Vaginal Dysbiotic Microbiome in Women With No Symptoms of Genital Infections

Rinku Pramanick†, Neelam Nathani‡, Himangi Warke§, Niranjan Mayadeo∥ and Clara Aranha*†

† Department of Molecular Immunology and Microbiology, Indian Council of Medical Research (ICMR)-National Institute for Research in Reproductive and Child Health, Mumbai, India.‡ School of Applied Sciences & Technology (SAST-GTU), Gujarat Technological University, Ahmedabad, India.§ Department of Obstetrics and Gynecology, King Edward Memorial Hospital and Seth Gordhandas Sunderdas Medical College, Mumbai, India.

The vaginal microbiome plays a critical role in determining the progression of female genital tract infections; however, little is known about the vaginal microbiota of Indian women. We aimed to investigate the vaginal microbial architecture of women with asymptomatic bacterial vaginosis (BV) (n=20) and normal microbiota (n=19). Microbial diversity was analyzed in vaginal swabs from regularly menstruating women (18-45yrs) by 16S rRNA V3-V4 amplicon (MiSeq Illumina) sequencing. Rarefaction analysis showed a higher number of species in normal flora compared to BV. Alpha diversity as measured by Pielou’s evenness revealed microbial diversity was significantly greater in BV samples than normal microbiota (p= 0.0165). Beta diversity comparison using UniFrac metrics indicated distinct microbial communities clustering between normal and BV flora. Firmicutes were the major phyla observed in vaginal specimens of normal microbiota whereas Actinobacteria, Fusobacteria, Bacteroidetes were significantly abundant in BV samples. Notably, the relative abundance of Lactobacillus was significantly high in normal microbiota. Conversely Gardnerella, Sneathia, Prevotella, Atopobium, Ureaplasma, Dialister significantly dominated dysbiotic microbiota. Relative frequency of Lactobacillus decreased significantly in BV (6%) as compared to normal microbiota (35.2%). L. fermentum, L. gasseri, L. iners, L. jensenii, L. mucosae, L. ruminis, L. salivarius, L. coleohominis was more exclusively present in normal microbiota. L. iners was detected from both the groups with a relative frequency of 50.4% and 17.2% in normal and BV microbiota respectively. Lefse analysis indicated Atopobium vaginae, Sneathia amnii, Mycoplasma hominis Prevotella disiens in the vaginal microbiota as a biomarker for dysbiosis and L. jensenii as a biomarker of a healthy microbiota. Firmicutes were negatively correlated to Tenericutes, Actinobacteria, Bacteroidetes, and Fusobacteria. Proteobacteria positively correlated to Tenericutes, and Bacteroidetes were shown to be positively correlated to Fusobacteria. Predicted functional analysis indicated differences in the functional profiles between BV and normal microbiota. Normal microbiota utilized pathways essential for phosphatidylglycerol biosynthesis I & II, peptidoglycan biosynthesis, geranylgeranyl diphosphate biosynthesis I, mevalonate...
INTRODUCTION

Reproductive Tract Infections (RTIs) caused by bacteria, viruses or protozoa have a profound impact on the reproductive and sexual health of men and women. The consequences of sexually transmitted infections/reproductive Tract Infections (STIs/RTIs) for reproductive health include pelvic inflammatory disease (PID), infertility (in women and men), ectopic pregnancy, and adverse pregnancy outcomes including miscarriage, stillbirth, preterm birth, and congenital infection (Mullick et al., 2005; Odogwu et al., 2021). STIs and RTIs are also known to increase the risk of HIV transmission (Taha and Gray, 2000; van de Wijgert et al., 2008; Ward and Rönn, 2010).

The healthy vaginal microbiota is predominantly dominated by lactobacilli which help in maintaining a healthy vaginal microenvironment and protecting the host from urogenital infections. Women with a healthy vaginal microbiome, are known to mostly harbor L. crispatus, L. gasseri, L. jensenii and L. iners which have been reported previously (Nunn and Forney, 2016; Pramanick et al., 2019). Based on the predominance of these species the vaginal microbiota has been classified as CST I, II, III and V, respectively (Ravel et al., 2011).

The vaginal microbiome has been studied in pregnancy (Romero et al., 2014; Mehta et al., 2020), cervical cancer (Champer et al., 2018; Chambers et al., 2021), and infertility (Zhang et al., 2021). Alteration in the vaginal ecology during which the commensal lactobacilli are reduced and displaced by the anaerobic species of the genera Gardnerella, Sneathia, Prevotella, Atopobium, can lead to bacterial vaginosis. The vaginal dysbiosis not only affects the quality of life in women but could lead to poor reproductive sequelae in IVF patients (Haahr et al., 2016; Skafte-Holm et al., 2021), adverse pregnancy outcomes and increase risk of cancer (Yang et al., 2020). This change in the microbial ecology has been attributed to various factors such as sex hormones (Brotman et al., 2014), hormonal contraception (Achilles and Hillier, 2013), sexual practices, personal hygiene and diet (Lewis et al., 2017). Significant differences in the vaginal microbiome composition related to races/ethnicity have been reported (Peipert et al., 2008; Fettweis et al., 2014). However, in 15- 84% of apparently healthy women BV could be asymptomatic (Koumans et al., 2007; Pramanick et al., 2019). While many studies have characterized the vaginal microbiota of symptomatic cases of BV, asymptomatic BV remained uncharacterized.

India has a varied population and diversified ethnic composition. The limited vaginal microbiota studies on Indian women have focused only on lactobacilli (Garg et al., 2009; Madhivanan et al., 2015; Das Purkayastha et al., 2019). These studies have often reported contrasting observations. Furthermore, the microbiome of non-pregnant women of reproductive age in India remains unexplored. In this work, we have characterized the vaginal microbiota of a healthy and asymptomatic BV microbiota.

METHODS

Study Participants and Recruitment

Vaginal swabs from 39 married, non-pregnant regularly menstruating women of the reproductive age group (18-45yrs) were collected for the study. The participants were recruited from the Gynecology and Obstetrics Out-Patients Department of King Edward Memorial Hospital and Seth Gordhandas Sunderdas Medical College, Mumbai. The study had the approval of the human ethics review board at the institute (Protocol Number EC/GOV-5/2012) and the collaborating institute (Protocol No EC/GOV-5/2012). Informed consent was obtained from all the participants. Inclusion criteria were women who were clinically healthy and in the age group of 18 - 45 years, had maintained sexual abstinence of at least 5 days, not using any vaginal hygienic products or lubricants, willing to get screened for STIs and other infections of the lower genital tract (vagina and cervix), have been diagnosed as normal with no infections of the lower genital tract based on the laboratory evidence, had not taken any antibiotics in the last six weeks, not menstruating and preferably 5 days following LMP, did not have any dysfunctional uterine bleeding or genital malignancy or genital prolapse. Pregnant women, women using hormonal contraceptives or OC’s, women with intrauterine devices, chronic drug intake, or with chronic medical illness were excluded from the study.

Nugent Scoring and Amsel Criteria

One swab was used to assess vaginal health by Amsel’s criteria (Amsel et al., 1983) and Nugent scoring (Nugent et al., 1991). The second swab was used for DNA extraction for sequencing. Nugent scoring was based on the microscopic examination of
different vaginal bacterial morphotypes and their enumeration on gram stained vaginal smears. Normal microbiota (score 0–3), intermediate (score 4–6), or BV microbiota (score 7–10) were the scores assigned by Nugent scoring. Amsel criteria were based on clinical symptoms and signs that include vaginal pH>4.5, the presence of clue cells (vaginal epithelial cells laden with coccobacilli) using wet mount microscopy, homogeneous white vaginal discharge and fishy odor of discharge (10% KOH amine test). A patient who satisfied three of these four criteria was diagnosed as positive for BV.

Sample Processing and DNA Extraction
Genomic DNA was extracted according to the instruction on MO BIO Powersoil DNA extraction kit (Qiagen, Catalog No. 12888). The extracted DNA was quantified and purity was determined on the NanoDrop reader. The samples were stored at -20°C until further use.

Amplicon Sequencing
The amplicons libraries were prepared using the Nextera XT Index kit (Illumina Inc.) as per the 16S metagenomic sequencing library preparation protocol. Primers for the amplification of the V3-V4 region of 16S rDNA were GCCTACGGGNGG CWGCAG and reverse ACTACHVGGGTATCATATCC. The amplicon libraries were purified by AMPureXP beads and quantified using a Qubit fluorometer. The libraries were sequenced on MiSeq using 2x300 bp chemistry.

Data Analysis and Statistics
Sequence read quality was assessed using Fastqc. Based on the observed quality, the reads were filtered out with the following parameters: all forward/reverse reads were trimmed by 10 bases from the left; from the right end, the reads were trimmed at length 280 for forward and length 220 for reverse. Reads were further pre-processed with DADA2 for denoising and eliminating chimera sequences and duplicates (Supplementary Table 1). The predicted ASVs were normalized by the minimum number of feature sequences in a sample from each of the study groups, respectively. Read pre-processing and taxonomic classification were performed in QIME2 framework (Bolyen et al., 2019) using the pre-trained classifier for V3-V4 region of 16S rRNA genes of the SILVA database. Beta diversity was calculated with unweighted and weighted UniFrac metrics to characterize using Illumina MiSeq sequencing of the V3-V4 region of the 16s rRNA gene with an average read length of 301bp. A total of 7041,312 reads were generated from 39 swabs with an average of 352,006 sequences per sample. On average 92.38% of sequences had a Phred score of >20.

amplicon sequence variants (ASVs) using Pielou’s evenness index. The microbial taxonomy was studied for statistical differences (p < 0.05) between the studied groups and the core microbiota was compared using Microbiome Analyst (Dhariwal et al., 2017). Functional attributes corresponding to the observed microbiota were assessed using the q2-picrust plugin (Douglas et al., 2020). The statistical differences between the normal and BV were studied by performing Kruskal–Wallis test in the STAMP v2.1 software (Parks et al., 2014).

Linear discriminant analysis (LDA) Effect Size (LEfSe) method (Segata et al., 2011) was employed to predict biomarker species and functional attributes (LDA score cutoff value of 4) with significant differences in abundance between the two groups. Linear discriminant analysis (LDA) Effect Size (LEfSe) method was employed to predict biomarker species and functional attributes (LDA score cutoff value of 4) with significant differences in abundance between the two groups.

Data Availability
The sequences were submitted in the NCBI database under the Bio project Accession No.: PRJNA674451. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA674451.

RESULTS
For this study, we recruited 39 regularly menstruating women of the reproductive age group of 18-45yrs, who were not on antibiotics. Nugent scoring of the vaginal swabs revealed, 19 women had normal microbiota and 20 had BV. The recruited participant’s characteristics are summarized in Table 1. There was no significant difference in age and the menstrual phases of participants recruited for each group (Table 1). Due to its cosmopolitan nature the population of Mumbai is characterized by people from different parts of the country. About 15.38% (6) participants were nonresidents of Mumbai whereas 10.26% (4) have migrated in the last one year from sample collection.

The vaginal microbial profile of these women was characterized using Illumina MiSeq sequencing of the V3-V4 region of the 16s rRNA gene with an average read length of 301bp. A total of 7041,312 reads were generated from 39 swabs with an average of 352,006 sequences per sample. On average 92.38% of sequences had a Phred score of >20.

**Table 1 | Characteristics of participants recruited for the study.**

| Groups | Study participants (N) | Vaginal pH (mean ± SD) | P value | Age (yrs) (mean ± SD) | P value | Menstrual phase | P value |
|--------|------------------------|-----------------------|---------|-----------------------|---------|----------------|---------|
| Normal | 19 | 3.85 ± 0.56 | 0.0001 | 30.79 ± 7.40 | 0.2402 | Follicular (n) 11 | 8 | 0.5273 |
| BV | 20 | 5.46 ± 0.69 | 33.26 ± 5.42 | 9 | 12 | 20 | 20 |
| Total | 39 | 3.85 ± 0.56 | 30.79 ± 7.40 | 0.2402 | 11 | 8 | 0.5273 |

Two-tailed unpaired t-test was calculated to evaluate the statistical differences between normal and BV group for vaginal pH and age. Statistical differences between normal and BV group for menstrual phases were calculated using Fisher’s exact two tailed test. Results were statistically significant at a p-value less than 0.05.
Comparison of Vaginal Microbiota Between Healthy and Asymptomatic BV Samples

The rarefaction analysis revealed a higher number of observed species in Normal microbiota compared to BV samples. However, the comparison difference revealed an FDR Adjusted P-value = 0.19 showing a less significant difference between Normal and BV samples. The rarefaction curve generated by the OTUs obtained from both groups indicated sufficient sequencing depth (Supplementary Figure 1). The alpha diversity measured by the Pielou evenness index revealed women with normal microbiota had less diverse communities as compared to women with asymptomatic BV. As observed from Figure 1A, a significant (p=0.0165) difference exists between the community evenness of BV and Normal samples. Beta diversity measures the amount of dissimilarity between the samples. Beta diversity comparison using UniFrac metrics, used to measure differences in microbial abundances between the groups, demonstrated distinct microbial communities between normal and BV samples (p = 0.0127). The first two components of the PCoA explained 78.32% of the variations among the samples in axes 1 (49.95) and 2 (28.41%) respectively. The cluster corresponding to the normal microbiota showed variation along axis 1, from the BV population (Figure 1B).

Firmicutes were the most abundant phyla in normal microbiota whereas Bacteroidetes, Actinobacteria, Fusobacteria were significantly abundant in the asymptomatic BV group (Figure 2A). Lactobacillus was the predominant genus in normal microbiota whereas Gardnerella, Sneathia, Prevotella, Atopobium, significantly dominated the BV microbiota (Figure 2B). The heatmap in Figure 3 depicts the abundance of the major genus present in each sample. The mean proportion of Lactobacillus and Gardnerella in normal microbiota was 87.2% and 3.1% as compared to 24% and 28.2% in BV samples respectively. Sneathia and Atopobium were absent in normal microbiota but constituted 12.1% and 4.9% in asymptomatic BV samples.

Lactobacillus was detected in all the samples of normal microbiota whereas it was completely missing from two samples with asymptomatic BV. L. iners (84.21%), (65%) and unidentified Lactobacillus spp. (84.21%), (80%) were the most frequently detected lactobacilli in both normal and BV samples respectively. Additionally, most of the women with normal microbiota harbored L. jensenii (36.84%) and L. gasseri (31.57%). Women with asymptomatic BV were frequently detected with L. gasseri (15%), L. salivarius (15%) and L. fermentum (15%). Other lactobacilli exclusively present in normal microbiota were L. coleohominis (10.52%) and L. ruminis (5.26%).

Classification of Microbiota Based on Bacterial Markers

Taxonomic biomarker identification in each group was carried out using LeSe analysis. Discriminate analysis using LeSe showed Lactobacillus jensenii, Comamonas, Weissella were enriched in normal samples while Atopobium vaginae, Sneathia amnii, Mycoplasma hominis were enriched in BV samples (Supplementary Figure 2).

Moreover, Cladogram represents differentially abundant genus and species (Figure 4). Lactobacillus jensenii was significantly abundant in normal microbiota as compared to asymptomatic BV. On the other hand, species such as Atopobium vaginae, Coriobacteriales bacterium DNF00809, Sneathia amnii were differentially elevated in asymptomatic BV samples.

Correlation of Microbial Members of the Vaginal Ecology

Firmicutes were negatively correlated to Tenericutes, Actinobacteria, Bacteroidetes and Fusobacteria. Proteobacteria positively correlated to Tenericutes, and Bacteroidetes were
shown to be positively correlated to Fusobacteria (Figure 5A). *Lactobacillus* highly negatively correlated to *Gardnerella* and other BV-related bacteria, but positively correlated to *Pseudomonas*. *Sneathia* positively correlated with *Enterococcus*, *Mycoplasma* and other pathogens such as *Dialister, Prevotella, Atopobium, Streptococcus* (Figure 5B) (Supplementary Table 2).

**Functional Profile of Vaginal Microbiota**

The normal microbiota was enriched in pathways involved in phosphatidylglycerol biosynthesis I & II, peptidoglycan biosynthesis, geranylgeranyl diphosphate biosynthesis I, mevalonate pathway, CoA biosynthesis pathway I and pyrimidine nucleotide salvage while the microbiota in BV women was enriched with aromatic amino acid biosynthesis, pentose phosphate pathway, carbohydrate degradation (Figure 6 and Table 2).

**DISCUSSION**

*Lactobacillus* which is the keystone bacteria of vaginal microbiota was significantly present in the normal sample as compared to asymptomatic BV microbiota. In contrast to our observation, a study on Estonia women of reproductive age has reported lactobacilli dominance in healthy as well as asymptomatic BV women (Drell et al., 2013). *Lactobacillus, Comamonas, Weissella* were identified as the biomarkers for a healthy microbiota. *Lactobacillus, Leuconostoc, Weissella, and Streptococcus* have been commonly identified in vaginal samples of other populations (Jin et al., 2007). Along with *Lactobacillus* Weissella spp. have been isolated in fermented food items and feces (Ruiz-Moyano et al., 2011; Gomathi et al., 2014; Zhang et al., 2014; Kang et al., 2020; Pabari et al., 2020) and demonstrated to have probiotic and anti-inflammatory...
FIGURE 3 | Genus level composition of the vaginal microbiota in normal and BV samples. The heatmap depicts the relative abundances of the most abundant genera. Each column represents one sample. Orange are samples with normal microbiota and Blue are BV samples.

FIGURE 4 | Cladogram representation of differentially abundant bacterial families detected using LEfSe. The cladogram diagram shows the microbial species with significant differences in the two groups. Red and green, indicate BV and normal microbiota groups respectively, with the species classification at the level of phylum, class, order, family, and genus shown from the outside to the inside. The red and green nodes in the phylogenetic tree represent microbial species that play an important role in the BV and normal microbiota groups, respectively. Yellow nodes represent species with no significant difference. Significantly abundant bacterial groups observed in the study are shown in the list on the right hand side.
properties (Fatmawati et al., 2020). Weissella is another lactic acid bacterium known to produce H₂O₂ in the genital tract of women (Jin et al., 2007) and shown to be a potential probiotic for vaginal health (Lee, 2005). Atopobium, Gardnerella, Sneathia were the biomarker genus for asymptomatic BV. The taxonomic biomarker identified in BV samples was similar to previously reported in Chinese women (Ling et al., 2010). All the enriched genera in asymptomatic BV samples have been reported as vaginal pathogens in various studies and contribute to the sequelae of spontaneous abortions, preterm birth, infertility and cancer (Agarwal et al., 2020). The presence of dysbiotic microbiota in vagina of asymptomatic women addresses the need for the characterization of the microbiome in those women who may have spontaneous abortions or infertility problems or those who go in for in vitro fertilization and embryo transfer (IVF-ET) procedures (Koedooder et al., 2019; Kong et al., 2020; Wang et al., 2020).

**FIGURE 5** Bacterial correlation (A) at the phylum level. Positive correlations are represented as red and negative correlations as blue (B) Network of associations between the bacteria at the genus level. Red are samples with normal microbiota and green are BV samples. The nodes represent genera of bacteria; the edges represent the correlation coefficients between genera. Node size indicates the number of subjects in which the genus was seen.

**FIGURE 6** Predicted functional profiling: Differential pathway abundance predicted by PICRUSt between normal and BV group. Pathways (BioCyc IDs) with an LDA score of 4 and above in normal (Green) and BV (Red) samples are represented.
et al., 2021). Our previous study on cultivable microbiota also reported a distinct microbial diversity of asymptomatic BV from the normal samples (Pramanick et al., 2019). The translational aspects of the vaginal microbiome and metabolome data could be exploited and based on the identification of specific biomarkers from BV and normal samples, new diagnostic point of care tests and treatment modalities could be developed.

The predicted key functional pathways in the normal microbiota were peptidoglycan biosynthesis, phosphatidylglycerol biosynthesis I and II, peptidoglycan biosynthesis IV and pyrimidine nucleotide salvage pathways. Increased cell wall organization and peptidoglycan biosynthesis in Lactobacillus dominated microbiomes have been associated with reduced FGT inflammation (Alisoltani et al., 2020) and in modulating the immune system (Song et al., 2018). Peptidoglycan biosynthesis IV and pyrimidine nucleotide salvage pathways have been further described during the proliferative phase of menstrual cycle and associated with increased bacterial proliferation (Chen et al., 2017). Our studies show that normal microbiota dominant in lactobacillus species showed mevalonate pathways. These strains are demonstrated to alleviate hyperlipidemia by modulating AMPK and downregulating cholesterol biosynthesis via the mevalonate pathway and Bloch pathway (Lew et al., 2020) and also increase the resilience of tissue cells to cholesterol-dependent cytolysins (Griffin et al., 2017). Geranylgeranyl diphosphate biosynthesis I (via mevalonate) forms geranylgeranyl diphosphate which is a crucial compound involved in the biosynthesis of a variety of terpenes and terpenoids, including central compounds such as ubiquinones and menaquinones. In addition, GGPP is also used in posttranslational modifications of proteins (geranylgeranylation) which is important for membrane adhesion as well as the function of some proteins (Jiang et al., 2017). Aromatic amino acid biosynthesis, carbohydrate degradation, pentose phosphate pathway that may cause preterm birth highlights the need for further research on its pathogenesis. The presence of these bacteria in women with asymptomatic BV is worrisome. Hence it is imperative to maintain homeostasis to prevent any future episodes of infections.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA674451.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by human ethics review board at the ICMR-National Institute for Research in Reproductive and Child Health (Protocol Number 215/2012) and the collaborating institute King Edward Hospital and Seth Gordhandas Sunderdas Medical CollegeGor(Protocol No EC/GOV-5/2012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RP and CA designed the study. NM and HW recruited the participants and carried out sample collection. RP carried out sample collection and its processing. NN performed the bioinformatic analysis. RP and CA interpreted the data. RP and CA wrote the paper. CA was involved in the acquisition of
SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.760459/full#supplementary-material

Figure 1 | Rarefaction curves showing the number of observed species for samples belonging to BV and Normal condition at each rarefaction depth starting from 100 seqs/sample to 80000 seqs/sample

Figure 2 | Differentially abundant bacterial taxa between normal and BV microbiota by LEfSe analyses Significant bacterial genera were determined by the Kruskal-Wallis test (P<0.05) with LDA score greater than 2. Normal and BV microbiota are represented by green and red bars respectively.

Table 1 | Sequence statistics of the samples after denoising by DADA2

Table 2 | Pearson correlation coefficients and P-values between bacterial taxa.
