [3, 21, 22].

It has been reported that genital infections associated with significant inflammation, namely gonorrhea, chlamydia, yeast, and herpes are associated with increased rates of genital HIV-1 RNA and DNA shedding; while treatment of these infections has been associated with a decrease in the detection of HIV-1 in genital secretions [23, 24]. However all women enrolled in our study were clinically asymptomatic. This indicates that genital HIV-1 shedding in these women combined with reduced lactobacilli may increase chances of female-to-male sexual transmission of HIV-1 and acquisition of genital infections, including infection by new HIV strains [25] and has implications for the use of Lactobacillus-based vaginal probiotics to maintain vaginal health in them.

Our study had limitations. All enrollments were done at a single urban clinic and hence the possibility of self-selected population cannot be ruled out, likewise we were not able to determine the effect of all systemic determinants of HIV-1 shedding in our participants. Also due to the cross-sectional nature of the study we could not identify the causal relationship between the presence of lactobacilli and reduction in HIV transmission.

To conclude, our findings suggest that exogenously applied Lactobacillus-based vaginal strategies with L. crispatus, L. gasseri and L. jensenii species hold promise for maintaining/restoring vaginal health and can aid prevention of HIV-1 transmission among Indian women; however confirmation by future prospective studies is indeed warranted.

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### Conflicts of interest
The authors declare that there are no conflicts of interest.

### Ethical statement
The study was approved by the institutional ethics committee (Name: Ethics committee of the National AIDS Research Institute, Pune, India) (Protocol approval number: NARI EC/2013–05), and all participants gave written informed consent for the study.

### References


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