



## Vaginal & gut microbiota diversity in pregnant women with bacterial vaginosis & effect of oral probiotics: An exploratory study

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**Background & objectives:** The vaginal microbiota undergoes subtle changes during pregnancy and may affect several aspects of pregnancy outcomes. There has been no comprehensive study characterizing the gestational vaginal and gut microbiota and the dynamics of the microbiota with oral probiotics among Indian women. Hence, the study was aimed to explore the microbiota of pregnant women with normal microbiota and bacterial vaginosis (BV) environments and the effect of oral probiotics on the microbiota and the BV status in these women.

**Methods:** Using high-throughput Illumina-MiSeq sequencing approach, the *16S rRNA* gene amplicons were analyzed and the vaginal and gut microbiota of pregnant women with and without BV and pre- and post-probiotics (*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14) intervention for a month was characterized.

**Results:** The study revealed a compositional difference in the vaginal and gut microbiota between BV and healthy pregnant women. The vaginal microbiota of healthy women was characteristically predominated by *Lactobacillus helveticus*, followed by *L. iners* and *L. gasseri*; in contrast, women positive for BV harboured higher  $\alpha$ -diversity and had lower abundance of *L. helveticus*. Similarly, *Prevotella copri*, a gut microbe, associated with normal environment was detected in the vaginal samples of all pregnant women without BV, it remained undetected in women with the infection, while all women with BV had *Gardnerella vaginalis*, which decreased significantly with probiotic treatment. Gut microbiota also revealed dominant abundance of *P. copri* in healthy women, whereas it was significantly lower in women with BV. The bacterial clade, *P. copri* abundance increased from 9.17 to 16.49 per cent in the probiotic group and reduced from 7.75 to 4.84 per cent in the placebo group.

**Interpretation & conclusions:** This study showed gestational vaginal and gut microbiota differences in normal and BV environments. With probiotic treatment, the dynamics of *L. helveticus* and *P. copri* hint towards a possible role of probiotics in modulating the vaginal microbiota.

**Key words** Bacterial vaginosis - dysbiosis - gut microbiota - *Lactobacillus* - maternal health - Nugent's score - preterm birth - probiotics and vaginal microbiota

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A healthy woman vaginal microbiota, generally dominated by *Lactobacillus* species, plays a key role in reproductive health and disease<sup>1</sup>. Studies using high-throughput sequencing tools have revealed that vaginal microbial communities could be categorized into five community state types (CSTs) based on the dominance of specific *Lactobacillus* species (CST I, II, III and V) or the prevalence of a somewhat diverse, *Lactobacillus*-poor configuration (CST IV)<sup>1-4</sup>. Vaginal lactobacilli are known to play a beneficial/symbiotic role for the host by restraining the growth of potential/opportunistic pathogens, thereby maintaining a healthy vaginal microbiota<sup>5</sup>. However, a vaginal microbiota deficient in lactobacilli and populous with facultative anaerobes such as *Gardnerella vaginalis*, *Atopobium vaginae* and *Mycoplasma hominis* may represent a somewhat abnormal (dysbiotic) vaginal microbiota configuration and may also prognosticate predisposition to bacterial vaginosis (BV) and other adverse health outcomes including preterm births<sup>6,7</sup>. It has been reported that there is 40 per cent of increased risk for preterm labour and illustrated the complex process of BV pathogens in triggering the early labour process, including inflammatory responses<sup>5</sup>. Lamont *et al*<sup>8</sup> presented a review comparing the aetiology of urogenital tract infections (especially BV) causing preterm births. The advent and expansion of high-throughput sequencing have been remarkably advantageous and efficacious in revealing new insights of the dynamics of the vaginal microbiota of pregnant<sup>9,10</sup> as well as non-pregnant women<sup>11-13</sup>. In this context, we employed the MiSeq sequencing approach to examine the diversity and features of the vaginal and gut microbiota in pregnant Indian women with normal vaginal microbiota versus BV and the effect of probiotics.

Probiotics have been in usage to prevent and/or ameliorate various inflammatory conditions and infections of the gut as well as the genital-urinary niches<sup>5</sup>. Probiotic lactobacilli have been shown to restore the vaginal *Lactobacillus* population and ameliorate BV<sup>5</sup> and other urinary tract infections<sup>5,14,15</sup> in non-pregnant women. In addition, oral probiotics, *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14, have been found to augment the vaginal load of lactobacilli and diminish the occurrence and recurrence of BV in non-pregnant women<sup>16</sup>. However, whether these strains (*i.e.*, GR-1 and RC-14) could also confer beneficial effects in pregnant women with BV remains to be tested. This testing becomes particularly

important in the context to the findings that vaginal microbial signatures are more stable and resilient during pregnancy than at other stages during adulthood<sup>1,17,18</sup>. Thus, the effect of oral administration of live GR-1 and RC-14 was examined on the composition of vaginal microbiota in pregnant Indian women diagnosed with a normal or high Nugent score, with an aim to test whether this could be beneficial in ameliorating BV in these women. Although the point of interest was the changes in vaginal microbiota status but basic changes in the gut microbiota were also checked to understand gut colonization of the orally supplemented vaginal probiotic strains.

### Material & Methods

This was a double-blind, randomized, placebo-controlled intervention trial in pregnant women with and without BV. The recruitment process was conducted for one year (2014-2015) at Modern Government Maternity Hospital, Hyderabad, India, after obtaining necessary ethical approvals. Pregnant women with gestation age between 26 and 30 wk, who agreed to participate, were included after taking written informed consent. The trial was registered with Clinical Trial Registry, India (CTRI/2013/01/003337).

The inclusion criterion was pregnant women in their third trimester and with normal or abnormal vaginal microbiota (positive for BV infection by Nugent score and Amsel's criteria). Women participating in another clinical study, HIV infected with multiple gestation, cervical incompetence, foetus with major congenital malformations in current gestation, insulin-dependent diabetes mellitus, systemic arterial hypertension under medication, chronic asthma requiring intermittent therapy and continuous or recent corticosteroid therapy (or any other medical or surgical complications in present gestation) were excluded. Further, women aged less than 19 or more than 35 yr and those who had intercourse in the last 24 h, were also excluded. Although 140 pregnant women were randomized to receive either probiotic or placebo, but next-generation sequencing (NGS) analysis was performed at the end of the intervention in a randomly selected sub-sample of 16 (8 women each from probiotic and placebo group). NGS analysis was also performed in 16 women with normal vaginal microbiota for the control group.

*Sample collection and intervention:* During the first visit, pregnant women were screened and those who fulfilled the inclusion criteria and had abnormal

vaginal microbiota based on Nugent's score and Amsel's criteria<sup>19</sup> participated in the study. During the second visit, past and present obstetric details were documented for all the enrolled women and they were randomized into two groups, *i.e.*, probiotic and placebo. Pregnant women who agreed to participate in the study had a speculum examination to collect the vaginal samples under direct visualization from the posterior vaginal fornix by an obstetrician using a sterile cotton swab (HiMedia Labs, Mumbai) and smeared on glass slide. These slides were Gram stained and categorized using the Nugent score. Those with a score of 0-3 were reported as having a low score, whereas those with scores of 4-6 and 7-10 were categorized as intermediate and high, respectively<sup>19</sup>. Vaginal pH was measured using the pH strips (Qualigens Fine Chemicals, Mumbai) and scored according to the manufacturer's instructions using a scale ranging from 4.0 to 7.7. Vaginal discharge, amine or fishy odour when KOH was added to vaginal discharge and the presence of clue cells (epithelial cells with borders obscured by pathogenic microbial cells) were also observed to confirm BV. Clindamycin vaginal cream two per cent, 5 g at bedtime intra-vaginally for seven days was advised for all the positive women as per the WHO guidelines<sup>20</sup>. The probiotic capsules (procured from CHR HANSEN, Denmark, Germany) containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 ( $10^9$  cfu in equal proportion) or matching placebo (dextrose) were given once daily for 30 days from the third trimester to BV-positive pregnant women. The women participants were handed over a bottle containing probiotic/placebo capsule for 15 days. Compliance was checked by reminding them over phone, and the women were asked to return the empty bottles when they visited the centre to collect the next 15 days dosage.

The vaginal swab samples were collected (at baseline and 30 days of intervention) and suspended in transport buffer (Amines liquid medium) and stored at  $-20^{\circ}\text{C}$  until assayed. Stool samples were also collected before and after the intervention. These swabs were thawed on ice and thoroughly mixed using cyclomixer (Remi CM 101, Remi Lab Instruments, Mumbai). Cells were transferred to a sterile DNase/RNase free 2 ml tube where an enzymatic lysis step was carried out for 1 h at  $37^{\circ}\text{C}$  as per the published methods with minor modifications<sup>1,21,22</sup>. Samples were placed for additional mechanical disruption using a bead beater (FastPrep instrument, Qbiogene, Carlsbad, CA, USA) set at 6.0 m/sec for 30 sec. The resulting lysate was

processed and purified using QIAamp DNA Mini kit (Qiagen, Manchester, UK), and the DNA was eluted in 100  $\mu\text{l}$  of TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0. Stool DNA was isolated by using QIAamp DNA stool kit (Qiagen, Manchester, UK) as per the manufacturer's protocol.

*Next-generation sequencing (NGS) on Illumina MiSeq platform:* The MiSeq analysis was performed on Illumina platform (Illumina Inc., San Diego, CA, USA) on a subsample of a prospective study of pregnant women with and without BV to evaluate the effect of probiotics on vaginal microbiota. Due to budget constraint, the minimum sample size taken for NGS analysis was 16 participants in each category which was similar to earlier studies<sup>23,24</sup>, and probiotic intervention was carried out only in pregnant women with BV.

The V3 hypervariable regions of 16S rRNA genes were amplified for sequencing using forward and reverse fusion primers. The forward primer was constructed with the Illumina i5 adapter (5'-3') (V3\_Faatgatacggcgaccaccgagatctacactctttcctacacgacg ctcttccgatctcctacggg aggcagcag, V3\_F modified2 aatgatacggcgaccaccgagatctacactctttcctacacgacgct ctccgatctNNNN cctacgggagggcagcag), and the reverse primer was constructed with (5'-3') the Illumina i7adapter(V3\_Rcaagcagaagacggcatacagatcgtagtgac tggagttcagactgtgtgctcttccgatctattaccgcggtgctgctgg) with 6 bp barcode<sup>25</sup>. Amplifications were performed in 25  $\mu\text{l}$  reactions with Ex Taq DNA polymerase (DSS Takara Bio India Pvt Ltd.), 1  $\mu\text{l}$  of each 5 pM primer and 50 ng of template. Reactions were performed on T100 Thermal cycler (Biorad, USA) under the following thermal profile:  $95^{\circ}\text{C}$  for five minutes, then 25 cycles of  $95^{\circ}\text{C}$  for one minute,  $60^{\circ}\text{C}$  for one minute,  $72^{\circ}\text{C}$  for one minute, followed by one cycle of  $72^{\circ}\text{C}$  for seven minutes and  $4^{\circ}\text{C}$  hold. The PCR products were confirmed by gel electrophoresis in three per cent agarose gels and visualized with ethidium bromide staining under Syngene G box (Syngene, Cambridge, UK). Products were cleaned with Agencourt AMPure XP (Beckman Coulter, Indianapolis, Indiana, USA) and quantified using Qubit Fluorometer (Thermo Fisher Scientific, India).

The 96 multiplexed samples were pooled into a single library for sequencing on the MiSeq. The pooled library containing indexed amplicons were loaded onto the reagent cartridge and then onto the

instrument along with the flow cell. Automated cluster generation and paired-end sequencing with dual index reads of  $2 \times 250$  bp (base pairs) were performed in a single 39 h run. On the instrument, the global cluster density and the global passed filter per flow cell were generated. The MiSeq Reporter software (Illumina) determined the percentage indexed and the clusters passing the filter for each amplicon or library. For each cluster that passes filter, a single sequence is written to the corresponding sample's R1 FASTQ file, and for a paired-end run, a single sequence is written to the sample's R2 FASTQ file. All the procedures of NGS and analysis were performed at the Sandor Life Sciences Pvt Ltd., Hyderabad, Telangana, India.

**Sequencing data analysis:** Microbial community analysis was done using Quantitative Insights into Microbial Ecology (QIIME) software package (<https://qiime.org>). The short-read sequencing data sets were normalized and analyzed using the operational taxonomic unit approach. The trimmed sequences in FASTQ file were then uploaded to metagenomic RAST server (MG-RAST). The analysis was performed in the MG-RAST server within ribosomal database project and taxonomic assignment was carried out with 97 per cent homology match. Bacterial abundance data at phylum, class, order, family, genus and species levels were downloaded from the MG-RAST server ([www.mg-rast.org](http://www.mg-rast.org)).

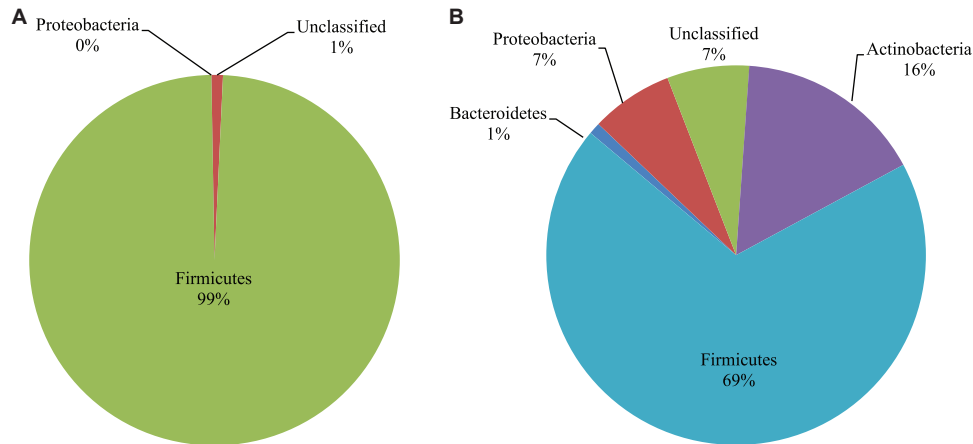
**Statistical analysis:** Principal coordinate analysis (PCoA) was performed to find clusters of similar groups of samples by QIIME. PCoA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions and reflects the similarity of the biological communities<sup>26</sup>. A matrix using the UniFrac metric (unweighted) for each pair of environments was calculated. The first three principal dimensions were used to plot a three-dimensional graph. To test whether the results were robust to sample size, a sequence-jackknifing technique<sup>27</sup> was used in which a smaller number of sequences were chosen at random from each sample (1000 sequences). Unweighted distance metric accounts for presence/absence of taxa. Bacterial groups at phylum-, genus- and species-level comparisons between control and BV and between baseline and end line after probiotic and placebo intervention were performed using independent t test and paired t test. The alpha diversity and beta diversity of annotated samples were estimated from the distribution of the species-level annotations and were

presented by PCoA plot analysis. Jackknife-supported confidence ellipsoids analysis<sup>27</sup> was also done to compare microbiota diversity among groups.

## Results

**Vaginal microbiota diversity in pregnant women with normal microbiota and bacterial vaginosis:** The age of the women participated in the study was  $23.31 \pm 1.74$  yr (mean  $\pm$  standard deviation). The mean gestational age at the time of sampling was  $28.2 \pm 1.31$  wk. They belonged to low socio-economic status as per the modified Kuppuswamy's guidelines<sup>28</sup>. The Nugent's scoring decreased from 6.7 to 3.3 and 6.2 to 4.8 in probiotic and placebo groups, respectively. For microbiota analysis, vaginal and faecal samples were collected from 16 pregnant women with normal vaginal microbiota and 16 with BV using sequence-based method. Using *t* test,  $\alpha$  diversity (Shannon matrix) (indicating bacterial diversity across women within a group) ( $P < 0.001$ ) and  $\beta$  diversity (indicating diversity of bacterial species within an individual) were found to be more in women with BV in comparison with normal pregnant women ( $P < 0.001$ ). Pregnant women with normal microbiota had minimal diversity when compared to women with BV as indicated by the jackknife-supported confidence ellipsoids analysis. At phylum level, *Firmicutes* was the most abundant in normal pregnant women (98.2%) compared to the women with BV (68.7%) (Fig. 1) and the next most abundant phylum in BV subjects was *Actinobacteria* (16%), whereas in pregnant women with normal vaginal microbiota, *Actinobacteria* was negligible (0.31%).

At species level, the vaginal microbiota showed *L. iners* to be the most frequent bacteria present in all women with or without BV. Other species such as *L. helveticus*, *L. gasseri* and *L. reuteri* were frequently present in women with or without BV; *L. crispatus* and *Prevotella copri* were completely absent in women with BV, though all women with normal microbiota had *P. copri*. In contrast, *L. acidophilus* was completely absent in women with normal microbiota but present in 73 per cent women with BV. As for relative abundance, *L. helveticus* ( $44.1 \pm 45.275\%$ ;  $19.2 \pm 32.494\%$ ) followed by *L. iners* ( $24.5 \pm 24.341\%$ ;  $17.3 \pm 19.14\%$ ) were the most abundant species in women with normal microbiota and BV; however, *L. reuteri* ( $P = 0.055$ ) was more abundant in normal women compared to those with BV. *Gardnerella vaginalis*, one of the causative bacteria of BV, showed more ( $P < 0.01$ ) abundance ( $9.86 \pm 13.86\%$ ) in women with BV compared to normal



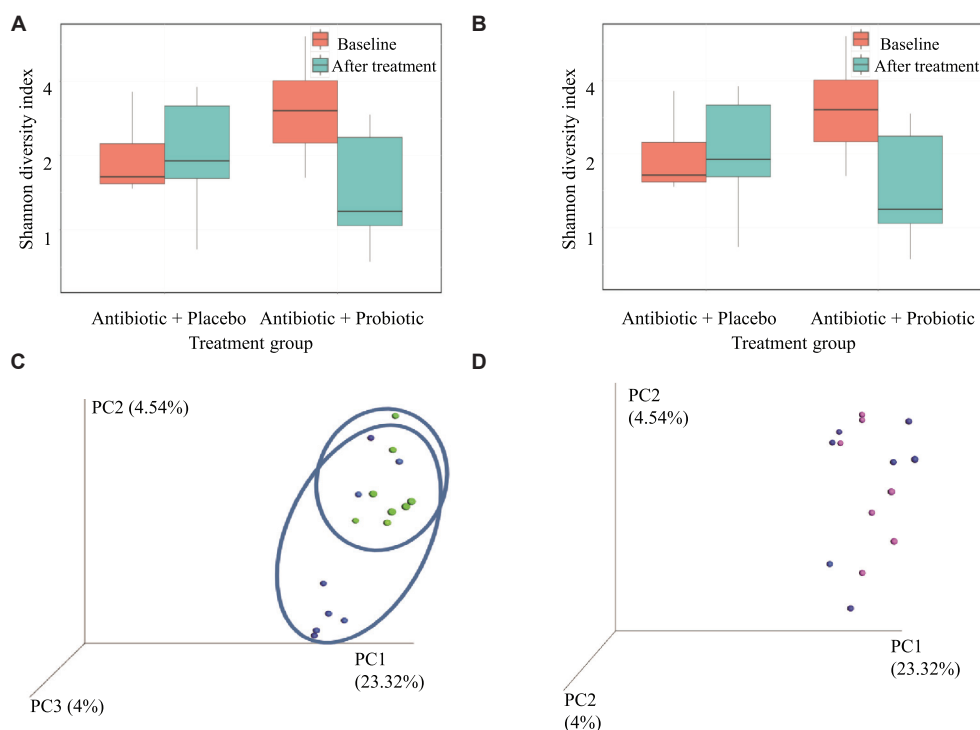
**Fig. 1.** Relative abundance of the bacterial groups representing the vaginal microbiota of normal and bacterial vaginosis (BV) positive women. Pie charts showing the phylum-level distribution of bacterial relative abundance (%) in the vagina of the (A) normal women and (B) BV positive women.

microbiota ( $0.084 \pm 0.062\%$ ). *Acidaminococcus* sp. D21 was also significantly ( $P < 0.05$ ) more in women with BV. Other species such as *Butyrivibrio fibrisolvens*, *Prevotella bivia* and *Pseudomonas rhodesiae* were not detectable in normal women but were evident in women with BV. As expected, the mean Nugent score was significantly ( $P < 0.001$ ) lower (2.2) in normal pregnant women compared to pregnant women with BV (6.5).

**Vaginal microbiota diversity in pregnant women with BV after oral intervention with probiotic (GR-1 and RC-14):** All the pregnant women with BV ( $n=16$ ) were treated with local clindamycin for seven days and were randomized to either probiotic ( $n=8$ ) or placebo ( $n=8$ ) arms. The intervention group received one capsule containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 ( $2-5 \times 10^8$ ) once daily for 30 days, and the placebo group received identical capsule containing dextrose sugar for similar period. At the baseline (before the intervention), the pH and Nugent score were similar in clindamycin + probiotic (probiotic group) and clindamycin + placebo (placebo group). At baseline, the alpha diversity was non-significant ( $P=0.072$ ) between the intervention and placebo groups; however, some primary pathogens such as *G. vaginalis*, *Acidaminococcus* sp. D21 and *A. vaginae* were significantly higher in the probiotic group at baseline (Fig. 2). Similarly, at phylum level, the baseline *Firmicutes* was significantly less, while *Actinobacteria* and *Proteobacteria* were significantly more in the probiotic group compared to the placebo. These factors could not be controlled as microbiota data were not available before randomization.

A significant difference was found in  $\alpha$ -diversity ( $P < 0.05$ ) and species richness ( $P=0.015$ ) after 30 days of probiotic treatment when compared to baseline. In addition, noticeable difference in the  $\beta$ -diversity (PCoA plot analysis) was observed in the probiotic group (Fig. 2). On comparing the bacterial communities at phylum level, probiotics group showed significant increase in the relative abundance of *Firmicutes* from baseline (50.69%) to after treatment (91.21%). In contrast, the abundance of *Bacteroidetes* decreased from 0.96 to 0.59 per cent after probiotic treatment. Other phylum, *Actinobacteria* also decreased from baseline (25.07%) after probiotic treatment (4.28%). Phylum-level analysis in the placebo group showed no change in the relative abundance of *Firmicutes* after placebo (85.91%), as compared to baseline (88.10%); and other phylum, such as *Actinobacteria* (5.57%) and *Proteobacteria* (3.71%), increased to 6.032 and 6.07 per cent, respectively, in placebo group.

At species level, in the probiotic group, there was significant ( $P < 0.05$ , t test) decrease in the *G. vaginalis* (from 12.40 to 2.83%) and increase in *L. iners* ( $P=0.017$ ) and *P. copri* ( $P=0.011$ ) and insignificant but considerable increase in *L. crispatus* ( $P=0.075$ ) was seen. The supplemented strains GR-1 (0-0.025%) and RC-14 (0-0.021%) showed a non-significant increase in the probiotics group while placebo group did not alter much. The other lactobacilli such as *L. helveticus*, *L. gasseri* ( $P=0.011$ ) and *Megasphaera genomosp type 1* ( $P=0.004$ ), a genus under *Firmicutes* decreased in placebo group as compared to probiotics. Those pregnant women receiving placebo continued to have more diversity in vaginal microbiota, compared



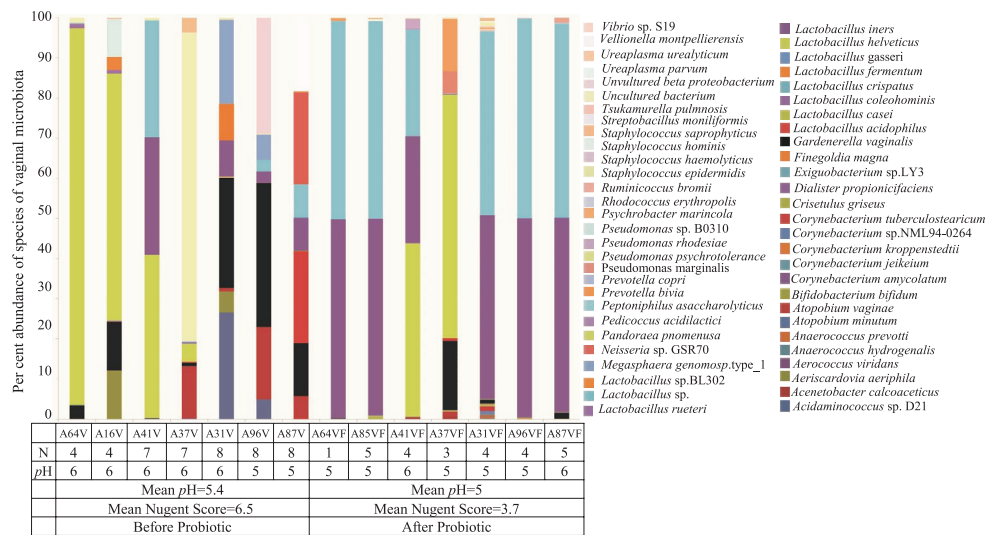
**Fig. 2.** (A) Shannon metrics of alpha diversity (B) Alpha diversity of observed species (C) 3D- principal coordinate analysis (PCoA) plot with unweighted UniFrac distance metric of vaginal swab of bacterial vaginosis (BV) positive subjects baseline (blue) and BV positive women with probiotic + clindamycin treatment (green) showing jackknife-supported confidence ellipsoids. (D): 3D-PCoA plot, principal components (PC) 1, 2 and 3 with unweighted UniFrac distance metric of vaginal swab of BV positive women baseline (blue) and BV positive women with probiotic + clindamycin treatment (pink) showing jackknife-supported confidence ellipsoids.

with women who received the probiotics, and there was a non-significant increase in *G. vaginalis* (from 2.609 to 3.81%) from the baseline in the placebo group (Fig. 3 and Table). Some species such as *Acidaminococcus* sp. D21 and *A. vaginae* showed a decreasing trend in both the groups with reference to baseline. After one month of intervention, the vaginal bacteria communities of probiotic treatment resembled normal vaginal microbiota.

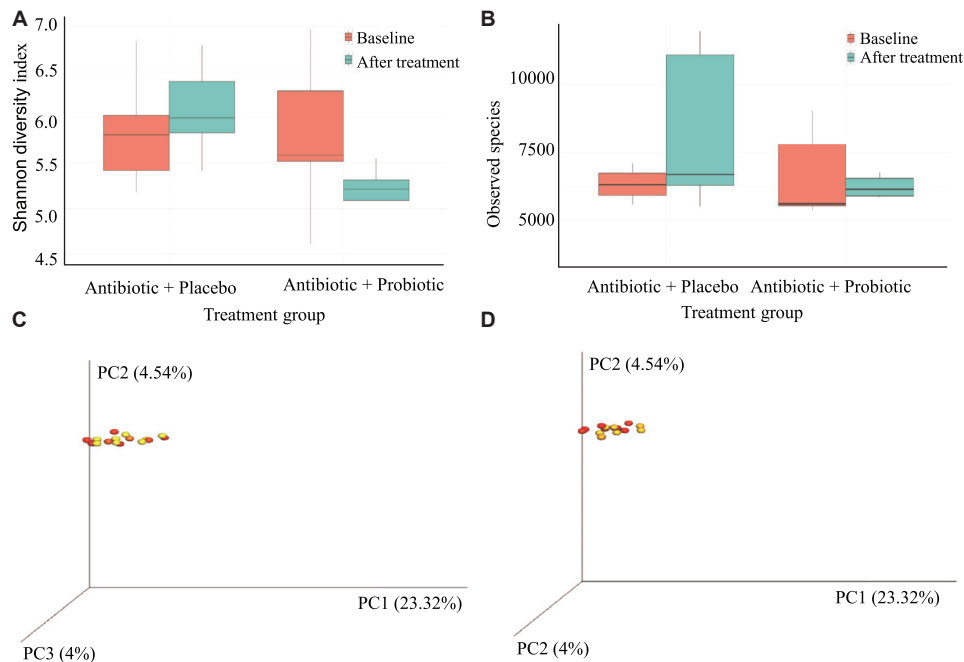
*Gut microbiota diversity in pregnant women with or without BV:* Analysis of gut microbiota signatures between BV-negative and BV-positive pregnant women revealed some differences in the faecal bacterial clades. For instance, the phylum-level analysis of the gut microbiota composition demonstrated a lower abundance of *Bacteroidetes* in BV-positive women as compared with the control group (11.6 vs. 24%); a relatively higher abundance of *Actinobacteria* was observed in women with BV compared to normal pregnant women (11.04 vs. 5.54%). The abundance of *Firmicutes*, however, was comparable between the two groups (60.9% control

group; 61.2% BV group). Species-level analyses revealed lower relative abundance of *P. copri* (8.2 vs. 16.7;  $P=0.11$ ), *Dialister invisus* (0.7 vs. 4.1;  $P=0.001$ ) and *Lactobacillus salivarius* (0.0 vs. 2.2;  $P=0.02$ ) in women with BV, while exhibiting a higher abundance of *Clostridium bifermentans* (5.2 vs. 0.0;  $P=0.0$ ), *Clostridium disporicum* (5.0 vs. 1.1;  $P=0.04$ ) and *Atopobium vaginae* (1.3 vs. 0.0;  $P=0.01$ ) compared to normal women (data not shown). Overall, the gut microbiota diversity in terms of Shannon-index appeared to be similar between the two groups; however, women with BV demonstrated significantly higher observed species richness as compared with normal women.

*Gut microbiota diversity in BV-positive pregnant women after oral probiotic intervention:* After 30 days of intervention, women receiving clindamycin + probiotics demonstrated lower diversity in the gut microbiota as compared with women receiving clindamycin + placebo (Fig. 4). The probiotic group demonstrated a significant reduction in  $\alpha$ -diversity ( $P=0.010$ ) and a relative, but insignificant



**Fig. 3.** Relative abundance of the species representing the vaginal microbiota of women with bacterial vaginosis (BV) before and after probiotics supplementation.



**Fig. 4.** (A) Shannon metrics of alpha diversity. (B) Alpha diversity of observed species. (C) 3D-principal coordinate analysis (PCoA) plot with unweighted UniFrac distance metric of faecal sample of bacterial vaginosis (BV) positive women baseline (red) and BV positive women with clindamycin + probiotic treatment (yellow) showing jackknife-supported confidence ellipsoids. (D) 3D-PCoA plot, principal components (PC) 1, 2 and 3 with unweighted UniFrac distance metric of vaginal swab of BV positive women baseline (blue) and BV positive subjects with probiotic + clindamycin treatment (pink) showing jackknife-supported confidence ellipsoids.

shrinkage in species richness divergence ( $P=0.160$ ) (Fig. 4) after the intervention.

Phylum-level analysis in the probiotic group demonstrated a slight increase in the abundance of *Bacteroidetes* (14.38-21.6%) and decrease in

*Actinobacteria* (13.15-3.56%) from baseline to after 30 days of probiotic, while showing a comparable relative abundance of *Firmicutes* at baseline (59.54%) and endpoint (60.41%). In contrast, the analysis in the placebo group showed a decrease in the abundance of *Firmicutes* (from 62 to 48%) and increase in the

**Table.** Relative abundance (%) of the species representing the vaginal microbiota of BV positive women (n=8) before and after probiotic supplementation

Vaginal microbiome	Baseline (mean)	Endpoint (mean)
<i>Acidaminococcus</i> sp. D21	4.005	0**
<i>Acinetobacter calcoaceticus</i>	0	0.154
<i>Aeriscardovia aeriphila</i>	2.198	0
<i>Aerococcus viridans</i>	0	0.012
<i>Anaerococcus hydrogenalis</i>	0	0.028
<i>Anaerococcus prevotii</i>	0	0.071
<i>Atopobium minutum</i>	0	0.012
<i>Atopobium vaginae</i>	5.281	0.178*
<i>Bifidobacterium bifidum</i>	0	0.014
<i>Corynebacterium amycolatum</i>	0	0.026
<i>Corynebacterium jeikeium</i>	0	0.012
<i>Corynebacterium kroppenstedtii</i>	0	0.027
<i>Corynebacterium</i> sp. NML94-0264	0	0.127
<i>Corynebacterium tuberculostearicum</i>	0	0.179**
<i>Cricetulus griseus</i>	0	0.227***
<i>Dialister propionificaciens</i>	0	0.013
<i>Exiguobacterium</i> sp. LY3	0	0
<i>Finegoldia magna</i>	0	0.02
<i>Gardnerella vaginalis</i>	12.409	2.832*
<i>Lactobacillus acidophilus</i>	3.312	0.101*
<i>Lactobacillus casei</i>	0	0
<i>Lactobacillus coleohominis</i>	0	0
<i>Lactobacillus crispatus</i>	0	0.015
<i>Lactobacillus fermentum</i>	0	0.011
<i>Lactobacillus gasseri</i>	0.03	0**
<i>Lactobacillus helveticus</i>	27.162	14.825
<i>Lactobacillus iners</i>	6.915	38.367*
<i>Lactobacillus reuteri</i>	0.208	0
<i>Lactobacillus</i> sp.	5.77	38.346*
<i>Lactobacillus</i> sp. BL302	1.147	0
<i>Megasphaera genomosp type_1</i>	3.517	0**
<i>Neisseria</i> sp. GSR70	3.226	0
<i>Pandoraea pnomenusa</i>	0	0
<i>Pediococcus acidilactici</i>	0	0.02**
<i>Peptoniphilus asaccharolyticus</i>	0	0.029
<i>Prevotella bivia</i>	0.438	0.015
<i>Prevotella copri</i>	0	0.021**
<i>Pseudomonas marginalis</i>	0	0.794
<i>Pseudomonas psychrotolerans</i>	0	0

*Contd...*

Vaginal microbiome	Baseline (mean)	Endpoint (mean)
<i>Pseudomonas rhodesiae</i>	0.014	0.373
<i>Pseudomonas</i> sp. B0310	1.207	0
<i>Psychrobacter marincola</i>	0	1.949
<i>Rhodococcus erythropolis</i>	0	0.024
<i>Ruminococcus bromii</i>	0	0.184
<i>Staphylococcus epidermidis</i>	0	0
<i>Staphylococcus haemolyticus</i>	0	0
<i>Staphylococcus hominis</i>	0	0.028
<i>Staphylococcus saprophyticus</i>	0	0.065
<i>Streptobacillus moniliformis</i>	0	0.028
<i>Tsukamurella pulmonis</i>	0	0.024
<i>uncultured bacterium</i>	11.12	0.223
<i>uncultured beta proteobacterium</i>	3.998	0
<i>Ureaplasma parvum</i>	0	0.1
<i>Ureaplasma urealyticum</i>	0.543	0.18
<i>Veillonella montpellierensis</i>	2.589	0
<i>Vibrio</i> sp. S19	0	0.012***

*P* < 0.05, \*\* < 0.01, \*\*\* < 0.001 compared to baseline

abundance of *Proteobacteria* (4-12%) after 30 days of placebo intervention. Species-level analyses showed some interesting differences between baselines and endpoint values between the two groups. Notably, at baseline, the bacterial clade, *P. copri* was 9.16 per cent at baseline in the probiotic group which increased following the probiotic intervention (16.495%); however, in the placebo group, its abundance reduced from 7.75 (baseline) to 4.84 per cent (endpoint, data not shown).

### Discussion

Data on vaginal and gut microbiota diversity in pregnant women with BV remain sparse. Furthermore, the influence of probiotics on BV-associated microbiota dysbiosis in pregnant women remains underexplored. Our results suggested potentially beneficial effects of oral probiotics on the vaginal and gut microbiota. The study revealed major compositional differences in the vaginal and gut microbiota between BV positive and healthy pregnant women. Oral probiotics for 30 days in pregnant women with BV changed the vaginal and gut microbiota profile to that of normal women.

In line with previous reports<sup>23</sup>, a relatively higher abundance of *G. vaginalis* was observed in



BV-positive women. However, in contrast to previous studies, which showed predominance of *L. iners* and *L. crispatus* among vaginal *Lactobacillus* community in women without BV, our study showed *L. helveticus* to be the predominant member followed by *L. iners*; a finding that might be attributed to differences in geographical and/or dietary habits<sup>6,7</sup>. This finding was not in line with previous cross-sectional studies of vaginal microbiota in non-pregnant women<sup>1,24</sup> and indicated that the relative abundance (or dominance) of *L. helveticus* might be an important factor in the predisposition to vaginal microbiota alterations, especially during pregnancy, thereby underpinning the importance of further evaluation of vaginal CSTs considering disparities among different population. *L. helveticus* abundance was associated with lower bacterial diversity that might suggest that *L. helveticus* is perhaps more exclusionary when more abundant than *L. iners*. These data also underscore the need for further exploration of dynamics of *L. iners* in vaginal microbial community, since it is well known to be a common member of the core vaginal microbiota<sup>23</sup>; however, little is known about its function and influence, especially during the last trimester of pregnancy<sup>29</sup>.

One week of local clindamycin therapy for women with BV in both probiotic and placebo groups led to reduction in Nugent score and Amsel's criteria; however, vaginal or gut microbiota was altered favourably only in the probiotic group. Considerable reduction in  $\alpha$ -diversity and species richness and reduction in *G. vaginalis* were observed in women taking oral probiotics. The vaginal microbial community in the probiotic group demonstrated that an increase in *L. crispatus*, *L. iners* and *P. copri*, a typical gut clade, was associated with women having normal vaginal microbiota (Fig. 3). These findings suggest that treatment with antibiotics (clindamycin) may ameliorate clinical signs and symptoms but may fail to restore normal microbiota, which is crucial for appropriate immunity, prevention of relapse and maintenance of good health. It was also intriguing to note how an oral probiotics can induce changes on the vaginal microbiota, thus suggesting that the oral administration of probiotic lactobacilli could affect bodily niches other than the intestine<sup>5,14,15</sup>. However, it remains unclear whether this influence is induced directly by live probiotic species reaching the vagina or through some secreted metabolites/compounds/molecules that may induce

alterations in the vaginal niche, thereby instigating changes in the vaginal microbiota. It would be interesting to see whether and how these effects are replicated in other population cohorts as well as to evaluate probiotic and the other strains influence on vaginal microbiota composition. In the current study, microbiota analysis after a long-term follow-up of the women might have given some information on relapse of BV but that could not be carried out due to some logistic reasons.

As for the gut microbiota, abundance of *Firmicutes* among all the pregnant women, irrespective of BV, was similar to earlier observations<sup>2</sup>. The most abundant bacterial clade was *P. copri*, which was associated with women with normal vaginal microbiota, and which increased further following probiotic intervention. This might point towards the possibility of an intriguing role of *P. copri*, a typical gut clade, in the modulation of vaginal microbiota that might have important implication and possible interactions between vaginal and gut microbiotas in the context to reproductive health and pregnancy outcomes. A comprehensive investigation of *P. copri* should be an interesting subject for such future studies. Analysis of faecal microbiota in the same woman demonstrated somewhat higher relative abundance of *P. copri* in normal versus BV-positive women, again pointing towards the possibility of some sort of translocation of this species from gut to vagina that might help in maintaining a healthy vaginal microbial configuration. Overall, the detection of *Prevotella* sp. was 100 per cent in vaginal microbiota of non-BV pregnant women (normal vaginal microbiota). Although *Prevotella* sp. has been previously demonstrated to be a common member of the vaginal community<sup>5</sup>, yet it seems that its prevalence has been rather underappreciated, and as a result, its precise significance and role in this vaginal community remains unclear. According to our data, this seems to be dependent upon the composition of *Prevotella* sp. community because we noted a different spectrum in this community between normal versus BV-positive women, viz. *P. copri* (0.03%, 14/14), *P. denticola* (0.006%, 2/14), *P. disiens* (0.006%, 1/14), *P. oris* (0.008%, 7/14) and *P. timonensis* (0.004%, 2/14) in normal women in comparison to *P. bivia* (0.34%, 8/15), *P. oris* (0.11%, 3/15), *P. buccalis* (0.10%, 4/15), *P. oulorum* (0.08%, 5/15) in women with BV. The only clade common in both the groups was *P. oris*; and its abundance was higher in BV-positive women.

Lamont *et al*<sup>8</sup> presented a deeper understanding of the vaginal microbiota based on the molecular techniques. Findings of the present study such as high diversity of the vaginal microbiota in BV-positive group than in the normal group and identifying the relative abundance of some under detected species (like *L. iners*) were in line with this review which also highlighted the importance of molecular techniques in studying the vaginal microbiota to fill the gap of information, especially in pregnancy and delivery outcomes. However, smaller sample size was the limitation of the present study to emphasize the results in a broader way. Further, some important aspects such as diet and low socio-economic status of the study participants were not considered in the analysis which could have shown deeper understanding.

In conclusion, our study revealed altered vaginal and gut microbiota in BV and normal environments in pregnant women. The vaginal microbiota of healthy women was dominated by *L. helveticus*; and *P. copri*, a gut microbe, was detected in the vaginal samples of all pregnant women with normal microbiota. With probiotic treatment, there was a reduction in *G. vaginalis* and an increase in *P. copri*. These findings hint towards a possible role of *L. helveticus* and also *P. copri*, in modulating the vaginal microbiota. Probiotic intervention strategy can be further developed to promote and contribute towards maintaining healthy vaginal environment.

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