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The female reproductive tract microbiome - gatekeeper for sexual and reproductive health

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The female reproductive tract microbiome - gatekeeper for sexual and reproductive health

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Dr. Petrova is employed by Winclove Probiotics, and is the founder of Microbiome insights and Probiotics (MiP) Consultancy. The other Topic Editors declare no conflict of interest with regards to the Research Topic theme.

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Assessing the Concordance Between Urogenital and Vaginal Microbiota: Can Urine Specimens Be Used as a Proxy for Vaginal Samples?

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Background: The vaginal microbiota play a key role in defense against reproductive tract infections; however, many population-based women's health studies do not collect vaginal samples. Molecular examinations of urine samples have revealed common vaginal bacteria. We sought to assess the extent that community state type assignments of archived random-catch and clean-catch urine samples agreed with the paired vaginal samples in both reproductive-age and peri/post-menopausal women.

Results: Using archived samples, we evaluated the microbiota concordance among women in three studies: two with paired mid-vaginal/random-catch urine (N=91 reproductive-age participants and N=13 peri/post-menopausal participants), and one with paired mid-vaginal/clean-catch urine (N=99 reproductive-age participants). Microbiota composition was characterized by sequencing amplicons of the 16S rRNA gene V3-V4 regions and assigned to community state types. Similarity of paired samples was gauged using agreement of community state types and Yue-Clayton θ indices. Analysis of Composition of Microbiomes II indicated which taxa were differently relatively abundant in paired vaginal and urine samples. In reproductive-age women, random-catch and clean-catch urines were 89.0% and 86.9% concordant on five community state types with paired mid-vaginal swabs, and Kappa statistics indicated almost perfect agreement ($\kappa_{random\text{-}catch}$=.85, $\kappa_{clean\text{-}catch}$=.81, p<0.0001). A small number of pairs of samples were discordant (23/190, 12%), and discordant pairs tended to be between samples classified to *L. iners*-dominated and/or low-*Lactobacillus* states. Concordance and agreement remained similar when dichotomizing the microbiota to *Lactobacillus*-dominated *versus* low-*Lactobacillus* microbiota, as well as when evaluating separately the three subtypes of the low-*Lactobacillus* community state type IV. Median similarity of paired urine/vaginal samples was high ($\theta_{random\text{-}catch}$=.85, $\theta_{clean\text{-}catch}$=.88), and a comparison of the random-catch and clean-catch similarity scores showed no significant difference (p=.80). Concordance and similarity were lower for peri/post-menopausal women, but agreement

remained substantial (76.9% concordant, $\kappa_{random\text{-}catch}$= 0.64, $\theta_{random\text{-}catch}$=.62). Taxonomic-level analysis confirmed these findings.

Conclusions: Random-catch and clean-catch urine samples showed substantial agreement on bacterial composition to paired mid-vaginal samples, indicating that the genitourinary microbiota may be a reliable proxy for assessing the overall composition of the vaginal microbiota *via* community state types. This data suggests that urine samples can, with proper interpretation, be utilized as a surrogate for developing preliminary data and hypothesis-generating studies.

Keywords: 16S rRNA gene amplicon sequencing, clean-catch urine, random-catch urine, vaginal microbiota, community state type

INTRODUCTION

The composition of the vaginal microbiota has critical implications for susceptibility to sexually transmitted infections (STIs), miscarriage, and spontaneous preterm delivery (Martin, 2012; Aldunate et al., 2015; Gosmann et al., 2017; Klatt et al., 2017; Tyssen et al., 2018; Elovitz et al., 2019; Al-Memar et al., 2020). The mechanism is in part attributed to the action of *Lactobacillus* spp., many of which provide broad-spectrum protection *via* lactic acid (Boskey et al., 2001; Aldunate et al., 2013; Tyssen et al., 2018; Edwards et al., 2019). Recent studies applying culture-independent methods have allowed for the detection of a quantifiable and diverse urinary microbiota (Nelson et al., 2010; Dong et al., 2011; Wolfe et al., 2012; Pearce et al., 2014; Mueller et al., 2017; Adebayo et al., 2020), and these findings have been validated with quantitative enhanced culture methods (Hilt et al., 2014; Coorevits et al., 2017).

Several organisms commonly found in the vagina have been observed in urine samples (Siddiqui et al., 2011; Pearce et al., 2014; Thomas-White et al., 2017), and bacterial strains isolated from the bladder and vagina have been found to be functionally and phylogenetically similar (Thomas-White et al., 2018a). In one study, voided urine samples demonstrated more similarity to paired vaginal swabs than to paired supra-pubic needle aspirates or trans-urethral catheterized samples (Wolfe et al., 2012). This suggests that the microbiota of some types of urine samples may more closely resemble vaginal microbiota than other urine sample types; however, there is also similarity at the genus-level between paired vaginal and trans-urethral catheterized samples (Komesu et al., 2019). Given the overlap between the genitourinary and vaginal microbiota, we hypothesized that voided urine may be used as a proxy for vaginal community assessment in research studies utilizing 16S rRNA gene amplicon sequencing.

To evaluate the use of urine as a proxy for vaginal swabs, we sought to compare the microbiota of paired mid-vaginal swabs with the microbiota of urine samples collected using "clean-catch" (CC) or "random-catch" (RC) methods from reproductive-aged women, and paired mid-vaginal swabs and RC urine samples from peri/post-menopausal women. The first-void of the initial urine stream is collected for RC urine samples while, for urine collected *via* the CC method, the labia are cleaned with an antibacterial wipe and mid-stream urine is collected. While the microbiota of both RC and CC urine samples might be similar in composition to vaginal microbiota because of shared species, RC urine may contain a higher proportion of vulvovaginal bacteria due to contamination from the urine stream washing over the labia, resulting in a better proxy of the vaginal microbiota than CC urine.

To our knowledge, CC and RC urine samples have not been assessed in conjunction with the vaginal microbiota and, although the concordance between the urinary and vaginal microbiota of peri/post-menopausal women has been studied (Komesu et al., 2019), they have not been evaluated separately from reproductive-age women. Peri/post-menopausal women have different vaginal (Brotman et al., 2014; Gliniewicz et al., 2019; Shardell et al., 2021) and urinary (Ammitzbøll et al., 2021) microbiota compared to reproductive-age women, and may carry lower bacterial loads (Holm et al., 2019). These differences may affect the extent to which the genitourinary and vaginal microbiota overlap. We aim to fill this knowledge gap by determining whether concordance data support the use of voided urine specimens as a proxy for broadly assessing the composition of the vaginal microbiota.

MATERIALS AND METHODS

Sample Collection

We utilized archived mid-vaginal swabs and urine samples collected at the same study visit from reproductive-age and peri/post-menopausal women who had enrolled in three separate observational cohort studies. All women contributed a single observation day to the dataset.

First, RC urine samples and paired mid-vaginal swabs were collected from 113 reproductive-age participants enrolled in a study of nonpregnant women at the University of Alabama at Birmingham (ages 18 to 45) as previously described (Ravel et al., 2013). Participants were asked to self-collect a mid-vaginal Copan ESwab (Copan Diagnostics) that was stored in Amies liquid transport medium and collect a first-void urine sample. Urine samples were aliquoted and frozen within one hour of

collection, and all samples were stored at -80°C until DNA extraction.

Second, RC urine samples and paired mid-vaginal swabs were collected from 15 peri/post-menopausal participants enrolled in the Gynecology and Lubricant Effects Study at the University of Maryland School of Medicine (ages 44 to 69 years) as previously described (Crowder et al., 2019). Participants were asked to self-collect a mid-vaginal Copan Eswab (Copan Diagnostics) that was stored in equal parts Amies liquid transport medium and RNAlater (ID 230 only) or modified C2 (MoBio), and collect a first-void urine sample. Urine samples were aliquoted and refrigerated within 1 hour, and frozen within 24 hours of collection, and all samples were stored at -80°C until DNA extraction.

Third, CC urine samples and paired mid-vaginal swabs were collected from 123 reproductive-age participants enrolled in the Hormonal Contraceptives Longitudinal Study, a cohort study recruited at the Johns Hopkins University School of Medicine, Baltimore, MD (ages 16 to 35 years), as previously described (Tuddenham et al., 2019). Participants were asked to self-collect a mid-vaginal Copan ESwab (Copan Diagnostics) that was stored in Amies liquid transport medium. For the urine sample, participants were asked to wipe the labia with a standard wipe containing chlorhexidine and collect a mid-stream specimen. Urine samples were aliquoted and frozen within one hour of collection, and all samples were stored at -80°C until DNA extraction.

All participants provided informed consent. The Institutional Review Boards at the University of Alabama Birmingham, Johns Hopkins University School of Medicine and the University of Maryland School of Medicine approved the protocols.

Genomic DNA Extractions

Genomic DNA was extracted from mid-vaginal swabs with either the QS DSP Virus/Pathogen Midi Kit (Qiagen, Germantown, MD) on the QiaSymphony platform or with the MagAttract Microbial DNA Kit (Qiagen, Germantown, MD) using a custom automated protocol on the Hamilton Microlab STAR **(Table S4)**. For the QiaSymphony platform, vaginal swabs were thawed on ice and 500 µL of the Amies transport medium was used as input following the protocol described in Holm et al. (2019). For the MagAttract kit, swabs were thawed on ice and a 200 µL aliquot from the Amies transport medium was used as input for the kit following the manufacturer protocol adapted for use on the Hamilton STAR robot. Cells were lysed *via* shaking on a TissueLyser (Qiagen, Germantown, MD) at 20Hz for 20 min. Negative controls consisting of 200 µL distilled sterile water were extracted in the same manner as samples.

DNA extractions on the RC urine samples from reproductive-age participants were performed on 1.5 mL urine using the Quick-DNA Urine Kit (Zymo Research, Irvine, CA). After addition of the Urine conditioning buffer and clearing beads, pelleted cells were resuspended in 720 µL of Lysis solution. Two enzymatic lysis steps were performed: 1) 40 µg Lysozyme, 120 U Mutanolysin, and 2.5 µg Lysostaphin were added, and samples were incubated at 37°C for 30 min and 2) 160 µg Proteinase K, 0.5% (final concentration) SDS, and 16 µg RNase was added and samples were incubated at 55°C for 45 min. A mechanical lysis step was performed with samples in Lysing Matrix B (MPBio) tubes and processed at 6 m/s for 40 s in the FastPrep. Subsequent washing steps were performed according to the Quick-DNA Urine kit manufacturer's standard protocol.

To test the feasibility of DNA extractions on urine in a more high-throughput manner, extractions on the CC urine samples from reproductive-age participants, and on the RC urine samples from peri/post-menopausal participants were performed with a hybrid approach, starting with the Quick-DNA Urine kit and ending with the MagAttract Microbial DNA Kit (Qiagen, Germantown, MD). Briefly, a 1 mL aliquot of urine was mixed with 70 µL of Urine Conditioning Buffer and 10 µL of Clearing Beads, both from the Quick-DNA Urine kit, and centrifuged at 3,000 x *g* for 15 min. The resulting pellet was resuspended in 650 µL of the Lysis buffer from the MagAttract kit and samples were transferred to a PowerBead DNA plate containing 0.1 mm glass beads. The rest of the extraction was performed with the MagAttract kit following a protocol adapted for use on the Hamilton STAR robot.

Library Construction and Sequencing

Relative abundances of bacteria were assessed through amplification and sequencing of the 16S rRNA gene V3-V4 regions. PCR amplifications were carried out in either 1- or 2-step reactions and sequenced on the MiSeq or HiSeq platforms (Illumina, San Diego, CA) as described in Holm et al. **(Table S4)** (Holm et al., 2019).

Post-Sequencing Data Processing, Taxonomy and CST Assignments

Post-sequencing data processing was done separately for samples from reproductive-age and peri/post-menopausal participants. Sequence de-multiplexing, removal of barcode sequences, and further sample processing were carried out with QIIME-dependent scripts, TagCleaner, and the DADA2 Workflow for Big Data as previously described (Holm et al., 2019). Amplicon sequence variants (ASVs) generated by DADA2 were classified using the RDP Naïve Bayesian Classifier (Wang et al., 2007) trained with the SILVA v128 16S rRNA gene sequence database (Quast et al., 2013) as implemented in the *dada2* R package (Callahan et al., 2016). ASVs of major vaginal taxa were assigned species-level annotations using *speciateIT* (http://ravel-lab.org/speciateit/). Extraction and PCR negative controls were examined for contaminating taxa, and all reads from contaminating taxa were removed from the overall dataset (*Pseudomonas* and *Achromobacter* in samples from reproductive-age women, and none in samples from peri/post-menopausal women). Taxa present at less than 10^{-5} across all samples were removed and samples with fewer than 500 reads were removed from analysis.

Community state types (CSTs) were assigned using VALENCIA, an algorithm based on similarity to the centroid of each cluster (France et al., 2020).

Statistical Analysis

Community similarity at the CST-level was compared with Cohen's kappa coefficient as implemented in SAS Studio Version 3.8. Seven, five and two levels of CST were assessed. Similarity of microbial populations from paired vaginal swabs

and urine samples was computed with Yue-Clayton θ indices, which take into account the relative abundances of shared and non-shared species in each population (Yue and Clayton, 2005), and a Wilcoxon rank sum test was used to compare the distributions of similarity indices of paired mid-vaginal/RC urine samples to paired mid-vaginal/CC urine samples. Among reproductive-age women, ANCOM II was used to detect taxa with significantly different relative abundances comparing paired vaginal swabs and urine samples, accounting for random subject effects and adjusting for urine sample type (Kaul et al., 2017). The ANCOM II analysis was carried out using the script ANCOM v2.1 (https://github.com/FrederickHuangLin/ANCOM) in R Studio Version 1.0.143.

RESULTS

Demographic, health, and behavioral characteristics of participants are given in **Table 1**. Across all studies, amplicons sequencing yielded 25,592,992 high-quality sequences. After removing potential contaminants, low-abundant taxa, and samples with low read counts, 91 (80.5%) and 13 (86.7%) pairs of mid-vaginal/RC urine samples were retained for reproductive-age and peri/post-menopausal women, respectively (**Table S1**). Of the sample pairs dropped, 21 pairs of mid-vaginal/RC urine samples from reproductive-age women and 2 pairs of mid-vaginal/RC urine samples from peri/post-menopausal women were excluded because of low sequencing counts in the vaginal samples. A total of 99 pairs of mid-vaginal/CC urine samples (80.5%) were retained for analysis, and 24 pairs were dropped because of low sequence counts in the urine samples.

In the vaginal microbiota, CSTs may be dominated by a few specific organisms (Ravel et al., 2011; Gosmann et al., 2017; Mckinnon et al., 2019). VALENCIA identified five CSTs in the urine and vaginal samples, four of which were dominated by the indicated *Lactobacillus* species: CST I: *Lactobacillus crispatus*, CST II: *L. gasseri*, CST III: *L. iners*, and CST V: *L. jensenii*. CST IV is a low-*Lactobacillus* state and could be further divided into three sub-CSTs based on the most abundant organisms detected (CST IV-A: "*Ca. Lachnocurva vaginae*" and *Gardnerella*, CST IV-B: *Gardnerella*, *Sneathia sanguinegens*, and *Atopobium vaginae*, and CST IV-C: *Streptococcus* spp. and *Corynebacterium*).

Similar taxonomic compositions and overall community structures were apparent when comparing paired mid-vaginal/RC urine samples and paired mid-vaginal/CC urine samples in reproductive-age women (**Figure 1**). Principle coordinate analysis demonstrated that paired mid-vaginal/RC urine and mid-vaginal/CC urine samples clustered by CST, rather than sample type (**Figure S1**).

In reproductive-age women, there was an 89.0% concordance in 5-level CST between paired mid-vaginal/RC urine samples (**Table 2**) and an 86.9% concordance between paired mid-vaginal/CC urine samples (**Table 3**). Kappa statistics indicated almost perfect chance-adjusted agreement (**Table 4**, κ_{RC}=0.85, 95% CI=0.75-0.94, κ_{CC}=0.81, 95% CI=0.71-0.90). In peri/post menopausal women, there was a 76.9% concordance in 5-level CST between paired mid-vaginal/RC urine samples (**Table 2**), and the Kappa statistic indicated substantial chance-adjusted agreement (**Table 4**, κ_{RC}=0.64, 95% CI=0.31-0.97). **Figure 2** presents stacked taxonomic relative abundance plots of CST-concordant pairs; similar community structures were often observed between sample types.

In reproductive-age women, 11% (10/91) and 13.1% (13/99) of paired mid-vaginal/RC urine and mid-vaginal/CC urine samples were discordant for CST, respectively (**Figure S2**). The most common disagreement was between CSTs IV and III (9/23),

TABLE 1 | Demographic, health, and behavioral characteristics of participants in each study.

	Project					
	Reproductive-age women with random-catch urine (N=91)		Reproductive-age women with clean-catch urine (N=99)		Peri/post-menopausal women with random-catch urine (N=13)	
	N (%)	*Mean (SD)*	*N (%)*	*Mean (SD)*	*N (%)*	*Mean (SD)*
Age (years)		28 (6.4)		26 (4.3)		55 (6.4)
Race						
Black	59 (65)		33 (35)		9 (69)	
White	27 (30)		57 (58)		1 (8)	
Multiracial	1 (1)		6 (6)		0 (0)	
Other	4 (4)		3 (3)		3 (23)	
Ever had vaginal sex	85 (93)		95 (96)		13 (100)	
Currently using hormonal contraception[1]	26 (29)		44 (44)		0 (0)	
Currently experiencing menses or vaginal bleeding	10 (11)		7 (7)		1 (9)	
Clinical findings						
None	63 (69)		87 (88)		10 (77)	
Asymptomatic bacterial vaginosis	23 (25)		2 (2)		2 (15)	
Symptomatic bacterial vaginosis	0 (0)		2 (2)		0 (0)	
Yeast infection	3 (3)		1 (1)		0 (0)	
Other	2 (2)		3 (3)		1 (8)	
Missing	0 (0)		4 (4)		0 (0)	

[1]*Includes oral contraceptive pill, progesterone-containing intrauterine device, implant, injection, and patch.*

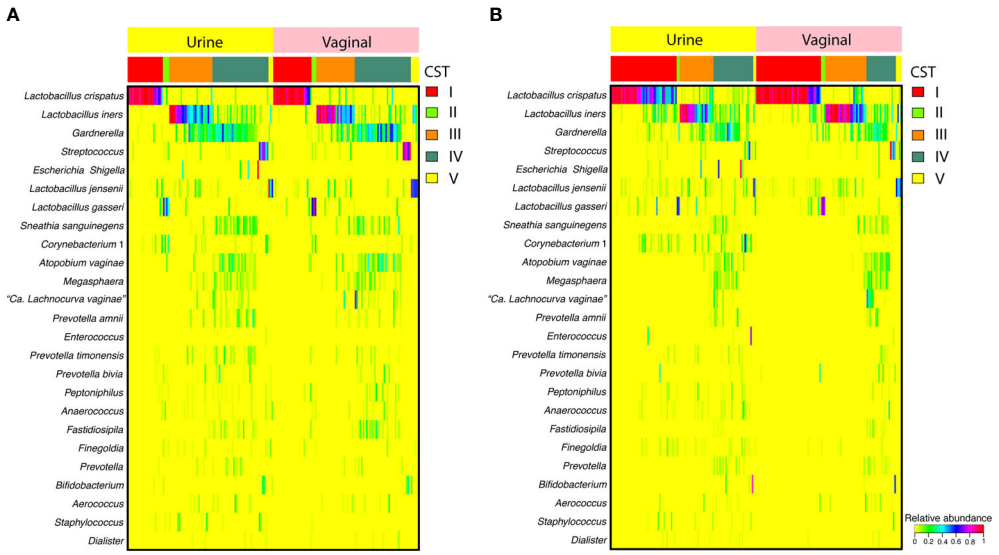

FIGURE 1 | Heatmap displaying the relative abundance of the 25 most abundant taxa in random-catch **(A)** or clean-catch **(B)** urine samples and paired vaginal swabs. Community state types (CSTs) are indicated in the second row from the top.

TABLE 2 | Concordance of random-catch urine to paired vaginal samples in reproductive-age (N=91) and peri/post-menopausal (N=13) women.

	Vaginal CST Reproductive-age women					Vaginal CST Peri/post-menopausal women				
	I	**II**	**III**	**IV**	**V**	**I**	**II**	**III**	**IV**	**V**
Urine CST										
I	**22**	0	0	0	0	**1**	0	0	0	0
II	1	**3**	0	0	0	0	**0**	0	0	0
III	1	0	**21**	3	2	0	0	**3**	0	0
IV	0	0	3	**32**	0	0	0	0	**6**	1
V	0	0	0	0	**3**	0	1	0	1	**0**

TABLE 3 | Concordance of clean-catch urine with paired vaginal samples in reproductive-age women (N=99).

	Vaginal CST				
	I	**II**	**III**	**IV**	**V**
Urine CST					
I	**41**	0	4	0	0
II	0	**2**	0	0	0
III	1	1	**21**	0	0
IV	2	0	3	**20**	2
V	0	0	0	0	**2**

and differences in the proportion of *L. iners* often accounted for the discordance (**Figure S2**). Other samples discordant for CST also shared similar community structures overall. In peri/post-menopausal women, 23.1% (3/13) of the paired mid-vaginal/RC urine samples were discordant for CST (**Figure S3**).

We carried out a sensitivity analysis to determine the robustness of urine to discriminate between CSTs when the three sub-CSTs of CST IV were identified. Paired mid-vaginal/RC urine samples were 82.4% concordant (**Table S2**), and paired >mid-vaginal/CC urine samples were 81.8% concordant (**Table S3**), with Kappa indicating substantial chance-adjusted agreement for both in reproductive-age women (**Table 4**, κ_{RC}=0.77, 95% CI=0.67-0.87, κ_{CC}=0.74, 95% CI=0.64-0.90). Paired mid-vaginal/RC urine samples in peri/post-menopausal women were 76.9% concordant (**Table S2**), with Kappa indicating substantial chance-adjusted agreement (**Table 4**, κ_{RC}=0.69, 95% CI=0.41-0.97).

We also evaluated how well urine samples broadly discriminate between communities dominated by *Lactobacillus* spp. *versus* communities with low levels of *Lactobacillus* spp. When CSTs I, II, III, and V were consolidated into a *Lactobacillus*-dominated group and CST IV was considered non-*Lactobacillus* dominated, the concordance for paired mid-vaginal/RC urine samples in reproductive-age and peri/post-menopausal women increased to 93.4% and 84.6%, respectively (**Table S4**, κ_{RC}=0.86, 95% CI=0.75-0.97, and κ_{RC}=0.69, 95% CI=0.30-1.00). The concordance for paired mid-vaginal/CC urine samples in reproductive-age women increased to 92.9% (**Table S5**, κ_{CC}=0.81, 95% CI=0.67-0.94).

The community-level similarities between paired mid-vaginal/urine samples were also estimated with the Yue-Clayton θ index (Yue and Clayton, 2005). The median similarity of RC and CC urine to paired mid-vaginal swabs indicates a high degree of similarity in reproductive-age women (θ_{RC}=.85 and θ_{CC}=.88), and a moderate degree of similarity in peri/post-menopausal women (θ_{RC}=.62). Comparison of the distribution of θ similarity scores for paired mid-vaginal/RC urine samples to mid-vaginal/CC urine samples in reproductive-age women showed no significant difference (p=0.80).

Figure S4A shows the taxa identified by ANCOM II as being most likely to be different in their relative abundance comparing

TABLE 4 | Agreement between urogenital and vaginal microbiota composition measured by Cohen's kappa statistic.

Categorical Analysis	Group	Urine Sample	κ	95% CI	p-value
5 levels:	Reproductive-age	RC	0.85	0.75-0.94	<0.0001
CSTs I, II, III, IV, V	Reproductive-age	CC	0.81	0.71-0.90	<0.0001
	Peri/post-menopausal	RC	0.64	0.31-0.97	<0.001
7 levels:	Reproductive-age	RC	0.77	0.67-0.87	<0.0001
CSTs I, II, III, IV-A, IV-B, IV-C, V	Reproductive-age	CC	0.74	0.64-0.85	<0.0001
	Peri/post-menopausal	RC	0.69	0.41-0.97	<0.0001
2 levels:	Reproductive-age	RC	0.86	0.75-0.97	<0.0001
Lactobacillus-dominated and low-*Lactobacillus*	Reproductive-age	CC	0.81	0.67-0.94	<0.001
	Peri/post-menopausal	RC	0.69	0.30-1.00	0.01

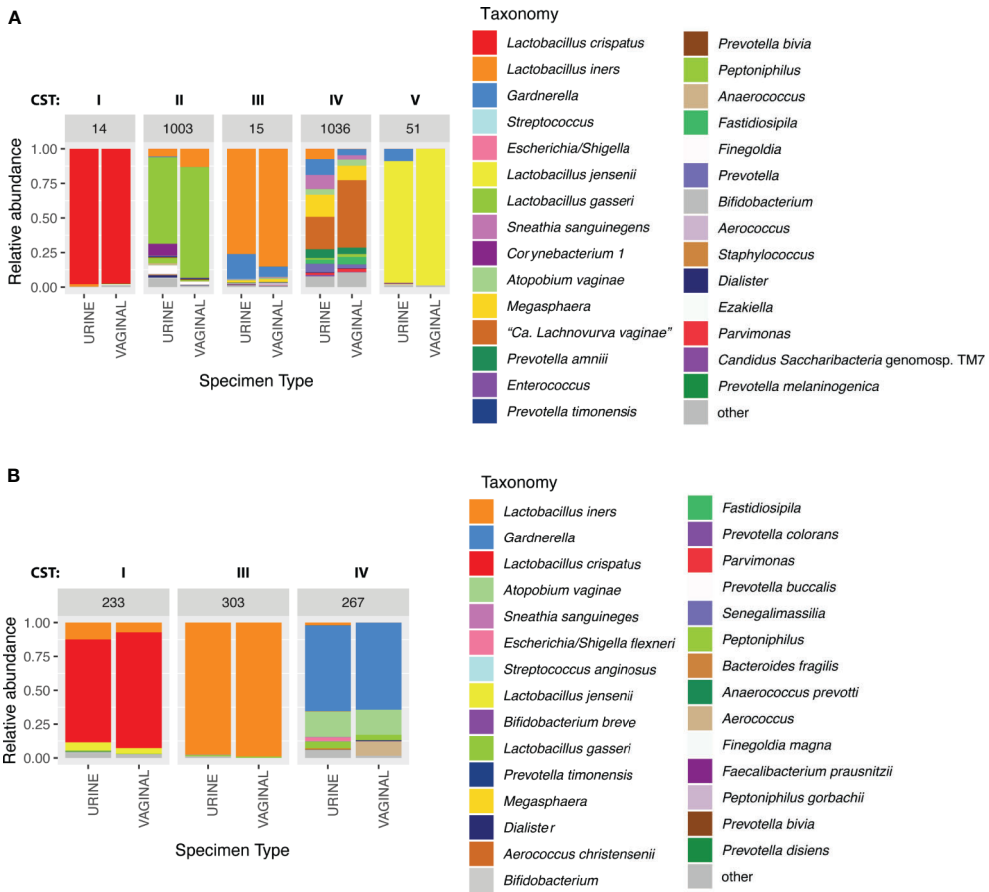

FIGURE 2 | Example taxonomic composition for paired urine and vaginal samples that were concordant on CST in **(A)** reproductive-age women (paired clean-catch urine IDs: 1003, 1036. Paired random-catch urine IDs: 14, 15, 51) and **(B)** peri/post-menopausal women (all random-catch urine). Assigned CSTs are indicated above each sample pair.

paired vaginal and urine samples, For each taxa, the W value represents the number of log-ratio hypothesis tests in which the ratio between that taxa and another taxa was significantly different in vaginal *versus* urine samples, with higher W values indicating stronger statistical evidence. The recommended cutoff for significance is a W value above the 70th percentile, although higher percentile cutoffs can be used for more conservative results and to decrease the false discovery rate. Ten of the top 25 most abundant taxa in reproductive-age women had W values above the 70th percentile, indicating their relative abundance was more likely to be differ by sample type compared to the relative abundance of other taxa. **Figure S4B** shows the distribution of paired differences in relative abundance (% abundance vaginal - % abundance urine) for the 10 most abundant taxa identified by ANCOM II. For each taxa, the median paired difference in relative abundance was approximately 0%. Mean paired differences in relative abundance ranged from 2.7% higher in urine samples (*Corynebacterium* 1) to 0.9% higher in vaginal samples (*Streptococcus*).

DISCUSSION

The vaginal microbiota play a major role in sexual and reproductive health, and ongoing studies continue to explore the mechanisms of protection and interventions to optimize the vaginal microbiome. However, not all women's health studies, particularly large population-based studies, include vaginal sampling. We assessed whether urine samples could serve as a proxy for vaginal samples in studies utilizing 16S rRNA gene amplicon sequencing to determine community state type (CST). We evaluated whether the CST assigned to a RC or CC urine sample was concordant with the CST assigned to the paired vaginal swab, and whether compositional measurements also indicated overall community similarity.

CST Concordance

For reproductive-age and peri/post-menopausal women, we found substantial agreement in the composition of the microbiota between both RC and CC urine samples and paired mid-vaginal swabs. This was true when comparing compositional similarity, when dichotomizing a woman's genitourinary microbiota to either *Lactobacillus*-dominated or low-*Lactobacillus*, as well as for higher resolution analyses using 5- or 7-level categorical CSTs. Importantly, clustering by CST allows identification of vaginal microbiota dominated by *L. iners* (CST III), which is often found in the vaginal microbiota of women with bacterial vaginosis (BV) (Zozaya-Hinchliffe et al., 2010; Srinivasan et al., 2012; Petrova et al., 2017). As we learn more about the functional and genomic diversity of *L. iners* (Macklaim et al., 2013), it is becoming evident that grouping all *L. iners*-dominated vaginal microbiota with other *Lactobacillus*-dominated CSTs may not be appropriate as it might not confer the same protection against adverse reproductive health outcomes (Van Houdt et al., 2018; Sarmento et al., 2021).

It is not entirely surprising that there is a high degree of similarity between the composition of the vaginal and genitourinary microbiota using CSTs. As previously mentioned, voided urine, particularly random-catch urine, is likely to be contaminated with vulvovaginal bacteria. Similar to our findings, a 2014 study also reported no significant difference in the accuracy of diagnosing BV using species-specific qPCR methods when comparing random-catch (first void) urine samples to paired vaginal swabs (Datcu et al., 2014). There are also demonstrated parallels between the vaginal microbiota and the microbiota of the bladder and lower urinary tract. One study found post-operative UTI was less likely among women with *Lactobacillus*, particularly *L. iners*, detected in their pre-operative urinary microbiota (Thomas-White et al., 2018b). This finding seems to suggest that, similar to what is seen in the vaginal microbiota with *Lactobacillus* spp. and reproductive tract infections, *Lactobacillus* spp. in the bladder may be associated with decreased risk of urinary tract infections, although more studies are needed to confirm these findings. Price et al. reported, as with the vaginal microbiota (Gajer et al., 2012), the composition of the lower urinary tract microbiota is also affected by sexual behaviors and menstruation (Price et al., 2020).

In menopause, urogenital tissues are affected by decreasing estrogen levels and are associated with recurrent UTIs, increased urinary frequency, genital dryness, thinning of the vaginal epithelium, and a loss of vaginal *Lactobacillus* spp. as well as a decrease in vaginal bacterial load (Hillier and Lau, 1997; Portman et al., 2014; Holm et al., 2019). These signs and symptoms, along with others, define the genitourinary syndrome of menopause (GSM) – a condition which affects approximately half of post-menopausal women (Portman et al., 2014). Because GSM affects both the vaginal microbiota and urinary tract (Thomas-White et al., 2020), and because lower vaginal bacterial loads may affect the ability of RC urine to collect vaginal microbes, we separately evaluated the microbiota of paired RC urine and vaginal samples collected from peri/post-menopausal women. Though limited in sample size, our results suggest that paired mid-vaginal/RC urine samples in peri/post-menopausal women share similar community structure with kappa agreement values indicating substantial agreement, although CST concordance was just a bit lower than reproductive-age women.

CST Discordance

Among all participants and sample types, we found 17.7% of urine samples were discordant for CST with the paired vaginal sample, including 11% of samples from reproductive-age women with RC urine (10/91), 13% of samples from reproductive-age women with CC urine (13/99), and 23% of samples from peri/post-menopausal women with RC urine (3/13). In reproductive-age women, discordance was found most often between CSTs III and IV [60% of discordant RC urine samples (6/10) and 23% of discordant CC urine samples (3/13)]. Still, urine samples assigned to CSTs III and IV were largely concordant with the CST assignment of the paired vaginal swab (84% for both). This is not surprising considering that *L. iners* is commonly found in a high relative abundance in CST IV, and that transitions are often observed between CST III and CST IV in longitudinal studies (Verstraelen et al., 2009; Gajer et al., 2012; Smith et al., 2012). Subtle differences in the relative abundance of *L. iners* affect assignment to CST III or CST IV, while overall community structures remain somewhat similar. Given that CST III samples often represent less optimal states compared to other *Lactobacillus*-dominated CSTs, future studies seeking to use urine samples as a proxy for the vaginal microbiota may consider categorizing CST III and CST IV samples together (92.6% concordant, reproductive-age women), rather than considering CST III with other *Lactobacillus*-dominated CSTs.

When considering the relative abundance of both shared and non-shared species using Yue-Clayton theta indices, our study identified strong similarities between paired RC and CC urine and vaginal microbiotas. ANCOM II did identify several key taxa as statistically likely to differ in their relative abundance, including *Gardnerella*, *Streptococcus, Corynebacterium* 1, and some species of *Prevotella*. However, it should be noted that ANCOM II defines significantly different taxa based on the strength of the evidence, and not the magnitude of the effect size. Among these significant taxa, we found small values for the

centered log-ratio (CLR) mean difference, which represents the log-fold change in the relative abundance of that taxa relative to the geometric mean composition. Given the small paired differences in relative abundance shown in **Figure S4B**, we would not expect these differences to significantly impact CST assignment. While *Gardnerella* in particular is an important taxa when defining CST, less than 2% of paired vaginal and urine samples had absolute difference of 30% or more in their relative abundance of *Gardnerella*, which might explain some of the discordance between CST III and CST IV. However, it is important to note that the relative abundance of *Gardnerella* did not appear to be biased by sample type. Some urine samples had a higher relative abundance of *Gardnerella* compared to the paired vaginal sample, while other urine samples had a lower relative abundance. Consequently, we would not expect to observe any systematic differences in the relative abundance of *Gardnerella* in studies using urine samples. In addition, all of the top 25 most abundant taxa could be found in both urine and vaginal samples.

Considerations for Clean-Catch Urine

The protocol for collecting a CC urine sample involves cleaning the labia and periurethral area before sample collection, and we had originally hypothesized that it might decrease the chance that urine collects vaginal microbes from the labia or introitus. It was noteworthy that both RC and CC urine samples demonstrated a similar bacterial composition to vaginal swabs. This finding could be explained by the urethra being colonized by similar organisms to the surrounding vulvovaginal environment, the proximity of the vaginal microbiota to the urethra, and the mechanical action of urine passing over the external vulva.

A high proportion of CC urine samples had fewer sequences than our quality control cutoff (19.5%; 24/123), compared to RC urine samples (0.8%; 1/128). This result may be attributed to an overall lower microbial burden in these samples due to the use of chlorhexidine wipes and collecting mid-stream urine. This observation is similar to another study utilizing CC urine that found approximately 14% of samples yielded undetectable bacterial DNA by 16S rRNA amplicon sequencing (Thomas-White et al., 2017). While our results suggest both RC and CC urine samples can be used as a proxy to study vaginal CSTs in reproductive-age women, RC urine may be favorable because of higher bacterial loads due to the contribution of vulvovaginal bacteria in the sample and not requiring mid-stream sampling and antibacterial wiping.

Limitations and Future Directions

A set of three convenience studies were utilized, and DNA extraction and sequencing methodologies were not consistent between studies (**Table S6**). The differences in extraction methodologies between urine types make it challenging to directly compare rates of success in sequencing CC *versus* RC urine samples. These methodological differences may offer an explanation as to why samples from reproductive-age women with paired RC urine were dropped due to low sequence counts in the vaginal sample, while no samples from reproductive-age women with paired CC urine were dropped due to low sequence counts in the vaginal sample. We have previously reported that HiSeq, which was used for all urine samples from reproductive-age women and all vaginal samples from reproductive-age women with paired CC urine, produces greater mean quality scores and number of sequences per sample compared to MiSeq, which was used for a large proportion of the vaginal samples from reproductive-age women with paired RC urine (Holm et al., 2019). In a prior study, we reported complete within-subject agreement in vaginal CST assignment when comparing data sequenced on HiSeq and MiSeq Ilumina instruments (Holm et al., 2019), and so we do not expect the differences in sequencing methodologies to have any effect on CST assignment or concordance. Additionally, we have reported that VALENCIA CST assignments are also largely unaffected by choice of bioinformatic pipeline or variable region (France et al., 2020).

The sample size for peri/post-menopausal samples was limited, and therefore, we are unable to confirm whether the concordance between genitourinary and vaginal CSTs was lower in peri/post-menopausal women compared to reproductive-age women. Studies seeking to use urine as a surrogate for vaginal samples in peri/post-menopausal women may benefit from broadly categorizing the microbiota as *Lactobacillus*-dominated (CSTs I/II/III/V) *versus* low-*Lactobacillus* (CST IV), or as CSTs I/II/V *versus* CSTs III/IV (84.6% concordance for both).

It would also be of interest to evaluate the concordance between genitourinary and vaginal microbiota using other molecular techniques such as qPCR for the quantitative detection of specific species of interest, and to evaluate whether paired urine and vaginal samples share similar metagenomic profiles. Lastly, future studies could determine whether longitudinal profiles of the genitourinary microbiota reflect paired longitudinal profiles of the vaginal microbiota, and if personal behaviors, menstruation, or use of medications, such as hormonal contraception, affect the degree to which the composition of the genitourinary microbiota overlaps with the vaginal microbiota.

CONCLUSIONS

Bacterial compositions of RC and CC urine samples demonstrated substantial agreement to paired mid-vaginal samples for both reproductive-age and peri/post-menopausal women. Urine samples may be a useful surrogate to evaluate broad community state type categories of microbiota, particularly for the purpose of hypothesis-generating research. Indeed, studies utilizing urine samples may provide important preliminary evidence to support conducting further research with vaginal samples.

DATA AVAILABILITY STATEMENT

The datasets are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive

(SRA) BioProject accession number PRJNA208535 (recruited at University of Alabama at Birmingham), and the Database of Genotypes and Phenotypes (dbGaP) accession numbers phs.002169.v1.p1 (recruited at Johns Hopkins University; Hormonal Contraceptives Longitudinal Study) and phs.002211.v1.p1 (recruited at University of Maryland School of Medicine; Gynecology and Lubricant Effects Study).

ETHICS STATEMENT

The studies were reviewed and approved by the Institutional Review Boards at the University of Alabama Birmingham, Johns Hopkins University School of Medicine and the University of Maryland Baltimore. The participants provided written informed consent.

AUTHOR CONTRIBUTIONS

SB: Conceptualization, formal analysis, and writing – original draft preparation. CR: Conceptualization, formal analysis, investigation, and writing – original draft preparation. MS: Formal analysis and writing – review and editing. JH: Data curation and writing – review and editing. JR: Funding acquisition, resources, and writing – review and editing. KG: Funding acquisition, resources, and writing – review and editing. RB: Conceptualization, funding acquisition, resources, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.671413/full#supplementary-material

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Conflict of Interest: JR is a co-founder of LUCA Biologics, a biotechnology company focusing on translating microbiome research into live biotherapeutic drugs for women's health.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-*Lactobacillus*-Dominated Vaginal Microbiota Is Associated With a Tubal Pregnancy in Symptomatic Chinese Women in the Early Stage of Pregnancy: A Nested Case–Control Study

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The features of the vaginal microbiota (VM) community can reflect health status, and they could become new biomarkers for disease diagnosis. During pregnancy, domination of bacteria of the genus *Lactobacillus* in the VM community is regarded as a keystone because they stabilize the VM by producing antimicrobial compounds and competing adhesion. An altered VM composition provides a marker for adverse pregnancy outcomes. This nested case–control study aimed to characterize the VM in women with a tubal pregnancy (TP) presenting with pain and/or uterine bleeding in early pregnancy. Chinese women with a symptomatic early pregnancy of unknown location were the study cohort. 16S rDNA gene-sequencing of V3–V4 variable regions was done to assess the diversity, structures, taxonomic biomarkers, and classification of the VM community. The primary outcome was the location of the early pregnancy. The VM community in women with a TP showed higher diversity (PD-whole-tree, median: 8.26 *vs*. 7.08, $P = 0.047$; Shannon Diversity Index, median: 1.43 *vs* 0.99, $P = 0.03$) and showed different structures to those in women with an intrauterine pregnancy (IUP) ($R = 0.23$, $P < 0.01$). Bacteria of the genus *Lactobacillus* were significantly enriched in the IUP group, whereas bacteria of the genera *Gardnerella* and *Prevotella* were significantly enriched in the TP group. *Lactobacillus* abundance could be used to classify the pregnancy location (AUC = 0.81). Non-*Lactobacillus*-dominated microbiota (≤ 0.85% *Lactobacillus*) was significantly associated with a TP (adjusted odds ratio: 4.42, 95% confidence interval: 1.33 to 14.71, $P = 0.02$). In conclusion, among women with a symptomatic early pregnancy, a higher diversity and lower abundance of *Lactobacillus* in the VM is associated with a TP.

Keywords: symptomatic early pregnancy, tubal pregnancy, vaginal microbiota, *Lactobacillus*, non-*Lactobacillus* dominated microbiota

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INTRODUCTION

An ectopic pregnancy occurs when a fertilized egg implants and grows outside the main cavity of the uterus. An ectopic pregnancy most often occurs within a fallopian tube. This type of ectopic pregnancy is called a "tubal pregnancy" (TP). TP is the leading cause of hemorrhage-related mortality, accounting for 2.7% of pregnancy-related mortality (Creanga et al., 2017).

Early screening of high-risk patients, as well as the early diagnosis and management of an unruptured TP, can reduce morbidity and mortality and preserve fertility. Although half of TP patients have no definite risk factor, several risk factors have been identified (Silva et al., 2006; Barnhart et al., 2011; ACOG, 2018). Pelvic inflammatory disease (PID) is one of the most important risk factors. Retrospective case–control studies have shown that the prevalence of fallopian tube damage increases after continuous exposure to PID (13% after one exposure, 35% after two exposures, and 75% after three exposures), which may lead to an increase in the incidence of TP (Bjartling et al., 2007; Bakken, 2008). However, assessment of the effect of a genital infection upon reproductive outcome is difficult due to the lack of reliable methods for measuring genital infections (Onan et al., 2005; AOCG, 2018; Elson et al., 2016). Conversely, one nested case–control study with a sample size of 2026 found that pain as the presenting symptom (odds ratio (OR): 1.16, 95% confidence interval (CI): 0.92–1.48) and bleeding as the presenting symptom (OR: 1.34, 95%CI:1.04–1.78) were risk factors for an ectopic pregnancy (Barnhart et al., 2006). More attention should be paid to such symptomatic women in the early stages of pregnancy. Therefore, finding inflammation-related biomarkers associated with TP risk in symptomatic women in early pregnancy is important.

DNA-sequencing (DNA-seq) methods have enabled culture-independent analyses of complex microbial communities. In this way, they have provided an integrative view and a much broader picture of the vaginal microbiota (VM). Ranging from nutrient acquisition, metabolic activity, to immune homeostasis, the VM, along with host cells, constitutes complex and interactive processes. The microbiota can affect female physiology, and female physiology can affect the composition and function of the VM (Smith and Ravel, 2017). The features of the VM community can reflect the health of women, and the VM could become a new biomarker for disease diagnosis.

In women with a normal pregnancy, the VM community shifts to become more dominated by *Lactobacillus*, less diverse, and highly stable, and this transition occurs in early pregnancy (Ravel et al., 2011; Hickey et al., 2012; DiGiulio et al., 2015; Freitas et al., 2017; Smith and Ravel, 2017; Blostein et al., 2020; Tsonis et al., 2020). During pregnancy, this dominance by *Lactobacillus* is regarded as a keystone in the VM community because it stabilizes the VM by producing antimicrobial compounds (e.g., lactic acid, hydrogen peroxide, and bacteriocins) and competing adhesion (Borges et al., 2014; Greenbaum et al., 2019). An altered VM composition provides a marker for adverse pregnancy outcomes. In early pregnancy, a decreased number of *Lactobacillus* alone with increased abundance of anaerobes and a highly diverse VM community is associated with miscarriage and preterm birth (Romero et al., 2014; DiGiulio et al., 2015; Callahan et al., 2017; Haque et al., 2017; Freitas et al., 2018; Fettweis et al., 2019; Al-Memar et al., 2020). Among women undergoing *in vitro* fertilization (IVF) treatment, infertility based on fallopian tube factors is more prevalent in those with an abnormal VM, which is characterized by a shift from a *Lactobacillus* -dominated state to a state of increased heterogeneous anaerobes (Haahr et al., 2016; Haahr et al., 2019). However, the association between the VM in early pregnancy and a TP is not known.

We wished to analyze whether particular characteristics of a VM community in early pregnancy were linked to a TP. We undertook a study using 16S rRNA gene sequencing (16S rRNA gene-seq) among women in early pregnancy who were initially considered to have a pregnancy of unknown location (PUL), and subsequently diagnosed with an intrauterine pregnancy (IUP) or TP. Taking into account the differences in vaginal microbiota between ethnicity and that this study only included Chinese women, the aim of this study is to evaluate the potential association between vaginal microbiota and pregnancy location.

MATERIALS AND METHODS

Ethical Approval of the Study Protocol

The study protocol was approved (ZYYECK2017-060; approval date: 6 February 2018) by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China). All participants provided written informed consent.

Inclusion Criteria

The inclusion criteria were pregnant women: (i) aged ≥18 years; (ii) with PUL [positive pregnancy test but no evidence on transvaginal ultrasound of an IUP or TP (Barnhart et al., 2011)]; (iii) who presented with pain and/or bleeding; and (iv) with a gestational age (from the last day of the last menstruation) of 4–8 weeks.

Exclusion Criteria

The exclusion criteria were pregnant women: (i) who had taken antibiotics within 30 days before sample collection; (ii) had sexual activity, vaginal douching, or recorded use of vaginal medications within 48 h before sample collection; or (iii) with vulvovaginal candidiasis, acute inflammation, or cancer.

Study Design

This nested case–control study was based on follow-up of a symptomatic cohort of Chinese women in early pregnancy. During May to December 2018, we enrolled women who were initially considered to have PUL at the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

Women were followed up until the definitive location of the pregnancy could be made. "Cases" were defined as women who were diagnosed definitively with a TP. "Controls" were defined as women who were diagnosed definitively with an IUP in the same cohort. The diagnosis of a TP and IUP were based on medical

history, clinical symptoms, physical examination, serial serum levels of human chorionic gonadotropin, and the findings of transvaginal ultrasound. An IUP was validated by a yolk sac or embryo within an intrauterine gestational sac (Barnhart et al., 2011). A TP was confirmed by laparoscopy and histopathology. Controls matched with cases for presenting with age (± 5 years) and gestational age of sample collection (± 7 days) at a ratio of 2:1 (Filion et al., 2016).

Collection of Clinical Data and Samples

At their initial visit, participants completed a questionnaire survey (including details on sociodemographic characteristics, past medical/reproductive history, and lifestyle), measurement of height and weight (without shoes or clothes), speculum examination, and collection of vaginal secretions. Pelvic inflammatory disease was defined as all outpatient, inpatient, and emergency treatment (Brunham et al., 2015). Collection of vaginal secretions from each participant was undertaken by a very experienced obstetrician. A sterile speculum without lubrication was inserted into the vaginal canal. Three sterile swabs (Improve Medical, Guangzhou, China) with triplicates were applied five times to both sides of the mid-vaginal canal. Swabs were placed in a sterile tube separately. One swab was used for screening and excluding vulvovaginal candidiasis by wet mount microscopy (Workowski and Bolan, 2015). One swab was sent immediately to measure the pH of the vaginal secretion. One swab was frozen at −20°C within 4 h after collection, transported to a laboratory, and then stored at −80°C until DNA extraction.

pH

Vaginal pH was measured using pH test paper (Sanaisi Scientific Instruments, Jiangsu, China) on an automatic vaginitis detection system (bPR-2014A; Bioperfectus Technologies, Jiangsu, China) ranging from 3.8 to 5.4 (incremental change was 0.2).

Extraction, Sequencing, and Data Processing of 16S rDNA

The main processes for extraction and sequencing of DNA were done in eight steps. First, genomic DNA was extracted using the spin-column method with the Hipure Bacterial DNA kit (Magen, Guangzhou, China). Second, quality control was undertaken using Qubit 2.0 (Agilent Technologies, Santa Clara, CA, USA) with a dsDNA HS Assay kit (Life Technologies) and agarose gel electrophoresis. Third, samples which passed the quality-control test were employed for library construction. Universal primers for the variable regions of 16S V3–V4 were amplified and purified by a multiplex polymerase chain reaction assay (NEBnext® Ultra™ II Q5® Master Mix, New England Biolabs, Ipswich, MA, USA). The quality of the library was checked by Qubit 2.0 and the 2200 Tapestation system (Agilent Technologies). Fourth, samples were barcoded and mixed before pooling. Fifth, paired-end sequencing was done on the MiSeq® Sequencing System (Illlumina, San Diego, CA, USA) with MiSeq Reagent Kit v3. Sixth, the raw sequencing data (raw reads) were denoised and filtered. Seventh, forward and reverse clean reads were coalesced into tags (paired-end reads) using fast length adjustment of short reads (FLASH) (Johns Hopkins University, Baltimore, MD, USA) (Magoc and Salzberg, 2011). Eighth, chimeras were removed using ultra-fast sequence analysis (USEARCH 61) (drive5; BioInformatics, Arlington, VA, USA). Finally, reads were filtered out if their length was <200 bp.

We pre-clustered the remaining high-quality tags into operational taxonomic units (OTUs) using a 2% single-linkage pre-clustering methodology to remove spurious OTUs (Huse et al., 2010). Next, we used the UCLUST algorithm to cluster the remaining tags into OTUs based on 97% nucleotide similarity (Edgar, 2010). SILVA (SILVA_132_QIIME_release; (Quantitative Insights Into Microbial Ecology (QIIME); http://qiime.org/index.html/) was used to classify the seed sequences of each OTU into specific taxa, even at the species-level classification of *Lactobacillus*.

Data Analyses

We used alpha diversity (phylogenetic diversity (PD)-whole-trees and Shannon Diversity Index) to analyze the within-community diversity. Beta diversity (weighted UniFrac distances) was employed to analyze the variation in community composition (Lozupone et al., 2011). Alpha diversity and beta diversity were calculated using QIIME (Caporaso et al., 2010). Principal component analysis (PCoA) was applied to further discover the community structure using statistical analysis of metagenomic profiles (STAMP). Analysis of similarities (ANOSIM) with 999 permutations was done using PRIMER-e 7.0 (www.primer-e.com/) to test the significant separation between groups in PCoA (Barrenha et al., 2017).

Linear discriminant analysis (LDA) effect size (LEfSe) methods with default parameters (alpha value for Wilcoxon tests was 0.05, the threshold on the logarithmic LDA score was 4.0) were implemented to further compare and visualize the significant differences in taxa (Segata et al., 2011).

In reference to the work of Moreno and colleagues (Moreno et al., 2016), to construct the classification of the VM in TP, we undertook five trials of 10-fold cross-validated classification and regression tree (CART) using the relative abundance of taxa in the Python Sklearn module (sklearn 0.21.3; http://scikit-learn.org/). The average area under the receiver operating characteristic curve (AUC) for five trials were used to compare the accuracy of various classifiers.

Multivariate logistic regression analysis was done to evaluate the associations between the VM and TP with EmpowerStats (www.empowerstats.com/). Non-adjusted and multivariate adjusted models were listed. Covariances were adjusted so that, upon addition to this model, the matched odds ratio was changed by ≥10% (Kernan et al., 2000).

A heatmap of the composition of VM taxa was drawn using Pearson correlation distance and complete-linkage hierarchical clustering with the pheatmap package within R (v1.0.8; http://cran.r-project.org/web/packages/pheatmap/). To better visualize the diversity found in all participants (even those with a highly skewed taxa proportion) we used the $\log_{10}$-transformed relative abundance of each taxon.

Statistical analyses for the cohort characteristics and indices of alpha diversity were undertaken using SPSS 22.0 (IBM, Armonk, NY, USA). Differences between groups were calculated by the Student's *t*-test for continuous variables with a normal distribution, Wilcoxon test for skewed continuous variables, and the chi-squared test or Fisher's exact test for categorical variables. $P < 0.05$ (two-tailed) was considered significant.

RESULTS

Characteristics of the Cohort and Samples

The cohort was comprised of 292 women with symptomatic early PUL, 46 of whom were diagnosed subsequently with a TP, and the remainder of whom diagnosed with an IUP. During the matching procedure, 32 women with a TP were 1:2 matched with 64 women with an IUP. However, one sample from a woman with an IUP failed the quality-control test and was excluded from the study. Ultimately, the final sample size for analysis was 32 cases and 63 controls (**Figure 1**).

The characteristics of the study cohort are described in **Table 1**. The mean age of the total study cohort was 29.6 ± 5.4 years. The gestational age of sample collection was 47.4 ± 7.6 days. Women with a TP were more likely to have uterine bleeding (81.2% *vs* 60.3%, $P = 0.04$), a shorter menstrual cycle (29.6 ± 3.8 *vs* 34.9 ± 10.2, $P < 0.01$), a previous ectopic pregnancy (18.8% *vs* 4.8%, $P = 0.03$), previous pelvic infection (53.1% *vs* 15.9%, $P < 0.01$), and higher vaginal pH (4.7 ± 0.4 *vs* 4.5 ± 0.3, $P < 0.01$) than those with an IUP. There were no significant differences in body mass index (BMI), gravidity, previous spontaneous abortion, previous surgery (uterine cavity, fallopian tube s, or pelvic), or tobacco-smoking status between the IUP group and TP group (P > 0.05).

TABLE 1 | Characteristics of subjects with tubal pregnancy compared with those with intrauterine pregnancy.

	Tubal pregnancy	Intrauterine pregnancy	*P* value
Subjects	32	63	–
Gestational age, days	46.4 ± 6.9	48.0 ± 8.0	0.34
Age, years	30.1 ± 5.7	29.4 ± 5.4	0.56
BMI, kg/m^2	22.0 ± 3.4	21.1 ± 2.8	0.15
Current smoker, yes	0	0	–
Uterine bleeding, yes	26 (81.2%)	38 (60.3%)	0.04*
Abdominal pain, yes	27 (84.4%)	42 (66.7%)	0.07
Menstrual cycle, days	29.6 ± 3.8	34.9 ± 10.2	<0.01*
Gravidity	3.0 (1.0-4.0)	2.0 (2.0-3.0)	0.41
Previous spontaneous abortion, yes	9 (28.1%)	29 (46.0%)	0.09
Previous ectopic pregnancy, yes	6 (18.8%)	3 (4.8%)	0.03*
Previous infertility, yes	6 (18.8%)	14 (22.2%)	0.70
Previous pelvic inflammatory disease, yes	17 (53.1%)	10 (15.9%)	<0.01*
Previous uterine cavity surgery, yes	18 (56.2%)	41 (65.1%)	0.40
Previous tubal surgery, yes	6 (18.8%)	4 (6.3%)	0.06
Previous pelvic surgery, yes	12 (37.5%)	21 (33.3%)	0.70
Vaginal environments			
Vaginal pH	4.7 ± 0.4	4.5 ± 0.3	<0.01*
non-*Lactobacillus* dominated microbiota (NLDM)	18 (56.2%)	8 (12.7%)	<0.01*

Continuous variables were presented as mean ± SD, categorical variables were expressed as percentages (%). P was calculated by t test for normally distributed continuous variables, chi-squared test, or Fisher's exact test for categorical variables.
BMI body mass index.
**P < 0.05*

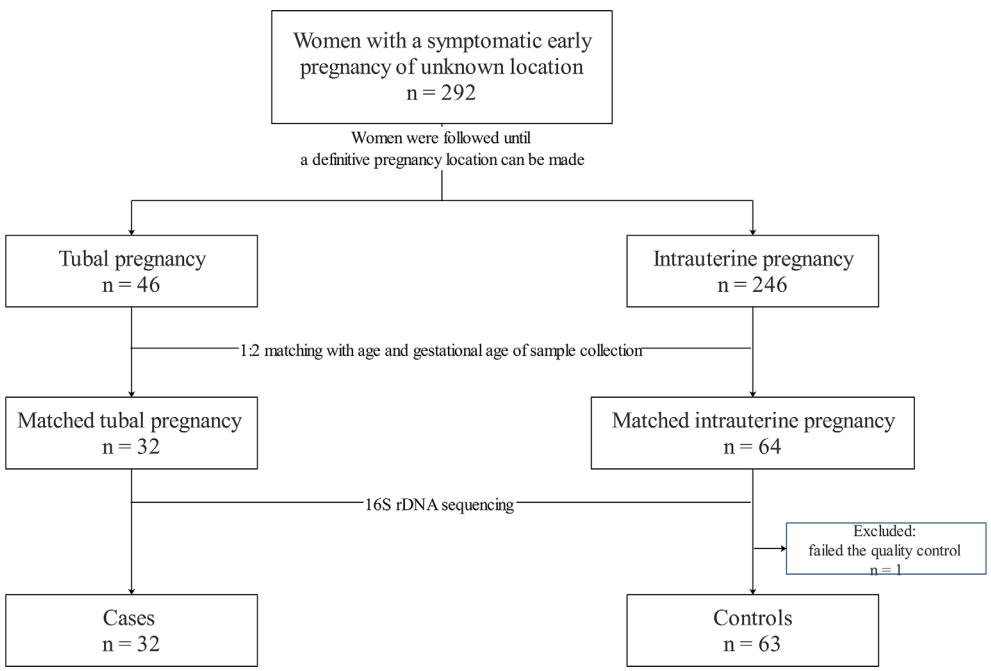

FIGURE 1 | Flowchart of the study.

Women With a TP Showed a Higher Diversity in VM Composition Than That in Women With an IUP

After sequencing, denoising, and filtering, an average of 381,558 high-quality reads per sample was obtained. This value was deep enough for intensive analysis of taxa, especially for rare species (Kim et al., 2011; Ravel et al., 2011).

At a sufficient sequencing depth, we compared alpha diversity (represented by PD-whole-trees and Shannon Diversity Index) in women with an IUP and women with a TP (**Figure 2A**). Compared with women with an IUP, women with a TP showed a higher PD-whole-tree value (median: 8.26 *vs* 7.08, $P = 0.047$) and Shannon Diversity Index (median: 1.43 *vs* 0.99, $P = 0.03$).

We undertook PCoA based on weighted UniFrac distances to further discover the diversity and community structure in women with an IUP or TP (**Figure 2B**). PCoA showed clustering, and the first principal coordinate (PC1) axes represented 79.3% of the total variations. ANOSIM showed that the dissimilarity between two groups was more significant than that within groups, which confirmed that the clustering was significant ($R = 0.23$, $P < 0.01$). These data indicated that, by considering the phylogeny as well as the abundance, women with a TP showed a different VM composition to that of women with an IUP.

The predominant taxa in the VM in women with an IUP were almost identical to those in women with a TP, but the relative abundance between the two groups was different (**Figure 3**). Firmicutes was the predominant phylum in women with an IUP (relative abundance: 91%) or a TP (relative abundance: 71%), but Actinobacteria was also common in TP (relative abundance: 18%). *Lactobacillus*, *Gardnerella*, and *Prevotella* were the predominant genera in both groups. In the IUP group, the relative abundance of the aforementioned genera was 89%, 4%, and 2%, respectively but, in the TP group, the relative abundance was 62%, 12%, and 6%. Bacteria of the genera *Atopobium* (4%), *Sneathia* (3%), and *Megasphaera* (2%) were also common in the TP group. Within the genus of *Lactobacillus*, *Lactobacillus iners AB-1*, *uncultured_bacterium*, and *uncultured Lactobacillus* were the major species in both groups.

Non-*Lactobacillus* Dominated VM Was Associated With a TP

We wished to further discover the potential taxonomic biomarkers which characterize the differences between an IUP and TP. We carried out LEfSe with a logarithmic LDA value of 4.0 (**Figure 4A**). We found genus *Lactobacillus*, order Lactobacillales, family Lactobacillaceae, and species *uncultured_bacterium* to be significantly enriched in the IUP group. Genus *Gardnerella*, phylum Actinobacteria, order Bifidobacteriales, and species *Gardnerella_vaginalis_00703Bmash* were significantly enriched in the TP group. Genus *Prevotella*, phylum Bacteroidetes, class Bacteroidia, order Bacteroidales, and family Prevotellaceae were also significantly enriched in the TP group. In addition, class Clostridia, order Clostridiale, and phylum Fusobacteria were significantly enriched in the TP group. Class Fusobacteriia, order Fusobacteriales, and family Leptotrichiaceae were also significantly increased in the TP group.

According to these analyses, we selected the relative abundance of three genera (*Lactobacillus*, *Gardnerella*, and *Prevotella*) to classify the pregnancy location (intrauterine pregnancy or tubal pregnancy). We carried out five trials of 10-fold cross-validated CART to classify samples. The average AUC for five trials of cross-validated CART of *Lactobacillus*, *Gardnerella*, and *Prevotella* was 0.81, 0.77, and 0.75, respectively (**Figure 4B**). The relative abundance of *Lactobacillus* was the most significant variable, and the best threshold was 85%. With regard to this model, the rule for classifying the pregnancy location was: if the relative abundance of *Lactobacillus* was > 85% the classification was an IUP; if the relative abundance of *Lactobacillus* was ≤ 85%, the classification was a TP. Based on these classifications, the VM of 95 women was divided into two groups: a relative abundance of *Lactobacillus* > 85% was termed "*Lactobacillus*-dominated microbiota" (LDM); a relative

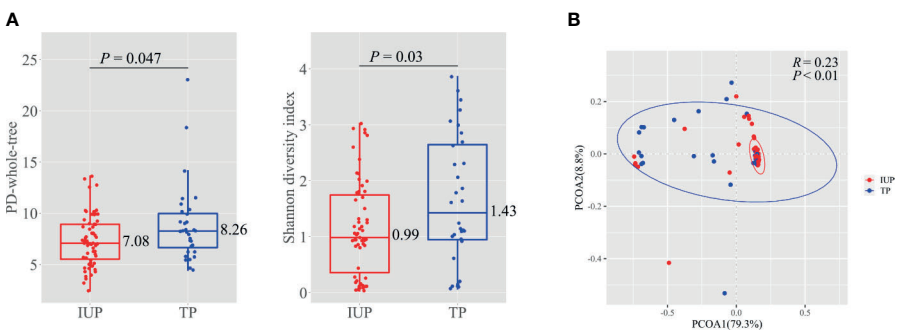

FIGURE 2 | Diversity measures in subjects with intrauterine pregnancy and with tubal pregnancy. **(A)** Alpha diversity represented by PD-whole-tree and Shannon diversity index in subjects with intrauterine pregnancy and with tubal pregnancy. Boxes with inside line represented interquartile range (IQR) and median, whiskers represented values within 1.5 × IQR of the first and third quartiles, points represented individual subjects. *P* was calculated by Wilcoxon test. **(B)** Principal coordinate analysis (PCoA) based on weighted UniFrac distances between the subjects with intrauterine pregnancy and with tubal pregnancy. Points represented individual subjects, and ellipses represented 95% confidence intervals around the cluster centroid. ANOSIM calculated *R* and *P* to determine the significance of clustering. Red indicated intrauterine pregnancy and blue indicated tubal pregnancy.

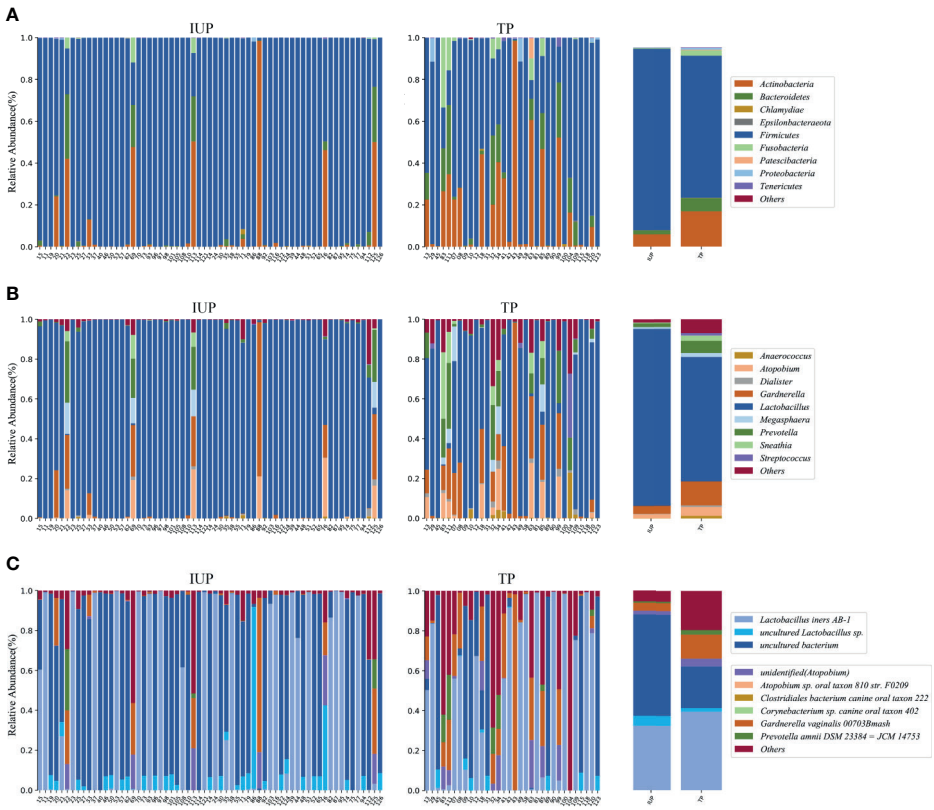

FIGURE 3 | Vaginal microbial composition in subjects with intrauterine pregnancy and with tubal pregnancy. **(A)** Phylum level; **(B)** Genus level; **(C)** Species level composition in subjects with intrauterine pregnancy and with tubal pregnancy. Bar charts showing the vaginal microbial taxa composition in mean values (on the right side) and individual subjects with intrauterine pregnancy and with tubal pregnancy (on the left side).

abundance of *Lactobacillus* ≤ 85% was classified as "non-*Lactobacillus*-dominated microbiota" (NLDM).

Compared with women with LDM, women with NLDM had a higher prevalence of a TP (56.2% *vs* 12.7%, $P < 0.01$). We used multivariate logistic regression analysis to evaluate the associations between NLDM and a TP. The association between NLDM and pregnancy location was robust in a crude model, minimally adjusted model, and fully adjusted model (**Table 2**). In the crude model, NLDM showed a positive association with an ectopic pregnancy [odds ratio (OR): 8.84, 95% confidence interval (CI): 3.19 to 24.48, $P < 0.01$]. In the minimally adjusted model (adjusted BMI), the result did not show an obvious change (OR: 9.46, 95%CI: 3.31 to 27.02, $P < 0.01$). Furthermore, in the fully adjusted model (adjusted for BMI, abdominal pain, vaginal pH, menstrual cycle, gravidity, previous spontaneous abortion, previous infertility, and previous uterine-cavity surgery), the correlation between NLDM and pregnancy location was stable (OR: 4.42, 95%CI: 1.33 to 14.71, $P = 0.02$).

With respect to alpha diversity, consistent with the alpha diversity between the IUP group and TP group, women with NLDM showed higher PD-whole-tree (median: 8.26 *vs*. 7.03 $P < 0.01$) and Shannon Diversity Index (median: 2.66 *vs* 0.95, $P < 0.01$) than those in women with LDM. In terms of beta diversity, PCoA on weighted UniFrac distances showed clustering in PC1 (representing 79.3% of the total variation), and ANOSIM confirmed the clustering to be significant ($R = 0.86$, $P < 0.01$) (**Figure S1B**).

We created heatmaps to visualize the taxa composition of the VM of the total cohort clustered by pregnancy location (intrauterine pregnancy or tubal pregnancy) and VM (NLDM and LDM) (**Figure 4C**). Subjects with NLDM were almost associated with tubal pregnancy while LDM were associated with intrauterine pregnancy. Consistent with this analysis, the VM of women with NLDM was characterized by greater diversity and a relatively higher abundance of bacteria of genera *Gardnerella*, *Prevotella*, *Atopobium*, *Megasphaera*, and *Sneathia*. Women with LDM showed high levels of skew dominated by bacteria of the genus *Lactobacillus* and a small proportion of bacteria of other genera. These data supported the notion that higher evenness was the cause of greater diversity in a TP and in NLDM. Bacteria of the genus *Lactobacillus* in NLDM and LDM could be subdivided into *Lactobacillus iners AB-1*, *uncultured_bacterium*, *uncultured Lactobacillus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*. Most women had more than one species of *Lactobacillus* in their VM.

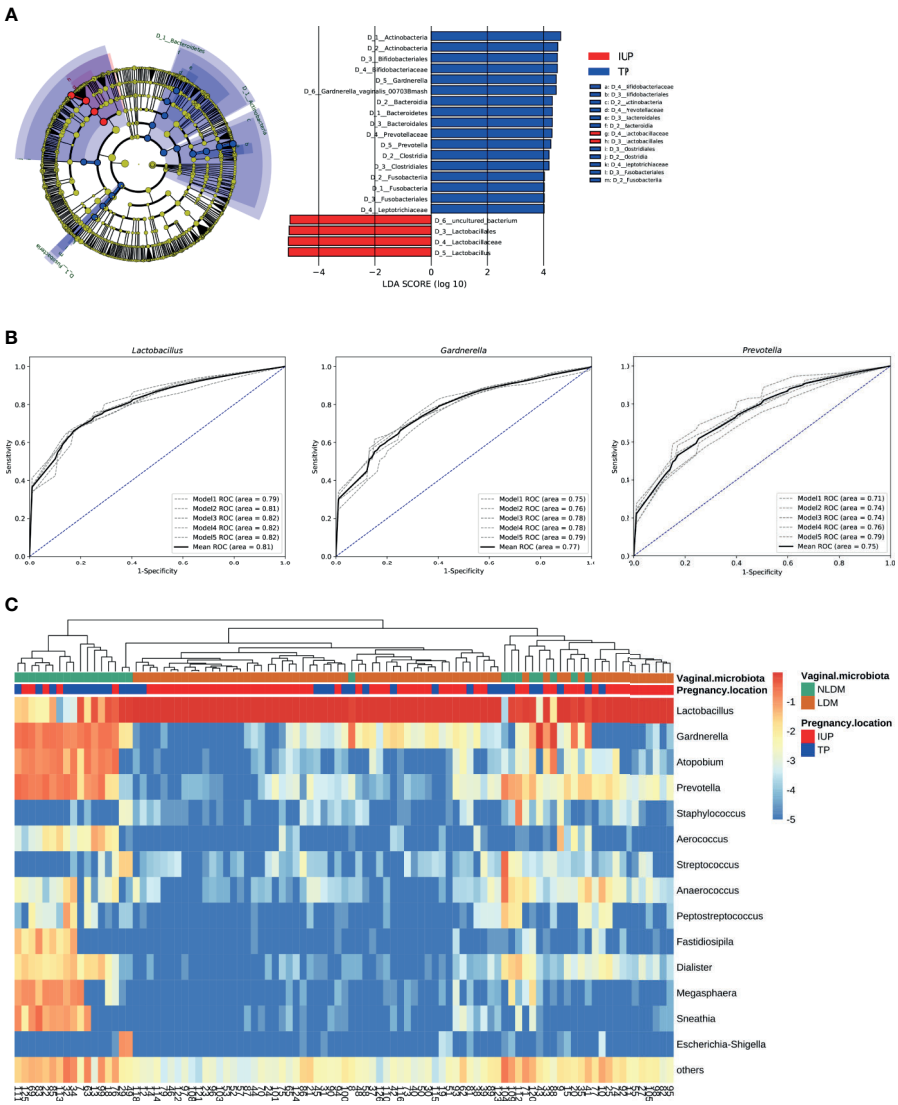

FIGURE 4 | The establishment of *Lactobacillus* dominated microbiota (LDM) and non-*Lactobacillus* dominated microbiota (NLDM). **(A)** Cladogram and scores identified by linear discriminant analysis (LDA) using LEfSe. The nodes of cladogram from the inner to the outer circles indicated the abundant taxa from the kingdom (D0) to the species (D6) level. Colors represented the groups in which differentially abundant taxa were enriched (red indicated intrauterine pregnancy, blue indicated tubal pregnancy, yellow indicated non-significant), and the diameter of each node was proportional to the taxon's abundance. The threshold on the logarithmic LDA score was 4.0. **(B)** Receiver operating characteristic curves for five trials of cross-validated CART of *Lactobacillu*, *Gardnerella*, and *Prevotella*. Black curve indicated the average ROC of the five trials (grey curves). The diagonal lines mark an area under the receiver operating characteristic curves of 0.5. **(C)** Heatmap of log10-transformed proportions of vaginal microbial taxa composition of 95 subjects clustered by pregnancy location and vaginal microbiota. The lower bar showed the pregnancy location of each subject (red indicated intrauterine pregnancy, blue indicated tubal pregnancy), while the upper bar showed vaginal microbiota (green indicated non-*Lactobacillus* dominated microbiota, orange indicated *Lactobacillus* dominated microbiota). The histogram showed the taxon $\log_{10\%}$ relative abundance (darkest red indicated greatest abundance; light red and yellow indicated relatively less abundance; and green indicated low abundance or not present). Fourteen of the most abundant genera were showed.

DISCUSSION

Before the pregnancy location could be visualized, the community structure of the VM in a TP was different to that of an IUP among women presenting with pain and/or uterine bleeding. A greater diversity and lower abundance of *Lactobacillus* in the VM was associated with a TP. As a potential valuable biomarker, the relative abundance of bacteria of the genus *Lactobacillus* could be used to classify the pregnancy location using a threshold of 85%. NLDM (≤85% *Lactobacillus*) was positively associated with a TP.

A TP is the main cause of hemorrhage-related mortality in early pregnancy (Creanga et al., 2017). Most studies have

TABLE 2 | Multivariate logistic regression analysis for the relationship between non-Lactobacillus dominated microbiota and tubal pregnancy.

LDM	Crude Model	Minimally adjusted model	Fully adjusted model
	OR, 95% CI, *P* Ref	OR, 95% CI, *P* Ref	OR, 95% CI, *P* Ref
NLDM	8.84 (3.19, 24.48) <0.01	9.46 (3.31, 27.02) <0.01	4.42 (1.33, 14.71) 0.02

Crude Model adjusted for: None.
Minimally adjusted model adjusted for: BMI.
Fully adjusted model adjusted for: BMI, abdominal pain, vaginal pH, menstrual cycle, gravidity, previous spontaneous abortion, previous infertility, and previous uterine cavity surgery.

suggested that previous infection in the fallopian tube (which results in adhesions or cilia damage) leads to a TP months or years later. Women with a previous ectopic pregnancy, PID, tubal surgery, and infertility are associated with an increased risk of a TP. However, ≤50% of patients with a TP have no definite risk factor (Barnhart et al., 2006; ACOG, 2018).

Development of NGS technologies has enabled more in-depth understanding of the VM. The key role of the commensal microbiome in the lower genital tract upon maternal and neonatal health has been documented. An increasing number of studies have shown the VM composition to be closely related with reproductive outcomes. A healthy pregnancy is characterized by a *Lactobacillus* species-dominated, low-richness, and low-diversity VM composition (DiGiulio et al., 2015). Al Memar and colleagues (Al-Memar et al., 2020) found that a lower vaginal abundance of *Lactobacillus*, high diversity, and high richness of bacteria was positively related with first-trimester miscarriage. This phenomenon was observable if the pregnancy seemed viable on ultrasound before miscarriage. Brown and coworkers (Brown et al., 2018) reported that VM disorders characterized as depletion of *Lactobacillus* and increased relative abundance of *Sneathia* were risk factors for subsequent preterm pre-labor rupture of fetal membranes. Freitas and collaborators (Freitas et al., 2017) observed that a higher abundance of *Mollicutes*, higher richness, and higher diversity of the VM was more likely to appear in women with spontaneous preterm birth compared with those in women who delivered at term. Moreover, Eckert and colleagues (Eckert et al., 2003) observed that a reduced vaginal abundance of *Lactobacillus* and bacterial vaginosis (BV) were related to early pregnancy loss (<6 weeks) and spontaneous pregnancy (10–16 weeks) after IVF. However, the association between pregnancy location and microbial disorders is poorly understood.

Consistent with those findings, we revealed a positive relationship between a TP and lower abundance of *Lactobacillus* as well as an increased prevalence of bacteria of the genera *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, and *Megasphaera*. A causal relationship between VM alterations and pregnancy location is not clear, but two main mechanisms have been proposed (Shaw et al., 2011; Borges et al., 2014; Witkin and Linhares, 2017; Amabebe and Anumba, 2018; Greenbaum et al., 2019).

First, depletion of *Lactobacillus* weakens protection of the reproductive tract. *Lactobacillus* in the vagina is thought to have great significance for reproductive health. *Lactobacillus* may promote embryo implantation and pregnancy. *Lactobacillus* produce lactic acid and hydrogen peroxide. Lactic acid can acidify the vagina, which deters pathogen proliferation. In addition, lactic acid reduces the production of proinflammatory mediators to prevent pathogens causing infection and damage (Witkin and Linhares, 2017). As an oxidizing agent, hydrogen peroxide is toxic to anaerobes. *Lactobacillus* inhibits pathogen colonization by competitively occupying their potential binding sites in the vaginal epithelium (Borges et al., 2014; Greenbaum et al., 2019). *Lactobacillus* have been shown to prevent sexually transmitted infections (STIs) (Witkin and Linhares, 2017; Amabebe and Anumba, 2018). STIs, including those caused by *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium*, are most frequently linked with PID and TP, which can be explained by inflammation, fibrosis, and subsequent scarring of fallopian tube (Borges et al., 2014; Greenbaum et al., 2019). Besides, Shaw and colleagues (Shaw et al., 2011) found that *C. trachomatis* infection increased fallopian tube expression of PROKRS mRNA, resulting in ectopic implantation in the fallopian tube.

Second, bacteria of the genera *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, and *Megasphaera* are thought to be involved in BV pathogenesis (Coleman and Gaydos, 2018; Muzny et al., 2019). As the most common cause of vaginal discharge, BV is closely correlated with different adverse pregnancy outcomes (Cauci and Culhane, 2011; Kindinger et al., 2016). BV also acts as a risk factor of tubal-factor infertility (van Oostrum et al., 2013; Haahr et al., 2019), STIs (Wiesenfeld et al., 2003; Brotman et al., 2010; Molenaar et al., 2018), and PID (Haggerty et al., 2016; Ravel et al., 2021). Many BV-associated bacteria produce sialidases. Sialidases are thought to enhance ascending infection in the reproductive tract by hampering the host's ability to recognize them, thereby facilitating bacterial attachment and inducing inflammatory reactions (Cauci and Culhane, 2011; Kindinger et al., 2016; Ravel et al., 2021).

Our study revealed the potential value of using the VM to classify the pregnancy location. However, translating these complex ecological metrics to the clinical setting is a challenge. Classification and categorization of the VM could greatly reduce the complexity of the biological dataset and be beneficial to epidemiological investigations and disease diagnoses (Cauci and Culhane, 2011; Kindinger et al., 2016; Ravel et al., 2021). Ravel and colleagues (Cauci and Culhane, 2011; Kindinger et al., 2016; Ravel et al., 2021) were the first to categorize the VM community in women of reproductive age based on the dominant bacterial species (using a threshold of 50% relative abundance) by clustering samples. That was a milestone study, and five types were found: four were dominated by *Lactobacillus* species (*L. crispatus, L. gasseri, L. iners*, and *L. jessenii*), and the remaining one was typified by a large proportion of anaerobic

bacteria of genera *Prevotella, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia, and Mobiluncus*. Gajer and colleagues (Gajer et al., 2012) used hierarchical clustering to refine and improve this classification and proposed the term "community state types" (CSTs). Since then, CSTs have been applied widely in clinical research. Several studies have pointed out that women who have experienced preterm delivery would be classified as "*Lactobacillus*-poor CST 4" (DiGiulio et al., 2015; Tabatabaei et al., 2019; Chang et al., 2020).

However, CSTs have their limitations, one of which is that they are built on four definite dominant *Lactobacillus* species. Recent studies have found that, in early pregnancy, some VM communities are dominated by other or more than one type of *Lactobacillus* species. By contrast, classifications based on the relative abundance of *Lactobacillus* at the genus level could separate samples clearly and may appraise the relationship between the VM community and pregnancy outcomes more accurately (Haahr et al., 2019; Al-Memar et al., 2020; Chang et al., 2020). Besides, there is no exact method, standard clustering algorithm, or consentaneous threshold for CST assignment, and the different rules for CST generation result in different grouping outcomes (Koren et al., 2013; Loeper et al., 2018). Robinson and coworkers (Robinson et al., 2016) pointed out that using machine-learning algorithms instead of clustering for classification could overcome such drawbacks. Machine learning has improved bioinformatics analysis dramatically for making the microbial-community groups independent of samples and aiding comparability between studies (LeCun et al., 2015). Nevertheless, few studies have applied machine learning to VM classification.

Moreno and collaborators (Moreno et al., 2016) selected the relative abundance of four genera in the endometrial fluid as variables. Then, they applied two supervised machine-learning models—a CART model and a generalized linear model by logistic regression—to predict the pregnancy outcome of women undergoing IVF. Both models came to the same conclusion: *Lactobacillus* was the only available genus. Based on CART, the endometrial microbiota could be classified into two groups: LDM and NLDM. A receptive endometrium with NLDM in women undergoing IVF tended to produce poor pregnancy outcomes.

In this context, to establish an effective and robust classification, we undertook LEfSe to obtain potential vaginal taxonomic biomarkers. LEfSe is an algorithm which emphasizes statistical significance and biological relevance for identification of high-dimensional biomarkers (Segata et al., 2011). We found bacteria of the genus *Lactobacillus* to be significantly enriched in IUPs whereas bacteria of the genera *Gardnerella* and *Prevotella* were enriched in TPs. Then, we undertook five trials of 10-fold cross-validated CART to establish VM community groups. CART is a powerful machine-learning algorithm. On account of its validity for categorizing subjects in groups, as well as handling the multicollinearity and interactions of variables, CART has become increasingly popular in clinical research (Marshall, 2001; Lemon et al., 2003; Henrard et al., 2015). We showed that the relative abundance of *Lactobacillus* could be used to discriminate a TP from an IUP with high accuracy. Also, the VM could be classified into LDM and NLDM. Establishment of a classification system for the VM could simplify microbial structure.

The was the first comparative study of VM between IUP and TP groups using NGS. We also established a readily accessible model which could accurately classify a TP before a diagnosis using transvaginal ultrasound based on identification of key taxa and a machine-learning model. Furthermore, we used a specific population of clinical interest—a group of women with symptomatic early PUL—presenting for care. We used a well-defined study population, so the results will be limited to women with pain and/or uterine bleeding. Also, we assessed Chinese women; studies have pointed out that ethnicity can impact the VM composition (Ravel et al., 2011; MacIntyre et al., 2015; He et al., 2019; Serrano et al., 2019). Also, our study was associative; we focused on describing the different VM compositions between IUPs and TPs. Although we suggest that the relative abundance of *Lactobacillus* might be a valuable biomarker to classify a TP, the causality and mechanism remains to be determined. Longitudinal studies are required to demonstrate a causal relationship between the VM and pregnancy location. Hence, further large-scale studies that incorporate asymptomatic populations as well as different ethnic groups are needed. Moreover, due to the limitation of 16S rRNA gene sequencing, it cannot distinguish microbiota well at the species level and cannot identify other important organisms such as viruses and fungi.

CONCLUSIONS

We showed, for the first time, that a higher evenness, greater diversity, and lower abundance of *Lactobacillus* in the VM was associated with a TP. The relative abundance of *Lactobacillus* in the VM could be a diagnostic marker. Our data provide a first glimpse and "snapshot" of the VM in early pregnancy, offering groundwork for further studies.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Sequence Read Archive, accession number PRJNA737055.

ETHICS STATEMENT

Our study was approved by the Ethical Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine (ZYYECK2017-060, approval date 06/02/2018), and all participants provided written informed consent. The patients/

participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG, G-PD, and S-PL designed and founded the study. Y-XH and X-JL recruited participants. X-FR, Y-XZ, SC, and X-RL collected clinical data and samples and analyzed and interpreted data. X-FR, Y-XZ, and SC generated figures and tables. X-FR wrote the first draft, which was developed by Y-XZ, F-FZ, and SC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.659505/full#supplementary-material

Supplementary Figure 1 | Diversity measures in subjects with non-*Lactobacillus* dominated microbiota and with *Lactobacillus* dominated microbiota. **(A)** Alpha diversity represented by PD-whole-tree and Shannon diversity index in subjects with NLDM and LDM. Boxes with inside line represented interquartile range (IQR) and median, whiskers represented values within 1.5 × IQR of the first and third quartiles, points represented individual subjects. *P* was calculated by Wilcoxon test. **(B)** Principal coordinate analysis (PCoA) based on weighted UniFrac distances between the subjects with NLDM and LDM. Points represented individual subjects, and ellipses represented 95% confidence intervals around the cluster centroid. ANOSIM calculated *R* and *P* to determine the significance of clustering. Green indicated NLDM, orange indicated LD

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Alterations of Vaginal Microbiota in Women With Infertility and *Chlamydia trachomatis* Infection

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Chlamydia trachomatis (*C. trachomatis*) is the most common etiological agent of bacterial sexually transmitted infections (STIs) worldwide and causes serious health sequelae such as cervicitis, pelvic inflammatory disease, and even infertility if ascending from the lower to the upper female genital tract. Previous studies have revealed the pivotal role of vaginal microbiota in susceptibility to STIs. However, alterations in the vaginal microbiota in women who are infertile and infected with *C. trachomatis* remain unknown. This study used metagenomic analysis of sequenced 16S rRNA gene amplicons to examine the vaginal microbial profiles of women with tubal infertility who were *C. trachomatis*-negative and those who were *C. trachomatis*-positive pre- and post-antibiotic treatment. Women who were *C. trachomatis*-negative and deemed healthy were recruited as references of eubiosis and dysbiosis. Women with tubal infertility and *C. trachomatis* infection presented a unique *Lactobacillus iners*-dominated vaginal microbiota rather than one dominated by *Lactobacillus crispatus* and displayed a decrease in *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Atopobium*, and *Streptococcus*, accompanied by decreased levels of cytokines such as interferon (IFN)-γ and interleukin (IL)-10. This altered vaginal microbiota could be restored with varying degrees after standard treatment for *C. trachomatis*. This shift could be a predictive vaginal microbiota signature for *C. trachomatis* infection among females with tubal infertility, while no significant differences in phylum, class, and operational taxonomic unit (OTU) levels were observed between women with tubal infertility who were *C. trachomatis*-negative and healthy controls. This is the first study to provide data on the association of vaginal microbiota with *C. trachomatis* infection among women with tubal infertility and highlights unprecedented potential opportunities to predict *C. trachomatis* infection.

Keywords: *Chlamydia trachomatis*, infertile women, vaginal microbiota, 16S rRNA gene sequencing, *Lactobacillus*

INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is an obligate intracellular parasitic bacterium that can infect both genital and non-genital sites including the cervix, rectum, and eyes (Mishori et al., 2012; Morrison et al., 2020). Genital *C. trachomatis* infection is a leading cause of bacterial sexually transmitted disease, responsible for more than 131 million emerging infections worldwide, and may manifest as mucopurulent cervicitis with a watery or purulent discharge and easily induced bleeding with a swab (Wiesenfeld, 2017). More than 60%–80% of infected women remains asymptomatic, which facilitates the spread of the pathogen and may lead to the development of a chronic infection (Woodhall et al., 2018). Furthermore, untreated *C. trachomatis* infection during labor can be vertically transmitted, resulting in conjunctivitis and pneumonitis in infants (Manavi, 2006).

C. trachomatis has a biphasic life cycle comprising a metabolically active noninfectious reticulate body (RB) and an infectious environmentally resistant elementary body (EB). The RB replicates by binary fission within the confines of the inclusion and differentiates into EBs at the end of the infectious replication cycle, while the EBs are closely followed by releasing from the cell to initiate new infection *via* cytolysis or endocytosis (Morrison, 2003; Chumduri et al., 2013). Various factors such as antibiotic treatment, host immunological response, or nutrient starvation disturb the *C. trachomatis* developmental cycle, and under such conditions, the EBs can convert to enlarged noninfectious aberrant bodies (ABs). This so-called "viable but non-cultivable growth stage" is associated with chronic and repeat infections that can lead to serious complications in women, including obstructive infertility, ectopic pregnancy, and preterm birth (den Hartog et al., 2006; Ziklo et al., 2016). Besides, persistent *C. trachomatis* infection enhanced the expression of *C. trachomatis* Hsp60 (cHsp60), capable of activating mononuclear cells or monocyte-derived macrophages producing E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 and amplifying the ongoing inflammatory process by secreting pro-inflammatory cytokines (Kol et al., 1999; Contini and Seraceni, 2012). Moreover, this infection could also provoke the release of human heat shock protein (HSP)60, very similar to chlamydial heat shock protein (cHsp)60, which was firstly produced by early-stage embryos. In this regard, cross-reactive cHsp60 peptides elicited an immune response that can recognize the human hsp60 and increase the pathogenesis of genital chlamydial infection (Pockley, 2003; Witkin et al., 2017).

The female genital tract harbors a plethora of microorganisms that play an emerging role in human health and are influenced by host lifestyle, antibiotic use, and hormonal contraception (Ziklo et al., 2016). *Lactobacillus*-dominated vaginal microbiota is considered a marker of health status for healthy women (Ma et al., 2012; Pekmezovic et al., 2019), owing to its ability to produce lactic acid and multiple bacteriostatic and bactericidal compounds to protect against extraneous pathogenic bacteria (Valenti et al., 2018; Tamarelle et al., 2019). Women infected with *C. trachomatis* were hypothesized to undergo an alteration in their vaginal microbiota dominated by *Lactobacillus iners* or by diverse anaerobic bacteria (van der Veer et al., 2017).

Previous studies have revealed that *C. trachomatis* and human papillomavirus (HPV) might serve as mutual risk factors to increase the risk of infections (Seraceni et al., 2014; Chen et al., 2020). Consequently, we hypothesize that some vaginal microbiota may also accelerate or inhibit *C. trachomatis*-induced infertility among females. There are currently no data regarding alterations in the vaginal microbiota in women with tubal infertility who are infected with *C. trachomatis*. Therefore, the present study aimed to use 16S rRNA gene amplicon-based metagenomic analysis to characterize vaginal microbiota diversity in healthy women who were *C. trachomatis*-negative and receiving an annual physical examination, women with tubal infertility who were *C. trachomatis*-negative, and women with tubal infertility who were *C. trachomatis*-positive pre- and post-antibiotic treatment. Deciphering vaginal microbiota alterations induced by *C. trachomatis* in women with infertility will enhance the understanding of the potential interaction(s) of distinct bacterial communities with *C. trachomatis* infection.

MATERIALS AND METHODS

Ethics Statement

All participants provided their written informed consent for participation in this study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Chenzhou No. 1 People's Hospital (CZ/1128).

Study Design

From January 2019 to January 2020, women diagnosed as tubal infertile plus *C. trachomatis*-negative (CT-N) or -positive (CT-P) and seeking assistance at the assisted reproductive technology center (ART) and women who were *C. trachomatis*-negative (CT-C) and receiving an annual physical examination in the physical examination center (PEC) of a teaching hospital in Chenzhou were enrolled in the study. Participants with tubal infertility who were *C. trachomatis*-positive had study visits scheduled 60 days after standard treatment with azithromycin (CT-PT), a frontline antibiotic used to treat *C. trachomatis* infection. Standard treatment is 1 g azithromycin in a single oral dose according to *C. trachomatis* treatment guidelines approved by the Centers for Disease Control and Prevention (CDC) (Workowski et al., 2015).

All enrolled women were 20–49 years old (hence of reproductive age), not pregnant, out of menstruation, and had no prior common sexually transmitted infections (STIs) including HPV, *Treponema pallidum*, and *gonococcus*. During routine gynecological inspection, female vaginal discharge was collected for *C. trachomatis* screening (Chen et al., 2020), leukorrhea routine detection, cytokine measurement, and vaginal microbiota analysis. Women who were infertile and *C. trachomatis*-positive were asked to revisit 60 days after treatment.

Clinical Samples

All participants were asked to abstain from unprotected sex or vaginal lavage for 48 h before sampling and avoid taking antibiotics and/or antiviral drugs in the 2 weeks prior to sampling. The peeping tube was not coated with lubricant when removing secretions. Four vaginal swabs were collected by doctors or trained nurses: (1) one for *C. trachomatis* screening to be completed within 24 h; (2) one for leukorrhea routine detection to be completed within 4 h; (3) one for vaginal flora diversity analysis, stored at -80°C until use; and (4) one for cytokine measurement, stored at -20°C until use.

Leukorrhea Routine Detection

Place and mix the vaginal swab vigorously in a tube with 1 ml sterile phosphate-buffered saline (PBS). A "wet mount" can be made with one to two drops of the vaginal discharge specimen and immediately examined under an optical microscope for observation of epithelial cells, white blood cells, clue cells, *Trichomonas vaginalis*, and fungi. Cleaning degree of leukorrhea includes grade I, II, III, and IV, ≥ grade II of which are considered abnormal leukorrhea according to the guide to Clinical Laboratory Procedures of China (Shang et al., 2015). One drop of the homogeneous mixture was also transferred to a grease-free dry slide to make a smear, and the dry smear was fixed by passing the slide quickly through a flame 3–4 times with the smear side facing up. The smear was then stained, following the instructions of the Gram Staining Kit (Baso, ZhuHai, China), to observe bacterial morphology.

Cytokine Measurements

Each vaginal swab was placed in a tube with 1 ml PBS and centrifuged at 12,000 *g*, 30 min at 4°C. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, and IL-10 in the resulting supernatant were measured using ELISA kits (Biolegend, CA, USA) following the manufacturer's instructions. Final cytokine concentrations were calculated in pg/ml from a standard curve based on prepared dilutions of recombinant cytokines in each experiment (Chen et al., 2017).

16S rRNA Amplicon Sequencing

Genomic DNA was extracted by using a Genomic DNA Mini Preparation Kit (Tiangen, Beijing, China), and the resulting concentration was diluted to 1 ng/μl with sterile water. The V3–V4 hypervariable regions of 16S rRNA genes were amplified by PCR using specific primers (806R 5′-GGACTACNN GGGTATCTAAT-3′, 341F 5′-CCTAYGGGRBGCASCAG-3′) with a barcode. All reactions were conducted in a 30-μl volume comprising 0.2 μM forward and reverse primers, 15 μl PCR Master Mix, and 10 ng DNA template. The PCR conditions were initially one cycle of 1 min at 98°C; 30 cycles of 10 s at 98°C, 30 s at 50°C, and 30 s at 72°C, followed by a final extension at 72°C for 10 min.

Dominant PCR products of 400–450 bp were selected for further experiments and were mixed in equidensity ratios, purifying with the GeneJET Gel Extraction Kit (Thermo Scientific, USA). Following the manufacturer's recommendations, sequencing libraries were conducted using Library Prep Kit for Illumina, and index codes were added. Library quality was assessed on the Agilent Bioanalyzer 2100 system and Qubit@ 2.0 Fluorometer (Thermo Scientific, USA). The library was ultimately sequenced on an Illumina HiSeq platform generating 250-bp paired-end reads.

Data Analysis

The operational taxonomic units (OTUs) clustering and species classification were analyzed at 97% identity level based on effective data discarding low-quality reads. Each OTU was annotated on the basis of OTU clustering to obtain the species-based abundance distribution and corresponding species information. The relative abundance, alpha-diversity calculation, Venn plot, and petal plot analysis of OTUs were carried out to obtain the information of species richness and evenness within the sample, as well as the information of common and special OTUs among different groups/samples. As well, multisequence alignment of OTUs can be carried out and phylogenetic trees can be constructed. Through dimension-reduction analysis such as principal coordinate analysis (PCoA), principal component analysis (PCA), nonmetric multidimensional scaling (NMDS), and sample cluster tree, the differences between community structure among distinct samples or groups can be explored. To further determine the differences in community structure among grouped samples, statistical analysis methods such as Student's *t*-test, Simper, Metastat, Linear discriminant analysis Effect Size (LEfSe), analysis fo similarities (Anosim), and multi-response permutation procedure (MRPP) were used to test the significance of differences in species composition and community structure among samples.

qRT-PCR

Bacterium-specific qRT-PCR assays were conducted by amplifying species-specific regions of the 16S rRNA gene. Primers specific to 17 dominant microorganisms characterized by the 16S rRNA amplicon were designed or obtained from the literature (Blackwood et al., 2005; Yang et al., 2015). Each subject was amplified with all primer pairs and was subjected to a human β-globin PCR to ensure the quality of amplifiable DNA and to monitor for PCR inhibitors. Each 20-μl qRT-PCR mixture contained 10 μl 2× Taq Plus MasterMix (TaKaRa, Dalian, China), 2 μl template DNA, and 0.2 μM of each primer (**Table S1**). The PCR reaction conditions were as follows: one cycle of 5 min at 95°C, 40 cycles of 30 s at 95°C, 35 s at 60°C–65°C (depend on the melting temperature of the primers), and 45 s at 72°C, then a final extension at 72°C for 10 min. Melting curve analysis was then conducted at 95°C for 15 s, 60°C for 50 s, and 95°C for 15 s before the reaction was terminated.

Serial dilutions of 1, 1:4, 1:16, 1:64, and 1:256 vaginal bacterial total DNA were used to determine primer amplification efficiencies. Quantitative analysis of vaginal bacterial communities was performed using the following formula:

$$X = \frac{(Eff \cdot Univ)^{Ct\ univ}}{(Eff \cdot Spec)^{Ct\ spec}} \times 100$$

where "Ct spec" and "Ct univ" represent Cycle threshold, "Eff. Spec" represents the efficiency of various genus-specific primers, and "Eff. Univ" represents the efficiency of bacterial universal

primers. "X" represents the percentage of bacterial species-specific gene copy number existing in a sample.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8.0 and SPSS 22.0. Differences of age in the study population were tested using a Student's *t*-test, and leukorrhea characteristics were analyzed using the chi-square, Fisher's exact tests. One-way analysis of variance in combination with Tukey's *post-hoc* tests was used to determine the differences in univariate data between the samples including cytokine measurements, DNA yield, Bray–Curtis dissimilarity, richness, and qRT-PCR results. Statistical significance of alpha-diversity measures was calculated using Qiime software, and beta-diversity parameters were determined using the R Studio package "phyloseq."

RESULTS

Characteristics of Study Subjects

Thirty samples from 26 women of reproductive age enrolled in the study were obtained. These were nine women who were CT-N and eight women who were CT-P from ART plus nine women classed as CT-C from PEC. Two CT-P participants discovered to be pregnant during the sampling visit were excluded from the study.

Five samples (two CT-C, one CT-N, and two CT-P samples) were excluded from 16S rRNA amplicon-based sequencing because of their failure to pass the sequencing and quality control. Consequently, 25 cervical samples comprising seven CT-C, eight CT-N, six CT-P, and four CT-PT can be applicable for downstream analysis. No significant differences were observed in the age distribution between the different groups of women (**Table 1**).

Leukorrhea and Cytokine Production in Vaginal Discharge

Leukorrhea abnormal features were found in 66.6% (4/6) of the samples in the CT-P group but only in 28.6% (2/7) for CT-C, 12.5% (1/8) for CT-N, and 25.0% (1/4) for CT-PT groups. However, there were no significant differences between the groups ($p > 0.05$). Similar results were found for lekcorrhea leukocytes (**Table 1**). Fewer vaginal bacterial species were observed in the CT-P group samples compared with the CT-C and CT-N groups, but this was restored to some extent following treatment (**Figure S1**).

Vaginal inflammation induced by *C. trachomatis* was further investigated by measuring the production of IFN-γ, TNF-α, IL-6, and IL-10. There were significantly higher levels of IFN-γ and IL-10 in the CT-P group than in the CT-C, CT-N, and CT-PT groups ($p < 0.05$), while there were no significant differences in TNF-α and IL-6 levels ($p > 0.05$; **Figure 1**). This indicated that *C. trachomatis* genital tract infection might induce a local inflammatory response.

Overview of the Vaginal Microbiota Sequencing Results

Illumina Nova sequencing was applied to determine the vaginal microbiota diversity. A total of 1,949,887 raw tags were obtained. After preprocessing, there was a final average of 65,600 high-quality sequences per sample for downward analysis, which clustered into 4,385 OTUs at the 97% similarity level for further species annotation. The number of shared OTUs among the samples was 221, which accounted for 5.4% of the 4,384 total OTUs (**Figure S2**), suggesting that there were large differences in the populations during *C. trachomatis* infection. In addition, the Good's coverage index value of each sample exceeded 98% (**Table 2**), suggesting the sequencing depth covered over 98% of the bacterial phylotypes and precisely reflected the microorganisms contained in each sample. The plot species accumulation curve became an asymptote after a sharp rise (**Figure S3**). This slow rise indicated that the species in this environment would not increase significantly with an increase in sample size and suggested that additional data volume would only produce a small number of new species. This further verified that the sampling and sequencing data were reasonable and sufficient.

Alpha- and Beta-Diversity Analyses

To evaluate the richness and diversity of vaginal microbial communities in samples, alpha-diversity analyses were conducted. Alpha-diversity metrics, including Shannon and observed species indices, indicated significant differences in biodiversity between the different groups ($p < 0.01$ and $p <$

TABLE 1 | Leukorrhea routine detection and characteristics of the study population.

Classification	CT-C (n = 7)	CT-N (n = 8)	CT-P (n = 6)	CT-PT (n = 4)	p
Age, years	33.6 ± 3.7	33.9 ± 4.2	34.1 ± 4.8	33.8 ± 4.6	>0.05
Cleaning degree					>0.05
I	3 (42.8%)	4 (50.0%)	0 (0%)	1 (25.0%)	
II	2 (28.6%)	3 (37.5%)	2 (33.3%)	2 (50.0%)	
III	2 (28.6%)	1 (12.5%)	2 (33.3%)	1 (25.0%)	
IV	0 (0%)	0 (0%)	2 (33.3%)	0 (0%)	
Leukocyte					>0.05
I	5 (71.4%)	6 (75.0%)	2 (33.3%)	3 (75.0%)	
II	2 (28.6%)	1 (12.5%)	2 (33.3%)	1 (25.0%)	
III	0 (0%)	1 (12.5%)	2 (33.3%)	0 (0%)	

CT-C, healthy women with C. trachomatis-negative from the PEC; CT-N, infertile women with C. trachomatis-negative from the ART; CT-P, infertile women with C. trachomatis-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin; ART, assisted reproductive technology center; PEC, physical examination center.

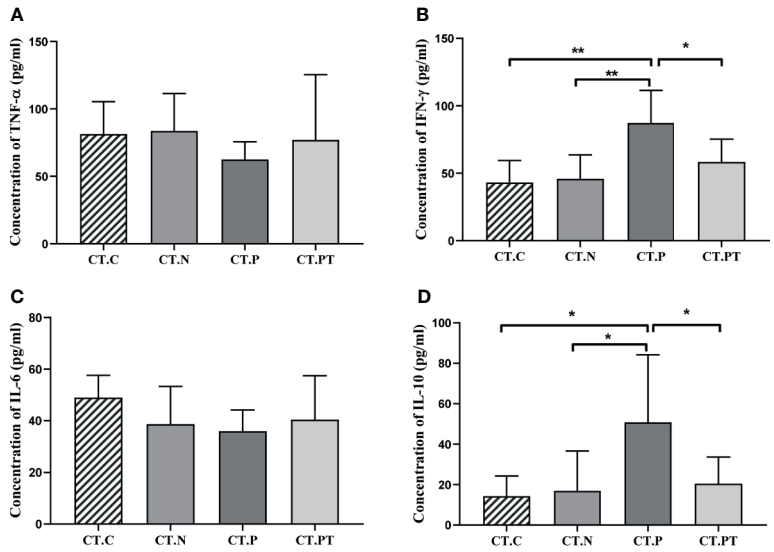

FIGURE 1 | Cytokine production in vaginal secretions in each group. **(A)** Tumor necrosis factor (TNF)-α, **(B)** interferon (IFN)-γ, **(C)** interleukin (IL)-6, and **(D)** IL-10 levels were detected in vaginal discharge samples using ELISA kits. Each bar represents the mean ± SD of the cytokine levels (pg/ml). *p < 0.05 and **p < 0.01. CT-C, women from the physical examination center (PEC) who were healthy and *C. trachomatis*-negative; CT-N, women who were infertile and *C. trachomatis*-negative from the assisted reproductive technology center (ART); CT-P, women who were infertile and *C. trachomatis*-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin.

0.05). A significantly lower Shannon index was observed in CT-P samples compared with CT-C ($p < 0.01$) and CT-N ($p < 0.05$) samples (**Table 2**), indicating that genital infection with *C. trachomatis* may be associated with a decreased diversity of the vaginal microbiota in women who were infertile. However, there were no statistical differences in Simpson, Chao1, and abundance-based coverage estimators (ACE) (**Table 2**).

Beta diversity was employed to understand the divergence in community composition between samples. PCoA based on Bray–Curtis dissimilarities showed no significant segregation of the groups on either weighted (**Figure 2A**) or unweighted Unifrac distance (**Figure 2B**). However, the CT-P vaginal samples were clustered and separated from the other samples when subjected to NMDS analysis based on unweighted Unifrac distance (**Figure 2C**). Moreover, a heatmap of beta-diversity index (**Figures 2D, E**) revealed that there was a statistically significant species diversity of CT-P compared with the other groups ($p < 0.01$). The unweighted pair group method with arithmetic mean (UPGMA) clustering tree, using Euclidean distance matrices with Ward linkage (**Figure 2F**), confirmed that vaginal samples in the CT-P group exhibited a tendency toward clustering and relatively diverged from the samples from the CT-C, CT-N, and CT-PT groups. This suggested that women in the CT-P group possessed a unique microbial composition that differed from that of healthy women, and that alterations in relative taxa abundance occurred after treatment for *C. trachomatis* infection.

Taxonomic Composition of Vaginal Microbiota

Bacterial taxa identified in the 25 studied vaginal samples comprised a total of 21 phyla, 30 classes, 66 orders, 115 families, and 203 genera. To further explore the taxonomic composition of vaginal microbiota in each group, the phylotypes were clustered according to their correlation profiles and the vaginal bacterial communities were grouped according to community composition. The heatmap in **Figures 3A, B** representing log10-transformed relative abundances of microbial taxa highlights the variation of microbial composition among the individuals in the study and the diversity in all vaginal bacterial communities. Of the 25 samples, 10 had *L. iners*-dominated

TABLE 2 | Alpha-diversity index for each group.

Group	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage
CT-C	427.86 ± 182.55	2.34 ± 1.44	0.53 ± 0.29	777.73 ± 327.01	892.58 ± 377.19	0.99 ± 0.00
CT-N	384.88 ± 374.46	1.82 ± 1.14	0.41 ± 0.27	689.01 ± 550.35	737.67 ± 532.83	0.99 ± 0.01
CT-P	258.33 ± 210.96*	1.32 ± 1.29*	0.32 ± 0.36	468.74 ± 469.55	559.80 ± 677.34	0.99 ± 0.01
CT-PT	243.25 ± 164.68*	1.59 ± 1.02	0.33 ± 0.33	491.67 ± 353.47	575.41 ± 410.79	0.99 ± 0.00

*CT-C, women from the PEC who were healthy and C. trachomatis-negative; CT-N, women who were infertile and C. trachomatis-negative from the ART; CT-P, women who were infertile and C. trachomatis-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin; ART, assisted reproductive technology center; PEC, physical examination center; ACE, abundance-based coverage estimators. *p < 0.05 compared with CT-N.*

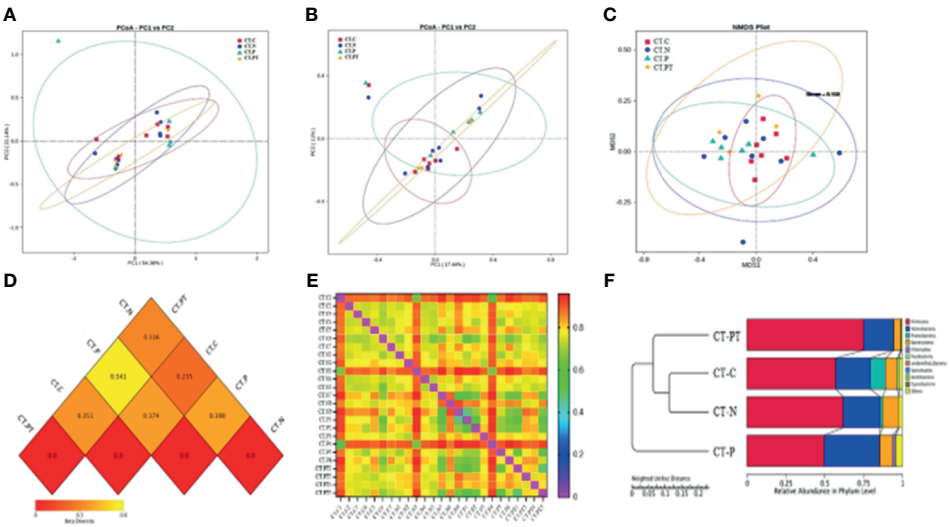

FIGURE 2 | Comparative analysis of beta diversity. Principal coordinate analysis (PCoA) based on weighted UniFrac **(A)** and unweighted Unifrac **(B)** distance. **(C)** Nonmetric multidimensional scaling analysis of each sample. Heatmap of beta-diversity index for each group **(D)** and samples **(E)**; the legend below the heatmap represents each participant. **(F)** Unweighted pair group method with arithmetic mean (UPGMA) clustering tree of each group. CT-C, women from the physical examination center (PEC) who were healthy and *C. trachomatis*-negative; CT-N, women who were infertile and *C. trachomatis*-negative from the assisted reproductive technology center (ART); CT-P, women who were infertile and *C. trachomatis*-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin.

microbiota and the rest had a community dominated by *Lactobacillus reuteri*, *Bifidobacterium breve*, *Lactobacillus gasseri*, *Atopobium vaginae*, or Clostridiales bacterium.

At the phylum level, the vaginal microbiota of all women was mainly dominated by Firmicutes, with abundance ranging from 75.2% in CT-PT to 48.8% in CT-P, followed by Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria, Chlamydiae, Spirochaetes, and Acidobacteria, accounting for an average of 0.1%–25.1% of the total relative abundance (**Figure 3C**). This was congruent with previously reported vaginal microbiota types (Filardo et al., 2017). Moreover, the relative abundance of the phylum Firmicutes was reduced in women who were *C. trachomatis*-positive when compared with participants in the CT-C (57.1%) and CT-N (61.9%) groups. Conversely, the phylum Acidobacteria was significantly enriched in the CT-P group (34.4%) compared with the CT-C (22.2%), CT-N (23.7%), and CT-PT (19.1%) groups. No statistically significant differences were observed in relation to phylum distribution between the CT-C group and the CT-N group.

At the family taxonomic level, Lactobacillaceae, as well as Enterobacteriaceae, Prevotellaceae, Atopobiaceae, Streptococcaceae, Veillonellaceae, and Lachnospiraceae, were less abundant in CT-P samples than in CT-N samples. In contrast, Bifidobacteriaceae was more abundant in CT-P samples compared with CT-N samples (median relative abundance 33.1% and 21.4%, respectively; **Figure 3D**).

Analysis of the vaginal microbiota composition at the lower taxonomic level of the genus revealed that eight out of the top 10 taxa in terms of relative abundance were significantly different between CT-N and CT-P samples (**Figure 3E**). These included *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Atopobium*, *Streptococcus*, and *Alistipes*, which were all more abundant in the CT-N samples, and *Chlamydia*, *Gardnerella*, and *Megasphaera*, which were more abundant in CT-P samples, suggesting that these bacteria, especially *Lactobacillus*, might be predictive of *C. trachomatis* infection. These results were in agreement with findings that growing strict and facultative anaerobes and depleted of *Lactobacillus* in vaginal microbiota could increase the risk of *C. trachomatis* infection (Balle et al., 2018). Notably, vaginal samples from the CT-PT group showed a robust increase in *Lactobacillus* (70.0%) compared with CT-P samples (37.1%), and this was even higher than that of CT-N samples (49.2%). Furthermore, other microorganisms including *Bifidobacterium*, *Enterobacter*, *Atopobium*, and *Streptococcus* were also restored with varying degrees in the CT-PT samples. Moreover, no genome sequences from *Chlamydia* were detected in the CT-PT samples, indicating the great effectiveness of treatment of *C. trachomatis* infection with azithromycin.

Alterations of Core Microbiomes in Women With Infertility and *C. trachomatis* Infection

To verify the predictive core microbiomes of *C. trachomatis* infection beyond the vagina, a bacterium-specific qRT-PCR approach capable of normalizing the variation in the absolute DNA yields of each sample was employed. As shown in **Figure 4**, the genus *Lactobacillus* dominated the vaginal microbiota of all women, with abundances ranging from 75.2% in CT-PT samples to 48.8% in CT-P samples. The abundances of most *Lactobacillus*, including *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. reuteri*, and *L. aviaries* and *B. breve*, *Prevotella bivia*, and *A.*

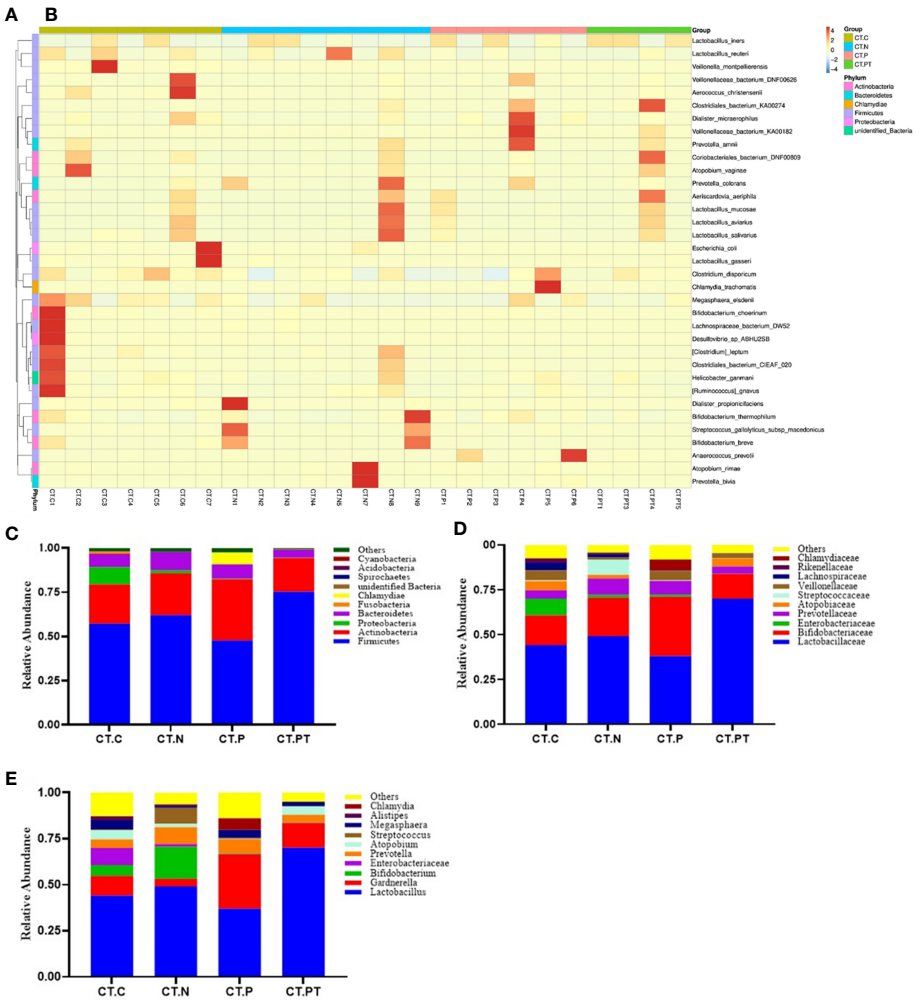

FIGURE 3 | Taxonomic composition of the vaginal microbiota. **(A)** Complete linkage clustering of sample relationships generated from Bray–Curtis dissimilarity matrix. **(B)** Heatmap of the log10-transformed proportions of the top 35 bacterial taxa, in terms of relative abundance, found in the vaginal microbiota. Stacked bar charts of top 10 taxa in terms of relative abundances at **(C)** phylum, **(D)** family, and **(E)** genus level for each group. Remaining taxa are grouped in the "Other" category. CT-C, women from the physical examination center (PEC) who were healthy and *C. trachomatis*-negative; CT-N, women who were infertile and *C. trachomatis*-negative from the assisted reproductive technology center (ART); CT-P, women who were infertile and *C. trachomatis*-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin.

vaginae in CT-P samples showed a decrease with respect to CT-C, CT-N, or CT-PT samples, whereas the proportions of *L. iners* and *Veillonellaceae bacterium* KA00182 were significantly increased in CT-P samples compared with CT-C and CT-N samples. This was in agreement with corresponding high-throughput 16S rRNA gene amplicon sequencing data (**Figure 3**).

DISCUSSION

C. trachomatis is recognized as a leading cause of infertility in women (Nsonwu-Anyanwu et al., 2015). Recent *in vivo* studies demonstrated the link between vaginal microbiota dysbiosis and *C. trachomatis* infection (van Houdt et al., 2018). The present study firstly investigated the associations between vaginal microbiota and female infertility with *C. trachomatis* infection. Participants enrolled in this study included women with tubal infertility and proven *C. trachomatis* infection pre- and post-antibiotic treatment, women with tubal infertility who were *C. trachomatis*-negative, and women who were deemed healthy and *C. trachomatis*-negative. The vaginal microbiota composition in each group of women was analyzed using next-generation sequencing of 16S rRNA gene amplicons, and the diversity and richness of vaginal microbiota from women with tubal infertility and *C. trachomatis* infection were compared with those without such infection. Data from this study highlighted notable alterations in the vaginal microbiota in women with tubal infertility and *C. trachomatis* infection. In particular, the vaginal microbiota of these women tended to show a decrease in *Lactobacillus* species dominated by *L. iners* rather than *L. crispatus*

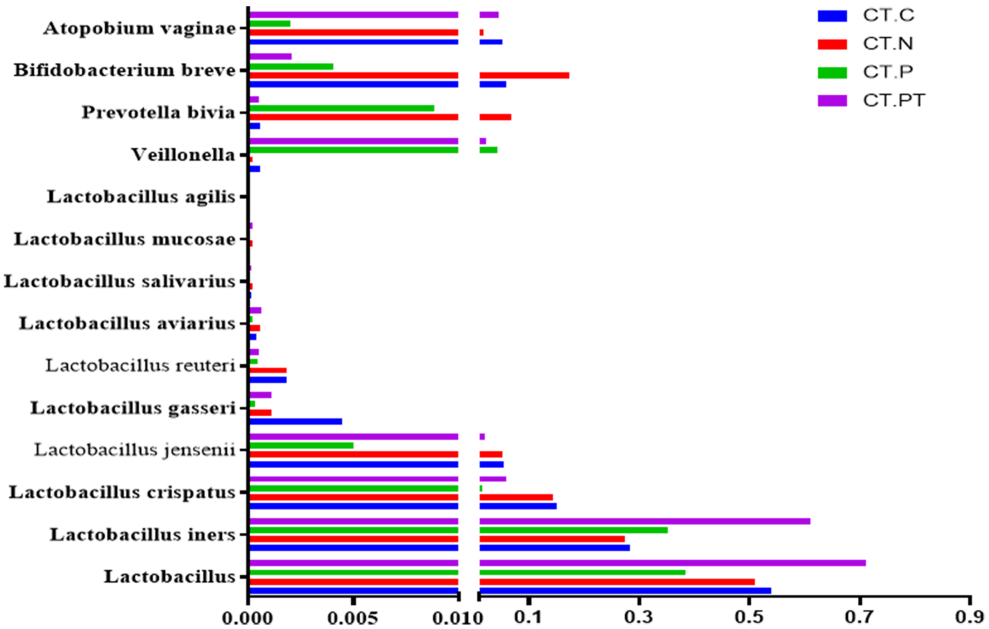

FIGURE 4 | Detection of dominant vaginal flora by specific qRT-PCR. CT-C, women from the physical examination center (PEC) who were healthy and *C. trachomatis*-negative; CT-N, women who were infertile and *C. trachomatis*-negative from the assisted reproductive technology center (ART); CT-P, women who were infertile and *C. trachomatis*-positive from the ART; CT-PT, CT-P women post-treatment with azithromycin.

compared with control samples. However, samples from women with tubal infertility and *C. trachomatis* infection treated with azithromycin showed a significant increase in *Lactobacillus* species dominated by *L. iners*.

The current study did not reveal any significant differences in phylum, class, and OTU levels between women with tubal infertility who were *C. trachomatis*-negative and healthy controls, suggesting an equivalent baseline of overall bacterial community compositions among these women. This may explain the *L. iners*-dominated vaginal microbiota in women with infertility and *C. trachomatis* infection, as well as in healthy women of reproductive age with *C. trachomatis* infection (Edwards et al., 2019). Similarly, van Houdt et al. (2018) emphasized that *L. iners*-dominated vaginal community could increase the risk for acquiring *C. trachomatis* genital infection among Dutch women. As anticipated, most vaginal microorganisms in CT-P women were also recovered to varying degrees when treated with azithromycin (Tamarelle et al., 2020). Surprisingly, *L. iners* accounted for more than 70% of the total vaginal bacteria among them, which was supposed to be at the same level as that in healthy controls (about 45%). This might be attributed to the high level of azithromycin resistance in *L. iners* (Edwards et al., 2019; Tamarelle et al., 2020). These findings support the postulation that *L. crispatus* is associated with stronger protection than *L. iners* against *C. trachomatis* infection (Caselli et al., 2020).

The vagina is an elastic, yet muscular, canal that harbors a large number of microorganisms including *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Escherichia coli*, and several anaerobes, which compose the vaginal microecosystem and maintain its self-cleaning property (Gupta et al., 2019). However, the vaginal microbiota varies greatly among individuals due to host intrinsic factors such as age, diet, ethnicity, menstrual cycle, and external factors such as geographic location and genital diseases (Mendling, 2016). This variation was also further confirmed by the within-sample diversity of vaginal microbiota found in the current study and could explain the outliers following dimension-reduction analysis.

To acquire a deeper insight into the variability in overall bacterial community compositions among CT-C, CT-N, CT-P, and CT-PT subjects, alpha diversity was applied to measure the average species diversity within a sample community and beta diversity was applied to test the divergence in community composition. A significantly different microbial diversity was found between the CT-N group and the CT-P group, as evidenced by robust decreases in Shannon and observed species indices among *C. trachomatis*-infected women, as well as a decrease in other alpha-diversity metrics (Simpson, Chao1, and ACE), although these were not significant. These observations of alpha diversity were coupled with greater beta diversity compared with healthy controls and were also consistent with the previously reported similar alpha diversity of endocervical microbiota between women who were *C. trachomatis*-positive and those who were uninfected (Balle et al., 2018).

In the current study, each group could be characterized by a unique fingerprint as evidenced by the variation presented in the relative abundance of each taxon, although the taxa composition is basically the same, in accordance with a pilot study indicating comparable taxa composition between women who were *C. trachomatis*-positive and healthy subjects (Filardo et al., 2017). For example, Lactobacilli, thought to be instrumental in host defense of the vagina and ectocervix owing to their ability to produce lactic acid, were frequently found in all female vaginal

microbiota in the current study and ranged from 70.0% to 45% in individuals (Edwards et al., 2019). The sequencing technology applied to this study could not exactly reflect all the "species" and "strains" of the microbial community (Yang et al., 2015); thus, a species-specific qRT-PCR was employed to verify the abundances of dominant vaginal bacteria Lactobacilli. The data demonstrated a reasonable concordance with amplicon sequencing findings that *L. iners* and *V. bacterium* were increased, and *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. reuteri*, *L. aviaries*, *L. salivarius*, *L. mucosae*, *L. agilis*, *P. bivia*, *B. breve*, and *A. vaginae* decreased in the vaginal microbiota of women with tubal infertility who were *C. trachomatis*-positive. These data further confirmed the evidence that *L. iners*-dominated vaginal microbiota strongly increased the risk for genital *C. trachomatis* infection (van Houdt et al., 2018). This may be due to the fact that *L. iners* is incapable of downregulating histone deacetylase 4 and does not sufficiently reduce cell proliferation to protect against *C. trachomatis* infection (Petrova et al., 2017; Edwards et al., 2019). Conversely, other species of the genus *Lactobacillus* such as *L. jensenii*, *L. crispatus*, and *L. gasseri* are capable of producing D-lactic acid, bacteriocins, and other antimicrobial compounds to protect against sexually transmitted pathogens, including *C. trachomatis*, *Neisseria gonorrhoeae*, and HPV (Brotman et al., 2014; Placzkiewicz et al., 2020). In particular, *L. crispatus* was reported to suppress the adhesion and infectivity of *C. trachomatis* in human epithelial cells (Nardini et al., 2016).

C. trachomatis is an intracellular pathogen that generally triggers a strong host T-helper 1 (Th1) cell and IFN-γ response by the release of chemokines upon infection, and in turn, this could magnify the inflammatory response by recruiting *Chlamydia*-specific immune cells (Elwell et al., 2016; Vicetti Miguel et al., 2016). In addition, *C. trachomatis* mediated production of IL-10 both *in vitro* and *in vivo* (Ohman et al., 2006). Therefore, it is not surprising that in the current study, women with tubal infertility who were *C. trachomatis*-positive had significantly higher vaginal levels of IFN-γ and IL-10 compared with those in healthy control subjects.

There were numerous strengths of the current study. Firstly, the strict inclusion criteria for population selection greatly diminished the impact of the confounding bias, allowing a clear judgment to be made. Secondly, both next-generation sequencing and species-specific qRT-PCR technology were applied to guarantee the accuracy of the relative abundance of taxa in vaginal microbiota. A third strength is that women with tubal infertility who were *C. trachomatis*-positive were enrolled pre- and post-antibiotic treatment, which facilitated a better understanding of the relationship between vaginal microbiota and *C. trachomatis* infection. However, the vaginal microbiota composition prior to *C. trachomatis* infection in these participants is not known and this is a limitation of the study. The study is also limited by the low number of participants, precluding exploration of the unique taxa directly associated with *C. trachomatis* infection.

In summary, this study provides the first demonstration that women with tubal infertility and *C. trachomatis* infection are prone to have an *L. iners*- rather than *L. crispatus*-dominated vaginal microbiota and have a decrease in *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Atopobium*, and *Streptococcus*, which could be restored with varying degrees by azithromycin treatment. Findings from the study contribute valuable information for epidemiological and fundamental research on *C. trachomatis* and further illuminate the potential of probiotics treatment for *C. trachomatis* infection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. The 16S rRNA amplicon sequencing data presented in this study are available in the GenBank sequence database, accession number PRJNA725638. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Chenzhou No. 1 People's Hospital (no. CZ/1128). This study was performed in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Conceptualization: HC, LW, LZ and ZL. Methodology: LW, LL, SM and LZ. Software: HC and LW. investigation: HC, LZ and SM. Data curation: LW, LL, SM, YW, WL, and MS. Writing—original draft preparation: HC and LW. Writing—review and editing: ZL. Funding acquisition: HC and ZL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.698840/full#supplementary-material

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Characterization of Vaginal Microbiota in Women With Recurrent Spontaneous Abortion That Can Be Modified by Drug Treatment

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Zhao F, Chen Y, Gao J, Wu M, Li C, Wang Z, Huang N, Cui L, Du M and Ying C (2021) Characterization of Vaginal Microbiota in Women With Recurrent Spontaneous Abortion That Can Be Modified by Drug Treatment. Front. Cell. Infect. Microbiol. 11:680643. doi: 10.3389/fcimb.2021.680643

Objective: The role of vaginal microbiota in recurrent spontaneous abortion (RSA) remains unknown. The purpose of this study was to investigate characteristics of vaginal microbiota and the effects of drug treatment on vaginal microbiota of patients with RSA.

Methods: A case-control study was performed, in which non-pregnant patients who experienced RSA were selected and divided into untreated and drug-treated groups. Drug-treated patients were subdivided into the metformin group, metformin plus aspirin group, and other drugs group. Healthy women who had live births and never experienced spontaneous abortion were enrolled in the control group. Characteristics of vaginal microbiomes of patients with RSA and healthy women and the impact of drug treatment on the microbiome was evaluated *via* 16S rRNA gene sequencing of the V3-V4 region using the Illumina MiSeq platform.

Results: Women who underwent RSA had lower microbial richness than healthy women. Compared to controls, the relative abundance of seven taxa (*Megasphaera*, *Sneathia sanguinegens, Pseudomonas*, *Sphingomonas, Rhodococcus, Burkholderia-Caballeronia-Paraburkholderia*, and *Corynebacterium_1*) in the patient's vaginal microbiota changed significantly, which may be closely related to RSA. The composition of the vaginal microbial community in RSA patients was altered by drug treatment. Metformin combined with aspirin treatment significantly increased the relative abundance of vaginal *Lactobacillus* spp. in patients.

Conclusion: An altered vaginal microbiome composition might be associated with RSA, which could be modified by drug treatment. The effect of metformin combined with aspirin on vaginal *Lactobacillus* is worthy of attention.

Keywords: recurrent spontaneous abortion, RSA, vaginal microbiome, aspirin, metformin, 16S rRNA gene

INTRODUCTION

The American Society for Reproductive Medicine and the European Society of Human Reproduction and Embryology (ESHRE) defines recurrent spontaneous abortion (RSA) as two or more failed pregnancies. RSA affects approximately 1%–5% of couples trying to conceive (Practice Committee of the American Society for Reproductive Medicine, 2012; ESHRE Guideline Group on RPL et al., 2018). Existing research shows that the etiology of RSA includes genetic, anatomic, infective, thrombophilic, endocrine, and immune factors (Evaluation and treatment of recurrent pregnancy loss: a committee opinion, 2012; Rai and Regan, 2006; ESHRE Guideline Group on RPL et al., 2018; Khalife et al., 2019). Unfortunately, 50% patients do not have any of the above conditions and are hence considered idiopathic (Khalife et al., 2019; Green and O'Donoghue, 2019). This adds to patients' financial and psychological burdens and also limits effective interventions and therapeutics. To date, international groups have not reached a consensus on the standard evaluation of RSA (Khalife et al., 2019). Various therapeutic strategies have been evaluated to decrease the rate of pregnancy loss. Low-dose aspirin, low-dose steroids, heparin, levothyroxine, progesterone, intralipid, and metformin are generally chosen to treat women with RSA through single or combined use (Rai and Regan, 2006; Vesce et al., 2014; Khalife et al., 2019; Green and O'Donoghue, 2019; Ou and Yu, 2019). The reported effects of the above-mentioned drug treatments are inconsistent. In-depth research on the etiology of RSA to determine the true causes will ensure a unified and standardized treatment in the future, thereby avoiding unreasonable treatment and reducing the financial and psychological burden of families with RSA.

With the development of high-throughput sequencing technology, an increasing number of microbiota inhabiting different parts of the human body have been associated with various diseases, which has expanded our understanding of the underlying mechanisms involved in these conditions. Although most of these studies focused on the gastrointestinal microbiome, the role of the vaginal microbiome for female reproductive health has attracted the attention of researchers over time (Chow and Mazmanian, 2010; Nikoopour and Singh, 2014; Younes et al., 2018). Two scientific conferences were held in Amsterdam to discuss the topic of women and vaginal microbiota in 2015 and 2016 to emphasize the particularity of the female microbiome and summarize the current research (Younes et al., 2018). Studies showed that the vaginal microbiota of healthy women are mainly colonized by *Lactobacillus* spp. including *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* (Aroutcheva et al., 2001). Notably, some healthy women do not have *Lactobacillus* dominance, especially South African black women. The causative factors leading to ethnic and geographic differences in vaginal bacteria remain unclear (Anahtar et al., 2018). Ravel et al. analyzed the vaginal microbiota of women of childbearing age in North America and proposed five vaginal community structure types (CSTs) that have been commonly adopted in vaginal microbiome studies. The vaginal microbiota is affected by some physiological factors such as menarche, menstrual cycle, pregnancy, menopause, and other hormonal changes (Achilles et al., 2018; Greenbaum et al., 2019). Hickey et al. suggested that vaginal pH in healthy premenarche girls after menarche was usually higher than the level in healthy adult women, even with high proportions of *Lactobacillus* (Hickey et al., 2015). The overall diversity of the vaginal microbiome during pregnancy decreases while the stability increases. In addition, the abundance of the dominant *Lactobacillus* in the vagina increases significantly during pregnancy, which reduces the vaginal pH and strengthens the vagina's ability to resist pathogenic microorganisms. After delivery, the microbiota revert to be similar to those in non-pregnant women (Neuman and Koren, 2017). Vaginal microbiota composition is also affected by exogenous factors including hygienic practices, contraceptive method, sexual behavior, stress, diet, exercise, drugs, and rectal colonization (Muzny and Schwebke, 2016; Abdelmaksoud et al., 2017; Achilles et al., 2018; Turpin et al., 2019; Song et al., 2020). According to existing literature reports, imbalanced vaginal microflora is related to the following diseases, including but not limited to, bacterial vaginosis, sexually transmitted infections, preterm birth (PTB), gynecological cancers, preterm pre-labor rupture of the fetal membranes, recurrent implantation failure, and polycystic ovary syndrome (PCOS) (Brotman, 2011; Muzny and Schwebke, 2016; Champer et al., 2017; Freitas et al., 2018; Brown et al., 2018; Elovitz et al., 2019; Fu et al., 2020; Hong et al., 2020). The relationship between PTB and the vaginal microbiome was recently described in several studies, with few reports on the vaginal microbiome of RSA (Zhang et al., 2019).

In the past, based on traditional serology and culture methods, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Listeria monocytogenes*, *Gardnerella vaginalis*, and other less frequent pathogens were identified more often in patients experiencing spontaneous abortion (Penta et al., 2003; Kuon et al., 2017). However, Contini et al. proposed that *Mycoplasma hominis* and *Ureaplasma urealyticum* may not cause embryo loss after analyzing bacterial DNA in the aborted tissues of women with early pregnancy loss and women underwent voluntary interruption of pregnancy *via* quantitative real-time PCR methods (Contini et al., 2018). With the development of technology, we will have a better understanding of the pathogenesis of abortion. Recent application of 16S rRNA gene-based metataxonomics for exploring the association between vaginal bacterial composition and abortion suggested that vaginal bacterial composition of abortion in the first trimester was related to decreased *Lactobacillus spp.* (Al-Memar et al., 2019). However, the authors could not conclusively show when the loss of *Lactobacillus* spp. occurs. In addition, a recent study has found that *Atopobium*, *Streptococcus*, and *Prevotella* were significantly more abundant in patients with unexplained recurrent miscarriage, while two (*Lactobacillus* and *Gardnerella*) taxa were overrepresented in controls (Zhang et al., 2019). As this study was limited by a small sample size, a definitive conclusion could not be drawn.

In our study, 108 patients with RSA were enrolled to characterize the vaginal microbiota composition in non-pregnant patients with RSA and assess the effects of drugs on bacterial composition using 16S rRNA gene-based metataxonomics.

MATERIALS AND METHODS

Subject Recruitment and Ethical Approval

Non-pregnant patients in the Department of Reproductive Immunity, Obstetrics, and Gynecology Hospital of Fudan University who had experienced RSA were selected and divided into untreated and drug-treated groups. Drug-treated patients were subdivided into the metformin group, metformin plus aspirin group, and other drugs group. Healthy women who had live births and never experienced spontaneous abortion were enrolled in the control group. The exclusion criteria were as follows: women who were menstruating or had sexual intercourse in the last 72 h. Participants on any antibiotic treatment or who underwent vaginal lavage in the 2 weeks prior to swab collection were also excluded. All participants provided written informed consent and gave permission for collection of their vaginal specimens and related clinical information. The study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University.

Sample Collection and Physiological and Biochemical Analyses

Vaginal specimens were collected from each participant from the lateral wall of the vagina using a sterile swab. One sample was used for physiological and biochemical testing immediately including hydrogen peroxide, pH, leukocyte esterase, and sialidase activity according to manufacturer's instructions (Bioperfectus Technologies, Jiangsu, China). Other swabs were placed immediately in an ice box and stored at -80°C until further analysis. Women enrolled in this study were asked to fill out a questionnaire to collect additional demographic and medical information (age, body mass index [BMI], drug use, gynecological and obstetric history, exercise intensity, smoking, hygiene habits, and mental health conditions).

DNA Extraction and 16S rRNA Sequencing

Total nucleic acid extracted from each vaginal swab was performed using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Ohio, USA) according to manufacturer's instructions. DNA purity and concentration of each sample were measured with the NanoDrop2000 (Thermo Fisher Scientific, Wilmington, USA). The quality of extracted DNA was determined by 1% agarose gel electrophoresis. The V3/V4 regions of the 16S rRNA gene were amplified by the PCR system with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') to further verify the quality of the sample DNA. The PCR system was performed in triplicate with 20 μL mixtures containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM deoxynucleoside triphosphates, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, 10 ng of template DNA, and double-distilled water to make up the total volume. PCR reactions were performed on ABI GeneAmp® 9700 (Thermo Fisher, Waltham, MA, USA) using the following cycling parameters: 95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. The amplicons were extracted from a 2% agarose gel purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified by QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's instructions. Equimolar quantities of purified amplicons were pooled and paired-end sequenced (2 × 300) on the Illumina MiSeq PE300 (Illumina, San Diego, CA, USA) according to the manufacturer's specifications for MiSeq Reagent Kit v3.

Bioinformatics and Microbiota Analysis

Statistical analyses were performed using 'R' language and other packages. Raw read-pairs were merged with FLASH and quality filtered by Trimmomatic (Illumina). Sequence alignment and classification was performed using the Silva bacterial database (www.arb-silva.de/) and the RDP (Ribosomal Database Project) database reference sequence files (http://rdp.cme.msu.edu). The taxonomy of operational taxonomic units (OTUs) clustering at a 97% sequence identity threshold was analyzed by the RDP Classifier algorithm (https://sourceforge.net/projects/rdp-classifier/, version 2.11) at each classification level (kingdom, phylum, class, order, family, genus, species). The vaginal CSTs of each sample was categorized as follows. CSTI is dominated by *L.crispatus*, CSTII by *L. gasseri*, CSTIII by *L. iners*, and CSTV by *L.jensenii.* CSTIV had a diverse set of facultative and strict anaerobes.

Alpha-diversity was inferred by the Chao1 estimator and Shannon–Wiener index. Differences in community richness and diversity between sample groups were determined with nonparametric one-sided Wilcoxon rank-sum tests. PCoA was used to estimate differences in beta diversity. Differentiation of the overall microbial community structure of each group was assessed with nonparametric multivariate analyses of variance (MANOVA). The nonparametric factorial Kruskal-Wallis rank-sum test was used to identify taxa showing differentially abundant features between two groups. Linear discriminant analysis (LDA) was used to estimate the contribution of differentially abundant taxa to group differentiation with the LEfSe software. Fisher's exact test was used to analyze whether there was a significant difference in the frequency of five CSTs between each group. Statistical analyses were performed using SPSSv.17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical Characteristics of the Participants and Sequencing Results

A total of 120 patients diagnosed with recurrent miscarriage and 20 healthy women were recruited and assigned to the case and control groups, respectively. We successfully analyzed 126 specimens with 16S rRNA gene sequencing, including 108 samples from the RSA group and 18 from the control group. The clinical and demographic information of participants are

shown in **Table 1**. All participants in the control and case were non-smokers and non-vegetarians. Of the enrolled 108 cases, 65 (60.18%) were newly diagnosed patients not taking medication and 43 (39.82%) were under treatment including metformin (n = 9), metformin combined with aspirin (n = 9), and other drugs (n=25). Other than age and leukocyte esterase (LE) activity (patients vs. controls, $p < 0.05$), there were no significant differences in other parameters including BMI, hygiene, exercise intensity, self-rating depression scale (SDS), self-rating anxiety scale (SAS), H_2O_2, sialidase activity, and pH (all $p > 0.05$). The MANOVA was used to evaluate the influence of age on differences between groups, we did not detect significant differences ($p = 0.396$).

After preprocessing, 5,758,981 reads were obtained from all samples, with an average read count of 45,706 reads per sample (range: 30,182–92,508). After clustering, the Shannon–Wiener curve of each sample was nearly a straight horizontal line, which demonstrated that the sequencing depth of each sample was sufficient (**Supplementary Figure S1**).

The Diversity of Vaginal Microbiomes in RSA and Response to Drug Treatment

Individual comparison showed that community richness decreased in the no-medication and drug treatment groups compared to the control group ($p < 0.05$); meanwhile, there was no significant difference in richness between the drug-treated and no-medication groups ($p = 0.67$), indicating reduced richness in the cases (**Figure 1A**). Community diversity was calculated by the Shannon–Wiener index (0.49 ± 0.54 for the no-medication group, 0.36 ± 0.39 for the drug-treated group, and 0.56 ± 0.46 for the control group) and showed significant differences in the drug-treated and control groups ($p = 0.01$) but not between the no-medication and control groups ($p = 0.24$) (**Figure 1B**). The Shannon–Wiener index values showed similar microbial diversity in the no-drug treatment cases and controls. However, the diversity of vaginal microflora decreased after drug treatment. These results revealed a decrease of RSA community richness and a similar diversity with that of

TABLE 1 | Clinical and demographic characteristics of the study population.

	NM group	DT group	P value (NM *vs.* DT)	Control group	P value (NM *vs.* Control)	P value (DT *vs.* Control)
Total number	65	43		18		
Age (years, mean ± SD)	31.5 ± 4.2	30.2 ± 3.6		36.6 ± 3.6		
≤29	25(38.5)	20(46.5)	0.19	1(5.6)	0.00	0.00
30–35	28(43.1)	20(46.5)		4(22.2)		
≥36	12(18.4)	3(7)		13(72.2)		
BMI (kg/m^2 mean ± SD)	22.4 ± 3.2	22.8 ± 3.4		22.5 ± 3.8		
Underweight (<18.5)	4(6.1)	2(4.7)	0.60	2(11.1)	0.86	0.62
Normal weight (18.5–24)	44(67.7)	28(65.1)		11(61.1)		
Overweight (>24)	17(26.2)	13(30.2)		5(27.8)		
Hygiene (frequency of washing the vulva)						
Once a day	54(83.1)	38(88.4)	0.45	14(77.8)	0.60	0.29
Clean once every 2-3 days	11(16.9)	5(11.6)		4(22.2)		
Exercise intensity						
Low	56(86.2)	36(83.7)	0.72	15(83.3)	0.76	0.97
Medium	9(13.8)	7(16.3)		3(6.7)		
SDS						
Positive	14(21.5)	7(16.3)	0.51	4(2.2)	0.95	0.58
Negative	51(78.5)	36(83.7)		14(7.8)		
SAS						
Positive	5(7.7)	2(4.7)	0.53	3(6.7)	0.25	0.12
Negative	60(92.3)	41(95.3)		15(3.3)		
RSA						
2	36(55.4)	24(55.8)	0.78			
3	21(32.3)	16(37.2)				
≥4	8(12.3)	3(7)				
H_2O_2						
Positive	64(98.5)	42(97.7)	1	18(100)	1	1
Negative	1(1.5)	1(2.3)		0(0)		
pH						
>4.5	10(15.4)	6(14.0)	1	2	1	1
≤ 4.5	55(84.6)	37(86.0)		16		
Sialidase activity						
Positive	0(0)	0(0)		0(0)		
Negative	65(100)	43(100)		18(100)		
Leukocyte esterase activity						
Positive	1(1.5)	2(4.7)	0.656	0(0)	0.001	0.001
Weakly positive	29(44.6)	20(46.5)		1(5.6)		
Negative	35(53.9)	21(48.8)		17(94.4)		

NM, no medication; DT, drug treatment; BMI, body mass index; SDS, self-rating depression scale; SAS, self-rating anxiety scale; RSA, recurrent spontaneous abortion.

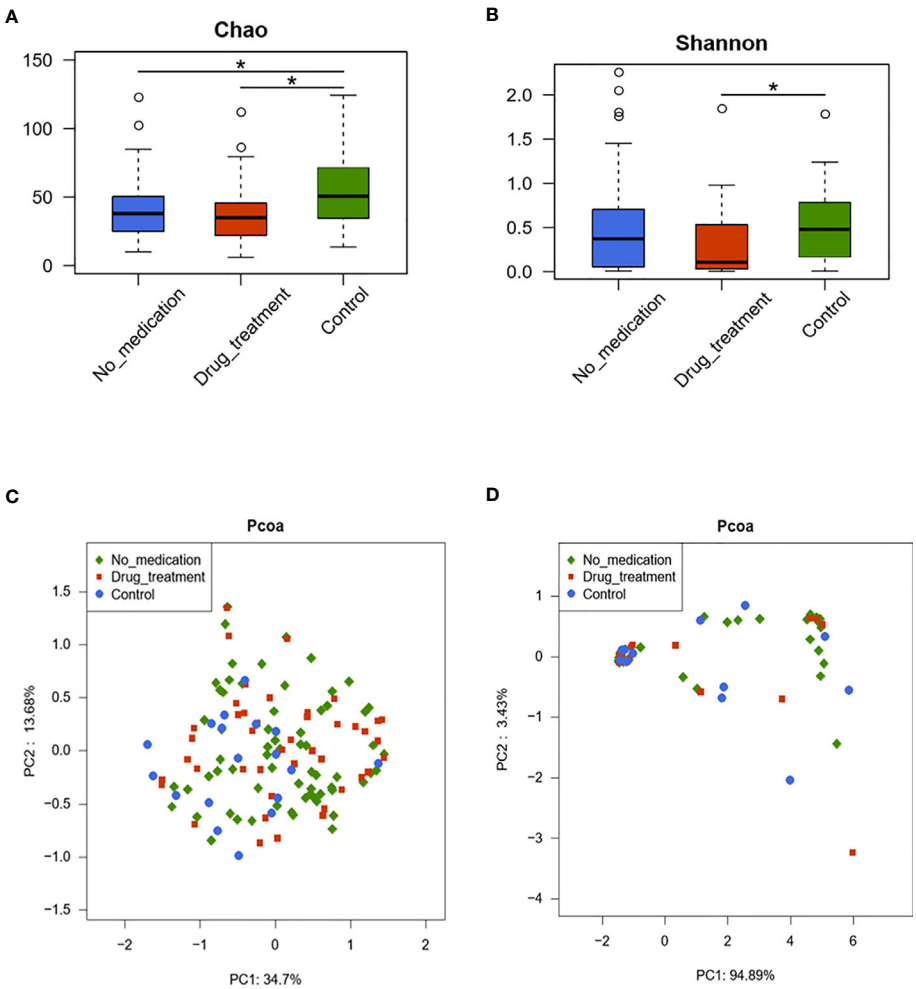

FIGURE 1 | The diversities of vaginal microbiota in the three groups are shown by the Chao index **(A)**, Shannon–Wiener index **(B)**, and principal coordinate analysis (PCoA) plot created based on the unweighted **(C)** and weighted **(D)** UniFrac distances. The values show the percentages of total community variation explained. **(A, B)** *p*-values were calculated using the one-sided Wilcoxon rank-sum tests. *$p < 0.05$.

healthy women, but this diversity would be reduced after drug treatment.

Two-dimensional principal coordinate analysis (PCoA) based on unweighted and weighted UniFrac distances was applied to illustrate characteristics of beta diversity between the case and control groups. **Figure 1C** shows the unweighted PCoA plot, which revealed clustering of the no-medication and drug-treated groups away from the control group. However, overall microbiota profiles clustered together based on analyzing with weighted UniFrac PCoA (**Figure 1D**). These findings were statistically confirmed with MANOVA (**Table 2**). These results suggested that RSA did alter the vaginal microbiome constituents.

TABLE 2 | Results from nonparametric MANOVA analysis based on UniFrac distance.

Comparison	*p*-value (UniFrac distance)	
	Unweighted	Weighted
NM *vs.* Control	0.012	0.498
DT *vs.* Control	0.021	0.287
NM *vs.* DT	0.487	0.174
Metformin *vs.* Control	0.534	0.518
Aspirin plus Metformin *vs.* Control	0.078	0.270
Other medication *vs.* Control	0.033	0.467
Aspirin plus Metformin *vs.* Other medication	0.543	0.295
Aspirin plus Metformin *vs.* Metformin	0.540	0.540
Metformin *vs.* Other medication	0.349	0.866

NM, no medication; DT, drug treatment.

Comparison of Vaginal Microbial Relative Abundance in Samples

At the genus level, differences in the abundance of vaginal microflora between cases and controls were evaluated by one-sided Wilcoxon rank-sum tests. Overall, at the genus level, the top eight dominant taxa were *Lactobacillus*, *Gardnerella*, *Prevotella*, *Streptococcus*, *Atopobium*, *Bifidobacterium*, *Escherichia/Shigella*, and *Pseudomonas* (**Figure 2**). In the no-

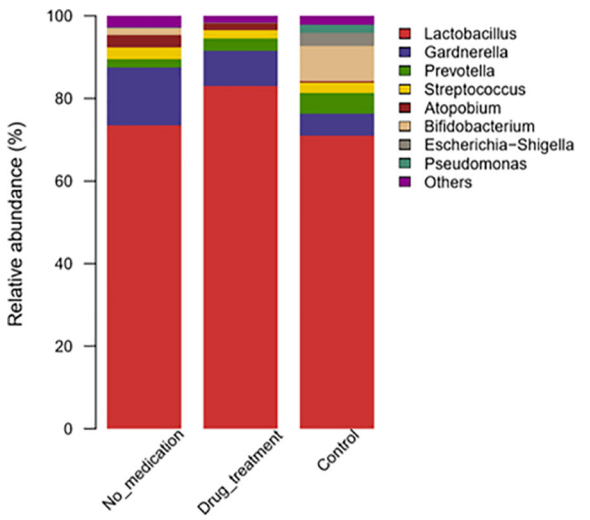

FIGURE 2 | Taxonomic classification of the vaginal microbiota at the genus level among different groups.

medication group, *Lactobacillus* and *Gardnerella* were found in 73.52% and 14.01% subjects, respectively, and in the control group in 70.89% and 5.48% subjects, respectively. In patients receiving medication, the *Lactobacillus* genus constituted 82.92% of total bacteria and *Gardnerella*, 8.64%. *Prevotella* was present in low percentages in the no-medication (2.03%) and drug-treated groups (2.88%) compared to the controls (4.87%). In general, there was no significant difference in these top eight genera among groups.

Megasphaera and *Sneathia* were more abundant in the no-medication group than the control group (p = 0.013 and p = 0.051, respectively) (**Supplementary Table S1**). The relative abundances of *Lactobacillus* and *Sneathia* were high in the medication group compared to the control group (p = 0.051 and p = 0.071, respectively). After the subdivision of *Sneathia* species, it was found that the statistical difference between *Sneathia* in RSA group and the control group was caused by *Sneathia sanguinegens* (**Supplementary Table S2**).

The relative abundances of 14 genera were significantly reduced in the no-medication group, and that of 23 genera were decreased in the medication group compared to controls (**Supplementary Tables S1, S3**). When the no-medication and medication groups were compared, nine genera with significantly different abundances were detected, of which eight were reduced in the medication group (**Supplementary Table S4**). LDA was used to identify the contribution of differentially abundant taxa to group differentiation. Five genera including *Corynebacterium_1*, *Rhodococcus*, *Burkholderia_Caballeronia_Paraburkholderia*, *Pseudomonas*, and *Sphingomonas* were significantly more abundant at the genus level in controls than in the no-medication group (**Figure 3A**). It is worth noting that after drug treatment, the relative abundance of *Lactobacillus* in patients was significantly higher than that in healthy women. In addition, when the patients were treated with medication, the relative abundance of *Pseudomonas* was the same as in controls (**Figures 3A, B**). These results indicated that *Megasphaera, Sneathia sanguinegens, Corynebacterium_1, Rhodococcus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Pseudomonas*, and *Sphingomonas* were strongly associated with RSA. This situation was altered to a certain extent after drug treatment.

The Effect of Metformin Monotherapy or Combined With Aspirin on Vaginal Microbiota

Bacterial richness and diversity (**Figures 4A, B**) remained unchanged when patients were treated with metformin. When patients received aspirin combined with metformin, the diversity of the microbiota was decreased (**Figure 4B**). The microbiota profiles of the metformin and control groups clustered together after weighted and unweighted UniFrac analyses (**Figures 4C, D**). These results were confirmed using the LEfSe (Linear Discriminant Analysis Effect Size) algorithm (**Figure 4E**). PCoA plots indicated that clustering of the metformin combined with aspirin group and control group could be separated to a certain degree by unweighted UniFrac analysis (p = 0.078) (**Figure 4C**). At the genus level, seven genera (*Lactobacillus*, *Aeromonas*, *Megasphaera*, *Bacteria_unclassified*, *Streptococcus*, *Sphingomonas, and Corynebacterium*) with significantly different relative abundances were detected in the metformin combined with aspirin group and control group (**Supplementary Table S5**). According to the LEfSe results, *Lactobacillus*, *Corynebacterium_1*, *Streptococcus*, and *Sphingomonas* played a key role regarding the differences between the two groups (**Figure 4F**). These findings showed that the composition of the patient's vaginal microbiota tended to approach the vaginal microbial composition of the controls after metformin and aspirin exposure, and the abundances of *Lactobacillus* spp. were significantly increased after treatment with metformin plus aspirin.

Characteristics of Vaginal Microbiome Community Structure Types (CSTs)

Vaginal microbiota of 126 samples were identified as five vaginal CSTs (**Figure 5**). Overall, frequencies of CSTs in all samples were as follows: CST I, 30.95% (39/126), CST II, 4.76% (6/126), CST III, 38.89% (49/126), CST IV, 23.02%(29/126), and CSTV, 3/126 (2.38%). CST I was present in 32.31% (21/65) of the no-medication (NM) group, 39.53% (17/43) in the drug-treatment (DT) group and 5.56% in control group. CST I was present in 32.31% (21/65) in the NM group, 39.53% (17/43) in the DT group, and 5.56% in control group. The CST II cluster included 4.62%(3/65) of NM patients, 2.33% (1/43) of DT patients, and only 11.11% (2/18) of the heathy controls. CST III accounted for 35.38% (23/65) of the NM group, 41.86% (18/43) in the DT group, and 44.44% (8/18) in the control group. CST IV included 26.15% (17/65) of the NM group, 16.28% (7/43) in the DT group, and 27.78%(5/18) in the control group. Only one case of CSTV was found in the NM group, none in DT group, and two cases in control group. In the NM group, only one case of CSTV was found, accounting for 1.53% (1/63); this type was not found in

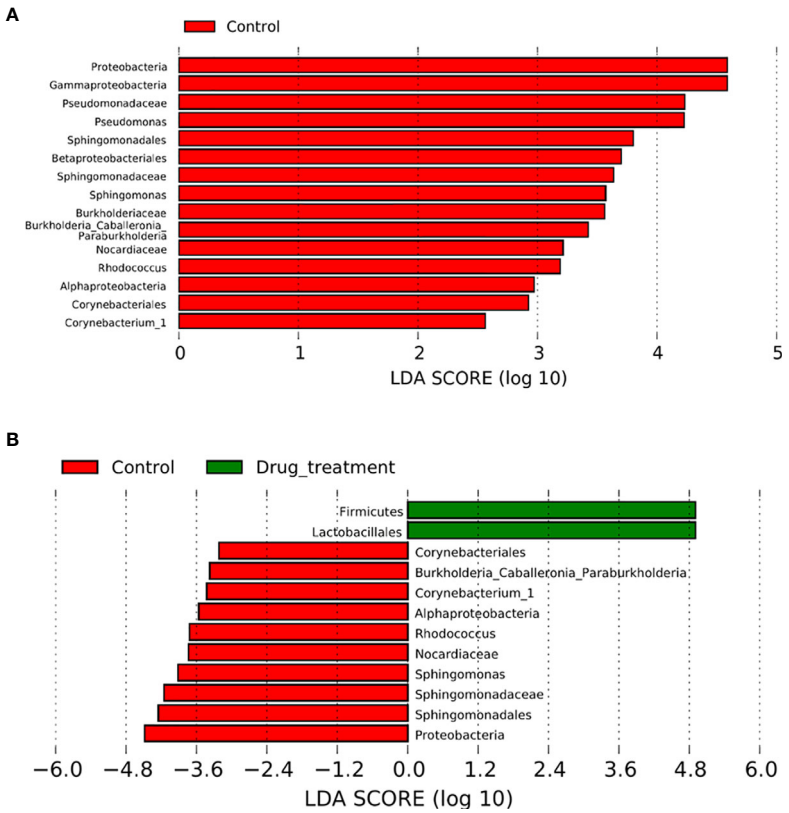

FIGURE 3 | Comparison between patients with recurrent spontaneous abortion (RSA) and controls. The effect size for each differentially abundant taxon was computed using linear discriminant analysis (LDA), which indicated its contribution to group differentiation. OTUs are presented in red and green when the taxa were significantly more abundant in the controls and RSA, respectively. **(A)** Differentially abundant vaginal taxa detected in patients not taking any medicine and controls. **(B)** Differentially abundant taxa detected in patients who did take medicine and controls.

the DT group. Two cases were found in the controls, accounting for 11.11% (2/18). There was no statistical difference in the distribution of CST between the NM group and the NT group. Comparing the CST distribution of the control group with the NM and DT groups respectively, there was significant statistical significance (p = 0.042 and p = 0.006, respectively), with a statistically significant increase of CST I in NM and DT patients compared to the control group (5.56% *vs.* 32.31%, $p < 0.05$; 5.56% *vs.* 39.53%, $p < 0.05$).

DISCUSSION

The pathogenesis of RSA is complex, and at least half of the cases do not seem to have any concrete underlying cause. The use of next-generation sequencing technology to determine the characteristics of vaginal microbes in patients with RSA may provide new clues for exploring the etiology. A comprehensive understanding of the characteristics of vaginal microbiota in RSA and its response to drug treatment is essential to improve diagnosis and treatment strategies. Our study showed that patients with RSA have decreased richness of the vaginal microbiome, along with unchanged dominant *Lactobacillus* spp. and increased abundances of *Megasphaera* and *Sneathia sanguinegens*. Additionally, the vaginal microecology composition in the patients was different from that of healthy women. The difference was attenuated to a certain extent after drug treatment. Treatment with metformin combined with aspirin might significantly increase the abundance of vaginal *Lactobacillus* spp. in patients.

According to previous reports, factors including age, BMI, hygiene habits, diet, exercise intensity, smoking, and perceived stress can affect the vaginal microbiome (ESHRE Guideline Group on RPL et al., 2018; He et al., 2019; Khalife et al., 2019; Turpin et al., 2019; Song et al., 2020). In this study, all factors except for age were similar among the experimental groups (**Table 1**). This is likely because healthy women in the control group had successfully conceived twice and were older than those in the case group. Based on the results of statistical analysis, the difference in age between the experimental groups has no effect on the comparison of differences in microbiota between groups.

As the dominant organism of the vaginal community, *Lactobacilli* play a key role in maintaining vaginal health.

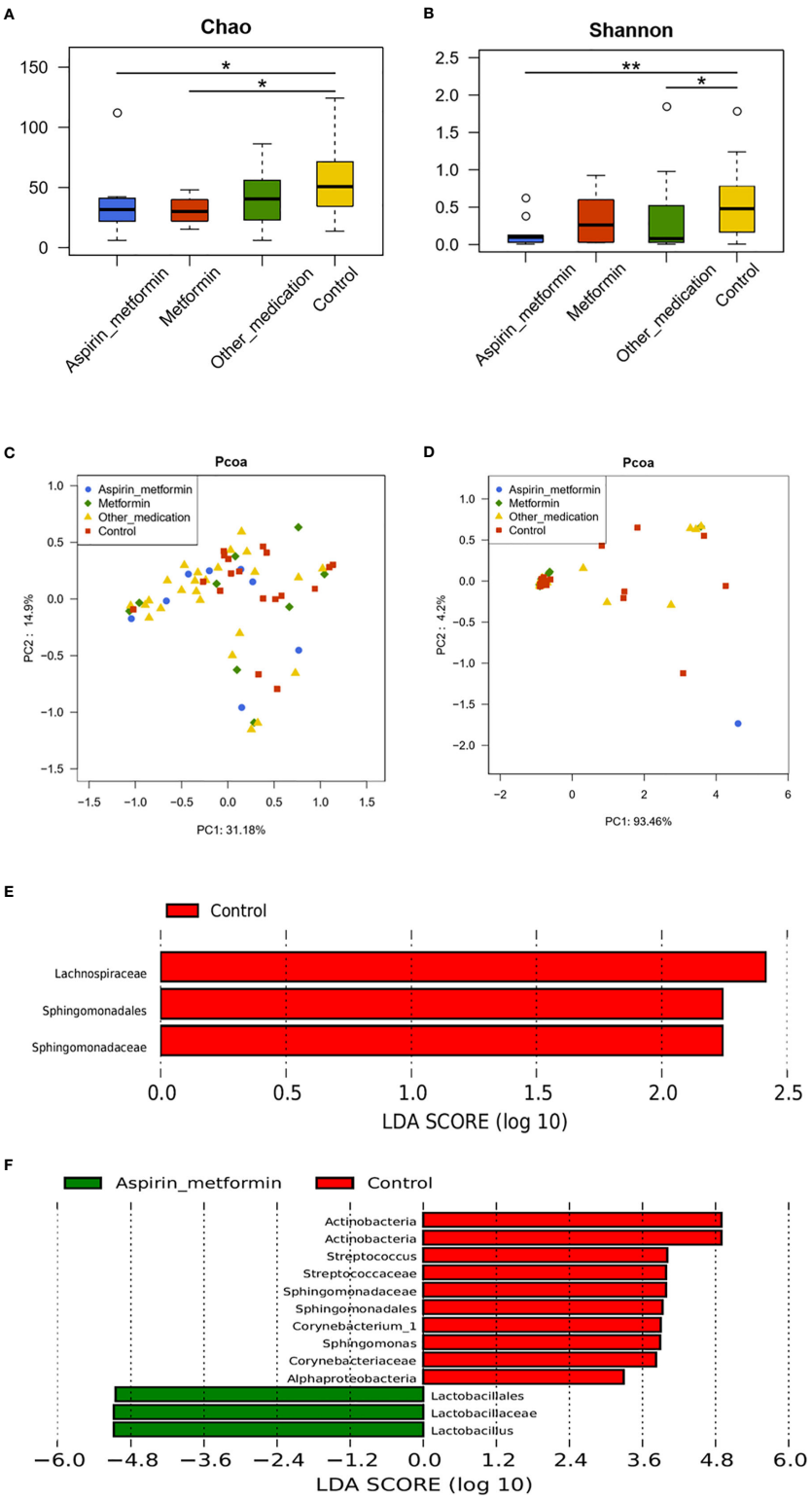

FIGURE 4 | The effect of different medications on vaginal microbiota. The diversity of the vaginal microbiota in each group is shown by the Chao index **(A)**, Shannon–Wiener index **(B)**, and principal coordinate analysis (PCoA) plot created based on the unweighted **(C)** and weighted **(D)** UniFrac distances. **(E)** Differentially abundant vaginal taxa detected using LDA in samples taken from the metformin and control groups. **(F)** Differentially abundant taxa detected in patients who received metformin combined with aspirin and controls. **(A, B)** P values were calculated using the one-sided Wilcoxon rank-sum tests. $^{*}p < 0.05$ and $^{**}p < 0.01$.

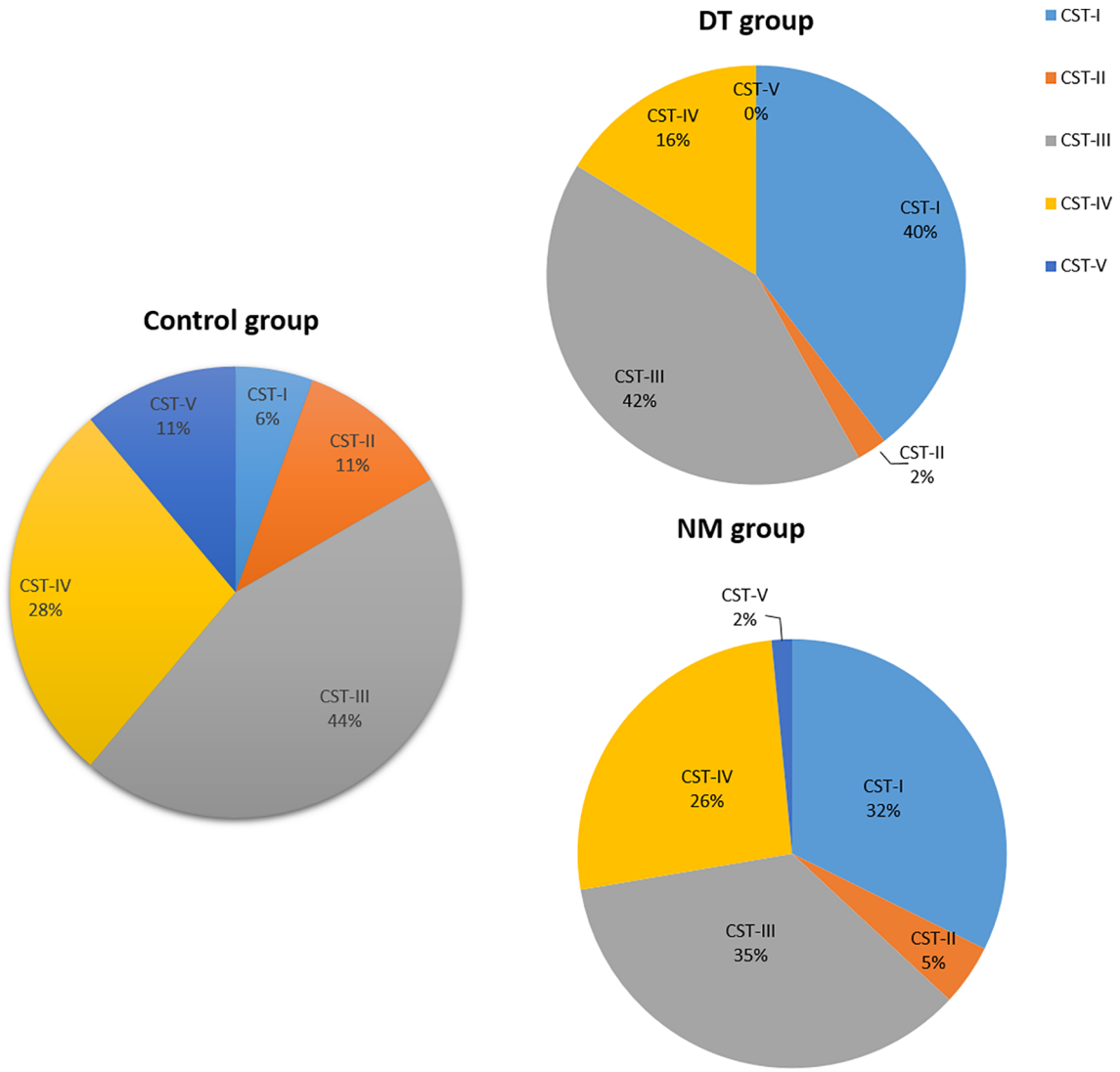

FIGURE 5 | The proportion of the Community State Types (CSTs) in each group; NM group (*n* = 65), DT group (*n* = 43), control group (*n* =18).

Lactobacillus species inhibit the growth of other microorganisms by producing lactic acid through metabolizing extracellular glycogen to lower the pH of the vaginal environment, producing bacteriocins and hydrogen peroxide (H_2O_2), and competing for nutrients and space, thereby protecting the vaginal ecosystem from harmful microbial communities. (Aroutcheva et al., 2001; Caselli et al., 2020). Therefore, vaginal pH (greater than 4.5) is one of the important indicators for the diagnosis of bacterial vaginosis in clinical practice (Verstraelen and Verhelst, 2009). H_2O_2 is generally considered to prevent bacteria associated with bacterial vaginosis (Eschenbach et al., 1989; Beigi et al., 2005). However, the ability of different types of *Lactobacillus* to produce organic acids, bacteriocins, and H_2O_2 is not completely the same (Eschenbach et al., 1989; Aroutcheva et al., 2001). Herein, there was no statistical difference in the measurement of vaginal pH and H_2O_2 in all participants between each group. This might be due to the similar distribution of vaginal *Lactobacillus* species, which can produce organic acids and H_2O_2 in each group. Sialidases are enzymes widely present in bacteria, fungi, viruses, mycoplasma, and animals that catalyze the removal of sialic acid from various glycoconjugates (Taylor, 1996). Many of these pathogens use sialidase to assist their pathogenesis and/or nutritional requirements. The detection of vaginal sialidase activity has also been used in the auxiliary diagnosis of bacterial vaginosis (Myziuk et al., 2003; Paladine and Desai, 2018). In the present study, vaginal sialidase activity was negative in all subjects. LE, an indicator of inflammatory cells present in tissues or samples, is used to evaluate urogenital tract infections and periprosthetic joint infection (Abbasi et al., 1985; Mårdh et al., 2003; Lee et al., 2017). It was found that LE activity of RSA group was higher than that of the control group, and the difference was statistically significant. No significant

difference was found between no-medication group and drug treatment group in the test of LE activity. LE tests were weakly positive in 46.2% of patients with RSA. In the control group, there was only one positive case of LE test, accounting for 5.5%, and no weak positive case was found. This finding suggested that nearly half of the patients in case group may have mild inflammation in the vagina. The reason of this mild inflammation might have to do with changes in their vaginal microbiome composition.

Recent studies have found that the diversity and richness of vaginal microbiota in women who miscarried in the first and second trimesters were significantly higher than those of normal controls (Al-Memar et al., 2019). Our results showed that compared with healthy women, non-pregnant patients with RSA have reduced vaginal microbiome richness and unchanged vaginal flora diversity. In contrast, Zhang et al. showed that there was no significant difference in flora richness and diversity compared with the control group (Zhang et al., 2019). This could be attributed to the significant difference in the number of patients in the two articles, and the fact that their study focused on 10 patients with unexplained miscarriage. Flora diversity was decreased when patients received aspirin combined with metformin treatment or other medications including immunomodulators, low-dose steroids, and/or Chinese medicine, except metformin.

At the genus level, predominant microbiota, including *Lactobacillus*, *Gardnerella*, *Prevotella*, *Streptococcus*, *Atopobium*, *Bifidobacterium*, *Escherichia/Shigella*, and *Pseudomonas* were not significantly different between the no-medication patient and control groups. However, Zhang et al. reported that *Atopobium*, *Prevotella*, and *Streptococcus* were more abundant in 10 patients and *Lactobacillus* and *Gardnerella* were overrepresented in 10 controls (Zhang et al., 2019). Kuon et al. indicated that patients with RSA had more colonization by *Gardnerella vaginalis* and Gram-negative anaerobes including *Prevotella*, *Bacteroides*, and *Veillonella* species, based on culture-dependent methods (Kuon et al., 2017). We speculate that the reason for the inconsistent conclusions of these two studies is because of differences in sample size and research methods.

According to the prevalence of *Lactobacillus* and other bacteria in the vaginal flora, CSTs are divided into five types. Among these five CSTs, four of them are predominated by species of *Lactobacillus* (CST I, *L. crispatus*; CST II, *L. gasseri*; CST III, *L. iners*; and CST V, *L. jensenii*). CST IV is composed of diverse bacteria dominated by anaerobic bacteria (Ravel et al., 2011). According to the analysis of the vaginal microbiome characterization of all subjects, it was found that there were statistical differences in the classification of the vaginal flora between the control group and the case group including no-medication and drug-treatment groups. No significant difference was found between no-medication patients and drug-treatment patients in the frequencies of CSTs. This may be caused by a different vaginal microbiota structure in patients than in controls. It is in line with the conclusion that RSA is associated with the changes in vaginal microbiome constituents as analyzed by PCoA. The prevalence of each CST within the three groups was compared and no statistical differences was found except in CST I. The prevalence of CST I was higher in the patient group than in the control group. The protective role of *Lactobacillus* species, including *L. crispatus*, was demonstrated in many previous studies. (Aroutcheva et al., 2001; Hočevar et al., 2019; Caselli et al., 2020). This difference may be attributed to the small sample size of control subjects and the effect of drug therapy on patients' vaginal microbiome profile. Prevalence of CST IV in the first trimester stages was correlated with gestational age at delivery (DiGiulio et al., 2015). By contrast, Elovitz et al. suggested that the prevalence of *Lactobacillus* spp. was not associated with spontaneous preterm birth after conducting a study involving a prospective cohort of 2,000 single pregnancy women (Elovitz et al., 2019). In future, a larger sample size is needed to analyze the characteristics of vaginal CSTs between patients with RSA and healthy controls.

The abundances of 15 genera were significantly different between the no-medication and control groups ($p < 0.05$). However, in untreated patients, *Megasphaera* abundance was significantly increased. The abundance of *Sneathia sanguinegens* in untreated patients was also higher than in controls, and this difference showed a trend toward statistical significance ($p = 0.051$). Therefore, *Sneathia sanguinegens*, *Megasphaera*, and the reduced 14 genera might be related to RSA. *Megasphaera* was also previously shown to have a positive association with PTB in studies by of Hočevar et al. and Nelson et al. (Nelson et al., 2014; Hočevar et al., 2019). *Sneathia* spp., which can adhere to cervical epithelial cells and have a high cytotoxic potential, were previously associated with serious pregnancy complications including spontaneous abortions, preterm labor, and preeclampsia resulting from invasion into the uterine cavity and amniotic sac (Harwich et al., 2012; Seo et al., 2017; Fettweis et al., 2019). Gentile and colleagues recently discovered a novel virulence-related CptA produced by *S. amnii* that can permeabilize chorionic trophoblast cells and lyse human red blood cells (Gentile et al., 2020). Our findings showed that *Sneathia sanguinegens* might have a specific role in RSA. Of the 14 reduced bacterial genera, 5 (*Corynebacterium_1*, *Rhodococcus*, *Sphingomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Pseudomonas*) contributed the most to the difference between controls and cases on in-depth analysis by LDA, meaning that they were most closely related to RSA. All five genera are composed of aerobic bacteria, and further studies are needed to explore the underlying mechanism of this phenomenon.

Metformin is an effective antidiabetic drug that is also used to treat endocrine diseases caused by PCOS (e.g., polycystic ovary-related recurrent miscarriage) and normalize endocrine, metabolic, and reproductive functions (Rai and Regan, 2006; Practice Committee of the American Society for Reproductive Medicine, 2012; Khalife et al., 2019; Wu et al., 2020). Metformin has attracted much attention in recent years because of its anticancer effects on several human solid tumors (Yu et al., 2019). Some scholars speculated that the benefits of metformin in cancer prevention and treatment might be mediated by the intestinal flora (Wu et al., 2020). Given its antiplatelet and anti-

inflammatory properties, aspirin is often used to prevent and treat cardiovascular diseases, as well as for the treatment of unexplained recurrent miscarriage and autoimmune-related recurrent miscarriage. Aspirin has also been proven to have antitumor effects, especially in colorectal cancer; this might be due to the relatively favorable migration of the intestinal microbiome caused by aspirin (Wu et al., 2020). In our study, the vaginal microbiota composition of patients significantly recovered when they received metformin alone. After administration of metformin plus aspirin, patients' vaginal microbial composition was partially restored. Specifically, at the genus level, the difference between the cases and controls was reduced from the original difference of 15 genera to 6 genera. It is worth noting that when patients were treated with metformin and aspirin, the abundance of vaginal *Lactobacillus* spp. increased significantly. To our knowledge, this has not been reported in past studies and provides new ideas for the future treatment of diseases caused by the decrease or absence of *Lactobacillus spp*. Ideally, further in-depth research studies should be performed on larger cohorts of subjects with RSA to verify our results and explore related mechanisms.

CONCLUSION

Patients experiencing RSA presented a less rich vaginal microbiome with decreased abundance of *Pseudomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, *Corynebacterium_1*, *Rhodococcus*, and *Sphingomonas*, along with increased abundance of *Megasphaera* and *Sneathia sanguinegens*. Drug treatment affects vaginal microbiota composition. Metformin alone or in combination with aspirin might normalize microbiota composition. Metformin plus aspirin might significantly increase the abundance of vaginal *Lactobacillus* spp.

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: NCBI SRA; PRJNA683172.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CY, MD, and FZ conceived and designed the study. JG and ZW contributed to obtaining ethical approval. YC was responsible for financial management. Participant recruitment and sample collection were conducted by MD and FZ. Experiments and data collection were performed by ZW, CL, YC, NH, and FZ. Data were analyzed by JG, MW, MD, FZ, CY, and LC. Figures and tables were generated by FZ and LC. The manuscript was written by FZ and reviewed by CY and MD. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.680643/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antigen Presenting Cells Link the Female Genital Tract Microbiome to Mucosal Inflammation, With Hormonal Contraception as an Additional Modulator of Inflammatory Signatures

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The microbiome of the female genital tract (FGT) is closely linked to reproductive health outcomes. Diverse, anaerobe-dominated communities with low *Lactobacillus* abundance are associated with a number of adverse reproductive outcomes, such as preterm birth, cervical dysplasia, and sexually transmitted infections (STIs), including HIV. Vaginal dysbiosis is associated with local mucosal inflammation, which likely serves as a biological mediator of poor reproductive outcomes. Yet the precise mechanisms of this FGT inflammation remain unclear. Studies in humans have been complicated by confounding demographic, behavioral, and clinical variables. Specifically, hormonal contraception is associated both with changes in the vaginal microbiome and with mucosal inflammation. In this study, we examined the transcriptional landscape of cervical cell populations in a cohort of South African women with differing vaginal microbial community types. We also investigate effects of reproductive hormones on the transcriptional profiles of cervical cells, focusing on the contraceptive depot medroxyprogesterone acetate (DMPA), the most common form of contraception in sub-Saharan Africa. We found that antigen presenting cells (APCs) are key mediators of

microbiome associated FGT inflammation. We also found that DMPA is associated with significant transcriptional changes across multiple cell lineages, with some shared and some distinct pathways compared to the inflammatory signature seen with dysbiosis. These results highlight the importance of an integrated, systems-level approach to understanding host-microbe interactions, with an appreciation for important variables, such as reproductive hormones, in the complex system of the FGT mucosa.

Keywords: HIV, female genital tract, microbiome, inflammation, mucosal immunology, hormonal contraception, host-microbiome interaction

INTRODUCTION

The microbiome of the female genital tract (FGT) has been implicated in a range of reproductive health outcomes. We have previously defined four discrete FGT microbial communities, termed cervicotypes (CTs), in South African women of reproductive age (Anahtar et al., 2015; Gosmann et al., 2017). Communities dominated by *Lactobacillus* species have been associated with favorable outcomes, while those with diverse anaerobes and low *Lactobacillus* abundance are associated with negative sequelae. Bacterial vaginosis (BV) is a clinical diagnosis in which these diverse communities are accompanied by vaginal discharge and associated symptoms. BV impacts up to 58% of women globally and is associated with a higher risk of sexually transmitted infections (STIs), including gonorrhea, chlamydia, herpes simplex virus type 2, and HIV [reviewed in (Brotman, 2011; Anahtar et al., 2018)], as well as preterm labor (Hillier et al., 1995; Meis et al., 1995; Anahtar et al., 2018). In sub-Saharan Africa, where HIV incidence remains high, particularly among young women, these dysbiotic vaginal communities are significantly more common than in the United States (Anahtar et al., 2015; UNAIDS, 2019). High prevalence of dysbiotic FGT communities has also been observed in other low and middle-income countries (Marconi et al., 2020), where women continue to face a disproportionate burden of global reproductive health challenges (Smid et al., 2016; Hull et al., 2020). The precise mechanistic links between the FGT microbiome and mucosal inflammation, the presumed link to these poor clinical outcomes, remain unclear.

Women's health around the world, particularly in areas with limited access to healthcare, has been significantly improved through effective and accessible contraception. Due in part to its efficacy, low cost, and discrete nature, depot medroxyprogesterone acetate (DMPA) has remained a popular contraceptive option. In South Africa, more than a quarter of women use DMPA, accounting for nearly half of all contraception use in the country (Tsui et al., 2017; United Nations, 2020). DMPA has been suggested to modulate both the FGT microbiome and immune environment, complicating analyses of host-microbiome interactions in this setting (van de Wijgert et al., 2013; Roxby et al., 2016; Wessels et al., 2019; Noel-Romas et al., 2020). Progestin-only contraception, including DMPA, is associated with a decreased risk of BV (Vodstrcil et al., 2013) but with an increased FGT inflammatory state (Chandra et al., 2013; Morrison et al., 2014; Deese et al., 2015; Byrne et al., 2016; Morrison et al., 2018; Edfeldt et al., 2020). Although reproductive hormones and the microbiome are both known to modulate the FGT immune environment, it remains unclear how these paths might converge on genital inflammation.

In this study, we characterize transcriptional programs of specific cellular lineages in the endocervix that shed light on the host pathways contributing to microbiome-induced inflammation at this key mucosal barrier. We identify antigen presenting cells (APCs) as an important sensor of vaginal microbiota. We additionally address the effects of hormonal contraception on FGT inflammation and provide evidence that DMPA induces significant host transcriptional changes in multiple cell lineages. These results demonstrate that dysbiosis and DMPA modulate the mucosal environment of the FGT in distinct ways, highlighting the importance of using an integrated approach to more fully understand host-microbe interactions in the context of human clinical and behavioral covariates.

METHODS

Sample Collection

Study specimens were obtained from participants in the Females Rising through Education, Support, and Health (FRESH) cohort, a prospective observational cohort study that enrolls 18- to 23-year-old, HIV-uninfected, non-pregnant women in Umlazi, South Africa. The cohort and its inclusion and exclusion criteria have been previously described in detail (Anahtar et al., 2015; Gosmann et al., 2017; Dong et al., 2018). The study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN; Ethics Reference Number BF131/11) and the Massachusetts General Hospital Institutional Review Board (2012P001812/MGH). Participants provided informed consent.

All genital tract sampling was performed at the FRESH clinical research site in an exam room dedicated to pelvic examination. Sampling was performed by the same two trained research nurses throughout the study. Sampling was performed outside the menstrual period. Sample collection was performed under direct visualization by speculum exam. Lubricants were not introduced into the vagina prior to or during the speculum exam. The plastic single-use speculum was wet with tap water prior to insertion. Upon speculum insertion, swab samples (Puritan 6" Sterile Standard Foam Swab 126 w/Polystyrene Handle) were collected from the mid-vaginal wall, then from

the ectocervical mucosa. A cervicovaginal lavage with 5 mL of sterile 0.9% saline (Adcock Ingram) was then performed, followed by collection of a cellular cytobrush (CooperSurgical, Medscand Cytobrush Plus, Catalog #C0005), which was rotated 360° in the cervical os. Cytobrushes were put into a 15 mL conical tube containing 5 mL of RPMI-1640 media supplemented with 10% v/v heat-inactivated fetal calf serum, 1% v/v Penicillin-Streptomycin-Amphotericin B 10K/10K/25ug mixture (Lonza Bioscience), L-glutamine (Lonza Bioscience, 2 mM final concentration), and HEPES (Lonza Bioscience, 10 mM final concentration). The tube was immediately placed on ice, and transferred to the processing lab at the HIV Pathogenesis Programme (HPP), Doris Duke Medical Research Institute, UKZN, for further processing (see details below). One cervical swab was used to make a microscope slide preparation for Gram stain analysis. The remaining cervical and vaginal swabs were placed in individual sterile vials on ice. A cervical swab was transferred, along with the prepared slide, to Neuberg Global Laboratories, Durban, South Africa, an accredited commercial laboratory diagnostics company, where polymerase chain reaction (PCR) testing was performed from the cervical swab for the sexually transmitted infections (STIs) *Neisseria gonorrheae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, and *Mycoplasma genitalium*. BV status was determined from the prepared slide by trained laboratory technologists at Neuberg Global Laboratories using the Gram-stain-based Nugent scoring method (Nugent et al., 1991). The remaining cervical and vaginal swabs were transferred to the HPP processing lab and stored at -80°C for subsequent sequence-based microbiome analysis.

Samples for host transcriptomic analysis were selected from participants who were STI-negative (chlamydia, gonorrhea, trichomonas, mycoplasma) at the time of sampling and who represented a distribution across the four CTs (**Table 1** and **Appendix 2**). Participants and samples were also selected based on maximal number of sorted cervical cells count, yielding comparable cell counts for each cell type across participants.

At the time of sampling, participants were asked about their current family planning methods and their last menstrual period (LMP). Women using no hormonal contraceptive were determined to be in the follicular phase of the menstrual cycle based on a self-reported LMP less than 14 days prior to sampling.

Microbiome Sequencing and Analysis

Total nucleic acids from cervical or vaginal swab samples were extracted with a phenol-chloroform method, which included a bead beating process to disrupt bacteria as previously described (Anahtar et al., 2015; Anahtar et al., 2016). The V4 region of the bacterial 16S rRNA gene was PCR-amplified following standard protocols (Caporaso et al., 2012; Anahtar et al., 2015; Hoang et al., 2020). Amplicons were pooled, purified, and prepared according to standard Illumina protocols, and single-end sequenced on an Illumina MiSeq using a v2 300-cycle sequencing kit with addition of custom Earth Microbiome Project sequencing primers (Caporaso et al., 2012).

Sequence demultiplexing was performed as previously described (Hoang et al., 2020). Demultiplexed sequences were then processed using dada2 version 1.6.0 (Callahan et al., 2016) with taxonomy assignments performed using the RDP training database supplemented by manual curation (Anahtar et al., 2015). Tables with amplicon sequence variant (ASV) taxonomy, per-sample ASV read counts, species read counts, and sequencing metadata are detailed in **Supplementary File 1**. The denoised dada2 results with final taxonomic assignment were analyzed in R using phyloseq version 1.30.0

TABLE 1 | Demographics of FRESH participants included in analysis.

Characteristic	CT 1/2 (n=13)	CT 3/4	p-value
Days since last sex (median, IQR)	18 [3, 32]	17 [6, 43]	0.600
Number of sexual encounters, last 30d (median, IQR)	2 [0, 2]	1 [0, 3]	0.762
Number of sex partners, last 30d (median, IQR)	1 [0, 1]	1 [0, 1]	0.581
Days since LMP (median, IQR)	45 [21, 315]	16 [1, 21]	0.009
Condom use during sex			0.550
Always	2	1	
Sometimes	4	7	
Never	3	2	
Drying agent use			0.613
Never	12	14	
Sometimes	1	3	
Contraceptive use			0.061
DMPA	4	4	
Nuristerate	3	0	
Implanon	1	0	
Lipez Loop	0	1	
None	5	12	

All women were enrolled between the ages of 18-23 years and all participants who were included in this analysis were HIV negative at the time of sampling. Data are presented as median [IQR] or number of participants, and p-values are calculated by Wilcoxon or Fisher's Exact test. One participant was excluded due to samples failing to meet quality metrics during RNA sequence data processing (see Methods).

(McMurdie and Holmes, 2013) and custom R scripts (available in **Supplementary File 2**.

Sample read counts after dada2 processing all exceeded 13,000 reads per sample. For additional analysis, we excluded ASVs that could not be defined at least to the taxonomic level of class, that were not represented by >10 reads in >2 different samples, or that had an abundance of <50 reads within the entire cohort. To explore variation between bacterial communities, counts from distinct ASVs belonging to the same species were merged (**Supplementary File 1**). Bray-Curtis distances (β diversity) between all samples were calculated and between-sample variation was examined by performing principal coordinate analysis (PCoA) in phyloseq (McMurdie and Holmes, 2013), then plotting the ordinations in R using ggplot2 (Wickham, 2016) from the tidyverse package (Wickham et al., 2019).

Raw sequence read files for genital tract 16S rRNA-gene profiling are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA738803 (all 29 BioSamples in the BioProject), plus BioSample SAMN19246318 from BioProject PRJNA730929. Custom R code with associated data files sufficient to reproduce the 16S-based microbiota analysis is available as a compressed supplementary file (**Supplementary File 2**).

Cytobrush Processing, RNA Sequencing, and RNA-Seq Analysis

Cells were gently dislodged from cytobrushes without use of enzymatic digestion, and samples were processed and sorted fresh on the day of collection. We used fluorescence-activated cell sorting to isolate four specific live (Invitrogen blue viability dye) cell populations from the cytobrush samples on a BD FACSAria Fusion using BD FACSDiva software: antigen presenting cells (APCs; CD19- (clone H1B19, BD), CD45+ (H130, BD), CD66b- (G10F5, Biolegend), HLADR+ (G46-6, BD), CD3- (UCHT1, BD), and CD11c+ (B-ly6, BD) and/or CD14+ (M5E2, BD)), CD4+ T cells (CD19-, CD45+, CD66b-, CD3+, CD4+ (SK3, BD)), neutrophils (CD19-, CD45+, CD66b+), and epithelial cells (CD19-, CD45-, large by FSC-A, EpCAM+ (94C, Biolegend)) (See **Appendix 1** for sorting strategy and **Appendix 3** for cell counts). Sorted cell populations were stored in Trizol Reagent at -80C. We then performed bulk RNA-seq on each sorted cell population from each of the 30 participants, totaling 117 samples across the four cell types. RNA was extracted using a Chloroform method and quality was assessed by TapeStation. Bulk RNA library prep was performed on these RNA samples using the SmartSeq2 protocol (Picelli et al., 2014). Size and concentration of the libraries were assessed with TapeStation and Qubit.

Pooled cDNA was sequenced three times on an Illumina NexSeq using paired-end sequencing with a 75-cycle kit to achieve adequate sequencing depth. We used fastqc (0.11.5) and multiqc (1.5) for initial quality control to flag samples with poor sequencing quality (Ewels et al., 2016). We then used STAR (2.5.2b) for alignment and mapping, eliminating samples with less than 20% uniquely mapped reads (Dobin et al., 2013). HTSeq (0.9.1) was used to count the number of reads per gene (Anders et al., 2015). All samples, across all three sequencing runs, were visualized using PCA to ensure there was no data skewing attributable to any run. There was no evidence of batch effect across sequencing runs (data not shown), so the data from the three runs was merged for analysis. Next, the mitochondrial transcriptional content of each sample was assessed using EnsDb.Hsapiens.v75 in R. Because the percentage of mitochondrial transcript content was significantly different between cell types, different mitochondrial content cutoffs were used in different cell types to filter out samples with a high proportion of dead or dying cells. For epithelial cells, which had significantly higher percentage of mitochondrial transcript, we used a cutoff of <50%. For all other cell types, we used a cutoff of <30% of mitochondrial transcript as a cutoff for samples to be included in downstream analysis. The gene count table was filtered to retain only genes with ≥0.5 counts per million (CPM) in at least two samples. Genes that were not approved in HGNC were also excluded. After performing all QC filtering, the transcriptome of 107 (out of 117) samples was analyzed across 18248 genes.

Differential expression analysis was conducted in R using DESeq2 version 1.30.0 (Love et al., 2014). A ranked gene list was compiled from this DESeq2 analysis and analyzed using GSEA v4.1.0 (Mootha et al., 2003; Subramanian et al., 2005). The ranked gene list was assessed for enriched gene sets using the hallmark gene list version 7.4 (Liberzon et al., 2015) and gene ontology (GO) biological processes version 7.4 (Ashburner et al., 2000; Gene Ontology, 2021). An FDR-adjusted p-value of 0.1 was used as the statistical significance cutoff. R scripts for host RNAseq analysis are available in **Supplementary File 3**.

RESULTS

Microbiome Composition Across Participants

All samples and data were collected from participants enrolled in the FRESH (Females Rising through Education, Support, and Health) study in Umlazi, South Africa, who were between the ages of 18 and 23 years at the time of study enrollment and were HIV-uninfected, non-pregnant, and did not have sexually transmitted infections (STIs) at the time of sampling. We selected samples from a subset of 30 participants for paired transcriptome-microbiome analysis to ensure distribution of participants across the four vaginal microbiota community types as determined by bacterial 16S rRNA gene sequencing (**Figure 1** and **Table 1**). We have previously established a classification system that divides the FGT microbial communities into four cervicotypes (CTs) (Gosmann et al., 2017; Munoz et al., 2021). CT1 is dominated by *Lactobacillus crispatus* and CT2 is dominated by *Lactobacillus iners*. CT3 and CT4 both have higher diversity. In CT3, *Gardnerella* is the most abundant taxon, while CT4 is dominated by other taxa, typically with a significant prevalence of *Prevotella* species (**Figure 1**). Prior work has shown that women with CT3 and CT4 communities exhibit higher levels of FGT inflammation, as measured by both inflammatory cytokines in cervicovaginal lavage and by an increased frequency of inflammatory cells, including HIV target cells (activated CCR5+ CD4+ T cells), in cervical cytobrush samples (Anahtar et al., 2015). CT3 and CT4

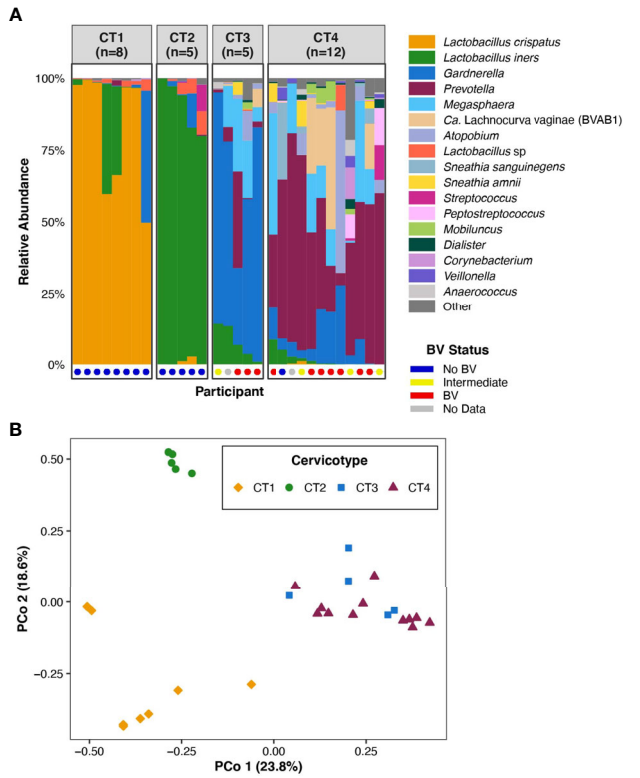

FIGURE 1 | FGT microbiota of the 30 participants included in the analysis. **(A)** Bar plot representation of the microbial taxa present in the FGT in all participants, grouped by CT. Nugent score was also calculated for 28 of the 30 participants and is represented at the base of the bar plot. **(B)** Principal coordinates analysis of FGT microbial taxa from the 30 participants.

are also associated with an increased risk of acquiring HIV (Gosmann et al., 2017).

The 30 participants examined in this analysis included eight in CT1, five in CT2, five in CT3, and twelve in CT4 (**Figure 1A**). We performed principal coordinates analysis (PCoA) based on Bray-Curtis distances to investigate the range of variation between participant vaginal microbial communities. The first two PCoA axes represented 42.4% of the total variance in the population (**Figure 1B**). Axis 1 fully segregated *Lactobacillus*-dominant (CT1 and CT2) communities from non-*Lactobacillus*-dominant (CT3 and CT4) communities, while Axis 2 fully segregated CT1 from CT2. The assignment of the four CTs (**Figure 1B**) was consistent with previous published work that studied larger sample sizes (Anahtar et al., 2015; Gosmann et al., 2017). The clear separation of FGT microbial communities based on *Lactobacillus* dominance or depletion (Axis 1, **Figure 1B**) highlighted the relevance of investigating host transcriptional differences between CT1/2 and CT3/4.

Demographic Characteristics Across Cervicotypes

We divided the cohort based on FGT *Lactobacillus* dominance, comparing *Lactobacillus* dominant (CT1/2) to *Lactobacillus* deficient (CT3/4) communities. No factors related to sexual practices differed between groups, including the number of days since last having sex, the number of sexual encounters over the previous 30 days, and the number of sexual partners over the past 30 days (**Table 1**). There was no significant difference in reported use of condoms, vaginal drying agents, or contraceptives; however, there was a non-significant trend toward increased use of the progestin-based long-acting reversible contraceptives (LARCs) DMPA, Nuristerate, and Implanon, in the CT1/2 group. The only significant difference was in days since last reported menstrual period (LMP) ($p=0.009$): women with CT1/2 had a median of 45 days elapsed since LMP, longer than a regular menstrual cycle and likely reflecting the amenorrhea associated with progestin-only contraceptive methods. Women with CT3/4 had a median of 16 days since LMP, and a range within that of regular menstrual cycles. No sampling occurred during menstruation.

Antigen Presenting Cells Exhibit a Strong Inflammatory Signature in the Non-*Lactobacillus*-Dominant FGT Microbiome

We sorted four cell lineages from each of participant: epithelial cells, APCs, CD4+ T cells, and neutrophils (**Appendix 1**, **Appendix 2**). These populations were selected based on their role in genital immune surveillance, barrier function, and HIV acquisition. We then performed bulk-RNA sequencing on each sorted cell population from each participant. In unsupervised clustering, the CTs did not cluster together (data not shown). Although we knew we were underpowered in this analysis, we proceeded with a hypothesis-generating comparison between *Lactobacillus*-dominant (CT1/2) and *Lactobacillus*-deficient (CT3/4) CTs. There were no significant differences in cell count per sample between CT groups for any cell type (**Appendix 3**) that might impact the differential gene expression analysis.

APCs demonstrated the greatest number of significantly differentially expressed genes (n=111) and the most inflammatory genes and pathways associated with non-*Lactobacillus*-dominant CTs (**Figure 2A** and **Appendix 4**; **Appendix 5**). The APC genes most strongly associated with CT3/4 include those involved in the interferon response (*e.g.*, *IFIT2*, *IFIT3*, *IFIT5*, *OASL*), class I major histocompatibility complex (*e.g.*, *HLA-S*, *HLA-G*, *HLA-H*, *HLA-L*), class II major histocompatibility complex (e.g., *HLA-DRB5*), genes related to innate immunity (e.g., *MEFV*, *PELI1*, *PTGS2*, *IL1RN*), and genes promoting T-cell mediated adaptive immunity (e.g., *TNFSF9*). Genes related to regulation of neoplastic cell growth were also upregulated in the CT3/4 group (e.g., *MYCL*, *DDIT4*, *PLAC8*, and *PMAIP1*); the genes upregulated in CT3/4 predominantly suppress cell growth and/or induce apoptosis. Hierarchical clustering of samples based on genes with significant differences in expression nearly completely segregated CT1/2 from CT3/4 samples (**Appendix 4A**).

In accordance with these observations, pathways identified as significantly enriched through gene set enrichment analysis (GSEA) using hallmark gene sets in CT3/4 included multiple

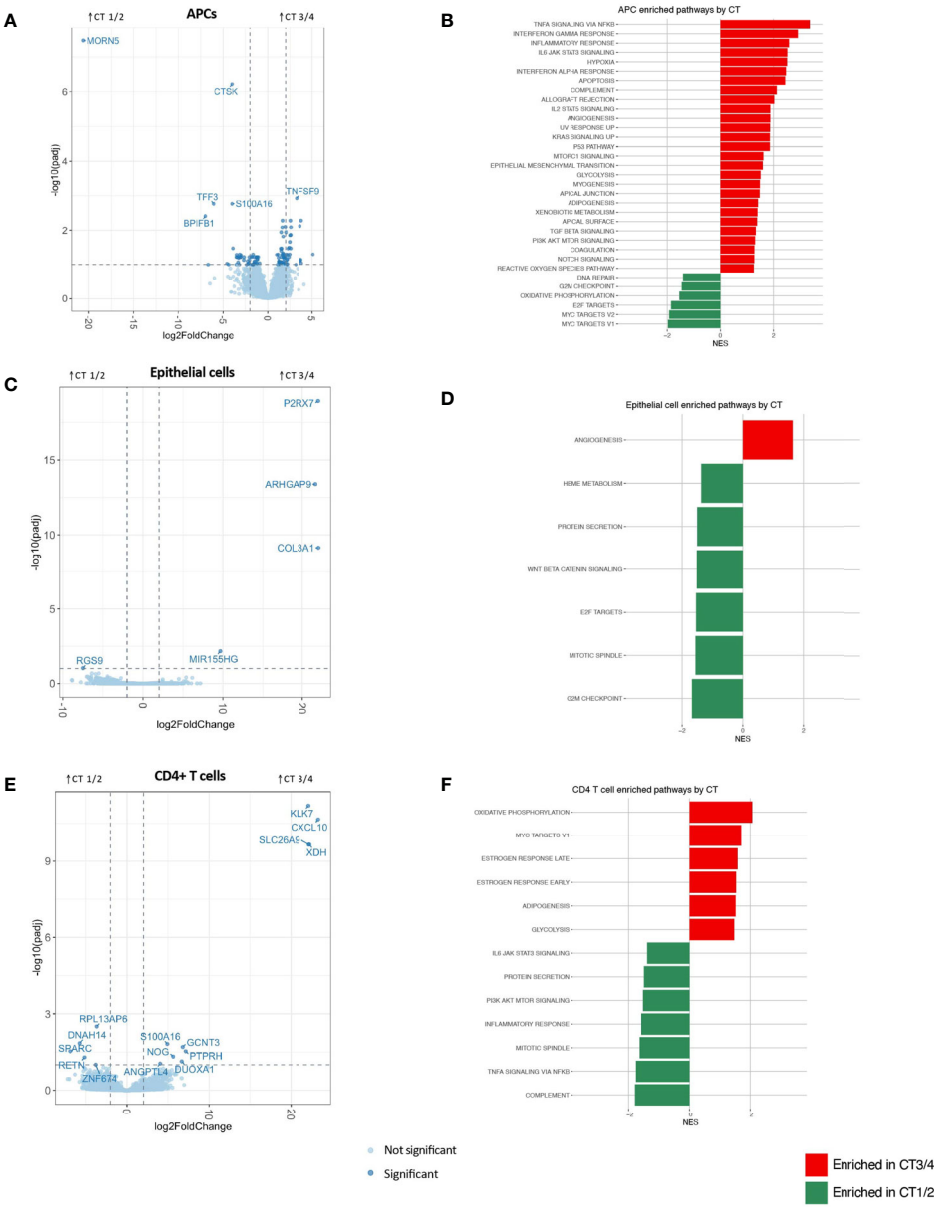

FIGURE 2 | APC **(A, B)**, epithelial cell **(C, D)** and CD4+ T cell **(E, F)** differential gene expression between CTs 3/4 and 1/2 visualized through volcano plots **(A, C, E)** and significantly enriched gene sets by GSEA using Hallmark gene sets (at FDR-corrected q value of 0.1) based on Hallmark gene lists **(B, D, F)**.

inflammatory pathways (e.g., TNFα signaling *via* NFkB, IFNγ response, inflammatory response, interferon alpha response, complement, allograft rejection, IL2 STAT5 signaling, and IL6 JAK STAT3 signaling) (**Figure 2B**). Pathways related to neoplastic suppression were also highlighted in this group (e.g., apoptosis, p53 pathway), as well as pro-proliferative pathways (KRAS signaling up, MTORC1 signaling, PI3K AKT MTOR signaling). Conversely, genes significantly enriched in CT1/2 included innate immune response genes (*BPIFB1*, *PELI3*), fractalkine (*CX3CL1*), genes related to mucosal epithelial integrity (*TFF3*, *CLDN10*, *MUC5B*, *LAMB1*, *RIPK4*), genes related to neoplastic invasion (*CTSK*, *KLK11*, *UCA1*), and few related to tumor suppression (S100A14). GSEA pathway analysis also highlighted proliferative signatures, such as progress through cell cycle checkpoints and Myc signaling, in CT1/2 as compared to CT3/4 (**Figure 2B**). Additional GSEA analysis performed with the gene ontology (GO) biological processes gene sets further supported APCs as drivers of dysbiosis-associated FGT inflammation, with 607 pathways upregulated in CT3/4, many of which were related to inflammatory signaling (**Appendix 5A**).

Epithelial cells represent another possible mediator of FGT inflammation. These cells exhibited very few differentially expressed genes based on microbiota composition (**Figure 2C**).

No GSEA hallmark-derived gene sets appeared to have any relevance for the inflammatory signal seen grossly in the setting of CT3/4 (**Figure 2D**). A few inflammatory pathways were significantly upregulated in CT3/4 based on GO terms, but they were more related to innate inflammation, such as response to fungus, eosinophil migration, monocyte chemotaxis, and granulocyte chemotaxis, and all had relatively low normalized enrichment scores (NES 1.96-2.36) (**Appendix 5B**).

CD4+ T cells are thought to be a final common pathway of inflammation that increases risk of HIV acquisition (Haase, 2005; Haase, 2010). In this analysis, CD4+ T cells had the second greatest number of significantly differentially expressed genes after APCs (15) (**Figure 2E**). However, hierarchical clustering of samples based on differentially expressed genes was unable to segregate samples from different CTs (**Appendix 4C**), unlike the clustering observed for APCs (**Appendix 4A**). Furthermore, although a non-*Lactobacillus*-dominant FGT microbiome is known to be associated with inflammation, inflammatory pathways were surprisingly upregulated in CD4+ T cells from women in CT1/2 as compared to CT3/4 (**Figure 2F**). GO term analysis also showed some inflammatory pathways in CT1/2, such as TLR2 signaling and myeloid leukocyte mediated immunity (**Appendix 5C**), possibly suggesting a host-mediated role in curating the FGT microbiome away from BV-associated bacteria (Mares et al., 2008). The most strongly upregulated pathways in CT3/4 were related to enhanced translation capabilities (e.g. cotranslational targeting to membrane (NES 2.79), establishment of protein localization to ER (NES 2.46), translation initiation (NES 2.28)), which might relate to a CD4+ T cell activation state previously observed with dysbiosis (Anahtar et al., 2015).

Neutrophils showed no significantly differentially expressed genes between CT groups (**Appendix 4D**). The hallmark gene set "TNFα signaling *via* NFkB" was significantly enriched in CT3/4, but IFNα response was significantly enriched in CT1/2 (**Appendix 4E**), and both had relatively low normalized enrichment scores. GO term analysis similarly showed a small number of inflammatory pathways upregulated in CT3/4 (**Appendix 5D**).

Overall, these results indicate a strong inflammatory signal through APCs in the setting of *Lactobacillus*-depleted vaginal microbial communities. While other cell types may contribute to the inflammatory milieu, the prominent influence of APCs in helping to orchestrate a dysbiosis-associated inflammatory milieu is clear.

DMPA Effects on Gene Expression

In order to explore whether hormonal effects also contribute to the transcriptional differences we observed between women with different vaginal microbiota, we compared samples from 7 women using DMPA (i.e., a high-progestin, low-estrogen state) with those from 6 women in the follicular phase of the menstrual cycle (i.e., a low-progestin, high-estrogen state; **Appendix 6**). Each hormone group was split relatively evenly by CT, resulting in 2-4 participants from each hormonal state in each CT group (**Appendix 6**). No demographic variables examined were significantly different between groups (**Appendix 6**). Due to the small sample size of an integrated analysis, we did not explicitly address the modifying effect of hormones on microbiota-associated inflammation.

Numerous transcriptional differences were observed across all cell types by hormonal state (**Figure 3** and **Appendix 8**). In APCs, epithelial cells, and CD4+ T cells, the pattern of differential gene expression nearly completely segregated women in the follicular phase from DMPA users (**Figures 3C, F, I**), suggesting a true and clear difference in transcriptional programs between groups.

In APCs, the hallmark gene set "inflammatory response" was significantly enriched (**Figure 3B**). In epithelial cells, one of the most highly differentially expressed gene across DMPA users was *CCL3L3*, which is a chemotactic cytokine for CCR5+ CD4+ T cells (**Figures 3D, F**). GO term analysis highlighted epithelial barrier differences in DMPA users (cornification, keratinization), while decreased leukocyte mediated immunity was observed in DMPA users, though with a low NES (**Appendix 9B**). Only five genes were significantly differentially expressed in CD4+ T cells (**Figures 3G, I**), but these genes were able, again, to perfectly segregate samples by hormone state.

These results demonstrate that DMPA is associated with significant changes in cellular pathways within FGT mucosal cell populations. Previous observations regarding DMPA-associated CD4+ T cell infiltrates may be partially explained by inflammation in APCs, chemotactic cytokines secreted by epithelial cells, and/or reduced epithelial barrier integrity.

DISCUSSION

Assessing differential gene expression characterizing cellular lineages known to be key in maintaining health of the FGT allowed us to more clearly define the transcriptional programs that underlie FGT mucosal inflammation in the context of a disrupted microbiome. Non-*Lactobacillus*-dominant vaginal microbial communities are associated with inflammation, but it has remained unclear which cell types and pathways modulate this response. Our analysis pointed strongly to APCs as a key modulator of the microbiome-associated inflammatory response. APCs exhibited upregulation of pro-inflammatory mediators in the setting of CT3/4, with increased expression of genes and pathways involved in priming of innate immunity and adaptive immunity (including T cell activation). This is consistent with previous work that highlighted APCs' inflammatory response when treated with bacterial products *in vitro* (Anahtar et al., 2015). APCs isolated from women with CT3/4 also upregulated genes involved in cancer suppression, which may be a reflection of the inflammatory state also upregulating antiviral immunity. Conversely, CT1/2 was associated with pathways involved in cellular checkpoint progression, suggesting a decreased antiviral state. A CT3/4 associated antiviral state likely relates to recruitment of HIV target cells, CD4+CCR5+ T cells, to the FGT mucosa, increasing susceptibility to infection (Haase, 2005; Haase, 2010). The other cell types assessed (CD4+ T cells, epithelial cells, and neutrophils) did not show a strong pro-inflammatory signature in the setting of CT3/4.

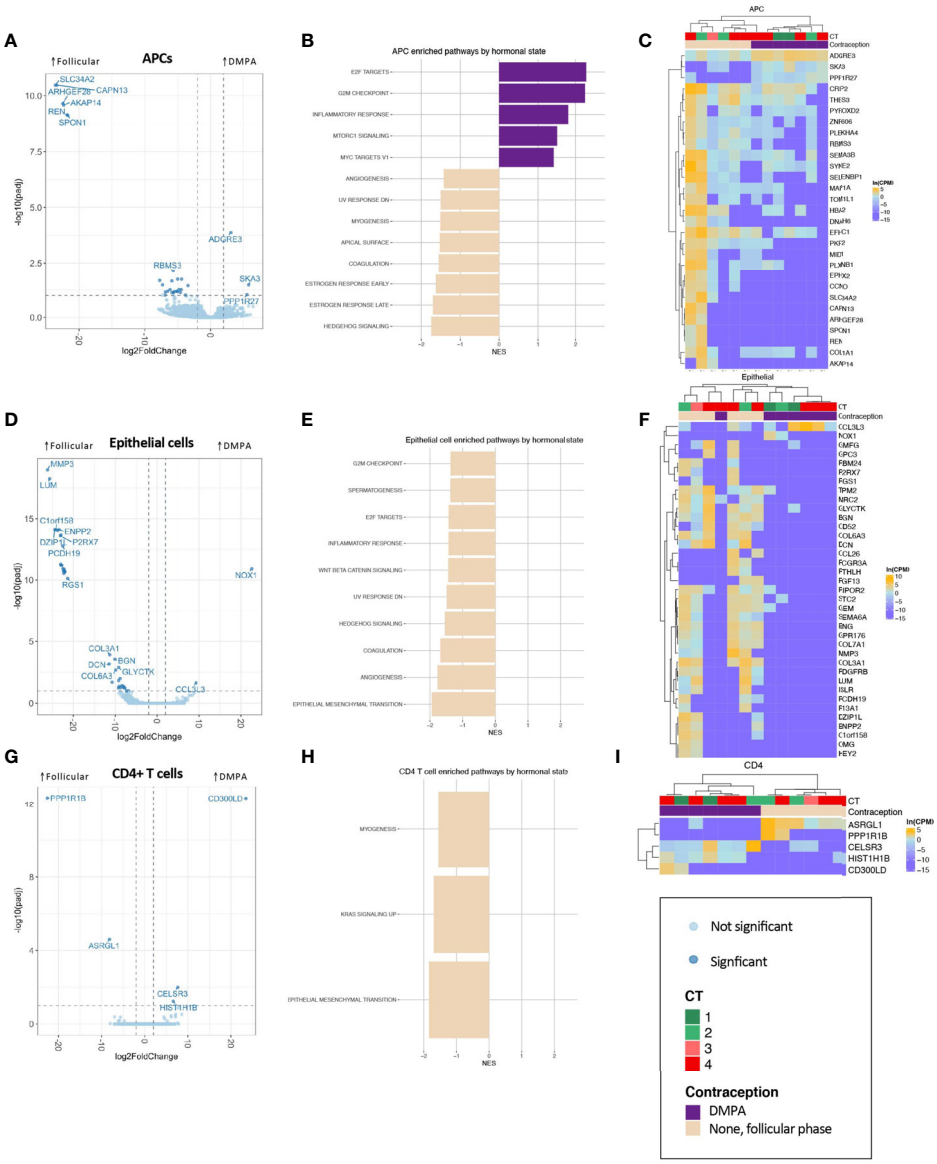

FIGURE 3 | Association of DMPA with FGT APC **(A–C)**, epithelial cell **(D–F)**, and CD4+ T cell **(G–I)** transcriptional landscape. **(A, D, G)** Volcano plots showing differentially expressed genes, with significantly differentially expressed genes (BH-corrected p-value of 0.1) shown in darker points. **(B, E, H)** Significantly enriched gene sets through GSEA based on the Hallmark gene set with an FDR-corrected q-value <0.1. **(C, F, I)** Significantly differentially expressed genes are shown in heatmaps, with clustering based on Spearman distances.

To more holistically characterize the transcriptional levers acting on the FGT mucosa, we further compared two extremes of the reproductive hormone spectrum: women in the follicular phase of the menstrual cycle (high-estrogen, low-progesterone) and women using DMPA (high-progestin, low-estrogen). We found that these hormone states were associated with a large number of significantly differentially expressed genes in all cell types. Furthermore, these differentially expressed genes were able to segregate samples by their associated hormone state almost perfectly for all cell types except neutrophils. These observations highlight the strong effect of reproductive hormones, including hormonal contraceptives, on the transcriptional landscape of the FGT. Epithelial cells in DMPA users expressed the T cell homing chemokine *CCL3L3*, suggesting a pathway by which CCR5+CD4+ T cells may traffic to the FGT in the setting of DMPA use, as has been previously described (Byrne et al., 2016; Edfeldt et al., 2020). Differences in epithelial cell cornification and keratinization may also help explain increased availability of CCR5+CD4+ T cells in the FGT, consistent with previous observations (Zalenskaya et al., 2018; Edfeldt et al., 2020;

Molatlhegi et al., 2020). Due to limited sample size and distribution of hormonal contraceptive methods in our cohort, we could not adequately assess for differences in transcriptional patterns between DMPA users and women in other high-progesterone, low-estrogen states, including users of other progestin-based long-acting reversible contraceptives (i.e. injectable nuristerate or Implanon), nor of women using no hormonal contraception who were in the luteal phase of the menstrual cycle. We hypothesize that many of the patterns we observe in cervix-derived cells from DMPA users would translate to these other progesterone-high states. However, interestingly, a study comparing the transcriptional and functional effects of treating cultured vaginal epithelial cells with estradiol or progesterone versus medroxyprogesterone acetate (MPA) found that MPA preferentially inhibited cell cycle progression and epithelial barrier function *in vitro* (Woods et al., 2021). Thus, larger-scale studies comparing transcriptional patterns in DMPA users versus individuals in other progesterone-high, estrogen-low states are needed to investigate whether DMPA exerts unique effects on key mucosal cell populations *in vivo*.

Together, our findings highlight the role of APCs in microbiome-associated FGT inflammation and point to the orthogonally important role of reproductive hormones in FGT inflammation. The small sample size of this analysis prohibited statistically meaningful assessment of interactions between the microbiome and hormones on the FGT transcriptional landscape. The transcriptional differences across these groups do, however, paint a compelling picture of APC-mediated inflammation due to microbiome influence, intersecting with inflammatory signals in certain hormonal contexts, with DMPA here.

As we further explore host-microbe interactions in the FGT, this study highlights the importance of considering the complex interactions that modify the FGT mucosa. The cellular effect of hormones like DMPA appears to be broader than the inflammatory pathways associated with the microbiome, primarily in APCs. Our study, in concert with previous literature, suggests that these two variables may influence FGT inflammation in lineage specific and interconnected ways to generate a common end result of FGT mucosal inflammation with increased availability of CD4+ T cells. Understanding specific cellular mechanisms of microbial and hormone associated inflammation may lead to interventions to improve reproductive outcomes, particularly in areas where the burden of these adverse outcomes is greatest.

DATA AVAILABILITY STATEMENT

Raw sequence read files for genital tract 16S rRNA-gene profiling are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA738803 (all 29 BioSamples in the BioProject), plus BioSample SAMN19246318 from BioProject PRJNA730929.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN; Ethics Reference Number BF131/11) and the Massachusetts General Hospital Institutional Review Board (2012P001812/MGH). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF, SB, NM, MH, and CG performed nucleic acid extractions and 16S rRNA gene sequencing from vaginal and cervical swabs. SB performed bacterial 16S rRNA gene sequencing analysis. CG developed the cell sorting panel, NX and CG performed the fluorescence activated cell sorting, and SB and NX performed the flow cytometry analysis. MF performed RNA extractions from sorted cells and RNA-Seq library preparations, and MF and JX performed sequencing of RNA-Seq libraries. KD, MD, TG, FC, TN, NI, CG, SB, NX, MG, CM and DK contributed to clinical trial design, trial performance, and/or sample acquisition and processing efforts. EB and DK conceptualized the analysis, and EB and BH performed the analysis. SH and A-CV provided analysis guidance. EB, SB, and DK wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Comprehensive Characterization of Microbial Community in the Female Genital Tract of Reproductive-Aged Women in China

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The microbiota in the human body play critical roles in many physiological and pathological processes. However, the diversity and dynamics of the female genital tract (FGT) microbiota have not been fully unveiled. In this study, we characterized the microbiome variations in reproductive-aged Chinese women, and we revealed that the cervicovaginal microbiota were dominated by *Lactobacillus*. Overall, the composition of microbiota in the uterine cavity was more diverse than that in the vagina and cervix. A positive correlation between *Lactobacillus iners* and *Lactobacillus crispatus* was observed in both the vagina and the cervix, suggesting that these two species might have a symbiotic relationship in the cervicovaginal microbiota. Moreover, we, for the first time, stratified the reproductive-aged Chinese women into subgroups, based on their microbiome profiles. Furthermore, we identified the bacteria whose abundance changed in the uterine cavity of infertile patients when compared with healthy controls, such as *L. iners* and *L. crispatus*. Functionally, the metabolism-related pathways, neurotrophin signaling pathway, and adipocytokine signaling pathway were predominantly dysregulated in the uterine cavity of infertile patients. In conclusion, we characterized a comprehensive microbial landscape in FGT, as well as their functional roles in female infertility of the Chinese population.

Keywords: female genital tract, microbiota, uterine cavity, infertile, *Lactobacillus*

INTRODUCTION

The microbiota in the human body play a critical role in maintaining our daily wellbeing and are associated with the pathogenesis of various diseases (Young, 2017). These communities of microorganisms can be found in the skin, respiratory tract, alimentary tract, and other tissue sites, each with their own functional capabilities (Group NHW et al., 2009).

Among the spectrum of microbial communities, the female genital tract (FGT) microbiota, mainly dominated by *Lactobacillus* species, are considered to be one of the simplest yet most

important microbial communities, as up to 9% of the human microbiota colonize the FGT, and the cervicovaginal microbiota have unique impacts on the reproductive health of women (Witkin and Linhares, 2017). For example, lactobacilli help regulate the pH of the vagina to inhibit the growth of other bacteria and to prevent undesirable microbial colonization and infection through their adhesion to the vaginal epithelial cells (Boris and Barbes, 2000). However, the composition, diversity, and dynamics of the microbiota in the uterine cavity of reproductive-aged women have not been fully unveiled, and as the uterine cavity is an essential part of the FGT, more efforts are needed to further illustrate the interaction between microbial communities in the vagina and uterus. Evidences have shown that FGT microbial communities are closely associated with gynecological diseases (White et al., 2011; Ma et al., 2012; Greenbaum et al., 2019). However, the impact of microbial communities in the uterine cavity on female fertility and the underlying mechanism are still unclear.

With 16S rRNA gene sequencing, a method that does not rely on microbial cultivation, it is possible to determine the composition of the microbiota in the FGT (Johnson et al., 2019). A previous study has investigated the microbial compositions in three sites of the vagina, including introitus, midpoint, and posterior fornix, and concluded that there was little variation in species across the three sampling sites, with *Lactobacillus* species being dominant in all sites (Human Microbiome Project C, 2012). Furthermore, a uterine microbiome study found that there was no difference in the microbial composition of infertile women who became pregnant and of those who did not undergo assisted reproductive technology (ART) (Franasiak et al., 2016). In addition, an analysis of the microbiota within the female reproductive tract revealed a microbiota continuum along the female reproductive tract, which is indicative of a non-sterile environment (Chen et al., 2017). In the present study, we measured the abundance of bacterial genera and species from the vagina, the endocervix, and the uterine cavity of reproductive-aged women; explored potentially competitive and symbiotic relationships within these microbial communities; and examined their biological functions, anticipating to uncover the association between microbial communities in reproductive-aged women and the female reproductive health.

MATERIALS AND METHODS

Sample Collection

A total of 184 reproductive-aged women visiting the Physical Examination Center of Shanghai Changzheng Hospital were recruited for the study. Subjects with presence of an intrauterine device (IUD), vaginal inflammation, any acute inflammation, concern for cervical or endometrial neoplasia, and endocrine or autoimmune disorders were excluded. The subjects had no recorded recent use of hormones, antibiotics, and vaginal medications; no cervical treatment, endometrial biopsy, IUD removal, or hysteroscopy within a week; no douching within 5 days; and no sexual activity within 48 h. None of the subjects were pregnant, lactating, or menstruating at the time of sampling. Age, current resident area, menstrual history, and fertility history were collected from all the participants. As for nulliparous subjects, we asked whether they were planning for pregnancy. Written informed consent was obtained from all participants, and this study has been approved by the ethics committee of the Shanghai Changzheng Hospital.

Samples from the lower reproductive tract (vaginal and endocervix) were taken with a swab on the day of the visit with no prior perturbation. Endometrial fluid samples were obtained by transcervical aspiration with a double lumen embryo transfer catheter (Kitazato ET Catheter, Kitazato Corporation Tokyo office). Specifically, an outer cannula was placed at the internal cervical os. Subsequently, the inner sheath absorbed intrauterine lavage fluid and then retracted into the outer cannula. Finally, the inner sheath and the outer cannulas exited from the cervix and vagina together, thereby avoiding the contamination from bacteria in the cervix and vagina (Franasiak et al., 2016). The specimens were collected in sterile tubes, then flash-frozen with liquid nitrogen, stored at −80°C, and transported in dry ice to BGI-Shenzhen.

DNA Extraction and 16S rRNA Amplicon Sequencing

Genomic DNA extraction was performed as previously described (Qin et al., 2012). DNA of high quality was used for PCR amplification, where the V1–V3 primers and the PCR master mix were used. It is well known that the V1–V3 regions of the 16S rRNA gene have a higher resolution for lower-rank taxa (genera and species); therefore, using primers targeting these regions allows for a more precise distance-based clustering of reads, which were then clustered into species-level amplicon sequence variants (ASVs) (Bukin et al., 2019). Finally, the PCR products were purified using the magnetic bead of Agencourt AMPure XP and dissolved in elution buffer. The range of the fragment in the library was tested by Agilent 2100 Bioanalyzer. The libraries passing quality control were sequenced by HiSeq 2000 platform. The primers for the V1–V3 regions were listed as follows: 8F-'AGAGTTTGAT[YM]TGGCTCAG', 518R-'ATTACCGCGGCTGCTGG'. Y and M represent bases C/T and C/G, respectively.

Quantitative Real-Time Polymerase Chain Reaction

Concentration of DNA from uterine cavity samples of 15 infertile patients and 15 healthy controls, and four reagents for DNA extraction, product purification, exonuclease, and DNA sequencing, were measured spectrophotometrically using a NanoDrop 2000 (Thermo Fisher, USA). The four reagents were used as negative controls. The real-time PCR assay was performed using primers to amplify the 16S rRNA genes and beta-actin. The primers were listed as follows (Ma et al., 2013): *Lactobacillus crispatus*: forward primer 5′-AGCGA GCGGAACTAACAGATTTAC-3′, reverse primer 5′-AGCTGATCATGCGATCTGCTT-3′; *Lactobacillus iners*:

forward primer 5′-AGTCTGCCTTGAAGATCGG-3′, reverse primer 5′-CTTTTAAACAGTTGATAGGCATCATC-3′; beta-actin: forward primer 5′-AAAAGCCACCCCACTTCTCT-3′, reverse primer 5′-CTCAAGTTGGGGGACAAAAA-3′. The 20-μl PCR mixture contained 1 μl of DNA sample, 1 μl of each primer, 6 μl of ultra-pure water and 12 μl of 2*SYBR Green Mix. The Eppendorf realplex system (Eppendorf, USA) was used with the thermal cycling profile of 95°C for 5 min, and 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Each sample had three technical replicates. The abundance of the bacterium was calculated by dividing the average CT value of 16S rRNA gene by that of beta-actin.

Cleaning the Raw Sequencing Data

The following steps were carried out to process the raw data: 1) discarding the reads of low base-quality: set 30 bp as the window length, and if the average quality of the window is lower than 17, truncate the end sequence of reads from the beginning of the window and remove the reads whose final read length is lower than 75% of the original read length; 2) discarding the reads contaminated by adapters: the default adapter sequence has an overlap of 15 bp with the read sequence, set it to 15 bp, and allow a mismatch of 3; 3) discarding reads with Ns; and 4) discarding reads with low complexity: the length of consecutive occurrences of a base in reads is ≥10. The resulting fastq format data were termed as clean data.

Amplicon Sequence Variants and Taxonomy Analysis

The 16S rRNA clean data were processed by DADA2 package (Callahan et al., 2016) in R. DADA2 provides a sensitive and specific workflow in amplicon sequencing. The DADA2 pipeline proceeds as follows: 1) filter and trim clean data: discarding reads at the first instance of a quality score less than or equal to 2; 2) remove duplicated sequence entries in fastq files; 3) merge paired reads; 4) learn the error rates and infer the sample composition using the error rates; 5) construct a sequence table; 6) remove chimeras; and 7) assign taxonomy using naive Bayesian classifier method and the RDP_16sRNA reference databases.

Normalizing the Relative Abundance of Bacteria

For each sample, we normalized the abundance of each bacterium at a genus or species level by dividing its raw count by the total number of read counts. The resulting proportions for each bacterium were used as the normalized abundance. Furthermore, the bacteria that accounted for less than 0.5% were combined and termed as "others". The bacteria that accounted for over 0.5% in at least two samples were retained for further data analyses.

Diversity Analysis

The alpha-diversity analysis was performed by R vegan package (Dixon, 2003). The Shannon index was used to evaluate the alpha-diversity of the microbiota. The Sorensen index was calculated by dividing the number of shared bacteria between the two samples by the total number of bacteria from those two samples.

The Functional Prediction of Microbiota

Tax4fun (Asshauer et al., 2015) package in R was used to estimate Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) scores for each sample, and the scores were further used to predict the relative activity of KEGG pathways.

The Differential Abundance Analysis

The microbiota abundances between two groups were compared using Wilcoxon rank-sum test. The *p*-values were adjusted by the Benjamini and Hochberg method to avoid multiple testing (Benjamini and Hochberg, 1995). The Kruskal–Wallis test was applied to make multiple comparisons between groups.

The Unsupervised Clustering Analysis

The unsupervised clustering analysis was conducted in R hclust. The Euclidean distance was used to measure the distance between samples based on the bacterial relative abundances. The dendrogram was determined by Ward clustering algorithm.

RESULTS

The Microbiome Landscape of the Vagina, Cervix, and Uterine Cavity

To explore the microbiome landscape in the genital tract of reproductive-aged women, we recruited 184 reproductive-aged women and collected the swab samples from the vagina, the cervix, and the uterine cavity. Since some subjects refused to be sampled from the uterine cavity, ultimately, we only retained 170 vaginas, 107 cervix, and 40 uterine cavity samples for 16S rRNA sequencing with a stringent quality control (**Supplementary Data 1**). As shown in **Figure 1A**, 97 subjects had matched vagina and cervix samples, while 36 paired vagina and uterine cavity samples were collected from the same patients.

As the microbial samples in the uterine cavity had low biomass, we first excluded the bacteria potentially contaminated by "kitome" from a previous study (Salter et al., 2014). The microbiome profile analysis revealed that *Lactobacillus* and *Prevotella* were dominant in the three sites of reproductive-aged women (**Figure 1B** and **Supplementary Data 2**). Specifically, in 119 out of 170 vagina samples and 57 out of 111 cervix samples, *Lactobacillus* genus was observed to account for over 90% of the community. Moreover, the bacterial diversity within the microbiota was much higher in the uterine cavity than in both the vagina and cervix (**Figure 1C**). The non-metric multidimensional scaling (NMDS) analysis of the relative abundance of bacteria revealed that the vagina and cervix showed high similarity in the microbiota composition, while both their relative abundance was significantly different from that of the uterine cavity (**Figure 1D**). Furthermore, we measured the similarities of the microbial composition across the three sites of the reproductive tract by calculating the Sorenson indices between the paired samples from each individual. Consistently, higher similarities were observed in paired cervix–vagina samples than

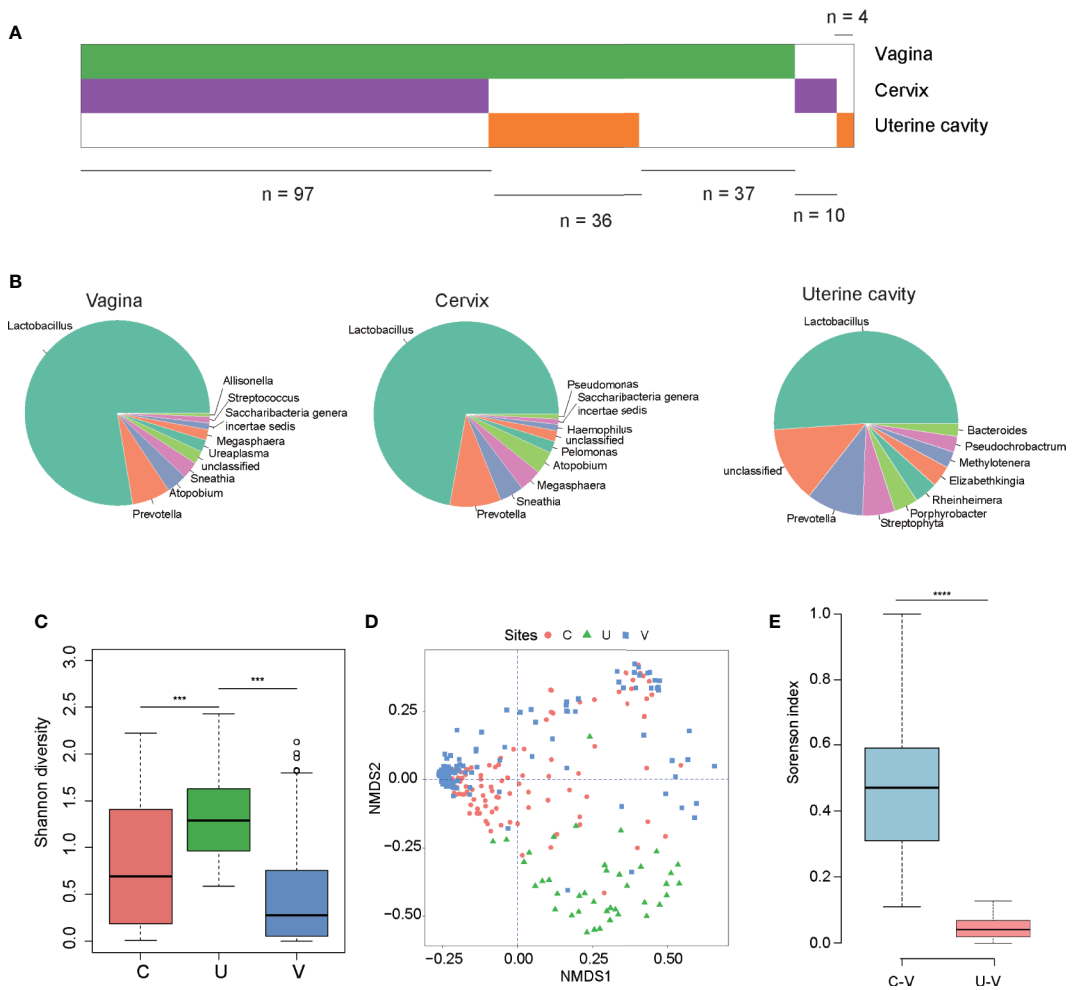

FIGURE 1 | Microbiome landscape in the genital tract of reproductive-aged women. **(A)** An overview of the sample collections (97 paired vagina–cervix samples and 36 paired vagina–uterine cavity samples). **(B)** Pie chart for 10 genera with the highest abundance at each site according to the mean relative abundance. **(C)** The Shannon diversity among the three sites of female genital tract (FGT). **(D)** Comparison of community for non-metric dimensional scaling (NMDS) ordination of the Bray–Curtis distance between sampling sites (V, vagina; C, cervix; U, uterine). **(E)** Intraindividual similarity between vagina–cervix and vagina–uterine. ***P-value < 0.001, ****P-value < 0.0001.

in the paired uterine cavity–vagina samples taken from the same recruits (**Figure 1E**, Wilcoxon rank-sum test, p-value < 0.05), suggesting that the microbial composition in the uterine cavity might be different from that in other sites of the female reproductive tract.

Clustering and Characterization of the Reproductive-Aged Women by the Genital Tract Microbiomes

To further investigate the composition of microbiota in the genital tract of reproductive-aged women, we first conducted hierarchical clustering analyses for the vagina, cervix, and uterine cavity samples, which were based on the relative abundance of the bacteria at the species level (**Supplementary Data 2**). Overall, the vagina samples were classified into four groups denoted by I, II, III, and IV with 36, 47, 68, and 19 samples (**Figures 2A, B**), respectively. Group I was characterized by a large proportion of *Atopobium vaginae*, *Mobiluncus mulieris*, *Prevotella timonensis*, *Prevotella amnii*, and *Prevotella buccalis*. The vagina microbiota in groups II and III were dominated by *L. crispatus* and *L. iners*, which accounted for over 90% of the community in 44/47 and 41/68 samples of these two groups, respectively. Notably, group III and *L. iners* subgroup, as described in a previous study, exhibited consistent microbial composition (Ravel et al., 2011). In addition, group IV was dominated by several microbiomes such as *Lactobacillus fornicalis*, *Lactobacillus gasseri*, *Lactobacillus delbrueckii*, and *Lactobacillus acidophilus*. As a result, the four groups were termed as *Heterogeneous*, *L. crispatus*, *L. iners*, and *L.Heterogeneous*.

Similarly, the cervix (**Supplementary Figure 1**) and uterine cavity samples were also stratified into four and three subgroups using unsupervised clustering, respectively. Among the

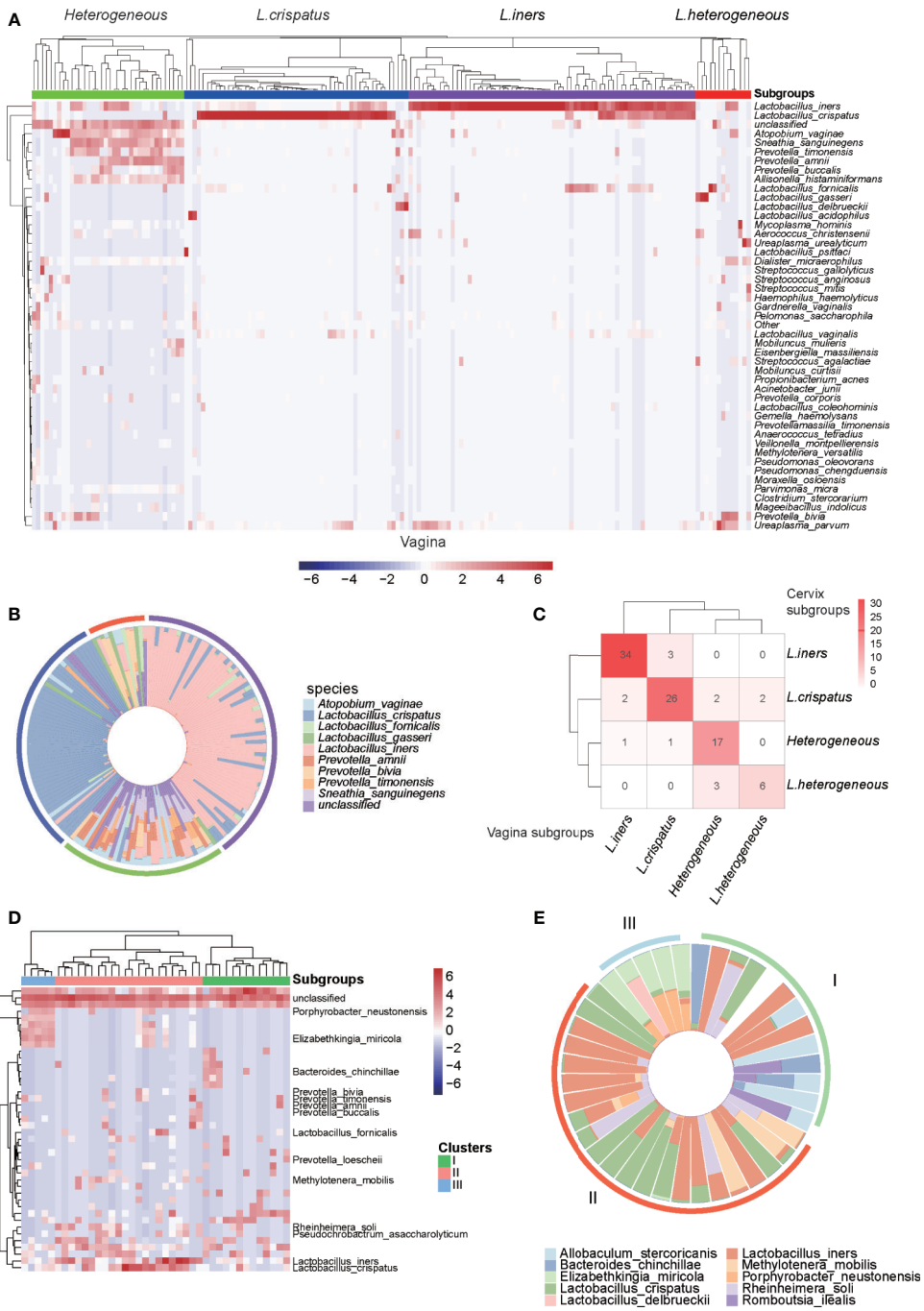

FIGURE 2 | Microbiome profiles of the reproductive-aged women. **(A)** Heatmap of log10-transformed relative abundance of species found in the vaginal bacterial communities. **(B)** Species-level vaginal microbiome composition in each vagitype (10 species with the highest mean relative abundance were selected). **(C)** "Confusion matrix" was used to evaluate concordance between groupings of the vagina and cervix. **(D)** Heatmap of the uterine bacterial communities. **(E)** Species-level vaginal microbiome composition in each uterine microbiota communities type (10 species with the highest mean relative abundance were selected).

97subjects with paired vaginal and cervical samples, 34, 26, 17, and 6 subjects were classified as *L. iners*, *L. crispatus*, Heterogeneous, and L.Heterogeneous subgroups, respectively, based on both vaginal and cervical microbiota. Taken together, they accounted for 85.57% of total subjects and suggested that the two groupings were highly consistent (**Figure 2C**). However, the bacterial samples from the uterine cavity were clustered into three subgroups (**Figure 2D**), which greatly differed from both vaginal and cervical subgroupings. The first subgroup (subgroup I) in the uterine cavity was characterized by *L. iners*

and *Allobaculum stercoricanis* (**Figure 2E**). Particularly, after investigating the clinical information of the samples, we found that all of those 10 infertile recruits were classified into this subgroup. The samples in subgroup II were colonized by two *Lactobacillus* species, *L. crispatus* and *L. iners* (**Figure 2E**). The samples in subgroup III were dominated by *Porphyrobacter neustonensis* and *Elizabethkingia miricola* (**Figure 2E**).

Furthermore, the alpha-diversity analysis of vaginal and cervical microbiomes revealed that the *L.Heterogeneous* and *Heterogeneous* groups showed higher diversity than the other two subgroups (**Supplementary Figure 2**, Kruskal–Wallis test, p-value < 0.0001). Similarly, the highest diversity was observed in subgroup I in the uterine cavity, followed by subgroups II and III (**Supplementary Figure 2**, Kruskal–Wallis test, p-value < 0.0001). From these results, we have disclosed that the microbiomes in the genital tract of reproductive-aged women varied greatly among individuals and body parts.

In addition, we also investigated the correlation between groupings and clinical characteristics. Specifically, we found that samples of *L. iners* had a slightly higher body mass index (BMI) than those of Heterogeneous subgroup (**Supplementary Figure 3**, t-test, p-value = 0.09).

Site-Specific Microbiota in the Female Genital Tract

As the microbiota in the three sites of the FGT differed from one another, we then compared the microbiota between the three sites at both genus and species levels. Specifically, the proportions of *Lactobacillus* were higher in the vagina and cervix than the uterine cavity (**Figure 3A**, Wilcoxon rank-sum test, p-value < 0.05). In contrast, samples from the cervix and the uterine cavity had a higher proportion of *Prevotella* than those from the vagina (**Figure 3A**, Wilcoxon rank-sum test, p-value < 0.05). Further analyses of the species of the two genera revealed that *L. iners* and *L. crispatus* were more abundant in the vagina and cervix than the uterine cavity, while the cervix had a higher proportion of *P. timonensis* than the vagina and the uterine cavity (**Figure 3B**, Wilcoxon rank-sum test, p-value < 0.05). These results further indicated that the uterine cavity had a microbiome profile distinct from that of the vagina and cervix.

Identification of the Microbiota With Changed Abundance in Infertile Women

As the 10 infertile patients were assigned to the same subgroup based on the composition of the uterine cavity microbiota, we then attempted to explore the microbiomes changed in these samples and their association with female infertility. As shown in **Figure 4A**, the infertile patients could be clearly differentiated from the healthy controls by their uterine cavity microbiota, but this difference was not observed when comparing their vaginal microbiota data using the principal coordinates analysis (PCoA). Similarly, the differences in the top two principal coordinates between infertile patients and healthy controls were also observed in the microbiota data of the uterine cavity (p-value < 0.05), but not in those of the vagina (the p-values of Wilcoxon rank-sum test for PC-1 and PC-2 were 0.16 and 0.98, respectively). These results indicated that it was not the microbiota in the vagina but the uterine cavity microbiota that have the potential to discriminate the infertile patients from healthy subjects and may be implicated in infertility.

Consistently, no certain bacterial species were observed to be significantly dominant or rare in the vagina of infertile patients (p < 0.05) when compared with healthy controls, further suggesting that the microbiota in the vagina of infertile patients and healthy reproductive-aged women showed no significant difference. On the contrary, *L. iners* and *L. crispatus* were significantly reduced in the uterine cavity of 10 infertile patients (**Figure 4B**, Wilcoxon rank-sum test, p-value < 0.05), suggesting that these bacterial species might be associated with female infertility. To verify the differential abundance of the two bacterial species between infertile patients and healthy controls, we quantified the abundance of these species in the uterine cavity

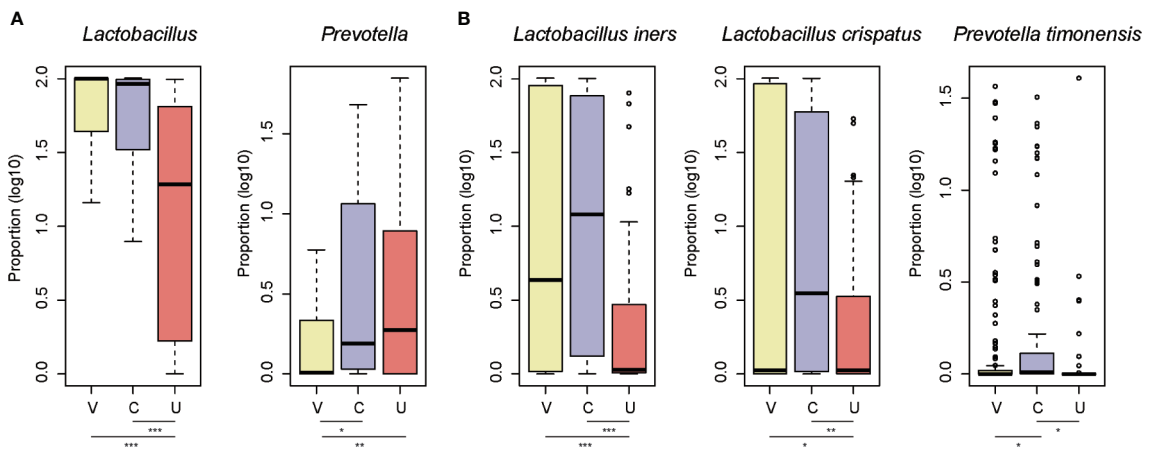

FIGURE 3 | The site-specific genus and species in the three sites of female genital tract. The signature genus for the vagina/cervix **(A)** and uterine cavity **(B)**. The three sites are abbreviated as V, C, and U. The points on the top of the boxes represent the outliers. *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001.

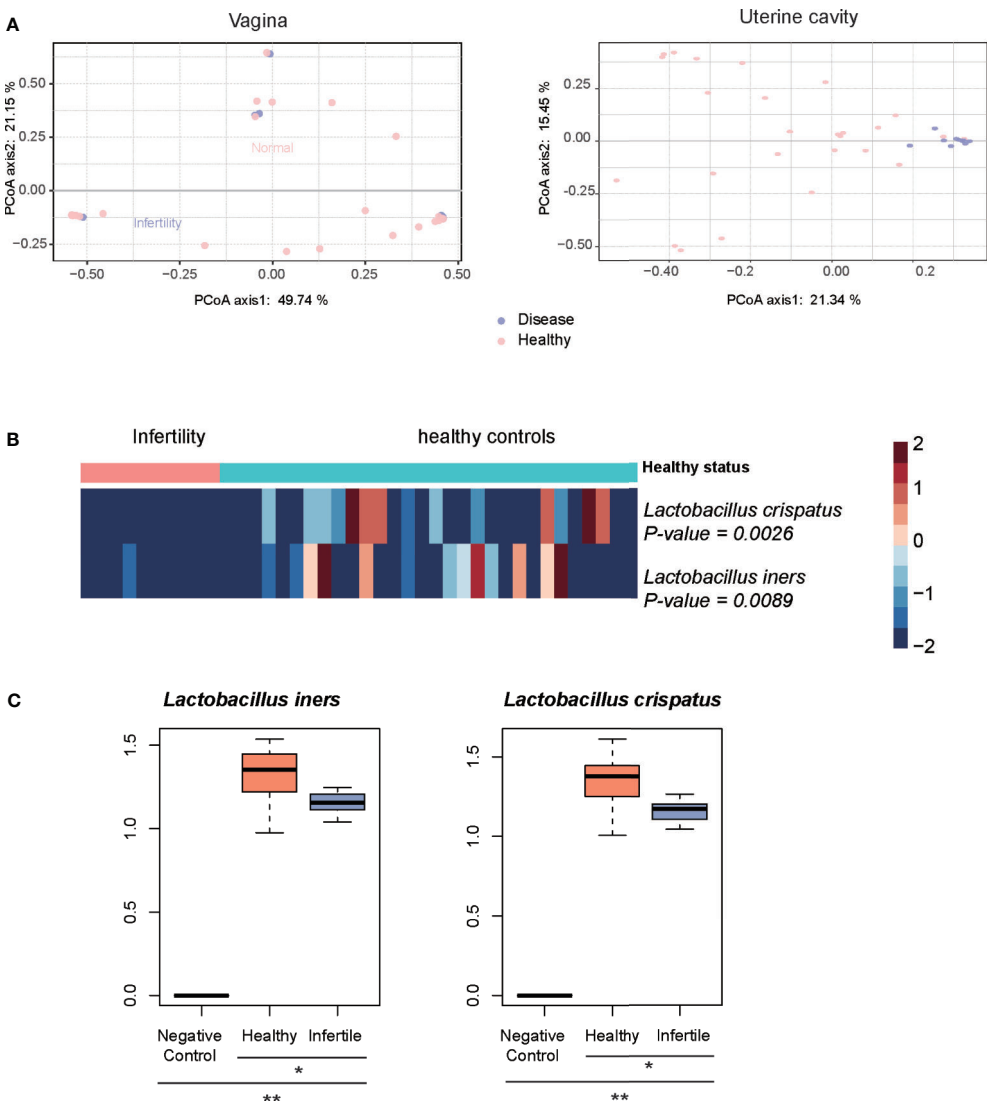

FIGURE 4 | Characteristics of microbiota in infertility of reproductive-aged women. **(A)** Principal coordinates analysis (PCoA) on the Bray–Curtis distance at the species level for all taxonomic profiles at the vagina and uterine. **(B)** Heatmap of relative abundance of species scaled from –2 to 2 in the infertility and healthy controls communities. **(C)** The difference of abundances of two *Lactobacillus* species between the infertile patients, healthy controls, and negative controls by qPCR method. *P-value < 0.05, **P-value < 0.01.

of 15 infertile patients and 15 healthy controls using qPCR. Consistently, the abundances of two *Lactobacillus* species, *L. iners* and *L. crispatus*, were significantly decreased in the infertile patients as compared with the healthy controls (**Figure 4C**, p-value < 0.05). These results indicated that reduced abundance of *L. iners* and *L. crispatus* in the uterine cavity might be associated with female infertility.

Functional Inferences of the Female Infertility by the Microbiota in the Uterine Cavity

As the microbiota in the uterine cavity were significantly altered in infertile patients, we aimed to explore the potential biological functions associated with this disease. The relative abundances of KO groups were estimated based on the microbiota in the uterine cavity by *tax4fun* method (**Supplementary Data 3**). Specifically, KOs in amino acid metabolism, metabolism of cofactors and vitamins, and biosynthesis of other secondary metabolites were observed to be more active in infertile patients (**Figure 5**). However, xenobiotics biodegradation and metabolism were observed to be downregulated in the uterine cavity, suggesting that the capability of degrading xenobiotics like chloroalkene and xenobiotics metabolized by cytochrome P450 in infertile patients might be decreased. Notably, two signaling pathways, neurotrophin signaling pathway and adipocytokine signaling pathway, were significantly enriched by those bacterial species

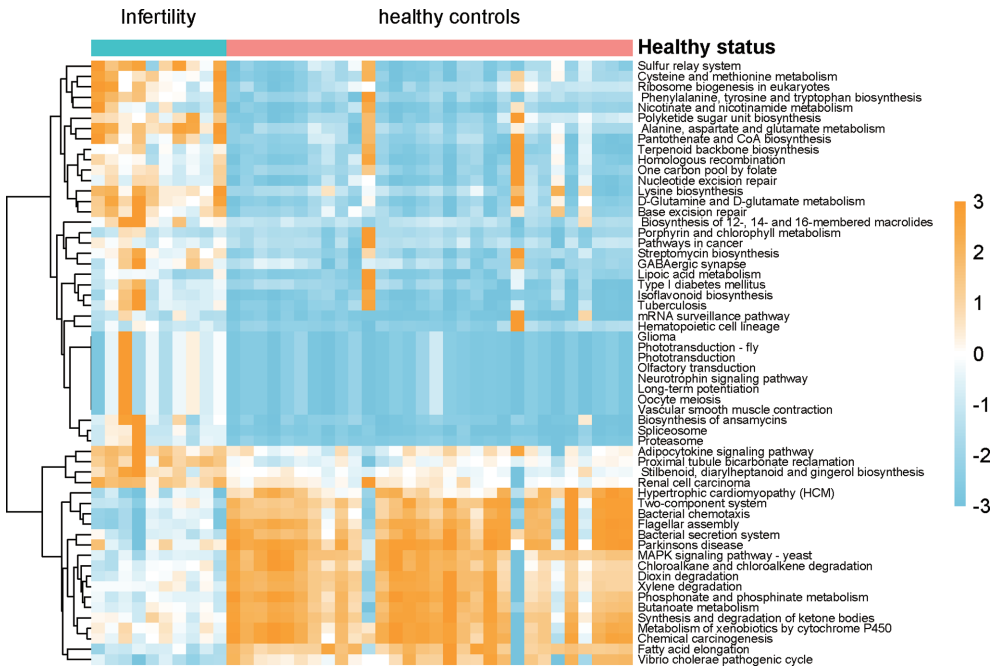

FIGURE 5 | The differences of biological function between infertility and healthy controls of reproductive-aged women. The pathways were predicted by the relative abundances of microbiota, Wilcoxon test was used the compare the differences, and the resulting *p*-values were adjusted with false discovery rate (FDR) method.

found in infertile patients. Both neurotrophin and adipocytokine were secreted proteins and involved in the regulation of several physiological and pathological processes, indicating their potential implications in the pathogenesis of infertility.

DISCUSSION

The microbiota in the human body play a critical role in maintaining our daily wellbeing and are associated with several physiological and pathological processes. As it is difficult to directly sample the upper reproductive tract, the diversity of the microbiota in the FGT has not been fully unveiled.

In this study, we conducted a strict sampling method to avoid the bacterial contamination between the vagina and the uterine cavity, aiming to characterize the microbiome variations of the female reproductive tract, especially of the uterine cavity, in reproductive-aged Chinese women. The microbiome profiling revealed that *Lactobacillus* accounted for the majority of the microbiota in the vagina and cervix of reproductive-aged women. In 119 of 170 vagina samples and 57 of 111 cervix samples, *Lactobacillus* was observed to comprise over 90% of detected species. It is well-recognized that *Lactobacillus* is a protective bacteria in the FGT, and its reduced proportion is associated with several gynecological and obstetric disorders like infertility (Moreno and Simon, 2018), preterm delivery (Elovitz et al., 2019; Fettweis et al., 2019), and even gynecological cancers (Wang et al., 2018; Nene et al., 2019).

The uterine cavity has been classically considered a sterile site (Simon, 2018). Based on the 16S rRNA gene sequencing analysis, we found that the uterine cavity was dominated by several microbiomes, such as *Lactobacillus*, *Prevotella*, *Streptophyta*, *Porphyrobacter*, and *Rheinheimera*. Compared with the vagina and cervix, the uterine cavity had a higher diversity and significantly distinct microbiota compositions. It has been reported that the uterine cavity microbial colonization originated from the vagina (Moreno and Franasiak, 2017), and cervical mucus with high concentrations of inflammatory cytokines, immunoglobulins, and peptides with antimicrobial properties could result in lower biomass and higher diversity in the uterine cavity (Franasiak and Scott, 2015). Accordingly, our data indicated that the alpha-diversity of the uterine cavity microbiota was significantly higher than that of the vagina and cervix (**Supplementary Figure 2**), suggesting that the high diversity might be associated with low biomass caused by the antimicrobial effect of inflammatory factors in the FGT. The comparison of the microbiota abundance between the three sites of the FGT revealed that two genera, *Lactobacillus* and *Prevotella*, as well as their species including *L. iners*, *L. crispatus*, and *P. timonensis*, were enriched in one of the three sites. A previous study reported that *Lactobacillus* was rare in the endometrium and that its presence might be contaminated by vaginal *Lactobacillus* (Winters et al., 2019). To avoid this contamination, we used embryo transfer catheter to take samples from the FGTs following a previous study (Franasiak et al., 2016). Therefore, we speculated that *Lactobacillus* genus was rare but dominant in the uterine cavity.

Based on the relative abundances of the microbiota, reproductive-aged women were stratified by the microbiota detected in three sites of the reproductive tract. To our knowledge, this is the first study to stratify reproductive-aged women by the microbiome profiles in Chinese population. In accordance with the high similarity of microbiota compositions between the vagina and the cervix, the groupings based on the microbiota between the vagina and the cervix were also highly concordant. The differential abundance analysis revealed that subgroup I in the vagina and cervix samples was characterized by a high proportion of strictly anaerobic bacteria like *A. vaginae*, *M. mulieris*, *P. timonensis*, *P. amnii*, and *P. buccalis*; and a high consistency was found between group I and group IV, which was defined by a previous study (Ravel et al., 2011). The remaining subgroups had a high proportion of *Lactobacillus* genus and were dominated by unique *Lactobacillus* species. Notably, group III was consistent with the *L. iners* group reported by a previous study (Ravel et al., 2011). Similarly, the uterine cavity subgroups were dominated by different microbiomes. Particularly, all 10 infertile patients were clustered into the same subgroup, suggesting that the uterine cavity of the infertile patients might have unique microbial composition. Furthermore, the comparison of the microbiota in the three sites between the infertile patients and healthy controls revealed that the microbiome of the uterine cavity had more obvious difference between the patients and healthy controls than that of the vagina or cervix. To explore the microbiota with changed abundance in the uterine cavity of infertile female, we compared the uterine cavity microbiota of the 10 infertile patients with those of healthy controls. Unfortunately, the potentially pathogenic bacteria of female infertility dominant in uterine cavity still could not be accurately identified in this study due to the low biomass in the uterine cavity. In contrast, the proportions of two probiotic bacteria, *L. iners* and *L. crispatus*, were significantly reduced in the uterine cavity of 10 infertile patients, which had been verified by qPCR method. Collectively, decreased proportions of *L. iners* and *L. crispatus* were associated with female infertility (Zhang et al., 2020).

Functionally, microbiota-related KO predictions for the uterine cavity revealed that metabolism-related pathways were predominantly dysregulated in infertile patients. Moreover, two signaling pathways, neurotrophin signaling pathway and adipocytokine signaling pathway, were significantly enriched by the microbiota in infertility patients. Notably, neurotrophins, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) in follicular fluid of women have been used for different infertility diagnoses (Sadeu et al., 2012).

However, the present study still has some limitations. First, the contaminations could not be excluded thoroughly due to lack of negative controls. Second, higher sequencing depth is necessary for the low-biomass samples. Overall, we characterized a comprehensive landscape of the microbiome in Chinese female reproductive tract, as well as explored the microbiota with changed abundance in infertile and biological functions potentially involved in female infertility. In summary, the present study provided microbiota reference for the healthy reproductive-aged women and had potential values for scientific and clinical applications in female infertility.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the The National Omics Data Encyclopedia (NODE) repository, accession number OEP002094.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Shanghai Changzheng Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WL and NS designed this study. NS, HD and HY conducted the experiments. NS, XX, WP, and XW conducted the data analysis. NS, HD, and QZ performed the data visualization. NS, YJ, and HY contributed to the writing of the paper and setting of figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.649067/full#supplementary-material

Supplementary Figure 1 | The microbiome profiles of the cervix in reproductive-aged women. **(A)** The heatmap of log10-transformed relative abundance of species found in the cervical bacterial communities. **(B)** Species-level vaginal microbiome composition in cervix (ten species with the highest mean relative abundance were selected).

Supplementary Figure 2 | The difference of alpha-diversity between the subgroups of vagina, cervix, and uterine.

Supplementary Figure 3 | The association of the groupings with the clinical characteristics.

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High Prevalence of *Lactobacillus crispatus* Dominated Vaginal Microbiome Among Kenyan Secondary School Girls: Negative Effects of Poor Quality Menstrual Hygiene Management and Sexual Activity

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The vaginal microbiome (VMB) impacts numerous health outcomes, but evaluation among adolescents is limited. We characterized the VMB *via* 16S rRNA gene amplicon sequencing, and its association with Bacterial vaginosis (BV) and sexually transmitted infections (STIs; chlamydia, gonorrhea, trichomoniasis) among 436 schoolgirls in Kenya, median age 16.9 years. BV and STI prevalence was 11.2% and 9.9%, respectively, with 17.6% of girls having any reproductive tract infection. Three community state types (CST) accounted for 95% of observations: CST-I *L.crispatus*-dominant (N=178, BV 0%, STI 2.8%, sexually active 21%); CST-III *L.iners*-dominant (N=152, BV 3.3%, STI 9.7%, sexually active 35%); CST-IV *G.vaginalis*-dominant (N=83, BV 51.8%, STI 25.3%, sexually active 43%). In multivariable adjusted analyses, sexually active girls had increased odds of CST-III and CST-IV, and use of cloth to manage menses had 1.72-fold increased odds of CST-IV *vs.* CST-I. The predominance of *L.crispatus*-dominated VMB, substantially higher than observed in prior studies of young adult and adult women in sub-Saharan Africa, indicates that non-optimal VMB can be an acquired state. Interventions to maintain or re-constitute *L.crispatus* dominance should be considered even in adolescents.

Keywords: vaginal microbiome, bacterial vaginosis (BV), sexually transmitted infection (STI), menstrual health, menstrual hygiene management, adolescents and youth, Sub-Saharan Africa (SSA)

INTRODUCTION

Globally, adolescent girls and young women account for at least one-third of the 357 million curable sexually transmitted infections (STIs) occurring each year (Blum and Nelson-Mmari, 2004; Newman et al., 2015; Population Reference Bureau, 2021). STIs are syndemic with HIV. UNAIDS reports that 15% of all women living with HIV are aged 15–24 years old, 80% of whom live in sub-Saharan Africa, and 31% of new HIV infections are among adolescent girls (Amornkul et al., 2009). In western Kenya, HSV-2 increases dramatically from 10% in 13-14 year-old girls to 28% in 15-19 year-olds (Otieno et al., 2015; Kenya Ministry of Health National AIDS and STI Control Programme (NASCOP), 2020). HIV prevalence increases from 1.2% among 15-19 year-old girls to 3.4% of those aged 20-24, compared to 0.5% and 0.6% for males of the same age (Amornkul et al., 2009). Among adolescent girls, the HIV/STI epidemic overlaps with broader reproductive health concerns. For example, to attend school and obtain necessities such as sanitary products, soap and underwear, girls often engage in exchange sex (Phillips-Howard et al., 2015). Menstrual hygiene management (MHM) is a pervasive problem across low- and middle-income countries and a lack of MHM materials negatively impacts girls' health and schooling (Sommer et al., 2016). Phillips-Howard et al. conducted a cluster randomized study of 644 girls aged 14-16 years old, comparing reusable menstrual cups to control condition of menstrual hygiene counseling (Zevin et al., 2016). After one year, menstrual cup use resulted in 35% reduction (p=0.034) in Bacterial vaginosis (BV) prevalence and 52% reduction (p=0.039) in STI prevalence compared to control condition. This decrease in STIs and BV may have been mediated by sexual practices, or the menstrual cups themselves.

Bacterial vaginosis affects 20-50% of general population women in sub-Saharan Africa (Torrone et al., 2018), and increases the risk of HIV acquisition and transmission (accounting for up to 15% of HIV infections) (Atashili et al., 2008), multiple adverse pregnancy outcomes (Eschenbach et al., 1984; Hay et al., 1994; Ralph et al., 1999), and is consistently associated with chlamydia, gonorrhea, HPV and HSV-2 (van de Wijgert et al., 2014). BV typically represents a vaginal microbiome (VMB) that is highly diverse (i.e., having many different types of bacteria) and depletion of *Lactobacillus* species (McKinnon et al., 2019). Using 16S rRNA gene amplicon sequencing, commonly occurring vaginal community state types (CST) have been identified (Ravel et al., 2011). CST-I (*Lactobacillus crispatus* dominated) has been considered an advantageous state, due to the demonstrated protective mechanisms of *L. crispatus* [e.g., maintaining acidic vaginal pH, inhibiting growth of pathogenic bacteria, activating immune cells, production of antibacterial substances, etc. (Lewis et al., 2017; Kovachev, 2018)], and due to the consistent protective association of *L. crispatus* against BV, HIV, HPV and other sexually transmitted infections (STIs) (van de Wijgert et al., 2014; McKinnon et al., 2019). On the other hand, CST-IV (a high diversity vaginal community that is usually depleted of lactobacilli) is considered a non-optimal vaginal microbiome, or "molecular BV" (McKinnon et al., 2019), and has been associated with epithelial barrier disruption and enhanced immune activation, even in the absence of clinical BV diagnosis (Zevin et al., 2016). The VMB in relation to menstrual hygiene practices and period characteristics (e.g., duration, cramping, flow) has not been rigorously assessed, and is especially lacking among adolescent girls.

We are currently evaluating the effect of menstrual cups on the VMB, BV, and STIs among secondary schoolgirls enrolled in a cluster randomized controlled trial in Siaya County, western Kenya (Zulaika et al., 2019). In the current analysis, we characterized the baseline VMB and factors associated with VMB composition in relation to sexual activity, MHM practices and menstrual characteristics, and presence of BV or STIs.

MATERIALS AND METHODS

This study was approved by the institutional review boards of the Kenya Medical Research Institutes (KEMRI, SERU #3215), Liverpool School of Tropical Medicine (LSTM, #15-005), and University of Illinois at Chicago (UIC, #2017-1301).

Study Setting

This study used baseline data and biological specimens from the Cups and Community Health (CaCHe, pronounced "Cash-Ay") study, a prospective cohort study of adolescent secondary school girls in Siaya County. The CaCHe study is nested in Cups or Cash for Girls (CCG), a large cluster randomized controlled trial assessing the impact of menstrual cups and cash transfer interventions on a composite outcome of school dropout, HIV and HSV-2 (Zulaika et al., 2019) (ClinicalTrials.gov NCT03051789). Siaya County area is positioned 400 km west of Nairobi, adjacent to Lake Victoria. Siaya County is largely rural and Bondo is the largest town with approximately 35,000 inhabitants. From the most recent Demographic and Health Survey, in Siaya County, women's median age of first sexual intercourse is 16.6 years (versus 19.3 years for Nairobi and 18.0 years nationally), 19.1 years for first marriage (versus 22.1 years for Nairobi and 20.2 years nationally), and 18.4 years for first birth (versus 22.7 years for Nairobi and 20.3 years nationally) (Kenya National Bureau of Statistics and Kenya Ministry of Health, 2014). Among adult women surveyed, 15.1% had completed secondary education or more in Siaya County, compared to 51.4% of women in Nairobi and 26.9% nationally. The prevalence of HIV among adult women in the area was estimated at 25.3% in 2015 (Kenya Ministry of Health, 2021). In Health and Demographic Surveillance (HDSS) rounds taking place 2011 to 2016, HIV incidence between rounds among 15-24 year-old women and girls was 8.9%, compared to 3.2% among boys and men of same age (Borgdorff et al., 2018).

Study Design and Participants

The CCG trial is an open-label, 4-arm, school-cluster randomized controlled superiority trial. Schools were allocated into 4 arms

(1:1:1:1) *via* block randomization: (1) provision of menstrual cups with training on safe cup use and care; (2) conditional cash transfer (CCT) based on >80% school attendance in previous term; (3) menstrual cup and CCT; and (4) usual practice. All girls received puberty and hygiene education.

For the CaCHe study, nested within the CCG trial, we aimed to enroll 20% of girls in the cup only and control arms of the CCG trial. Eligibility for CaCHe followed eligibility for CCG: attendance at a selected school, being a resident of the study area, provision of assent and parental/guardian consent, and girls had to report established menses (> 3 times). Girls were excluded if they declared pregnancy at baseline.

Data Collection

Following written informed parental consent and assent from minors, participants self-completed a tablet-based survey in their language of choice (English or DhoLuo) to obtain socio-demographic information and to assess sexual and MHM practices. Study nurses and counsellors trained in research and survey administration provided assistance or conducted interviews as needed. Socio-demographic data included age and assessment of household amenities, including water source, light source, latrine type, and possession of a television. Household mobile phone possession was 98% and was not used in the analyses. A household amenity score having range 0-4 was created, with one pint each for piped water source, electricity for light source, flush toilet, and possession of a television. Ever having sexual intercourse was assessed in two questions to differentiate forced sex from willing sex, and *via* a series of questions around exchange sex (sex in exchange for money, goods, or favors).

Sample Size

CaCHe was designed to estimate the effect of menstrual cups on girls' risk of BV, with an anticipated cumulative event rate of 30-40% among controls occurring over 30 months. In a design of 6 repeated measurements having AR(1) covariance structure, correlation between observations on the same subject ranging 0.25 to 0.4, and accounting for 20% loss to follow-up, group sample sizes of 220 in cup arm and 220 in control arm would achieve >80% power to detect 25% reduced prevalence of BV for the cup arm compared to control arm when BV prevalence is 30%, and 97% power when prevalence is 40% [p=0.05 two-sided test, two proportions in a repeated measures design; PASS v15 (Hintze, 2014)].

Specimen Collection

At baseline and each follow-up visit, girls were asked to take four self-collected vaginal swabs. The first swab obtained was for 16S rRNA gene amplicon sequencing (microbiome), the second for BV, the third for detection of *C. trachomatis* (CT) and *N. gonorrhoeae* (NG), and the fourth for detection of *T. vaginalis* (TV). Prior to vaginal swab collection, girls were given oral and graphic instruction on how to collect the swabs. Girls were instructed to insert each swab approximately 2-3 centimeters into the vaginal opening and to twirl the swab for 20 seconds. Each girl obtained her swabs in a private, enclosed area, with a nurse or female field assistant aiding girls with sample collection one on one. The nurses or research assistant handed the swabs to the girls sequentially, timing each collection for 20 seconds while girls were instructed to twirl, and then retrieving before passing the next swab. Nurses and research assistants prepared smears for BV immediately, with a lab assistant checking each slide for sufficiency after air drying. Swabs for amplicon sequencing, CT/NG, and TV were immediately placed on ice packs in coolers for transport.

Detection of Bacterial Vaginosis, Sexually Transmitted Infections, and HIV

Upon receipt at the lab, specimens for amplicon sequencing were placed at -80° C until shipment to Chicago for processing. Vaginal swabs for amplicon sequencing were collected using OMNIgene Vaginal kits (OMR-130; DNA Genotek^{TM}). Swabs for CT/NG were shipped weekly for processing at the University of Nairobi Institute for Tropical and Infectious Diseases (UNITID). Following manufacturer protocol, vaginal swabs were tested for CT/NG using the GeneXpert (Cepheid, Sunnydale, California, US). Swabs for TV were processed immediately upon receipt using the OSOM TV antigen detection assay (Sekisui, Lexington, MA, US). Air-dried smears prepared from self-collected vaginal swabs were Gram stained and evaluated according to Nugent's criteria within 48 hours of receipt; a score of 7-10 was defined as BV (Nugent et al., 1991). Finger-stick whole blood collected in EDTA tubes were tested for HIV according to Kenyan national guidelines (National AIDS and STI Control Programme, 2015). HIV positive girls were linked to care.

STI and BV Treatment

CT, NG, and TV were treated following Kenyan National guidelines (National AIDS et al., 2015). Treatment of BV was with 2g of tinidazole once daily for two days. While not a specified regimen in the Kenyan national guidelines, we followed this alternative treatment recommendation as per U.S. Centers for Disease Control and Prevention (CDC, 2015), British Association for Sexual Health and HIV (BASHH, 2021), and International Union against Sexually Transmitted Infections/ World Health Organization (IUSTI/WHO) (Sherrard et al., 2018), due to concerns of greater likelihood of gastrointestinal symptoms and decreased adherence with the longer duration regimens for metronidazole. While guidelines currently do not recommend treatment for asymptomatic BV, we treated all girls with Nugent score 7-10 due variability in recognition and reporting of symptoms (Lewis and Laurent, 2020), and potential benefits as reported in BASHH and IUSTI/WHO guidelines (Sherrard et al., 2018; BASHH, 2021), and due to the high proportion of girls reporting vaginal discharge (23%) overall, which did not differ by BV or STI status (**Table 1**). Treatment was documented for 48/49 (98%) of girls with BV, 27/27 (100%) with CT, 6/6 (100%) NG, and 13/14 (93%) TV.

TABLE 1 | Distribution of baseline characteristics by bacterial vaginosis (BV) status and sexually transmitted infection (STI) status.

Variables[3]	Total N=436 n (%)	BV Status[1]		P-value	STI Status[2]		P-value[4]
		Positive, N=49 n (%)	Negative, N=387 n (%)		Positive, N=43 n (%)	Negative, N=393 n (%)	
Socio-Demographics							
Median Age in years (IQR)[5]	16.9 (16.0-17.9)	17.7 (16.5-18.6)	16.9 (16.0-17.7)	<0.001	17.7 (16.0-18.5)	16.9 (16.0-17.8)	0.013
Age in years, categories				0.024			0.021
14-15	52 (11.9)	3 (6.1)	49 (12.7)		3 (7.0)	49 (12.5)	
16	113 (25.9)	9 (18.4)	104 (26.9)		8 (16.0)	105 (26.7)	
17	121 (27.8)	11 (22.5)	110 (28.4)		7 (16.3)	114 (29.0)	
18	96 (22.0)	13 (26.5)	83 (21.5)		16 (37.2)	80 (20.4)	
19-22	54 (12.4)	13 (26.5)	41 (10.6)		9 (20.9)	45 (11.5)	
Latrine Type				0.883			0.181
Flush toilet	50 (11.6)	5 (10.4)	45 (11.8)		7 (17.1)	43 (11.0)	
Traditional pit	197 (45.7)	23 (47.9)	174 (45.4)		17 (41.5)	180 (46.2)	
Ventilated improved pit	171 (39.7)	18 (37.5)	153 (40.0)		14 (34.2)	157 (40.3)	
Bush, field, other	13 (3.0)	2 (4.2)	11 (2.9)		3 (7.3)	10 (2.6)	
Water Source				0.408			0.611
Bore hole	69 (16.1)	4 (8.5)	65 (17.0)		6 (14.6)	63 (16.2)	
Surface	255 (59.3)	33 (70.2)	222 (58.0)		23 (56.1)	232 (59.6)	
Pipe in house	23 (5.4)	2 (4.3)	21 (5.5)		1 (2.4)	22 (5.7)	
Rainwater	83 (19.3)	8 (17.0)	75 (19.6)		11 (26.8)	72 (18.5)	
Source of Light				0.258			0.469
Electricity	95 (22.0)	7 (14.6)	88 (23.0)		8 (19.5)	87 (22.3)	
Kerosene	171 (39.7)	23 (47.9)	148 (38.6)		22 (53.7)	149 (38.2)	
Tin lamp	61 (14.2)	10 (20.8)	51 (13.3)		5 (12.2)	56 (14.4)	
Solar	83 (19.3)	6 (12.5)	77 (20.1)		5 (12.2)	78 (20.0)	
Other	21 (4.9)	2 (4.2)	19 (5.0)		1 (2.4)	20 (5.1)	
Has Television in Home	104 (24.1)	8 (16.7)	96 (25.1)	0.200	8 (19.5)	96 (24.6)	0.468
Median household amenities (IQR): summed score of flush toilet, piped water, electricity, television	0 (0-1)	0 (0-1)	0 (0-1)	0.251	0 (0-1)	0 (0-1)	0.894
Health Status							
HIV Positive	6 (1.4)	3 (6.3)	3 (0.8)	0.020	0 (0.0)	6 (1.5)	>0.999
Reports having vaginal discharge	98 (23.0)	14 (29.2)	84 (22.2)	0.282	10 (25.0)	88 (22.8)	0.753
Reports having pain on urination	27 (6.3)	6 (12.5)	21 (5.6)	0.063	6 (15.0)	21 (5.4)	0.018
Past 6 months, been to health facility	246 (57.1)	30 (62.5)	216 (56.4)	0.421	25 (61.0)	221 (56.7)	0.596
Reported antibiotic use past 30 days	85 (20.0)	9 (18.8)	76 (20.1)	0.825	7 (17.5)	78 (20.2)	0.683
Body mass index, median (IQR)	21.6 (20.0-23.3)	22.6 (21.4-24.5)	21.4 (19.9-23.2)	<0.001	21.5 (20.0-23.6)	21.6 (20.0-23.3)	0.799
Body mass index, category				0.038			0.085
Underweight (<18)	25 (5.8)	0 (0.0)	25 (6.6)		5 (12.2)	20 (5.2)	
Normal (18-25)	350 (81.8)	37 (78.7)	313 (82.2)		29 (70.3)	321 (83.0)	
Overweight or obese (>25)[a]	53 (12.4)	10 (21.3)	43 (11.3)		7 (17.1)	46 (11.9)	
Sexual Exposures							
Past 6 months: Touched indecently by a man or boy	69 (16.0)	12 (25.0)	57 (14.9)	0.072	8 (19.5)	61 (15.6)	0.520
Past 6 months: Harassed for sex outside of school	175 (40.6)	20 (41.7)	155 (40.5)	0.874	15 (36.6)	160 (41.0)	0.582
Past 6 months: Harassed for sex at school	45 (10.4)	8 (16.7)	37 (9.7)	0.135	2 (5.9)	43 (11.0)	0.290
Ever had sex willingly	100 (23.2)	22 (45.8)	78 (20.4)	<0.001	23 (56.1)	77 (19.7)	<0.001
Ever forced or tricked to have sex	70 (16.2)	9 (18.8)	61 (15.9)	0.617	12 (29.3)	58 (14.9)	0.017
Any sexual exposure ever				0.001			<0.001
No sex ever	301 (69.8)	25 (52.1)	276 (72.1)		16 (39.0)	285 (73.1)	
Had sex willingly	60 (13.9)	14 (29.2)	46 (12.0)		13 (31.7)	47 (12.1)	
Forced or tricked intercourse	30 (7.0)	1 (2.1)	29 (7.6)		2 (4.9)	28 (7.2)	
Had sex willingly, and forced or tricked intercourse	40 (9.3)	8 (16.7)	32 (8.4)		10 (24.4)	30 (7.7)	
Menstruation and Management							
Median age in years at first period (IQR)	14 (14-15)	15 (14-15)	14 (14-15)	0.126	14 (13.5-15)	14 (14-15)	0.537
Early menarche (first period < 13 years of age)	19 (4.6)	0 (0)	19 (5.2)	0.147	6 (14.6)	13 (3.5)	0.001
Had period in the past 6 weeks	405 (94.0)	46 (95.8)	359 (93.7)	0.755	40 (97.6)	365 (93.6)	0.495
Pad used at last period	405 (94.0)	46 (95.8)	359 (93.7)	0.755	39 (95.1)	366 (93.9)	>0.999
Cloth used for part or all of last period	108 (25.1)	14 (29.2)	94 (24.5)	0.486	13 (31.7)	94 (24.4)	0.302

[1] *BV is defined as Nugent score 7-10.*

[2] *STI is a composite of positive for C. trachomatis, N. gonorrhoeae, and/or T. vaginalis.*

[3] *Not all cells sum to N due to missing values.*

[4] *P-value by chi-square test unless otherwise noted; Fisher exact test used for categorical comparisons where any cell count was less than 5.*

[5] *Wilcoxon rank sum test used for comparison of non-normally distributed continuous variables.*

[a] *"Overweight and obese" includes n=4 girls with BMI >30.*

DNA Extraction and Amplicon Sequencing and Annotation

Genomic DNA (gDNA) was used as template for PCR amplification of the V3-V4 variable region of bacterial 16S rRNA gene according to a two-stage PCR protocol using primers 341F and 806R, as described previously (Naqib et al., 2018; Mehta et al., 2020). After pooling of barcoded samples, amplicons were sequenced on an Illumina MiSeq instrument, implementing V3 chemistry (600 cycles). DNA extraction, library preparation and sequencing were performed by the Genome Research Core (GRC) at the University of Illinois at Chicago (UIC). Forward and reverse reads were merged using the software package PEAR (Zhang et al., 2014). Quality and primer trimmed sequence data were then processed using a standard bioinformatics pipeline for chimera removal, and annotation was conducted by University of Maryland Institute for Genomic Science (UMD IGS) (Holm et al., 2019). Subsequently, a biological observation matrix was generated at the lowest taxonomic level identifiable. Vaginal CST were identified in a reference dataset using nearest centroid classification (*VA*gina*L* community state typ*E* *N*earest Centro*I*d cl*A*ssifier; VALENCIA) as described in (France et al., 2020). Data were filtered to retain taxa that contributed at least 0.05% of the total sequence reads, resulting in retention of 26 vaginal taxa. There were 5 observations with <5,000 sequence reads which were excluded from analyses.

Statistical Analysis

In this cross-sectional analysis, we examined two questions: (1) how the baseline VMB composition varied by whether girls were sexually active, and BV and/or STI presence; (2) how the baseline VMB composition varied by menstrual management practices and period characteristics.

Stacked bar plots summarizing taxa with highest relative abundance were created using Stata/SE 15. Alpha diversity indices were calculated at the amplicon sequence variant level using filtered data after rarefaction to a depth of 5,000 sequence per sample (*vegan*) (Gihring et al., 2012). We tested for global differences in vaginal community composition by BV and non-ulcerative STI status using analysis of similarity (ANOSIM) of the Bray Curtis resemblance matrix; ANOSIM is a non-parametric statistical test that assesses whether observations within a group are more similar to each other than to another group, in this way detecting differences between groups (Clarke, 1993). We visualized the relationship of global bacterial communities by BV and STI status using non-metric multidimensional scaling (NMDS) of bootstrapped averages of centroids with 100 replicates for each of the four groups representing outcome states (negative for both BV and STIs, positive for STI only, positive for BV only, positive for BV and STI). Bootstrapping is a resampling procedure that was used to estimate standard errors that allowed statistical inference on the differences between groups (Paliy and Shankar, 2016). ANOSIM and NMDS procedures were conducted in Primer-E, version 7, United Kingdom. We used multinomial logistic regression to quantify associations between explanatory factors (e.g., age, material used to manage menses, sexual activity) and CST, and Poisson regression with robust variance estimate (Barros and Hirakata, 2003) was used to quantify associations between explanatory factors and BV or STI. Because school was the unit of randomization and there were differences in distribution of socio-demographics, sexual activity, BV and STIs by school (**Supplementary Table 1**), we included a random effect for school in models of CST, BV, and STI. Multinomial logistic regression and Poisson regression were conducted in Stata/SE 15. Explanatory variables that were associated with outcomes at the p<0.10 level were entered in multivariable regression, and those with Wald p-value <0.05 were retained in multivariable models.

To identify specific taxa associated with Nugent BV and STIs, we used stability selection for feature selection [*stabs* package, implemented in R (Meinshausen and Bühlmann, 2010)]. In this approach, we applied ElasticNet regression to 250 randomly generated subsets of the vaginal microbiome data and used a cutoff of p<0.20 in combination with detection of a specific taxa in at least 60% of subsets. We chose ElasticNet regression as its ridge regression penalty supports inclusion of highly correlated variables while maintaining sparsity (Zou and Hastie, 2005). Prior to feature selection, data were center log ratio transformed following geometric Bayesian multiplicative prior imputation of zeros [*zCompositions* package, implemented in R (Palarea-Albaladejo and Martin-Fernandez, 2015)], to address sparsity while maintaining read depth. As a supplementary analysis, we also identified taxa that differed by BV and STI status using similarity of percentage analysis (Clarke, 1993), which determined the percent contributions of individual taxa to the Bray Curtis dissimilarity between groups (Primer-E, version 7, United Kingdom).

RESULTS

Study Population

The median age of girls was 16.9 years (interquartile range 16.0 – 17.9) (**Table 1**). The median household amenities score – a summed score of flush toilet, piped water, electricity, and television – was zero. Majority of girls reported traditional pit (45.7%) for latrine, surface water as main water source (59.3%), and kerosene for lighting (39.7%), with 24.1% having a television. Many girls reported having been to a health facility in the past 6 months, with 20% (n=85) reporting antibiotic use in the past 30 days, primarily for fever (n=64) and generally in combination with other symptoms (such as respiratory or diarrhea). Nearly one-third (30.2%) of girls reported any prior sexual intercourse and, of those, 54% reported that they had been forced or tricked to have sex. Among sexually active girls, just 8.5% reported using a hormonal contraceptive for family planning (n=6 injectable, n=4 implant, n=1 pill) and this sparsity prevented evaluating associations with BV, STIs, or CST.

Bacterial Community Composition Differed by BV and STI Status

Three vaginal CSTs accounted for 95% of VMBs: *L. crispatus* dominant CST-I (41%), *L. iners* dominant CST-III (35%), and non-optimal CST-IV (19%) (**Table 2** and **Figure 1**). There were 12 (2.8%) girls with *L. gasseri* dominant CST-II and 8 (1.8%) girls with *L. jensenii* dominant CST-V. In keeping with the associations between non-optimal CST-IV reported in the literature (McKinnon et al., 2019), the prevalence of BV (52.4%) and non-ulcerative STIs (24.4%) was high within CST-IV, lowest in CST-I (0% BV, 2.8% non-ulcerative STI), and intermediate in CST-III (3.3% BV, 9.8% non-ulcerative STI). Overall, 59.8% of girls within CST-IV were detected with BV and/or non-ulcerative STI, compared to 2.8% within CST-I and 12.4% within CST-III (**Table 2** and **Figure 2**).

The global difference in bacterial community composition by BV and STI outcome was statistically significant (ANOSIM test, p=0.001; **Supplementary Table 2**); all pairwise comparisons were statistically significant (p=0.001, each) except for the comparison of communities in which the participant was positive for both BV and STI versus positive for BV and negative for STI (p=0.871). This difference in VMB composition is visualized in non-metric dimensional plots of the bootstrapped averages of the centroids of the four possible states of outcome (**Figure 3A**). The distribution of CST differed by BV and/or STI outcome (**Figure 3B**) and results of stability selection identified specific taxa differences between BV and STI outcomes: *L. jensenii, Dialister succinatiphilus*, *Sneathia sanguinegens, Megasphaera*, and *Lactobacillus* spp. (*Lactobacillus* identified at the genus level, but species was not identified) were associated with BV, while *Megasphaera, Atopobium vaginae, S. sanguinegens*, and *L. crispatus* were associated with STI (**Table 3** and **Figure 3C**). In supplementary analysis, the taxa contributing most to Bray Curtis dissimilarity between BV status (**Supplementary Table 3**) and STI status (**Supplementary Table 4**) were similar, with notable differences: *G. vaginalis* had strong contribution to BV positive and STI positive status, and while ElasticNet identified *L. jensenii* but not *L. crispatus* in association with BV, *L. crispatus* was the top differentiating taxa by Bray Curtis dissimilarity while *L. jensenii* was not identified as an important taxon. Girls with BV and STI had higher alpha diversity metrics (Shannon, Simpson, evenness, richness) (**Figures 4, 5**), and this is in keeping with the greater frequency of diverse CST-IV among girls with BV and STIs.

The Distribution of Community State Type Varied by Sociodemographic and Behavioral Characteristics

Household amenities scores were higher for girls with CST-II and CST-V (p=0.041), though numbers are small in these CSTs (**Table 2**). The median BMI was marginally higher for girls with CST-IV (p=0.094), in keeping with the association we observed between BMI and BV. There were no statistically significant differences in MHM or period characteristics by CST, though cloth use was more common in CST-III (29.1%) and CST-IV (31.3%) than CST-I (19.3%) (p=0.143), and when restricted to these three CSTs the difference was statistically significant (p=0.050). Notably, any sexual activity (willing or forced) was reported by 43.8% of girls with CST-IV, compared to 21% of girls with CST-I, and 35.1% of girls with CST-III. Looking at this in transpose, among girls reporting never being sexually active, 46.6% had CST-I VMB, 32.9% with CST-III, and 15.1% with CST-IV, while among girls reporting having been sexually active, 28.7% had CST-I, 41.1% CST-III, and 27.1% CST-IV (**Figure 2**).

In multinomial logistic regression (**Supplementary Table 5**) examining one covariate at a time (i.e., unadjusted), for each one year increase in age, there was a 31% increase in odds of CST-IV relative to CST-I (OR=1.31; 95% CI: 1.08 – 1.59) and increasing household amenity score was inversely associated with CST-III (OR=0.71; 95% CI: 0.55 – 0.90) relative to CST-I. Ever having been sexually active was associated with increased likelihood of CST-III (OR=2.03; 95% CI: 1.56 – 2.65) and CST-IV (OR=2.92; 95% CI: 1.26 – 6.77), as was cloth use during last period (CST-III OR=1.72; 95% CI: 1.28 – 2.31; CST-IV OR=1.90; 95% CI: 1.07 – 3.37). Increasing BMI was associated with decreasing likelihood of being in CST-III (OR=0.93; 95% CI: 0.87 – 0.99) or CST-V (OR=0.84; 95% CI: 0.71 – 0.99) relative to CST-I. In multivariable multinomial logistic regression analyses simultaneously adjusted for all variables presented (**Table 4**), all of these associations remained statistically significant (p<0.05, two sided), with no evidence of strong confounding.

Prevalence of Bacterial Vaginosis and Sexually Transmitted Infections

The prevalence of STIs was 9.9% (3.0% TV, 6.2% CT, 1.4% NG), and the prevalence of BV was 11.2%. There was substantial co-infection with 31% of girls with BV having an STI, and 35% of girls with an STI also having BV (**Figure 6**).

Only two variables were associated with both BV and STI: increasing age and ever having had sex willingly (**Table 1**). Increasing BMI was associated with BV, but not with STIs. Unsurprisingly, ever reporting willing and/or forced/tricked sexual activity was more common among girls with detected BV or STI, though 52% of girls with BV and 39% of girls with STI reported never having had any type of sexual intercourse. Among girls who reported any sexual exposure, the distribution of condom use, number of lifetime partners, and age of most recent male sex partner did not differ by BV or STI status (**Supplementary Table 6**). No individual MHM practices were associated with BV or STI.

In multivariable adjusted log binomial regression analyses (**Table 5**), BV was more prevalent with increasing age (adjusted prevalence rate ratio [aPRR] = 1.24 per one year increase; 95% CI: 1.15 – 1.33), increasing BMI (aPRR = 1.13 per one unit increase; 95% CI: 1.11 – 1.15), and sexual intercourse (aPRR=2.17; 95% CI: 1.41 – 3.35), while increasing household amenities score was

TABLE 2 | Distribution of characteristics by vaginal microbiome community state type[1].

	CST-I, N=177 *L. crispatus* dominant n (%)	CST-II, N=12 *L. gasseri* dominant n (%)	CST-III, N=153 *L iners* Dominant n (%)	CST-IV, N=81 *G. vaginalis* dominant n (%)	CST-V, N=8 *L. jensenii* dominant n (%)	[3]P-value
Bacterial Vaginosis (BV)	0 (0.0)	1 (8.3)	5 (3.3)	43 (52.4)	0 (0.0)	<0.001
Sexually Transmitted Infection (STI)	5 (2.8)	2 (16.7)	15 (9.8)	20 (24.4)	0 (0.0)	<0.001
C. trachomatis (CT)	4 (2.3)	2 (16.7)	8 (5.2)	12 (14.6)	0 (0.0)	0.002
N. gonorrhoeae (NG)	1 (0.6)	0 (0.0)	3 (2.0)	2 (2.4)	0 (0.0)	0.522
T. vaginalis (TV)	0 (0.0)	0 (0.0)	5 (3.3)	9 (11.0)	0 (0.0)	<0.001
BV and/or STI (CT, NG, TV)	5 (2.8)	3 (25.0)	19 (12.4)	49 (59.8)	0 (0.0)	<0.001
Ever had sex, willingly and/or forced or tricked	37 (21.0)	3 (25.0)	53 (35.1)	35 (43.8)	1 (12.5)	0.001
Median age in years (IQR)[4]	17 (16-18)	17 (16-17.8)	17 (16-18)	17 (16 – 18)	16.3 (17.5 – 18)	0.284
Median material goods point score (IQR)[4]	0 (0-1)	1 (0.25-1)	0 (0-1)	0 (0-1)	0.5 (0-1.75)	0.041
Latrine Type						0.748
Flush toilet	24 (13.6)	3 (25.0)	13 (8.6)	9 (11.3)	1 (12.5)	
Traditional pit	74 (42.1)	5 (41.7)	75 (49.7)	36 (45.0)	5 (62.5)	
Ventilated improved pit	74 (42.1)	3 (25.0)	48 (38.4)	32 (40.0)	2 (25.0)	
Bush, field, other	4 (2.3)	1 (8.3)	5 (3.3)	3 (3.8)	0 (0.0)	
Water Source						0.425
Bore hole	30 (17.1)	0 (0.0)	29 (19.2)	10 (12.7)	0 (0.0)	
Surface	103 (58.5)	8 (66.7)	84 (55.6)	52 (65.8)	4 (50.0)	
Rain water	11 (6.3)	1 (8.3)	9 (6.0)	2 (2.5)	0 (0.0)	
Pipe in house	32 (18.2)	3 (25.0)	29 (19.2)	15 (19.0)	4 (50.0)	
Source of Light						0.385
Electricity	47 (26.7)	3 (25.0)	25 (16.6)	19 (23.8)	1 (12.5)	
Kerosene	67 (38.1)	6 (50.0)	60 (39.7)	33 (41.2)	4 (50.0)	
Tin lamp	20 (11.4)	0 (0.0)	23 (15.2)	16 (20.0)	1 (12.5)	
Solar	36 (20.5)	3 (25.0)	32 (21.2)	9 (11.3)	2 (25.0)	
Other	6 (3.4)	0 (0.0)	11 (7.3)	3 (3.7)	0 (0.0)	
Has television in home	48 (27.3)	3 (25.0)	26 (17.2)	22 (27.5)	4 (50.0)	0.062
Body mass index, median (IQR)	21.6 (20.1-23.5)	21.1 (18.7-22.8)	21.2 (19.7-23.0)	22.2 (20.8-23.5)	21.1 (19.2-22.2)	0.094
Body mass index, category						0.777
Underweight (<18)	8 (4.6)	0 (0)	13 (8.7)	4 (4.9)	0 (0.0)	
Normal (18-25)	143 (82.2)	10 (83.3)	121 (80.7)	65 (80.3)	8 (100)	
Overweight or obese (>25)[2]	23 (13.2)	2 (16.7)	16 (10.7)	12 (14.8)	0 (0.0)	
School						0.346
A	52 (29.4)	2 (16.7)	36 (23.5)	21 (25.9)	4 (50.0)	
B	16 (9.0)	2 (16.7)	11 (7.2)	10 (12.4)	1 (12.5)	
C	26 (14.7)	1 (8.3)	19 (12.4)	19 (23.5)	0 (0.0)	
D	42 (23.7)	2 (16.7)	34 (22.2)	14 (17.3)	2 (25.0)	
E	20 (11.3)	2 (16.7)	25 (16.3)	11 (13.6)	1 (12.5)	
F	21 (11.9)	3 (25.0)	28 (18.3)	6 (7.4)	0 (0.0)	
Any antibiotic use past 30 days	36 (20.7)	1 (8.3)	28 (18.8)	17 (21.5)	2 (25.0)	0.847
Menstruation & Management						
Median age in years at first period (IQR)	14 (14-15)	14 (14-15)	14 (14-15)	14 (14-15)	15 (14-15)	0.510
Early menarche (first period < 13 years of age)	5 (3.0)	0 (0.0)	10 (6.9)	3 (3.7)	0 (0)	0.498
Cloth used to manage last menstrual period	34 (19.3)	3 (25.0)	44 (29.1)	25 (31.3)	1 (12.5)	0.143
Period Characteristics						
Pain or cramps last period	106 (60.2)	6 (50.0)	106 (70.2)	47 (58.8)	7 (87.5)	0.114
Menstrual bleeding last period						0.055
Light	11 (6.2)	2 (16.7)	7 (4.6)	9 (11.3)	2 (25.0)	
Normal	139 (79.0)	10 (83.3)	110 (72.9)	59 (73.7)	5 (62.5)	
Heavy	26 (15.8)	0 (0.0)	34 (22.5)	12 (15.0)	1 (12.5)	
Median days of last period (IQR)	4 (3-5)	4 (3-4.75)	4 (3-5)	4 (3-5)	4.5 (3-5.75)	0.149
1-3 days	70 (40.0)	5 (41.7)	46 (30.7)	25 (31.7)	3 (37.5)	0.341
4-6 days	93 (53.1)	5 (41.7)	92 (61.3)	43 (54.5)	5 (62.5)	
7+ days	12 (6.9)	2 (16.6)	12 (8.0)	11 (13.9)	0 (0.0)	

[1]*Excludes n=4 participants with <5,000 total sequence reads.*

[2]*BMI category "overweight or obese" includes n=4 participants with BMI >30.*

[3]*P-value by chi-square test unless otherwise noted; Fisher exact test used for categorical comparisons where any cell count was less than 5.*

[4]*Wilcoxon rank sum test used for comparison of non-normally distributed continuous variables.*

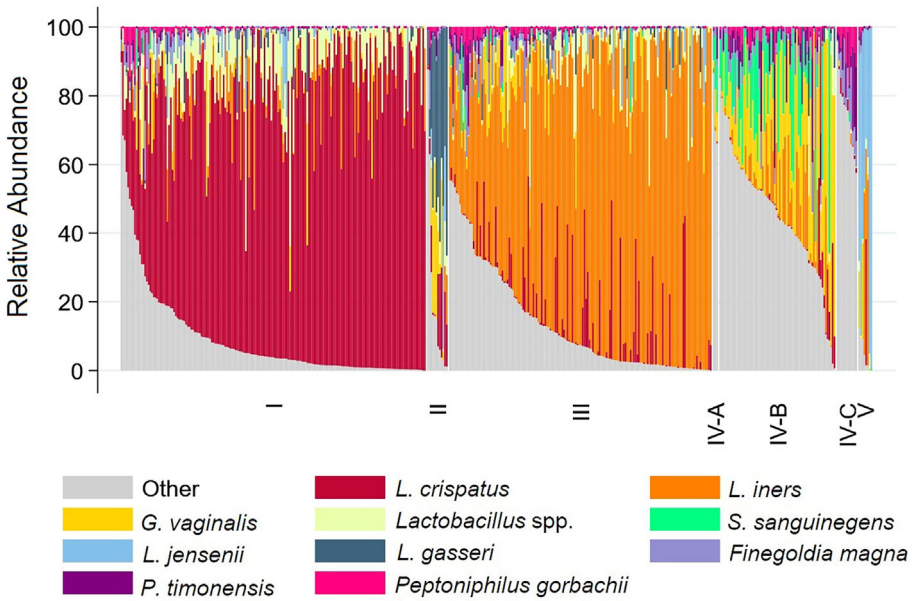

FIGURE 1 | Stacked bar chart showing relative abundance of 10 taxa with highest mean relative abundance by community state type for each participant. Legend: The relative abundance of the 10 taxa with the highest mean relative abundance is shown (y-axis), with individual subjects represented by individual bars (N=431), separated by Community State Type (x-axis).

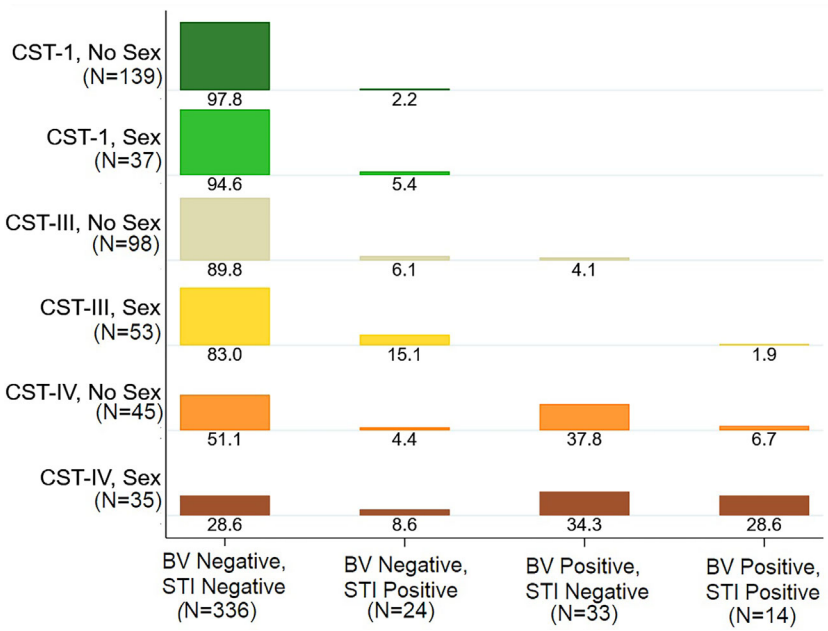

FIGURE 2 | Distribution of Bacterial vaginosis (BV) and Sexually Transmitted Infection (STI) Status by Vaginal Microbiome Community State Type (CST), Stratified by Sexual Activity. Legend: The plot shows the distribution of BV and STI status by vaginal CST, stratified by sexual activity ever. For example, among girls with CST-I who do not report sexual activity, 97.8% were negative for BV and STI and 2.2% were BV Negative and STI positive. Among girls with CST-IV who reported having sexual activity, 28.6% were negative for BV and STI, 8.6% with STI only, 34.3% with BV only, and 28.6% with both BV and STI.

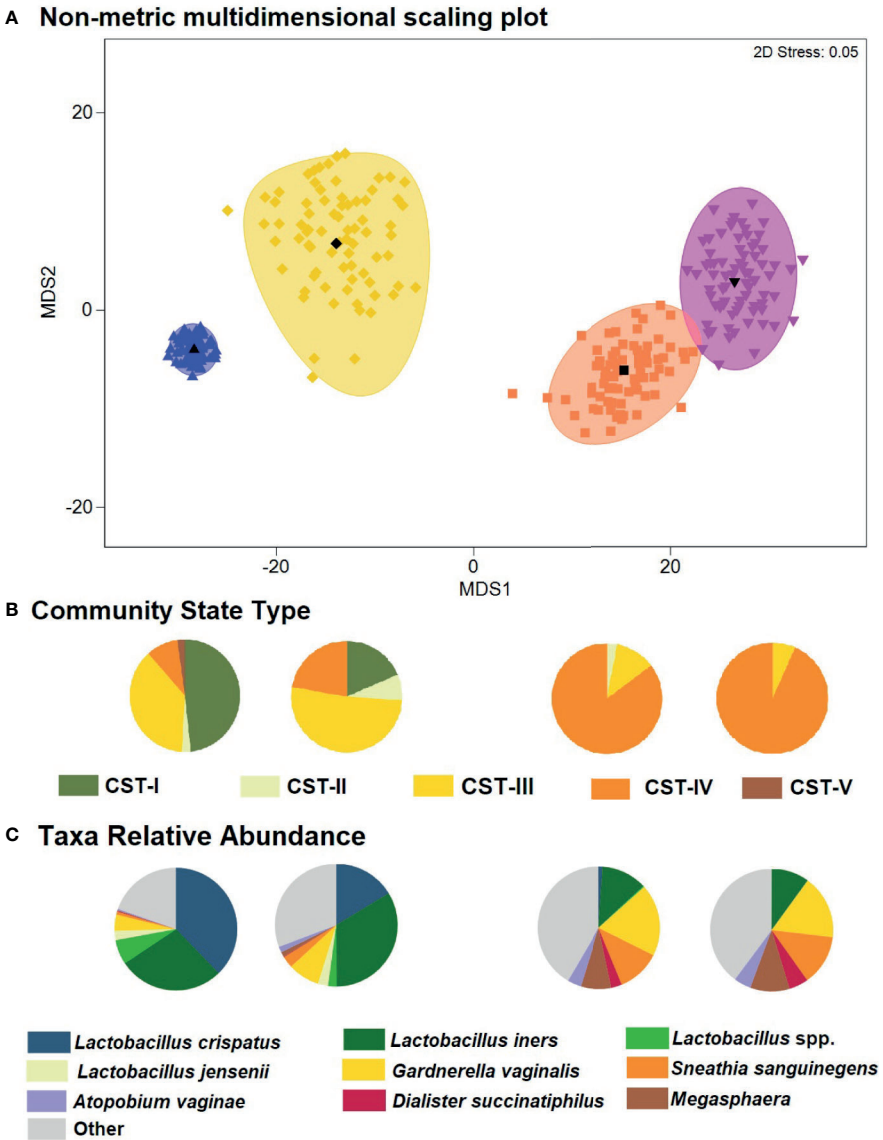

FIGURE 3 | Non-metric dimensional scaling plot for each of the four outcome states for Bacterial vaginosis (BV) or sexually transmitted infection (STI) and distribution of Community State Type (CST) and taxa relative abundance. **(A)** The four different colors represent the four outcome states for Bacterial vaginosis and sexually transmitted infection. STI is a composite of infection with any of *C. trachomatis, N. gonorrhoeae, T. vaginalis.* Blue = negative for BV and all STIs; yellow = STI positive, BV negative; orange = BV positive, STI negative; pink = both STI and BV positive. Each colored mark indicates one of 100 bootstrappings of the dataset. The matching shaded area represents the 95% coverage. The black symbol at the center of each colored shape represents the average centroid of the 100 bootstraps. **(B)** Pie charts below the non-metric dimensional scaling plot show the distribution of CST, aligned to outcome states for BV and STI (N=431 individuals represented). **(C)** Pie charts below the non-metric dimensional scaling plot show the distribution of mean relative abundance of taxa identified through stability selection in association with BV and STI (N=431 individuals represented).

protective of BV (aPRR=0.76; 95% CI: 0.60 – 0.97). In a multivariable log-binomial regression model simultaneously adjusted for all variables presented, only age (aPRR=1.23; 95% CI: 1.04 – 1.46) and sexual intercourse (aPRR=3.11; 95% CI: 1.10 – 8.77) were statistically significantly associated with STI (**Table 6**). While higher BMI was associated with lower likelihood of STI, this became insignificant once adjusted for age due to the positive correlation between BMI and age.

DISCUSSION

The major findings in our analyses are: (1) *L. crispatus* dominant CST-I was the most common vaginal community state type, and was more likely among girls who did not report sexual activity. (2) Girls who used cloth to manage their menses were more likely to have CST-III or non-optimal CST-IV than CST-I. (3) The prevalence of BV and STIs was high.

TABLE 3 | Results of stability selection with p=0.20 Error Bound.

Bacterial Vaginosis (BV)				Sexually Transmitted Infection (STI)			
Taxa	**Proportion of Bootstrap samples Identified in**	**Mean Relative Abundance**		**Taxa**	**Proportion of Bootstrap samples Identified in**	**Mean Relative Abundance**	
		BV present % (SD)	**BV Absent % (SD)**			**STI% present (SD)**	**STI% absent (SD)**
Lactobacillus jensenii	0.840	0 (0)	2.50 (10.6)	*Megasphaera*	0.612	4.43 (6.83)	0.86 (3.32)
Sneathia sanguinegens	0.918	12.0 (11.0)	0.96 (5.2)	*Atopobium vaginae*	0.762	2.59 (4.37)	0.63 (2.66)
Dialister succinatiphilus	0.920	3.55 (3.15)	0.20 (0.89)	*S. sanguinegens*	0.828	6.64 (8.45)	1.72 (6.73)
Lactobacillus spp.	0.996	0.19 (0.61)	6.14 (7.99)	*L. crispatus*	0.958	10.68 (24.7)	34.6 (36.9)
Megasphaera	0.998	8.78 (7.28)	0.25 (1.64)				

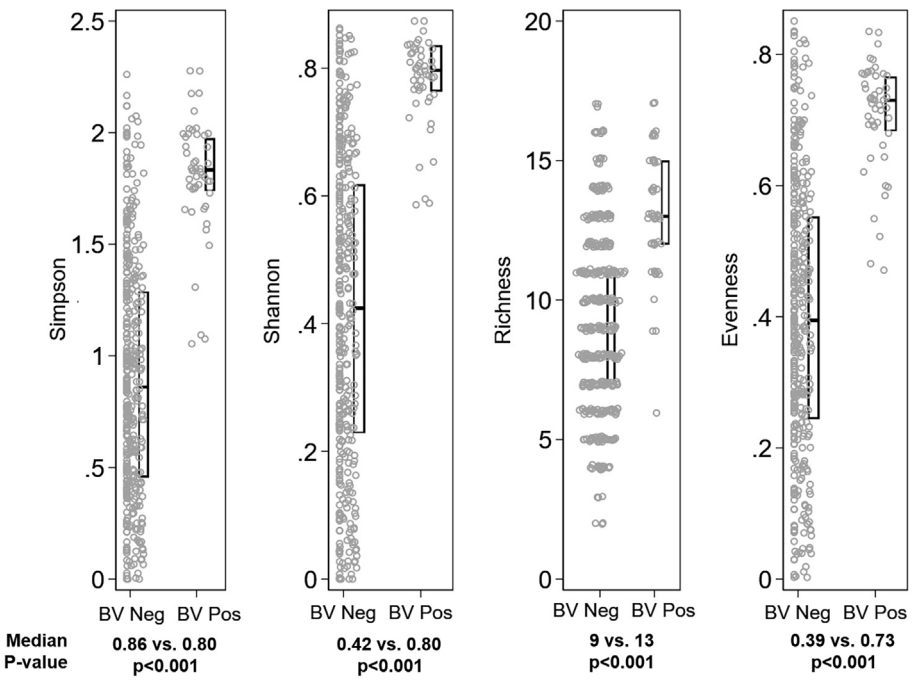

FIGURE 4 | Distribution of alpha diversity metrics by Bacterial vaginosis (BV) status. Legend: The distribution of alpha diversity metrics (Simpson, Shannon, Richness, and Evenness) are shown on the y-axis, separately for girls with Nugent score 0-6 ("BV Neg", N=382) and Nugent score 7-10 ("BV Pos", N=49) on the x-axis. Within panels, each grey dot represents a single observation. Box plots indicate the median (horizontal bar) and interquartile range (lower 25th percentile and upper 75th percentile). Below each graph, the median value for each alpha diversity metric is reported for "BV Neg" and "BV Pos" observations, with Wilcoxon rank sum p-value of the comparison reported beneath the medians.

The majority of girls had a *L. crispatus* (41%) or *L. iners* (35%) dominant vaginal CST. This is important because numerous studies show that women of African descent are more likely to have non-optimal CST-IV vaginal community type (Ravel et al., 2011; Lewis et al., 2017). In our study of adult women (median age 23 years) in long-term sexual relationships who resided in Kisumu (approximately 70 km from Siaya County), at baseline 8.7% had *L. crispatus* dominant CST-I, 42% had *L. iners* dominant CST-III, and 47.2% had non-optimal CST-IV (Mehta et al., 2020). That such a high proportion of native Kenyan adolescent girls in our current study had *L. crispatus* dominant CST-I clearly indicates that this is a common phenotype and is most likely altered as girls become sexually active, as reflected by the increased odds of association with CST-

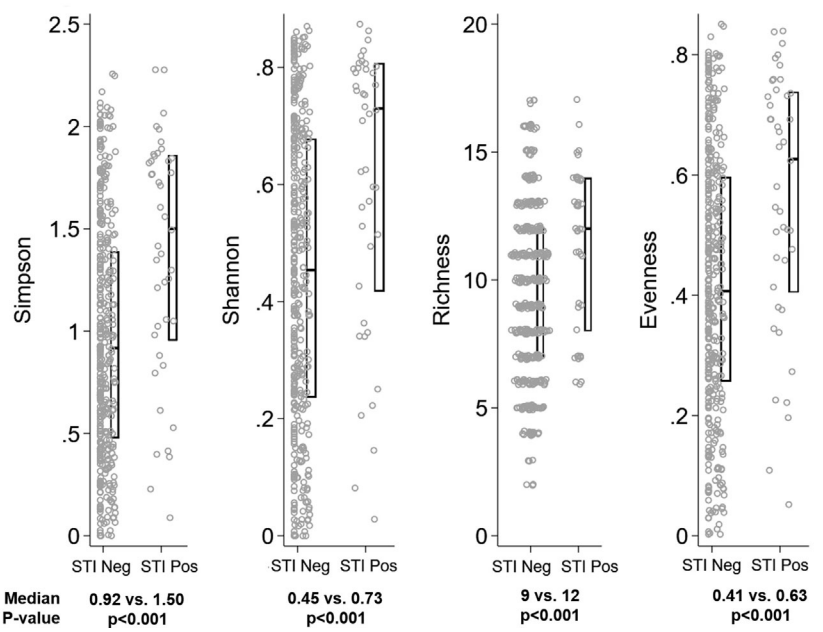

FIGURE 5 | Distribution of alpha diversity metrics by Sexually Transmitted Infection (STI) status. Legend: The distribution of alpha diversity metrics (Simpson, Shannon, Richness, and Evenness) are shown on the y-axis, separately for girls testing negative for all three STIs ("STI Neg", N=43) and testing positive for any STI ("STI Pos", N=388) on the x-axis. Within panels, each grey dot represents a single observation. Box plots indicate the median (horizontal bar) and interquartile range (lower 25th percentile and upper 75th percentile). Below each graph, the median value for each alpha diversity metric is reported for "STI Neg" and "STI Pos" observations, with Wilcoxon rank sum p-value of the comparison reported beneath the medians.

TABLE 4 | Results of multivariable adjusted multinomial logistic regression with random effect for school: factors associated with community state type, N=420.

	CST-II (*vs.* CST-I) OR (95% CI)	CST-III (*vs.* CST-I) OR (95% CI)	CST-IV (*vs.* CST-I) OR (95% CI)	CST-V (*vs.* CST-I) OR (95% CI)
Age in years, continuous	1.04 (0.66 – 1.63)	1.01 (0.87 – 1.17)	1.19 (1.02 – 1.38)[b]	1.26 (0.83 – 1.92)
Household amenities score, continuous	1.14 (0.73 – 1.79)	0.71 (0.56 – 0.90)[a]	0.90 (0.69 – 1.19)	1.07 (0.55 – 2.10)
Ever had sex, willingly and/or forced or tricked (*vs*. Never)	1.18 (0.19 – 7.36)	2.00 (1.63 – 2.45)[a]	2.58 (1.14 – 5.86)[b]	0.49 (0.02 – 10.1)
Cloth used during last period (*vs*. no)	1.51 (0.63 – 3.60)	1.59 (1.17 – 2.17)[a]	1.72 (1.03 – 2.86)[b]	0.72 (0.11 – 4.60)
Body mass index, continuous	0.91 (0.67-1.25)	0.93 (0.86-1.01)[c]	1.02 (0.94 – 1.11)	0.82 (0.70 – 0.98)[b]

[a] *p-value<0.01.*
[b] *0.01<p-value<0.05.*
[c] *0.05<p-value<0.10.*

III (aOR 2.00) and CST-IV (aOR=2.58) compared to CST-I for girls ever having had sexual exposure, adjusted for age, socioeconomic measure, and cloth use for menses. The association between older age and CST-III and CST-IV may represent that older girls have different types of sex partners, different sexual practices, or may have been sexually active longer. As the cohort is ongoing, our eventual longitudinal evaluation will be able to quantify this change over time as girls become sexually active, and among those becoming sexually active we will be able to examine the association with sexual practices and partner characteristics.

This finding has implications for the design of behavioral and biological interventions, indicating that a non-optimal VMB composition may be preventable and that adolescence could be a critical intervention point for preventing adverse reproductive health outcomes. Poor quality menstrual hygiene is modifiable, through provision of cheap accessible hygienic products instead of cloth use, and could have substantial biological consequence. Cloth use may promote non-optimal vaginal microbiome through facilitation of anaerobic bacterial growth, through improperly washed fabric (i.e., direct transfer of bacteria), or an occlusive environment. In a district level household survey of 577,758 women aged 15-49 years in India, those who used cloth during menses were more likely to report vaginal discharge in the past 3 months (aOR=1.30), adjusted for age, gynecologic factors, and socioeconomic indicators

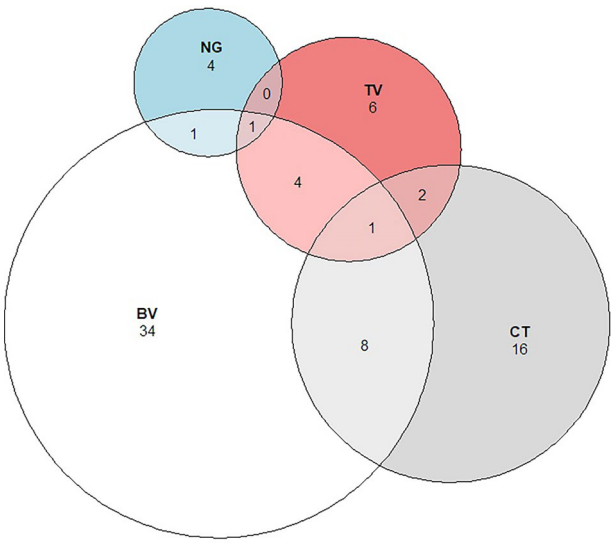

FIGURE 6 | Proportional Venn diagram showing the relationship between Bacterial vaginosis and Sexually Transmitted Infections. Legend: The figure above shows the number and overlap of individuals identified with *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), and Bacterial vaginosis (BV).

TABLE 5 | Results of poisson regression with robust variance estimate and random effect for school: crude and multivariable adjusted associations of sociodemographic and behavioral factors with bacterial vaginosis.

Variables	Crude Prevalence Rate Ratio [95% CI]	Adjusted Prevalence Rate Ratio [95% CI] N=423
Age in years, continuous	1.40 [1.26–1.55]	1.24 [1.15-1.33]
Household amenities score, continuous	0.78 [0.60-1.02]	0.76 [0.60-0.97]
Ever had sex, willingly and/or forced or tricked	2.32 [1.47-3.65]	2.17 [1.41-3.35]
Body mass index, continuous	1.15 [1.10–1.21]	1.13 [1.11–1.15]
Cloth used to manage last menstrual period	1.21 [0.78–1.86]	

Multivariable model simultaneously adjusted for all variables presented.

TABLE 6 | Results of poisson regression with robust variance estimate and random effect for school: crude and multivariable adjusted associations of sociodemographic and behavioral factors with non-ulcerative sexually transmitted infection.

Variables	Crude Prevalence Rate Ratio [95% CI]	Adjusted Prevalence Rate Ratio [95% CI] N=431
Age in years, continuous	1.35 [1.12-1.61]	1.23 [1.04-1.46]
Household amenities score, continuous	0.91 [0.74-1.12]	
Ever had sex, willingly and/or forced or tricked	3.62 [1.21-10.8]	3.11 [1.10-8.77]
Early menarche (first period before age 13 years)	3.59 [1.92–6.70]	
Body mass index, category		
Underweight (<18)	ref	
Normal (18-25)	0.41 [0.23 – 0.73]	
Overweight or obese (>25)	0.66 [0.22 – 1.97]	

(Anand et al., 2015). Cloth use for menses has also been associated with BV among women in Tanzania (Baisley et al., 2009) and in India (Torondel et al., 2018), and tampon use has been associated with VMB composition among women in the United States (Noyes et al., 2018). Alterations in vaginal flora during menses may be modified with other MHM products such as the menstrual cup. Menstrual cups are medical grade silicone bell chambers inserted vaginally to collected menstrual flow. Among 406 U.S. women having 4,750 collective days of menstrual cup use, colonization with *Lactobacillus* was maintained at pre-cup use levels with no change in pH or colonization with *S. aureus, G. vaginalis*, or *Bacteroides* spp (North and Oldham, 2011). Systematic review with meta-analysis suggests that menstrual cups are a safe option for menstrual hygiene in low-, middle-, and high-income countries (van Eijk et al., 2019). In a cluster randomized controlled feasibility study of 644 girls aged 14-16 years old, Phillips-Howard et al. randomized girls by school cluster 1:1:1 to reusable menstrual cups, disposable sanitary pads, or standard water, sanitation and hygiene counseling (Phillips-Howard et al., 2016). The prevalence of BV (Gram stain Nugent score 7-10) was reduced by 35% (aPRR=0.65; p=0.034) for menstrual cup users (13%) compared to pad users (20%) and control subjects (19%). Menstrual cup use also resulted in 52% (p=0.039) reduction in the prevalence of STIs (composite measure of *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*). In our current analysis, cloth use was more common among girls with BV (29.2%) than without BV (24.5%), and for girls with STI (31.7%) than without STI (24.4%), though neither difference was statistically significant. However, cloth use was significantly associated with CSTI-III (aOR=1.59) and CST-IV (aOR=1.72). While this may seem contradictory, this could reflect underreporting of cloth use, which could have attenuating effects on the measure of association with BV and STI, both having smaller sample size than CST-III and CST-IV. Of note, vaginal discharge was more commonly reported by girls using cloth (28.7%) than those without (21.1%), though not statistically significant (p=0.10; data not shown).

The prevalence of BV and STIs was high, with 9.9% of girls having STIs and 11.2% having BV. While BV is considered a sexually enhanced condition (Verstraelen et al., 2010), there are non-sexual risk factors including intravaginal and vaginal hygiene practices (Low et al., 2011), cigarette smoking (Nelson et al., 2018), and male sexual partner's circumcision status (Liu et al., 2015). Of girls who reported ever having had sexual activity, 37% reported not knowing the male partner's circumcision status and just 3% reported the male partner as uncircumcised [it is estimated that 40% of men in Siaya County are uncircumcised (McKinnon et al., 2019)], precluding meaningful analysis of this variable. Only one girl reported smoking cigarettes. It is a limitation that we did not ask about

intravaginal practices or application of substances to the vagina as it was felt by the local study team to be too invasive and that girls would not answer due to perceived stigma. Among girls who reported they had ever had sexual activity, we did not find factors that differentiated girls with BV or STI, though this analysis was biased by underreporting of sexual activity, as evidenced by 39% of girls with STI reporting never having been sexually active. Antibiotic use within the past 30 days was common (20%), and we did not find an association between recent antibiotic use and BV, STI, or CST. This may be due to misclassification (e.g., taking anti-malarial and reporting it as antibiotic use), underreporting of antibiotics, use of antibiotics class, dose, or duration that was not strongly influential to the VMB, or because the sample represented a mixture of antibiotic classes and indications, and therefore too much noise to detect a signal.

The VMB composition differed substantially by BV and/or STI status, as demonstrated by global community comparison (ANOSIM), distribution of CSTs, and distribution of specific taxa. These differences were in keeping with previous literature. Of note, *G. vaginalis* was not identified by ElasticNet implemented within stability selection as one of the specific taxa discriminating between BV and STI states, though it is considered a key taxa in BV pathogenesis (Schwebke et al., 2014) and was one of the top taxa by contribution to Bray Curtis dissimilarity analysis. Differences in results by machine learning and ecological approaches highlight the importance of using different analytic approaches to maximize information gain and robustness.

Limitations

There was substantial underreporting of sexual activity, as 39% of STIs occurred among girls who reported never having had sexual activity (willing or forced). Having a small number of girls infected with each STI, we analyzed STI as a composite of CT, NG, and TV; while data comparing the VMB composition by each pathogen are limited, the specific taxa associated with each pathogen may differ (Masha et al., 2019). Nevertheless, despite high co-infection of BV and STIs, we demonstrate that taxa associated with STIs differ from those associated with BV and longitudinal analyses will provide insight on the temporal occurrence of BV and/or STIs, and VMB composition and taxa in relation to specific STI pathogens. HIV prevalence at baseline was 1.4%, and while this is high given the young median age of girls, the number is small and we cannot relate HIV status to VMB in this analysis. Our results may not be generalizable to girls who are not in school. In this cross-sectional analysis of baseline data, we cannot be certain that exposures preceded outcomes.

CONCLUSIONS

Nearly half of adolescent girls had a *L. crispatus* dominant VMB, differing substantially from studies of young adult and adult women in Kenya and other parts of sub-Saharan Africa. This indicates that non-optimal VMB may be an acquired state for many women and girls, and interventions to maintain or re-constitute *L. crispatus* dominance should be considered, with adolescence being a potentially critical point. Menstrual cups may be a potential intervention for preventing non-optimal vaginal microbiome composition associated with non-hygienic menstrual management.

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/PRJNA540529.

ETHICS STATEMENT

This study was approved by the institutional review boards of the Kenya Medical Research Institutes (KEMRI, SERU #3215), Liverpool School of Tropical Medicine (LSTM, #15-005), and University of Illinois at Chicago (UIC, #2017-1301). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SM: Obtained funding, study conceptualization and design, statistical analysis inference, data visualization, drafted manuscript. GZ: Study oversight and management to ensure integrity to protocols and integration of CaCHe within CCG trial, data management and cleaning, critical review and revision of manuscript. FO: Study oversight and management to ensure integrity to protocols of CaCHe, critical review and revision of manuscript. EN: Study oversight and management to ensure integrity to protocols of CCG trial, critical review and revision of manuscript. WA: Development, implementation, and oversight of laboratory protocols in Kenya, acquisition of data, microbiologic analyses and interpretation, critical review and revision of manuscript. RB: Design and execution of statistical analysis approaches, critical review and revision of manuscript. SG: Development and oversight of protocols for amplicon sequencing, microbiologic analyses and interpretation, critical review and revision of manuscript. AE: Data management and cleaning, critical review and revision of manuscript. DK: Study oversight and management to ensure integrity to protocols of CCG trial and regulatory integration of CaCHe, critical review and revision of manuscript. PP-H: Obtained funding, study oversight and management to ensure integrity to protocols, critical review and revision of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.716537/full#supplementary-material

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Association of the Cervical Microbiota With Pregnancy Outcome in a Subfertile Population Undergoing *In Vitro* Fertilization: A Case-Control Study

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The microorganisms of the reproductive tract have been implicated to affect *in vitro* fertilization (IVF) outcomes. However, studies on the reproductive tract microbiota of infertile women are limited and the correlation between cervical microbiota and IVF outcome remains elusive. This study aimed to characterize the cervical microbiota of IVF patients undergoing embryo transfer (ET) and assess associations between the cervical microbiota and pregnancy outcomes while exploring the underlying contributing factors. We launched a nested case-control study of 100 patients with two fresh or frozen-thawed cleavage embryos transferred per IVF cycle. Cervical swabs were collected on the day of ET and divided into four groups according to clinical pregnancy outcomes. Variable regions 3 and 4 (V3-V4) of the 16S rRNA gene were amplified and sequenced on the Illumina MiSeq platform. In fresh IVF-ET cycles, the clinical pregnancy group (FP, n = 25) demonstrated higher α diversity ($P = 0.0078$) than the non-pregnancy group (FN, n = 26). Analysis of similarity (ANOSIM) revealed a significant difference in β diversity between the two groups ($R = 0.242$, $P = 0.001$). In frozen-thawed ET cycles, though not significant, similar higher α diversity was found in the clinical pregnancy group (TP, n = 27) compared to the non-pregnancy group (TN, n = 22) and ANOSIM analysis showed a significant difference between the two groups ($R = 0.062$, $P = 0.045$). For patients in fresh IVF-ET groups, *Lactobacillus*, *Akkermansia*, *Desulfovibrio*, *Atopobium*, and *Gardnerella* showed differentially abundance between pregnant and non-pregnant women and they accounted for the largest share of all taxa investigated. Among them, *Lactobacillus* was negatively correlated with the other genera and positively correlated with serum estradiol levels. Logistic regression analysis suggested that the composition of the cervical microbiota on the day of ET was associated with the clinical pregnancy in fresh IVF-ET cycles ($P = 0.030$). Our results indicate that cervical microbiota composition has an impact on the outcome of assisted reproductive therapy.

Keywords: 16S r RNA, IVF (*in vitro* fertilization), pregnancy, infertility, cervical microbiota

INTRODUCTION

At least a billion microorganisms settle on the female reproductive tract and interact with the host to maintain a series of physiological processes such as immunity and metabolism (Jie et al., 2021). Disruption of human microbial stability is the leading cause of infections and is also implicated in other diseases such as Crohn's disease, Subglottic stenosis, and periodontitis (Peñalver Bernabé et al., 2018; Curtis et al., 2020; Aronson et al., 2021; Siniagina et al., 2021). Cervicovaginal microbiota is known to play an important role in female reproductive function. Healthy cervicovaginal microbiota is often characterized by a low diversity of bacterial species, with *Lactobacillus* tending to be the dominant microbiota. In recent years, researchers divided the cervicovaginal microbiota of women at childbearing age into six main community state types (CSTs), of which four were predominated by either *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), *Lactobacillus iners* (CST III) or *Lactobacillus jensenii* (CST V), and two (CST IV-A and CST IV-B) comprised a wide array of strict and facultative bacterial anaerobes, where CST IV-A was characterized with the higher abundance of BVAB1 (Elovitz et al., 2019). The composition of the vaginal microbiota is affected by various factors including race, personal hygiene, sexual activity, and menstrual cycle (Gajer et al., 2012), with a shift to facultative or strictly anaerobic bacterial dominance causing the clinical syndrome called bacterial vaginosis (BV) (Mendling, 2016). Previous studies have demonstrated the association between BV and adverse obstetric outcomes such as late-term abortion and premature delivery (Nelson et al., 2007; Foxman et al., 2014).

Infertility refers to a couple's failure to become pregnant after one year of regular and unprotected intercourse (Cooper et al., 2009), affecting up to 10% of couples at childbearing age worldwide (Mascarenhas et al., 2012). Since the first live birth achieved by *in vitro* fertilization (IVF) in 1978, improving the pregnancy rate of IVF patients has become a major clinical challenge (De Geyter, 2019). Research has shown that—in addition to the known factors used in prediction models such as female age, sperm quality, and antral follicle count—IVF outcome might also be affected by the microorganisms of the female reproductive tract (Koedooder et al., 2019). The relatively few studies on the microbiota inhabited reproductive tract of infertile women have yielded inconsistent results on the correlation between vaginal microbiota and IVF outcome. Liversedge et al evaluated the vaginal swabs collected at the time of oocyte retrieval by Gram stain and found that the incidence of BV in tubal infertility was significantly higher than that in non-tubal infertility and that BV did not affect fertilization (Liversedge et al., 1999). Ralph et al. showed that BV was associated with an increased risk of miscarriage in the first trimester of women undergoing IVF (Ralph et al., 1999). In another study, researchers used Nugent score and polymerase chain reaction to diagnose BV in IVF patients and found no significant difference in obstetric results between the BV group and the non-BV group (Mangot-Bertrand et al., 2013). Results of the study conducted by Selim et al. showed that BV and lower concentrations of hydrogen peroxide-producing *Lactobacillus* may reduce the conception rate and increase the rate of failed pregnancy on women who were undergoing intracytoplasmic sperm injection (Selim et al., 2011). A recent meta-analysis conducted on IVF patients showed that BV was significantly associated with early spontaneous abortion, but had no significant effect on live birth rate and clinical pregnancy rate (Haahr et al., 2019).

With the development of high-throughput sequencing technology, emerged data have identified the existence of continuous changed microbiota along the female reproductive tract. In 2016, Moreno et al. demonstrated the presence of endometrial microbiota and found that its composition was associated with the reproductive results of IVF patients (Moreno et al., 2016). Chen et al. systematically sampled the microbiota in the reproductive tract of 110 women at childbearing age and performed 16S rRNA gene sequencing. The results revealed that the vaginal-uterine microbiota was a continuum and the microbiota in the cervical canal and uterus was different from the vaginal microbiota (Chen et al., 2017). The cervix, located at the transition zone between the lower and upper reproductive tract, serves as both a mechanical and chemical barrier to ascending bacteria. The state of the uterus is an important maternal factor which affects female fertility, but knowledge about endometrial microbiota was deficient owing to the invasiveness of uterine sample collection. Cervical microbiota detected from sampling of cervical mucosa, can be used to survey the status of the uterus and peritoneal cavity in the general population with minimally invasive procedures (Chen et al., 2017). Based on the special anatomy of the cervix, the risk of contamination at the sampling point is minimal (Schoenmakers and Laven, 2020). Our study aimed to characterize the cervical microbiota of 100 women undergoing IVF treatment and assess the impact of cervical microbiota composition on IVF clinical pregnancy outcomes.

MATERIALS AND METHODS

Patient Recruitment

This study recruited infertile female patients undergoing IVF treatment at the Reproductive Center of Shengjing Hospital of China Medical University from January 2019 to March 2019. The study was approved by the Ethics Committee of the Shengjing Hospital of China Medical University and all participants provided written informed consent (approval number: 2017PS269K). Inclusion criteria were as follows: 20–40 years of age; undergoing assisted reproductive technology (ART) treatment with their own gametes; transfer of two cleavage-stage embryos. Exclusion criteria were as follows: autoimmune diseases; endocrine diseases; cervical diseases; endometrial diseases (uterine fibroids, adenomyosis, moderate to severe endometriosis, unrecoverable uterine adhesion, etc.); blood contamination of collected samples. All participants followed the conventional ART protocol for treatment and the primary outcome of clinical pregnancy was defined as positive fetal heartbeat and fetal buds observed under ultrasound 35 days after embryo transfer (ET). The baseline characteristics collected for each patient included age, body mass index (BMI), duration of infertility, smoking history, drinking history, menstrual cycle

length, cause of infertility, previous fertility history, the total dose of Gonadotropin (Gn), duration of Gn administration, number of oocytes retrieved, endometrial thickness on the day of ET, estradiol (E_2) and progesterone (P) levels, and number of good-quality embryos transferred.

Sample Collection

In the operating room, two cervical samples were collected before ET: 1) a sterile cotton ball was used to clean the patient's vaginal secretions; 2) two sterile cotton swabs were used to access the patient's cervical canal and rotated to obtain cervical secretions. Both swabs were used for genomic DNA extraction. During this process, operator ensured that the cotton swab did not touch the patient's vaginal wall. After collecting the swab samples, ultrasound-guided ET was performed according to the established protocol. All samples were stored at –80°C for later analysis (Chen et al., 2017).

DNA Extraction and PCR Amplification

Microbial DNA was extracted from cotton swab samples using the PureLink microbiota DNA extraction Kit (ThermoFisher) according to the manufacturer's protocol. The V3-V4 region of the bacteria 16S ribosomal RNA genes was amplified by PCR (95°C for 3 min, followed by 30 cycles at 98°C for 20 s, 58°C for 15 s, and 72°C for 20 s and a final extension at 72°C for 5 min) using primers 341F 5'-CCTACGGGRSGCAGCAG-3' and 806R 5'-GGACTACVVGGGTATCTAATC-3'. PCR reactions were performed in 30 μL mixture containing 15 μL of 2 × KAPA Library Amplification ReadyMix, 1 μL of each primer (10 μM), 50 ng of template DNA, and ddH_2O.

Illumina MiSeq PE250 Sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instruction and quantified using Qubit®2.0 (Invitrogen, U.S.). After preparation of the library, these tags were sequenced on the MiSeq platform (Illumina, Inc., CA, USA) for paired end reads of 250bp, which were overlapped on their 3' ends for concatenation into original longer tags. DNA extraction, library construction, and sequencing were conducted at Realbio Genomics Institute (Shanghai, China).

Process of Sequencing Data

Pandaseq (version 2.8.1) was used for reads assemble and Realbio analysis platform (Shanghai, China) was responsible for quality control. Tags, trimmed of barcodes and primers, were further checked on their rest lengths and average base quality. 16S tags were restricted between 220 bp and 500 bp such that the average Phred score of bases was no worse than 20 (Q20) and no more than 3 ambiguous N. The copy number of tags was enumerated and redundancy of repeated tags was removed. Only the tags with a frequency of more than 1, which tend to be more reliable, were clustered into Operational Taxonomic Units (OTUs), each of which had a representative tag. OTUs were clustered with 97% similarity using UPARSE (http://drive5.com/uparse/) and chimeric sequences were identified and removed using Userach (version 7.0). Each representative tags was assigned to a taxa by RDP Classifer (version 2.12, http://rdp.cme.msu.edu/) against the RDP database (version 11.4, http://rdp.cme.msu.edu/) using a confidence threshold of 0.8. Analysis of α diversity (Chao1 index, Shannon index, Simpson index) and β diversity (unweighted UniFrac) was also achieved by python scripts of Qiime (version 1.9.1).

Statistical Analysis

Statistical analysis was conducted with R software version 3.5.1 and SPSS version 25.

We generated the OTU Venn diagram (in R) to illustrate the number of OTUs shared among four groups or unique to a group, according to the abundance of OTU in each sample. Shannon index and Simpson index were used to evaluate α diversity and P values were calculated using the Wilcox test function in R between the pregnancy group and non-pregnancy group. Analysis of similarity (ANOSIM) and Principal co-ordinates analysis (PCoA) was performed to compare the overall cervical microbiota composition between pregnancy and non-pregnancy groups in fresh and frozen-thawed cycles. Linear discriminant analysis effect size (LEfSe) analysis were performed with the LEfSe tool (http://huttenhower.sph.harvard.edu/galaxy). The cladogram was generated using the online LEfSe project. For the LEfSe analysis, we used the Wilcoxon test to detect significantly different abundances between the pregnancy and non-pregnancy groups in fresh cycles and performed Linear discriminant analysis (LDA) scores to estimate the effect size (threshold: ≥ 2) at all levels. Based on the LEfSe result, we selected the genera abundance with Top 30 and conducted the Spearman correlation heatmap between dominant genera through the corrplot package of R software to show the relationships among dominant genera. The correlation between differential abundances at genus level and serum sex hormone levels is calculated by the Spearman correlation test, and the thermal map is drawn by R software corrplot package so as to reveal the important relationship between differential abundant genera and sex hormone (E_2, P). In SPSS, Kruskal-Wallis H test, chi-square test, and One way ANOVA were used to compare the baseline data between groups. Univariate and multivariate logistic regression were used to evaluate the association of clinical factors and microbiota composition with clinical pregnancy. The continuous variables with normal distribution were expressed as the mean ± standard deviation (SD), and the variables with non-normal distribution were presented as the median (interquartile range). *P*-value of less than 0.05 is considered significant.

Accession Number

The sequence data in this study have been deposited in NCBI under BioProject number PRJNA693672.

RESULTS

Characteristics of the Participants

A total of 124 patients contributed cervical samples. Among them, the samples from 20 patients were contaminated with blood during the collection process, barring them from further analysis. Samples derived from four patients failed sequencing

due to low DNA content. Ultimately, 100 patients were included in the study. A total of 51 patients underwent fresh IVF-ET cycle, 25 of whom achieved clinical pregnancy (Group FP, FP01-FP25) and 26 were non-pregnant (Group FN, FN01-FN26). A total of 49 patients underwent a frozen-thawed ET cycle, 27 of whom were clinically pregnant (Group TP, TP01-TP27) and 22 were non-pregnant (Group TN, TN01-TN22). **Table 1** summarizes the baseline characteristics of these four groups.

Analysis of Cervical Microbiota Profiles

A total of 5 704 398 clean reads were generated by 16S rRNA gene sequencing. After using USEARCH to cluster and filter on a similarity of 0.97, 28 122 OTUs were obtained. **Figure 1A** shows the number and distribution of OTUs among the four groups. At the phylum level, Firmicutes predominated in infertile women, followed by Actinobacteria and Bacteroidetes (**Figure 1B**). At the genus level, the cervical microbiota of infertile women consisted primarily of *Lactobacillus*, followed by *Gardnerella*, *Desulfovibrio*, *Prevotella*, and *Bacteroides* (**Figure 1C**). It is worth noting that the average relative abundance of *Lactobacillus* in most cases of FP group (66.76%), FN group (85.82%), TP group (63.84%), and TN group (69.27%) was higher than 60%, with the difference between fresh goups was significant while not significant between frozen-thawed goups (**Table S1**). *Lactobacillus*, as the dominant bacterial genus, consisted of different classifications at the species level. **Figure 1D** showed the average relative abundance of microbiota in four groups at the level of species. Due to technical limitations, only some species have been detected. Among the five types of *Lactobacillus* species detected, *L. crispatus* has the highest average relative abundance. In fresh IVF-ET cycles, the relative abundances of *L. crispatus*, *L. jensenii*, and *L. gasseri* in the pregnancy group were all lower than those in the non-pregnancy group, but these differences were not statistically significant (**Table S1**). Similarly, in the frozen-thawed ET cycles, the relative abundance of *L. crispatus* in the pregnancy group was lower than that in the non-pregnancy group. While the relative abundances of *L. jensenii* and *L. gasseri* in the pregnancy group were higher than those in the non-pregnancy group, these differences again were not statistically significant (**Table S1**). The bar graph in **Figure 1E** shows the cervical microbiota distribution of 100 infertile women at the genus level. Among them, 84% of the samples presented *Lactobacillus* as the dominant bacteria (84/100); 48.8% of these samples were from clinically pregnant women (41/84). Of the remaining 16 samples dominated by other bacteria, 68.8% of patients were clinically pregnant (11/16). The abundance of *Gardnerella* in the cervical microbiota of four patients was greater than 60% (FP04, FN13, TN08, TN12).

The microbiota diversity of the samples from fresh IVF-ET cycles was significantly lower than that of the samples from frozen-thawed ET cycles. The α diversity index dilution curves Chao1 richness (**Figures 2A, B**) of the species abundance of both fresh and frozen-thawed ET cycles showed a smooth trend, indicating that the sequencing depth was sufficient to cover most of the microorganisms in each sample. Shannon and Simpson indices were used to evaluate α diversity. In fresh and frozen-thawed cycles, the α diversity of the pregnancy group was

TABLE 1 | Characteristics of the participants.

		Group FN (n = 26)	Group TN (n = 22)	Group FP (n = 25)	Group TP (n = 27)	*P*-value
Age (years)		33.0 ± 3.9	32.5 ± 4.0	31.2 ± 4.0	31.1 ± 4.4	0.247[a]
BMI (kg/m^2)		23.2 ± 3.2	23.2 ± 4.0	23.3 ± 4.1	22.8 ± 3.3	0.974[a]
Infertility duration (years)		3.4 ± 2.5	4.3 ± 2.3	3.4 ± 2.1	4.6 ± 2.9	0.238[b]
Smoking	Yes	0	2 (9.1%)	1 (4.0%)	2 (7.4%)	0.524[c]
	No	26 (100%)	20 (90.9%)	24 (96.0%)	25 (92.6%)	
Alcoholism	Yes	0	1 (4.5%)	0	0	0.220[c]
	No	26 (100%)	21 (95.5%)	25 (100%)	27 (100%)	
Menstrual cycle	regular	25 (96.2%)	18 (81.8%)	21 (84.0%)	25 (92.6%)	0.297[c]
	irregular	1 (3.8%)	4 (18.2%)	4 (16.0%)	2 (7.4%)	
Indication[d]	Male factors	10 (38.5%)	9 (40.9%)	13 (52.0%)	10 (37.0%)	0.704[c]
	Tubal factors	14 (53.8%)	16 (72.7%)	13 (52.0%)	19 (70.4%)	0.305[c]
	PCOS	4 (15.4%)	3 (13.6%)	3 (12.0%)	5 (18.5%)	0.962[c]
	Ovarian dysfunction	2 (7.7%)	2 (9.1%)	2 (8.0%)	2 (7.4%)	1.000[c]
	Unexplained	4 (15.4%)	1 (4.5%)	2 (8.0%)	2 (7.4%)	0.657[c]
	Others	1 (3.8%)	1 (4.5%)	4 (16.0%)	2 (7.4%)	0.459[c]
Previous pregnancy times		0.88	0.59	0.56	0.74	0.862[b]
Previous childbirth times		0.15	0.05	0.08	0	0.332[b]
Previous miscarriage times		0.42	0.50	0.36	0.59	0.884[b]
Previous ectopic pregnancy times		0.23	0.00	0.12	0.15	0.166[b]
Previous IVF cycle		0.42	0.50	0.24	0.81	0.082[b]

Values are given as mean ± SD, number (%), and mean. BMI, Body Mass Index; PCOS, polycystic ovary syndrome; IVF, In Vitro Fertilization; FN, fresh IVF-ET cycle non-pregnancy; FP, fresh IVF-ET cycle pregnancy; TN, frozen-thaw ET cycle non-pregnancy; TP, frozen-thaw ET cycle pregnancy.

[a]*By One way ANOVA.*

[b]*By Kruskal-Wallis H test.*

[c]*By chi-square test.*

[d]*Not 100% in total due to multiple diagnoses for some patients.*

**P < 0.05.*

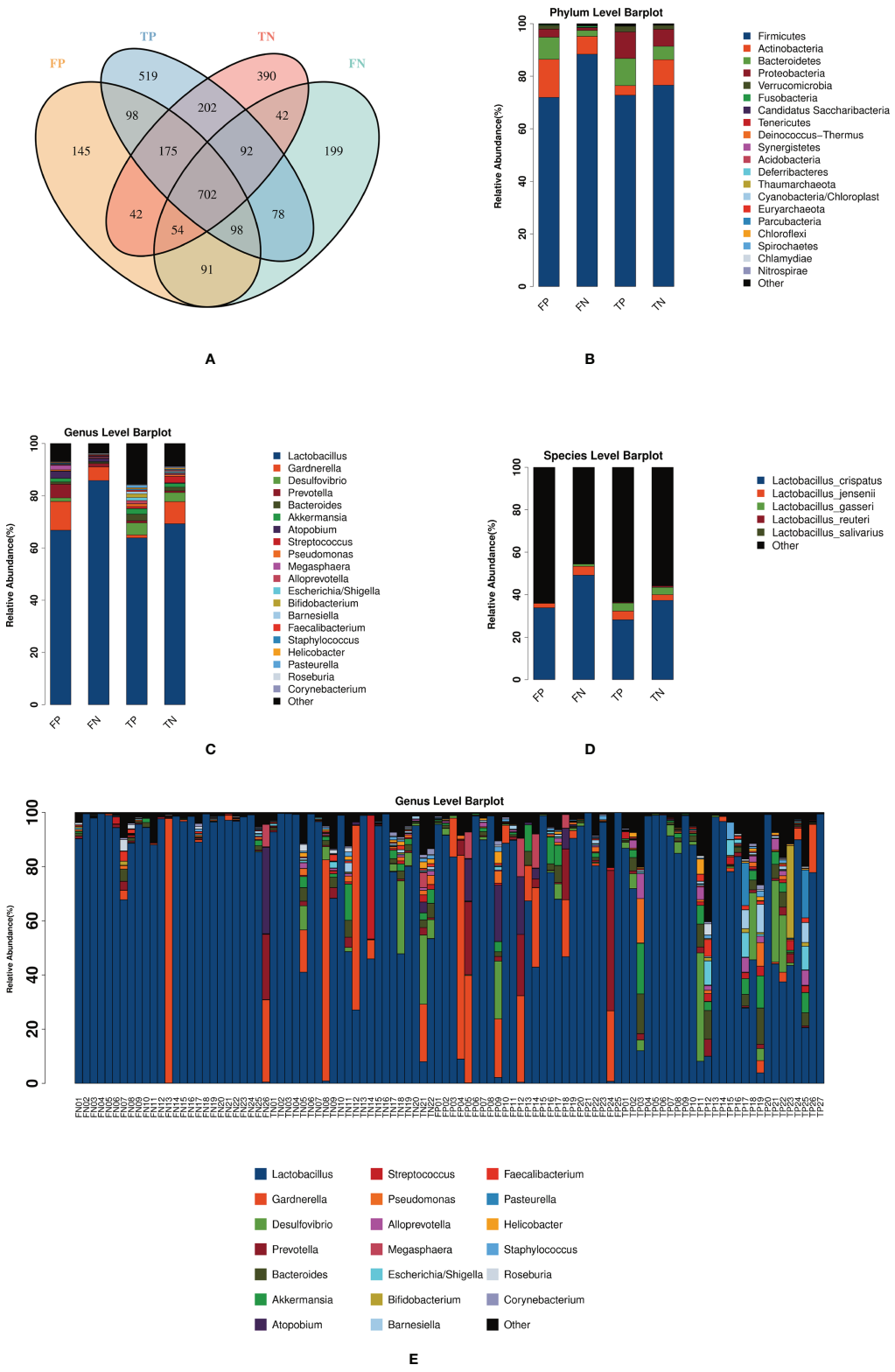

FIGURE 1 | Shows the distribution of the microbiota in the FP, FN, TP, and TN groups. **(A)** Venn diagram: represents the OTU distribution of the four groups. **(B–D)** The average relative abundance of the sample microbiota in four groups at the level of phylum, genus, and species. **(E)** The relative abundance of the 100 sample microbiota at the genus level. FN, fresh IVF-ET cycle non-pregnancy; FP, fresh IVF-ET cycle pregnancy; TN, frozen-thaw ET cycle non-pregnancy; TP, frozen-thaw ET cycle pregnancy.

higher than that of the non-pregnancy group; the difference was statistically significant between fresh groups. In fresh IVF-ET cycles, the Shannon index of the non-pregnancy group (mean = 1.345) was significantly lower than that of the pregnancy group (mean = 2.085), with $P = 0.0078$ (**Figure 2C**). The Simpson index of the non-pregnancy group (mean = 0.354) was significantly lower than that of the pregnancy group (mean = 0.537), with $P = 0.0117$ (**Figure 2D**). In the frozen-thawed ET cycle, the Shannon index (mean = 2.221) of the non-pregnancy group was lower than that of the pregnancy group (mean = 2.966), with $P =$

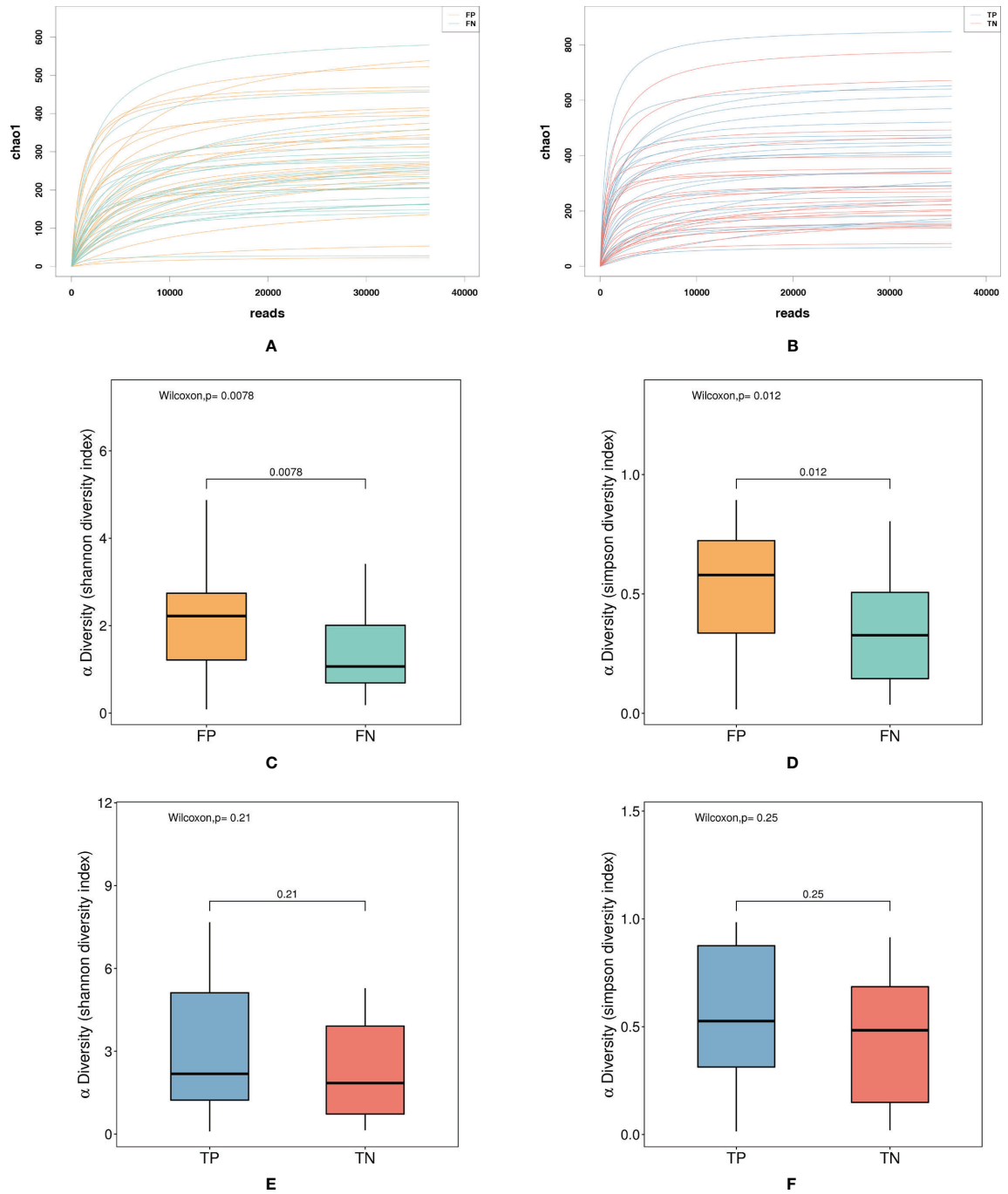

FIGURE 2 | Shows the α diversity of the FP, FN, TP, and TN groups. **(A)** Dilution curve: FP, FN group Chao diversity index dilution curve **(B)** Dilution curve: TP, TN group Chao diversity index dilution curve **(C)** Box diagram, FP, FN group Shannon diversity index **(D)** Box plot: FP, FN group Simpson diversity index **(E)** Box plot: TP, TN group Shannon diversity index **(F)** Box plot: TP, TN group Simpson diversity index. FN, fresh IVF-ET cycle non-pregnancy; FP, fresh IVF-ET cycle pregnancy; TN, frozen-thaw ET cycle non-pregnancy; TP, frozen-thaw ET cycle pregnancy.

0.2111 (**Figure 2E**). The Simpson index of the non-pregnancy group (mean = 0.455) was lower than that of the pregnancy group (mean = 0.556), with $P = 0.2503$ (**Figure 2F**).

We used PCoA and ANOSIM analysis to elucidate the β diversity of the microbiota jointly between pregnancy and non-pregnancy groups in fresh and frozen-thawed cycles. The β diversity analysis aimed to compare the overall microbiota composition between pregnancy and non-pregnancy groups. In fresh IVF-ET cycles, PCoA of the pregnancy and non-pregnancy group samples showed different distributions in the first and second principal coordinates (**Figure 3A**, $P = 0.004$), while in frozen-thawed ET cycles, PCoA revealed similar distributions between the pregnancy and non-pregnancy group samples (**Figure 3B**, $P = 0.072$). In the ANOSIM of both fresh and frozen-thawed ET cycles, $R > 0$ and $P < 0.05$ revealed significant differences between the pregnancy and the non-pregnancy groups (fresh IVF-ET cycle, $R = 0.242$, $P = 0.001$; frozen-thawed ET cycle, $R = 0.062$, $P = 0.045$); the difference between the fresh groups was greater than the difference between the frozen-thawed groups. In addition, $R = 0.062$ indicated that there was little difference between the pregnancy and non-pregnancy group samples in the frozen-thawed cycles (**Figures 3C, D**).

Given that the α diversity and β diversity in the fresh IVF-ET cycle groups were statistically different, we further conducted LEfSe analyses, in which LDA score was used to estimate the effect of the abundance of each component on a different effect. As shown in **Figure 4A**, the clustering result of bacterial taxa with different abundance at different levels can be observed. At the genus level, abundance of 35 genera was different between pregnancy and non-pregnancy groups (LDA scores more than 2.0, **Table S2**) in the fresh IVF-ET cycle. Among these genera, the LDA scores of *Lactobacillus*, *Akkermansia*, *Desulfovibrio*, *Atopobium*, and *Gardnerella* were

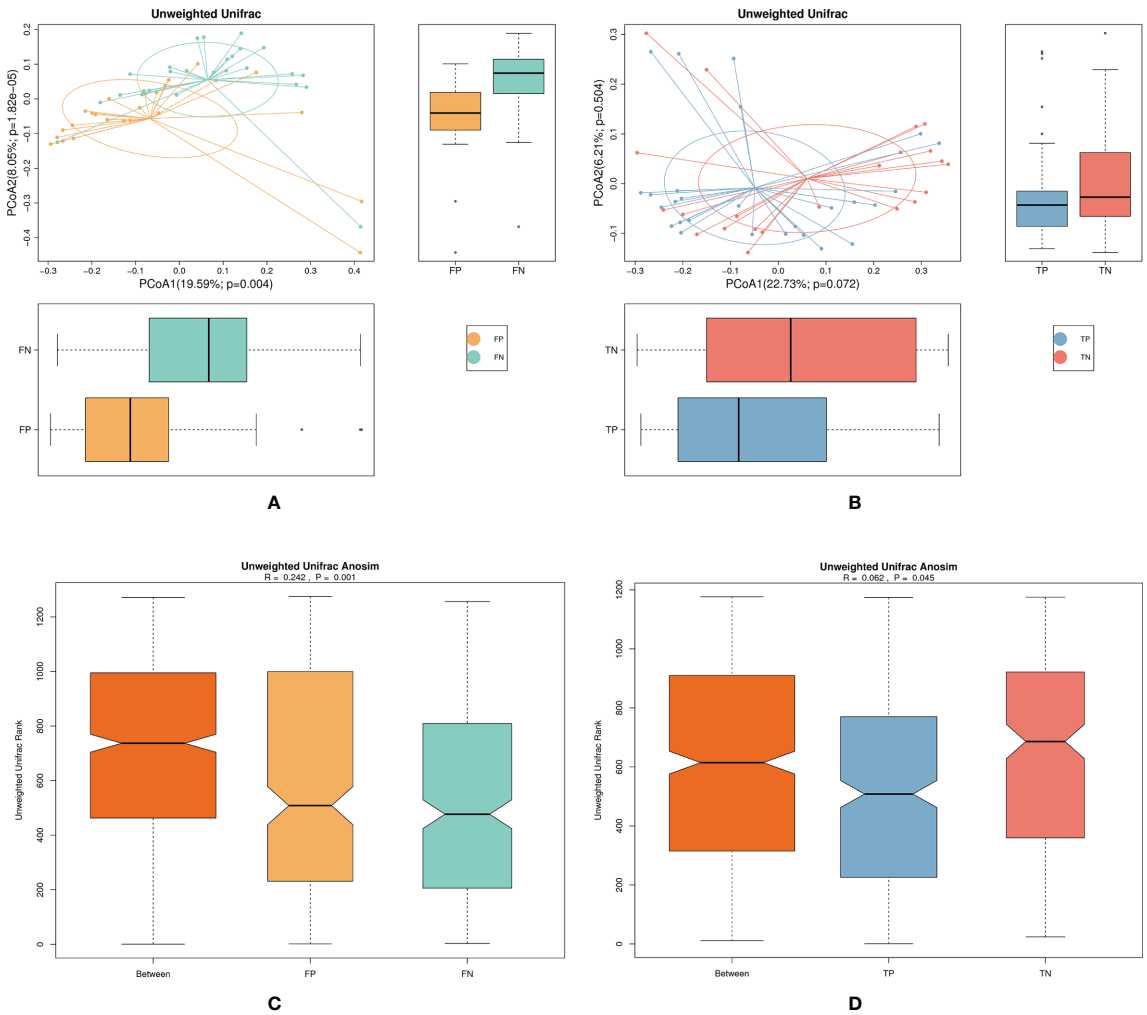

FIGURE 3 | Shows the β diversity of the microbiota in the pregnancy and non-pregnancy groups of the fresh and frozen-thawed cycles. **(A)** FP, FN group microbiota unweighted PCoA analysis chart **(B)** TP, TN group microbiota unweighted PCoA analysis chart **(C)** FP, FN group microbiota unweighted Anosim analysis chart **(D)** TP, TN group microbiota unweighted Anosim analysis chart. FN, fresh IVF-ET cycle non-pregnancy; FP, fresh IVF-ET cycle pregnancy; TN, frozen-thaw ET cycle non-pregnancy; TP, frozen-thaw ET cycle pregnancy, PCoA, Principal co-ordinates analysis; ANOSIM, Analysis of similarity.

greater than 3.6, indicating a relatively large degree of difference between the groups. Furthermore, Spearman correlation coefficient analysis of the top 30 genera exhibiting differential abundance demonstrated that *Lactobacillus* had a negative correlation with other genera, of which the strongest negative correlation was with *Gardnerella* and *Dialister* (**Figure 4B**). To identify the underlying contributing factors for the relative abundance of the 35 genera which were detected based on LEfSe analysis, we assessed the serum sex hormone levels (E_2, P) on the day of ET and conducted a Spearman thermal map analysis of the correlation between the serum sex hormone levels and genera abundance. The results showed that the abundance of these genera had a strong correlation with the serum sex hormone levels on the day of transplantation. As shown in **Figure 4C**, the abundance of 19 genera was correlated with the E_2 level (*Lactobacillus*, *Parabacteroides*, *Acetatifactor*, *Alloprevotella*, *Helicobacter*, *Parasutterella*, *Clostridium IV*, *Phascolarctobacterium*, *Akkermansia*, *Roseburia*, *Bilophila*, *Rhodococcus*, *Butyricimonas*, *Desulfovibrio*, *Neisseria*, *Fusobacterium*, *Anaerotruncus*, *Clostridium XVIII*, *Faecalibacterium*). Among them, the abundance of *Lactobacillus* was positively correlated with E_2, while the remaining genera were negatively correlated with E_2. The abundance of seven genera was negatively correlated with the P level (*Parabacteroides*, *Parasutterella*, *Acetatifactor*, *Alloprevotella*, *Helicobacter*, *Clostridium IV*, *Butyricimonas*).

Multivariate Analysis

In fresh IVF-ET cycles, the diversity between the microbiota of the pregnancy and non-pregnancy groups was statistically different; we ultimately selected five genera, (*Lactobacillus*, *Gardnerella*, *Atopobium*, *Akkermansia*, *Desulfovibrio*) with large differences between the groups based on the results of LEfSe analysis for further analysis. We defined Log (*Lactobacillus*/others) as the logarithmic conversion of the relative abundance ratio of *Lactobacillus* to the other four genera. In view of the fact that in addition to microbiota, embryonic and maternal factors may also have impacts on clinical pregnancy, we compared the endometrial thickness, E_2,

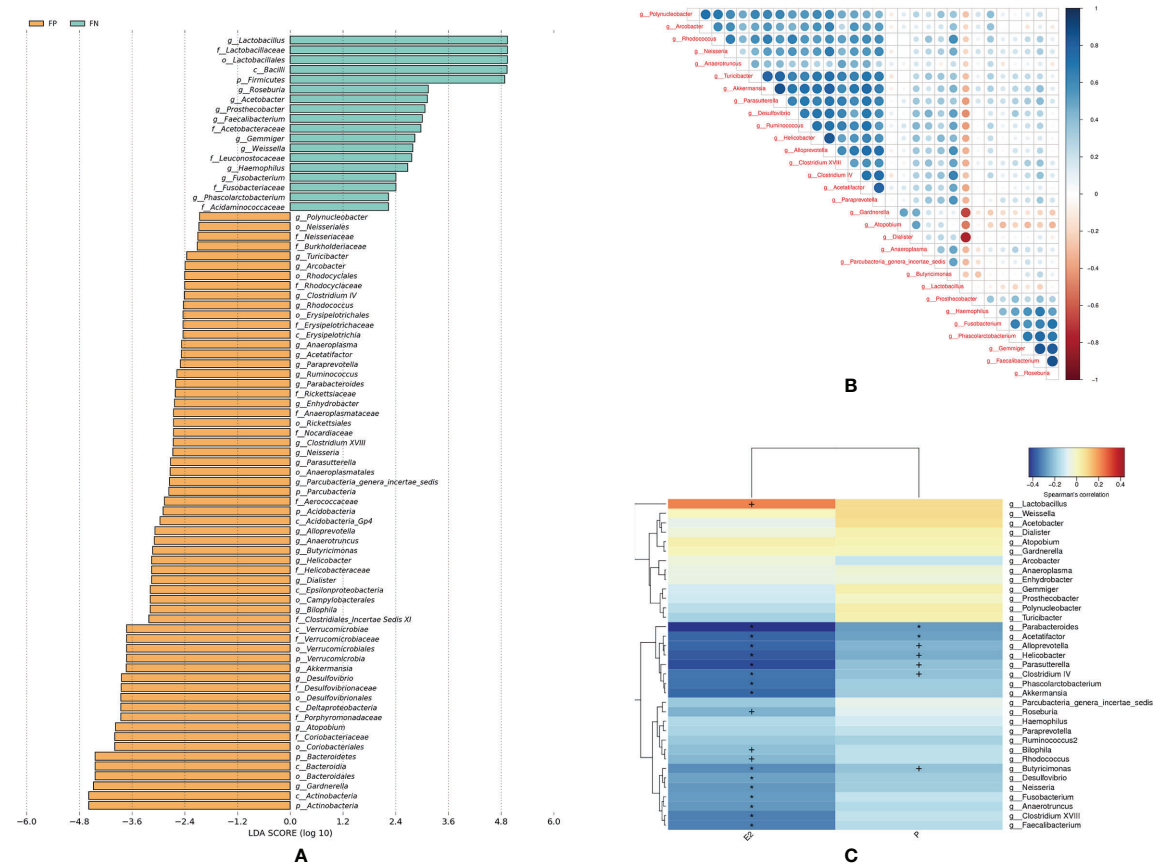

FIGURE 4 | Shows the differential abundance and association analysis among the cervical microbiota of the fresh pregnancy group and the non-pregnancy group. **(A)** LEfSe analysis chart: The LDA score of the genera which showed differentially abundance between pregnant and non-pregnant women in fresh IVF-ET cycle. **(B)** Spearman correlation coefficient analysis: the important patterns and relationships among the genera abundance with Top 30 in FP, FN groups **(C)** Spearman thermal map analysis of the correlation between the serum sex hormone levels and genera which were detected in LEfSe analysis. FN, fresh IVF-ET cycle non-pregnancy; FP, fresh IVF-ET cycle pregnancy. E_2, estradiol; LDA, Linear discriminant analysis; LEfSe, Linear discriminant analysis effect size analysis; P, progesterone. $^{+}P < 0.05$; $^{*}P < 0.01$.

embryo quality, and other related clinical factors between the pregnancy and non-pregnancy group (**Table 2**). Cleavage embryos graded above 8CII on day 3 were considered as good-quality embryos (Wu et al., 2020). Moreover, we conducted univariate logistic regression including age, BMI, endometrial thickness, and other factors that may affect the clinical pregnancy rate as independent variables. As shown in **Table 3**, Log (*Lactobacillus*/others) and the number of good-quality embryos transferred were correlated with clinical pregnancy. Consequently, we included these two variates in the multivariate logistic regression analysis (**Table 4**). The result revealed that the number of good-quality embryos transferred was associated with increased odds of clinical pregnancy and a lower ratio of *Lactobacillus* to other bacteria in the cervical microbiota was associated with decreased odds of clinical pregnancy, while only the latter was statistically significant.

TABLE 4 | Multivariate logistic regression assessing the association of the number of good-quality embryos transferred and microbiota composition with clinical pregnancy.

	B	OR	95% CI	*P*-value
Log (*Lactobacillus*/others)	-4.57	0.633	0.419-0.957	0.030*
Number of good-quality embryo transferred	1.174	3.234	0.984-10.634	0.053

*OR, odds radio; 95% CI, 95% confidence interval. *P < 0.05.*

DISCUSSION

Over the past decade, several researchers have assessed the reproductive tract microbiota of female patients undergoing IVF through 16S rRNA sequencing technology (Hyman et al., 2012; Moreno et al., 2016; Bernabeu et al., 2019). To the best of our knowledge, this is the first study on the cervical microbiota of IVF patients with different clinical pregnancy outcomes. Our results suggested that, whether in fresh or frozen-thawed ET cycles, *Lactobacillus* is the predominant genus present in the cervical microbiota of IVF patients. In fresh IVF-ET cycles, the cervical microbiota in the pregnancy and non-pregnancy groups showed differences in α diversity and β diversity. Moreover, *Lactobacillus*, *Akkermansia*, *Desulfovibrio*, *Atopobium*, and *Gardnerella* were differentially abundant between pregnant and non-pregnant women and they had the LDA scores of all taxa investigated. Among them, *Lactobacillus* was negatively correlated with other genera and positively correlated with serum E_2 levels. Ultimately, we found that both the composition of the cervical microbiota and the number of good-quality embryos transferred were significantly correlated with the clinical pregnancy rate in fresh IVF-ET cycles.

Our results revealed that the α diversity of the pregnancy group was higher than that of the non-pregnancy group, regardless of the cycle and the difference was statistically significant in fresh IVF-ET cycles. This result is inconsistent with the differences between endometrial and vaginal microbiota reported in previous studies (Moreno et al., 2016; Bernabeu et al., 2019). We consider the main reason for the differences in the conclusions of these studies to be related to the different sites for microbiota collection. Our understanding of the female

TABLE 2 | Supplement of baseline data for fresh IVF-ET cycle patients.

	Non-pregnancy (n = 26)	Pregnancy (n = 25)	*P*-value
E_2 (pg/mL)	1333 (689.75, 1778.75)	1548 (1111, 2352.5)	0.407
Total Gn dosage (IU)	2400 (1762.5, 3017)	2475 (1837.5, 3000)	0.741
Gn days (days)	10 (8, 11.25)	10 (8.5, 11.5)	0.901
Endometrial thickness (mm)	10 (8.75, 12)	10 (10, 12)	0.518
Number of follicles	8.5 (6, 15.25)	11 (8, 16)	0.160
Number of good-quality embryo transferred	2 (1, 2)	2 (2, 2)	0.024*

Values are given as median (25^{th}, 75^{th} percentile).
*Tested by Kruskal-Wallis H test. *P < 0.05.*
E_2, estradiol; Gn, Gonadotropin.

TABLE 3 | Univariate logistic regression assessing the association of clinical factors and microbiota composition with clinical pregnancy.

	B	OR	95% CI	*P*-value
Age (years)	-0.121	0.886	0.762-1.030	0.114
BMI (kg/m2)	0.011	1.011	0.868-1.077	0.893
Infertility duration	0.011	1.011	0.795-1.284	0.931
E_2 (pg/mL)	0.000	1.000	1.000-1.001	0.652
Total Gn dosage (IU)	0.000	1.000	0.999-1.001	0.714
Gn days (days)	0.000	1.000	0.777-1.286	0.998
Endometrial thickness (mm)	0.041	1.042	0.856-1.269	0.681
Number of follicles	0.074	1.077	0.975-1.188	0.143
Log (*Lactobacillus*/others)	-0.515	0.597	0.394-0.906	0.015*
Number of good-quality embryo transferred	1.269	3.557	1.178-10.738	0.024*

*BMI, Body Mass Index; E_2, estradiol; Gn, Gonadotropin; OR, odds radio; 95% CI, 95% confidence interval. *P < 0.05.*

reproductive tract microbiota is gradually changing. Previously, researchers speculated that vaginal bacteria colonized the upper genital tract through the cervix, thereby affecting pregnancy outcomes (Mitchell et al., 2015). In a 2016 study, the researchers conducted two samplings of vaginal and endometrial microbiota on women at childbearing age during the same menstrual cycle. The results showed that the vaginal microbiota was different from the endometrial microbiota, also indicating that the endometrial microbiota is not completely derived from the vagina (Moreno et al., 2016). In 2017, Chen et al. sampled and sequenced the reproductive-tract microbiota of 110 women at childbearing age. They found that the community types of some subjects were different in the cervix and endometrium; moreover, the microbiota from the vagina to the peritoneal fluid was continuous changing (Chen et al., 2017). The results of a study in 2020 validated the previous conclusion. The researchers collected samples from the lower third of the vagina, posterior fornix, cervical mucus, endometrium, and peritoneal fluid of patients with endometriosis for sequencing. The results showed that the cervical mucus of endometriosis patients began to show significant differences in community diversity that increased upward the reproductive tract (Wei et al., 2020). These continuous changes in the female reproductive tract microbiota indicate that research on the cervical microbiota must be more in-depth to provide a greater understanding of its potential impact on pregnancy outcomes.

Lactobacillus was still the predominant genus in the cervical microbiota of most IVF patients, which is consistent with the previous study (García-Velasco et al., 2017). *Lactobacillus* dominates the lower genital tract of women at reproductive age mainly *via* utilizing the glycogen deposited under the action of estrogen (Spear et al., 2014; Nasioudis et al., 2015) and impeding the growth of other bacteria through metabolized lactic acid (O'Hanlon et al., 2013; Roselló et al., 2013), competitive inhibition (Boris and Barbés, 2000) or bacteriocin and other substances production (Ojala et al., 2014). Of interest, we found that the relative abundance of *Lactobacillus* dominated patients in the non-pregnancy group was significantly higher than the clinical pregnancy group of fresh cycles, while non-significant difference was seen between frozen-thawed groups. In consistence, Bernabeu et al. reported that the abundance of vaginal *Lactobacillus* on the day of ET in women who achieved pregnancy was not significantly different from the control group after frozen embryo transfer (Bernabeu et al., 2019). It could be found that the E_2 level on the day of embryo transfer was higher in the fresh groups than in the frozen-thawed groups (**Table S3**). E_2 concentrations have been reported to decrease from the day of human chorionic gonadotropin (hCG) trigger to ET and this decrease was significantly slight in patients who did not have a live birth (Hyman et al., 2012). Fluctuations during ovulation and especially low levels during the periovulatory period of vaginal *Lactobacillus* in women was also observed (Zhao et al., 2020). These findings indicate that the high abundance of Lac*tobacillus* in *the* non-pregnancy group of fresh cycles may be partially due to the maintained high level of E_2 though its concentrations on the day of hCG were not recorded. Thus future longitudinal research that collecting more comprehensive information is necessary to clarify this phenomenon.

Moreover, compared with the results of Vergaro's research on vaginal bacterial communities (76.7% *Lactobacillus* dominance) (Vergaro et al., 2019), our 16S rRNA results showed a lower percentage of *Lactobacillus* dominance of the cervical microbiota (73%). Nor did we uncover a positive correlation between *L. crispatus* and clinical pregnancy outcome (**Table S4**). It has been reported by Chen et al. that distinct microbial communities exist in a continuum along the female reproductive tract, involving the vagina, cervical canal, fallopian tubes, uterus, and peritoneal fluid (Chen et al., 2017). The specific anatomy of the cervix and cervical mucus may function as a partial ascent filter (Mitchell et al., 2015), exerting an impact on the composition of the cervical microbiota. Regarding *L. crispatus*—validated by Koedooder et al. to be a predictor of IVF outcome—a favorable profile with < 60% *L. crispatus* dominance of the vaginal microbiota indicated the highest chance of pregnancy among the other grouping strategies (Koedooder et al., 2019). The results of this study suggest that *L. crispatus* may have a more complex relationship with the clinical pregnancy rate.

Beyond the predominance of *Lactobacillus*, non-*Lactobacillus*-dominanced samples were also detected in our pregnancy groups. Despite the fact that numerous preceding studies reported that communities lacking *Lactobacillus* as the dominant strain were not conducive to pregnancy outcomes (Moreno et al., 2016; Wee et al., 2018; Liu et al., 2019; Singer et al., 2019), non-*Lactobacillus*-dominanced microbiota was seen in healthy or pregnant people with less exceptional. Reid suggested that the presence of non-*Lactobacillus* organisms does not necessarily confer disease (Reid, 2016). A trial in 2019 also revealed that some patients achieved ongoing pregnancies with 0% *Lactobacillus* in the endometrium (Hashimoto and Kyono, 2019). This team failed to prove the obvious benefits of establishing an endometrium dominated by *Lactobacillus* in pregnancy outcomes (Kyono et al., 2019). These conflicting experimental results suggest that we must redefine the community most beneficial to IVF outcomes. When the cervical microbiota presents non-*Lactobacillus*-dominance, clinical pregnancy can still be achieved.

Age, duration of infertility, and other factors also have an impact on the clinical pregnancy rate (Brosens et al., 2004; Eijkemans et al., 2008). We ultimately used multivariate logistic regression analysis to analyze the impact of various factors on the clinical pregnancy rate, deviating from previous research (Moreno et al., 2016; Bernabeu et al., 2019). Based on the results of univariate logistic regression analysis, we selected two indicators—the ratio of *Lactobacillus* to other bacteria in the cervical microbiota and the number of good-quality embryos transferred—as independent variables. Finally, through multivariate regression analysis, both the ratio of *Lactobacillus* to other bacteria in the cervical microbiota and the quality of the embryos transferred contributed to the IVF clinical pregnancy rate.

Unfortunately, due to the small sample size used in our research, we cannot validate our model. In addition, our

research did not involve the collection of specimens at other time points and other locations in the reproductive tract, rendering some of our assertions unverifiable. By sequencing variable regions (V3-V4) of the 16S rRNA gene, the ability to characterize members of the cervical microbial community to species-level taxonomy was limited. These constitute the limitations of our experiment.

In conclusion, the results of this study indicate that the cervical microbiota has an impact on the outcome of IVF. Although the relative abundance of *Lactobacillus* may be related to the clinical pregnancy rate, it is impossible to conclude that non-*Lactobacillus*-based microorganisms are not conducive to pregnancy. Further studies must clarify the optimal abundance of *Lactobacillus* in patients undergoing IVF treatment and explore the mechanisms by which multiple microbiotas affect the IVF outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA693672.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Shengjing Hospital of China Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT and XH designed and were responsible for this project. JT and XH collected samples and conducted clinical studies. XH and SW performed statistical analysis on the data. XH and PL wrote this paper. XH and PL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Microbiome Compositions From Infertile Couples Seeking *In Vitro* Fertilization, Using 16S rRNA Gene Sequencing Methods: Any Correlation to Clinical Outcomes?

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Background: Bacterial infections are usually suspected in infertile couples seeking IVF with no clear understanding of the microbial compositions present in the seminal fluids and vaginal niche of the patients. We used next-generation sequencing technology to correlate microbiota compositions with IVF clinical outcomes.

Methods: Thirty-six couples were recruited to provide seminal fluids and vaginal swabs. Bacterial DNA was extracted, and V4 region of the 16S rRNA was amplified and sequenced in a pair-end configuration on the Illumina MiSeq platform rendering 2 × 150 bp sequences. Microbial taxonomy to species level was generated using the Greengenes database. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify biologically and statistically significant differences in relative abundance.

Results: Seminal fluid microbiota compositions had lower bacterial concentrations compared with the vagina, but species diversity was significantly higher in seminal fluid samples. Azoospermic subjects had more relative abundance of *Mycoplasma* and *Ureaplasma.* In Normospermic semen, *Lactobacillus* (43.86%) was the most abundant, followed by *Gardnerella* (25.45%), while the corresponding vaginal samples, *Lactobacillus* (61.74%) was the most abundant, followed by *Prevotella* (6.07%) and *Gardnerella* (5.86%).

Conclusions: Semen samples with positive IVF were significantly colonized by *Lactobacillus jensenii* (P=0.002), *Faecalibacterium* (P=0.042) and significantly less colonized by *Proteobacteria*, *Prevotella*, *Bacteroides*, and lower *Firmicutes*/*Bacteroidetes* ratio compared with semen samples with negative IVF. Vaginal samples with positive IVF clinical outcome were significantly colonized by *Lactobacillus gasseri*,

less colonized by *Bacteroides* and *Lactobacillus iners*. This study has opened a window of possibility for *Lactobacillus* replenishments in men and women before IVF treatment.

Keywords: seminal fluid, microbiome, *in vitro* fertilization - pregnancy, infertility, bacteria infections, vagina, 16S rRNA, sequencing

INTRODUCTION

Bacterial infections affecting the reproductive tracts of males and/or females with infertility have been documented in previous studies using culture methods (Eggert-Kruse et al., 1995; Gdoura et al., 2008). Some bacteria, fungi, viruses, and parasites are known to interfere with reproductive functions in both male and females of reproductive age, and infections of the genitourinary tract account for about 15% of male infertility cases. More than a few bacteria, including *Lactobacillus iners*, *Gardnerella vaginalis*, *Escherichia faecalis*, *E. coli*, and *Staphylococcus aureus*, have been found to be associated with male infertility as demonstrated using polymerase chain reaction (PCR) (Franasiak and Scott, 2015). Bacterial vaginosis (BV) has been found to be a major risk factor for infertility (Al-Awadhi et al., 2013). Some specific bacteria incriminated in BV, such as *Atopobium vaginae*, *Ureaplasma vaginae*, *U. parvum*, *U. urealyticum*, *Gardnerella vaginalis*, and reduced abundance of *Lactobacillus* species, are associated with infertility in women (Onemu and Ibeh, 2001). Women with endometrial dysbiosis have also been found to experience implantation failure leading to infertility (Momoh et al., 2007). While vaginal microbiota is normally under the influence of oestrogen (Ibadin and Kai, 2008), endometrial microbiota is not affected by hormonal fluctuations (Ekhaise and Richard, 2001).

There are several studies confirming that a vaginal microbiota replete with relative abundances of *Lactobacillus* species, devoid of bacterial vaginosis, leads to more positive IVF clinical outcomes (Nwadioha et al., 2016).

Besides other sexually transmitted infections, genital mycoplasmas are associated with poor reproductive health of women including but not limited to endometritis, cervicitis and pelvic inflammatory disease, and adverse pregnancy outcomes (Lis et al., 2015; Taylor et al., 2018). The pregnancy success rate of the various assisted reproductive health care such as *in vitro* fertilization (IVF) tend to be reduced as a result of prior *Mycoplasma* colonization of the female and male genital tract (Günyeli et al., 2011). One wonders whether genital *Mycoplasma* was the only pathogen associated with poor pregnancy outcome, although several studies have shown that *Mycoplasma* species can attach to spermatozoa and remain adherent to spermatozoa after assisted reproductive treatment washing procedures (Ibadin and Osemwenkha, 2013; Ahmadi et al., 2018).

Clinical studies have shown that bacterial contamination of the embryo transfer catheter has significant negative effect on the clinical pregnancy rates (Ikechebelu, 2003). Approximately 35% of infertile women are afflicted with post-inflammatory changes of the oviduct or surrounding peritoneum that interfere with tubal-ovarian function mostly as a result of infection and are likely to develop ectopic (tubal) pregnancy (Swift and Liu, 2014).

In Africa, especially Nigeria, little is known about the bacterial communities found in the seminal fluids of men seeking reproductive health care with next-generation sequencing technology. A recent pilot study of seminal fluid in a tertiary hospital revealed varying bacterial community diversities that are unique in each sample in contrast to culture-dependent methods (Ndiokwere et al., 2019). We have also previously shown that women with bacterial vaginosis (BV) had varying proportions of diverse bacteria including *Lactobacillus* species in all BV subjects, but the total number of all the BV-associated microbes (*Gardnerella*, *Prevotella*, *Magasphaera*, and others) outnumbered *Lactobacillus* genera (Anukam et al., 2019). In the present study, next-generation 16S rRNA gene sequencing method was used to compare seminal bacterial composition in couples seeking reproductive health care IVF. In addition, the study delineated semen quality, bacterial functional gene predictions, and correlated microbiota composition with clinical outcome of the IVF-assisted reproductive care.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the Ethics Review Committee on Human Research from Nnamdi Azikiwe University Teaching Hospital (Ref # NAUTH/CS/66/VOL11/175/2018/111).

Participation in the study was voluntary. Informed written consent was obtained from the patients. All methods were performed in accordance with the relevant guidelines and regulations.

Selection Criteria

Couples seeking reproductive health care at Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi campus, Anambra State, Nigeria, were referred to Life Fertility Center, Nnewi, for IVF/Embryo Transfer for self-cycle with cases of primary or secondary infertility after 1–12 years of uninterrupted sexual intercourse with partner.

Collection of Specimens

Two high vaginal swabs were collected by a qualified gynecologist with a non-lubricated sterile disposable plastic speculum. One of the swabs was agitated into a tube containing buffer for DNA preservation at ambient temperature, and the other was used for microscopy to detect leukocytes. Each semen sample was produced by masturbation after 5 days of sexual intercourse abstinence, and on the same day vaginal sample was collected.

Semen Analysis

The semen quality of the patients was analyzed with Semen Quality Analyzer-Vision (SQA-V) Gold (Medical Electronic Systems, USA), following the manufacturer's procedural instructions.

Extraction of Bacterial DNA From Vaginal Swabs/Semen Samples and Sequencing of the Amplified 16S rRNA Region

Bacterial DNA was extracted from the vaginal swabs/semen samples using a protocol developed by uBiome Inc. Briefly, samples were lysed using bead-beating, and DNA was extracted in a class 1,000 clean room by a guanidine thiocyanate silica column-based purification method using a liquid-handling robot. PCR amplification of the 16S rRNA genes was performed with primers containing universal primers amplifying the V4 region (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTA CHVGGGTWTCTAAT). In addition, the primers contained Illumina tags and barcodes. Samples were barcoded with a unique combination of forward and reverse indexes allowing for simultaneous processing of multiple samples. PCR products were pooled, column-purified, and size-selected through microfluidic DNA fractionation. Consolidated libraries were quantified by quantitative real-time PCR using the Kapa Bio-Rad iCycler qPCR kit on a BioRad MyiQ before loading into the sequencer. Sequencing was performed in a pair-end modality on the Illumina NextSeq 500 platform rendering 2 × 150 bp pair-end sequences (Almonacid et al., 2017).

16S rRNA Sequence Analysis

Raw sequence reads were demultiplexed using Illumina's BCL2FASTQ algorithm. Reads were filtered using an average Q-score >30. The paired-end sequence FASTQ reads were imported into MG-RAST pipeline for quality check (QC). EzBiocloud Microbiome Taxonomic Profile (MTP) pipeline (Yoon et al., 2017) was employed for alpha and beta diversity estimation using PKSSU4.0 version database and Open reference UCLUST_MC2 for OTUs picking at 97% cutoff. Sequences were prescreened using QIIME-UCLUST algorithms for at least 97% identity to ribosomal sequences from the RNA databases (Quast et al., 2013). Rarefication to 1,000 reads per sample was employed to calculate microbial diversity. Alpha diversity was calculated for species richness by Abundance Coverage Estimate (ACE), Chao1 and Jackknife method, while diversity indexes were calculated by Shannon, Non-parametric Shannon, and Simpson index. Principal coordinate analysis (PCoA) with Jensen-Shannon divergence distance metrices were used to evaluate beta diversity between vaginal and semen samples (Koren et al., 2013). Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was used to identify biologically and statistically significant differences in the OTU relative abundance. Phylogenetics Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the metabolic function of the metagenomes from the 16S rRNA gene dataset (Langille et al., 2013) with reference to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs categorizations (Kanehisa et al., 2014).

Availability of Data and Materials

The datasets used and or analyzed in the current study are available from the corresponding author on reasonable request.

RESULTS

Demographic Information, IVF Clinical Outcome, and Semen Quality

As shown in **Table 1A/Table 1B**, of the 36 men that were examined for semen quality and that had result for 16S rRNA gene sequencing, 11 were clinically diagnosed as having secondary infertility with duration of infertility ranging from 1 to 13 years, while 25 men were diagnosed with primary infertility, had duration of infertility from 1 to 8 years. The semen characteristics of the subjects were as follows: 11 samples were assigned as normospermia [>15×10 (6)], 7 had oligospermia [<15×10 (6)], 7 had azoospermia, 10 had asthenozoospermia, while 1 sample was classified as tetratozoospermia. The number of semen samples that had leukocytes (pyospermia) were 11, while 1 sample was assigned as oligoasthenozoospermia.

Semen and Vagina Microbiome Compositions

The first objective was to determine whether seminal fluid microbiota differ substantially with the vagina. In this regard, the alpha diversity that estimates the species richness typified by ACE, CHAO Jackknife, Shannon, and Simpson shows that seminal fluid microbiota composition is less in species richness (lower bacterial concentrations) compared with the vagina as shown in **Figure 1A**.

However, species diversity was significantly higher in seminal fluid samples represented in **Figure 1B**. The Principal Coordinate Analysis (PCoA) with Bray-Curtis metrices confirmed the differences in bacterial community diversities between semen and vagina as shown in **Supplementary Figure S1**. The EzBiocloud Microbiome Taxonomic Profile (MTP) pipeline was able to provide distinct taxonomic categories (Family, Genus, and Species) at 1% cutoff between semen and vagina as shown in **Figure 2**.

The rarefaction curve showing the number of reads between the semen samples and vaginal microbiota is presented in **Supplementary Figure S2**. For comparative purposes, we selected the corresponding semen and vagina bacterial communities in line with the three semen categories. At the genera taxonomic level, out of 621 genera identified, 6.8% were exclusive to normospermia, 20.5% to oligospermia, and 2.9% exclusive to azoospermia, while 41.5% were common to all the categories. When the genera taxa are examined with the three semen categories, *Mycoplasma* and *Ureaplasma* occurred more in relative abundance in azoospermic subjects (**Figure 3**).

At the species taxonomic level, out of 1,384 species identified, 10.3% were exclusive to normospermia, 26.4% to oligospermia, and 6.8% exclusive to azoospermia, while 27% were common to all the categories as shown in **Supplementary Figure S3**.

TABLE 1A | Demographic information and IVF clinical outcome.

Sample No.	Female age (years)	Clinical pregnancy outcome	Scan result	Sex of the baby	Live delivery	Male age (years)	Duration of marriage (years)	Duration of infertility (years)	Type of infertility
1	26–30	Negative	–	-		31–35	6	6	Primary
2	36–40	Negative	–	-		41–45	10	8	Secondary
3	36–40	Negative	–	-		46–50	6	6	Primary
4	31–35	Negative	–	-		36–40	10	10	Primary
5	26–30	Negative	–	-		36–40	5	5	Primary
6	26–30	Negative	–	-		26–30	4	4	Primary
7	36–40	POSITIVE	Singleton	Male	YES	46–50	9	8	Secondary
8	31–35	Negative	–	–		31–35	3	3	Primary
9	21–25	Negative	–	–		26–30	4	4	Primary
10	36–40	POSITIVE	Singleton	Female	YES	36–40	5	5	Primary
11	41–45	POSITIVE	Twins	Male, Female	YES	46–50	3	2	Secondary
12	36–40	Negative	–	–		41–45	4	4	Primary
13	26–30	Negative	–	–		26–30	1	1	Primary
14	31–35	POSITIVE	Singleton	Male	YES	36–40	2	2	Primary
15	26–30	Negative	–	–		26–30	5	3	Secondary
16	26–30	POSITIVE	Singleton	Male	YES	31–35	2	2	Primary
17	26–30	POSITIVE	Singleton	Male	YES	31–35	3	3	Primary
18	41–45	Negative	–	–		46–50	7	6	Secondary
19	31–35	POSITIVE	Twins	Male, Female	YES	41–45	2	2	Primary
20	26–30	Negative	–	–		31–35	6	6	Primary
21	36–40	Negative	–	–		46–50	9	9	Secondary
22	31–35	Negative	–	–		41–45	4	1	Secondary
23	36–40	POSITIVE	Twins	Male, Female	YES	46–50	12	6	Secondary
24	31–36	Negative	–	-		31–35	5	5	Primary
25	26–30	POSITIVE	Singleton	Female	No (Misca)	31–35	3	3	Primary
26	41–45	Negative	–	–		51–55	2	2	Primary
27	31–35	POSITIVE	Singleton	Male	YES	36–40	6	6	Primary
28	31–35	Negative	–	–		31–35	2	2	Primary
29	31–35	Negative	–	–		31–35	4	4	Primary
30	31–35	Negative	–	–		36–40	5	4	Secondary
31	46–50	Negative	–	–		56–60	13	13	Primary
32	36–40	Negative	–	–		46–50	6	6	Primary
33	41–45	POSITIVE	Singleton	Male	YES	51–55	5	5	Primary
34	36–40	Negative	–	–		51–55	10	7	Secondary
35	31–35	POSITIVE	Singleton	Female	YES	41–45	4	1	Secondary
36	36–40	Negative	–	-		36–40	6	6	Primary

Microbiota Compositions From Normospermia Couples

Twenty-four phyla were identified from subjects with normospermia, while 22 were identified from the corresponding vagina samples. *Firmicutes* accounted for 54.47 *vs* 75.89% relative abundance, followed by *Actinobacteria* (32.26 *vs* 8.72%), *Proteobacteria* (8.60 *vs* 7.20%), *Bacteroidetes* (2.29 *vs* 5.95%), *Chlamydiae* (1.32 *vs* 0.0003%), *Fusobacteria* (0.67 *vs* 2.09%), *Tenericutes* (0.30 *vs* 0.09%), and others represented in **Supplementary Figure S4**. At the genera taxonomic level, 451 genera were identified in normospermia samples, while 331 genera were found in the corresponding females. Interestingly, 282 genera were shared between couples. *Lactobacillus* (43.86%) was the most abundant genera in semen/vagina, followed by *Gardnerella* (25.45%), *Veillonella* (7.78%), *Corynebacterium* (3.73%), *Escherichia* (2.47%), *Haemophilus* (2.36%), *Prevotella* (2.03%), and others, while in the corresponding vagina samples, *Lactobacillus* (61.74%) was the most abundant genera, followed by *Prevotella* (6.07%), *Gardnerella* (5.86%), *Streptococcus* (5.84%), *Escherichia* (5.40%), *Megasphaera* (4.51%), *Sneathia* (2.13%), and others represented in **Supplementary Figure S5**.

At the species taxonomic level, 848 species were identified in normospermia samples, while 585 species were found in the corresponding vagina samples. *Gardnerella vaginalis* (31.93%) was the most abundant species identified in 10/12 of the semen samples, followed by *Lactobacillus iners* 8/12 (15.56%), *Lactobacillus pentosus* 1/12 (12.39%), *Veillonella montpellierensis* 8/12 (9.61%), *Lactobacillus japonicus* 3/12 (4.29%), *Haemophilus parainfluenzae* 9/12 (2.91%), *Corynebacterium tuberculostearicum* 11/12 (1.75%), *Lactobacillus jensenii* 3/12 (1.73%), and others. The corresponding vaginal samples had *Lactobacillus iners* 9/12 (49.06%) as the most abundant species, followed by *Lactobacillus jensenii* 2/12 (10.60%), *Peptostreptococcus stomatis* 6/12 (6.92%), *Actinocatenispora silicis* 5/12 (6.19%), *Pasteurella pneumotropica* 2/12 (4.09%), *Actinomyces naturae* 4/12 (3.32%), *Lactobacillus*

TABLE 1B | Semen Characteristics.

Sample #	Days of abstinence	Viscosity	Liquefaction	Volume (ml)	Progressive motility (%)	Non-progressive motility (%)	Non-motile (%)	Total motility (%)	Velocity (mic/s)	Sperm conc (m/ml)	Total sperm/volume (million)	Presence of pus cells
1	3	NORMAL	NORMAL	2	9	3	88	12	32	88	176.1	+
2	5	NORMAL	NORMAL	2.5	5	2	93	7	27	22.2	55.4	NIL
3	4	ABNORMAL	ABNORMAL	1.2	0	0	0	0	0	0	0	2+
4	3	NORMAL	NORMAL	2.1	2	9	89	11	0	2	0	NIL
5	3	NORMAL	NORMAL	1.2	27	6	67	33	41	120.5	144.6	NIL
6	4	ABNORMAL	ABNORMAL	1.2	0	0	0	0	0	0	0	2+
7	3	NORMAL	NORMAL	2.8	37	10	53	47	38	77.4	216.7	NIL
8	3	NORMAL	NORMAL	1	44	8	48	52	43	72	72	NIL
9	4	NORMAL	NORMAL	3	22	10	68	32	30	33.8	101.4	NIL
10	5	NORMAL	NORMAL	1.5	39	8	53	47	42	194.5	291.8	NIL
11	3	NORMAL	NORMAL	2.3	51	12	37	63	39	52.7	121.3	NIL
12	5	NORMAL	NORMAL	0.8	17	3	80	20	46	207.6	166.1	NIL
13	4	NORMAL	NORMAL	2	52	20	28	72	32	6.4	12.8	NIL
14	4	ABNORMAL	ABNORMAL	3	0	0	0	0	0	0	0	3+
15	3	NORMAL	NORMAL	3.2	0	1	99	1	0	16	51.1	NIL
16	5	NORMAL	NORMAL	2.2	35	8	57	43	42	77.2	169.8	NIL
17	3	NORMAL	NORMAL	1.5	11	7	82	18	25	66.4	99.7	NIL
18	3	ABNORMAL	ABNORMAL	2.5	0	0	0	0	0	0	0	2+
19	4	NORMAL	NORMAL	1.5	30	6	62	38	43	163.3	244.9	NIL
20	3	ABNORMAL	ABNORMAL	5	0	0	0	0	0	0	0	2+
21	4	NORMAL	NORMAL	3.2	46	9	40	60	44	50.3	160.9	NIL
22	5	ABNORMAL	ABNORMAL	2	0	0	0	0	0	0	0	2+
23	3	NORMAL	NORMAL	2	50	4	84	16	34	157	314	2+
24	3	NORMAL	NORMAL	3.2	47	10	40	60	45	61.6	197.2	NIL
25	3	ABNORMAL	ABNORMAL	2.1	0	0	0	0	0	0	0	2+
26	4	NORMAL	NORMAL	2	5	24	29	29	5	4.7	9.4	NIL
27	3	NORMAL	NORMAL	2.4	3	2	95	5	22	75.3	180	NIL
28	4	NORMAL	NORMAL	2	40	14	46	54	34	44.6	89.2	NIL
29	3	NORMAL	NORMAL	1	3	2	95	5	22	72.2	72.2	NIL
30	5	NORMAL	NORMAL	3	8	19	73	27	10	5.6	16.9	3+
31	3	NORMAL	NORMAL	3.2	1	11	88	12	4	11.3	36	NIL
32	4	ABNORMAL	ABNORMAL	2.1	39	22	39	61	26	5.1	10.8	2+
33	5	NORMAL	NORMAL	1.5	27	6	67	33	40	77.6	116.4	NIL
34	4	NORMAL	NORMAL	1	20	5	75	25	37	134.1	134.1	NIL
35	3	NORMAL	NORMAL	2.5	29	9	62	38	34	80.8	97	NIL
36	5	NORMAL	NORMAL	3	44	7	49	51	45	83.8	251.5	NIL

Key: NIL represents absence of pus cells.

taiwanensis 10/12 (2.78%), *Peptoniphilus asaccharolyticus* 8/12 (2.69%) and others as shown in **Figure 4**.

Microbiota Compositions From Oligospermia Couples

Among the oligospermic couples, 555 genera were identified in semen samples, while 403 genera were found in the corresponding vaginal samples. *Prevotella* (22.13%) was the most abundant genus in oligospermic semen, followed by *Escherichia* (21.33%), *Lactobacillus* (9.73%), *Shuttleworthia* (7.67%), *Serratia* (5.33%), *Megasphaera* (5.04%), *Gardnerella* (2.85%), *Sneathia* (2.79%), *Porphyromonas* (2.22%), and others. The corresponding vaginal samples had *Lactobacillus* (53.90%) as the most abundant genus, followed by *Streptococcus* (14.68%), *Gardnerella* (5.48%), and others as shown in **Figure 5A**.

Microbiota Compositions From Azoospermia Couples

The results from azoospermic couples show that 342 genera colonized the semen samples, while 309 genera were found in the corresponding vaginal samples. *Lactobacillus* (39.38%) was the most abundant genus in azoospermic men, followed by *Enterococcus* (28.52%), *Corynebacterium* (6.58%), *Veillonella* (5.53%), *Gardnerella* (4.25%), *Ureaplasma* (1.91%), *Prevotella* (1.82%), and others. The corresponding vaginal samples were colonized mostly by *Lactobacillus* (68.38%), *Prevotella* (7.37%), *Gardnerella* (5.69%), *Megasphaera* (5.23%), *Olsenella* (3.41%), *Sneathia* (2.69%), and others represented in **Figure 5B**.

Microbiota Compositions From Semen Samples With Leukocytes

We compared the microbiota compositions of nine semen samples with leukocytes (2+ to 3++) and nine semen samples without the presence of leukocytes. Semen samples with leukocytes tend to be significantly less colonized by *Lactobacillus reuteri* group, *Faecalibacterium*, and more inhabited by *Bacteroides* and *Prevotella* (**Supplementary Figure S6**). The corresponding microbiota at genera level from vaginal samples is presented in **Supplementary Figure S7**

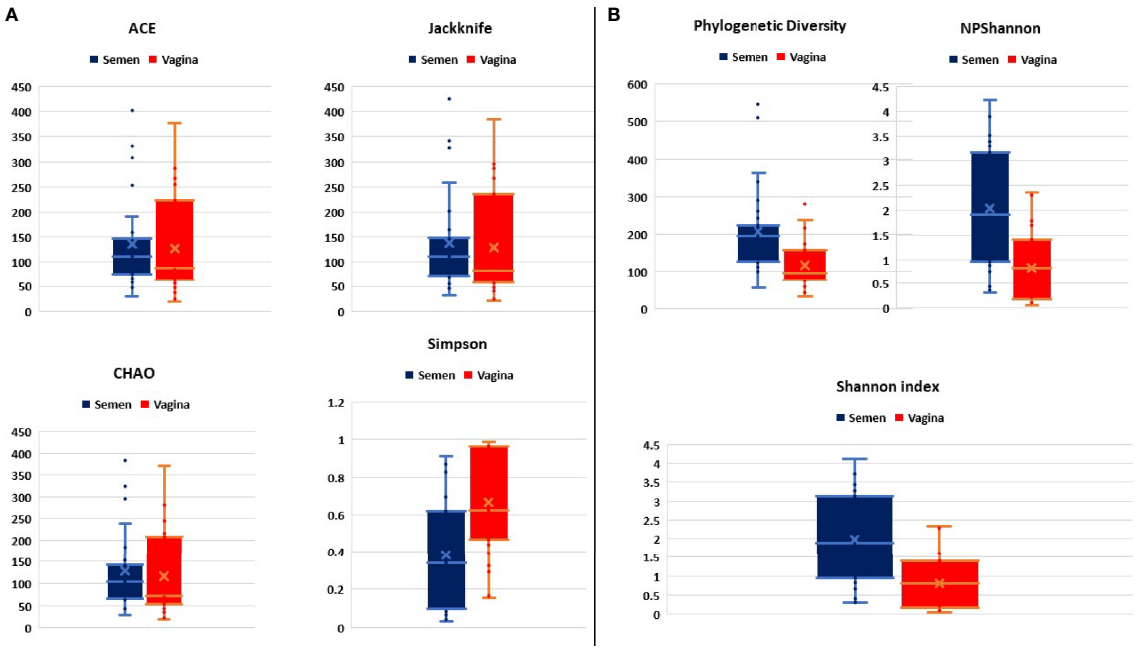

FIGURE 1 | **(A, B)** Comparative alpha diversities between semen and vagina showing species richness typified by ACE, CHAO Jackknife, Shannon, and Simpson index.

showing *Lactobacillus* and *Gardnerella* as the most relative abundance. At the species taxonomic level, semen samples with leukocytes had more relative abundance of *Lactobacillus iners* and *Enterococcus faecium* compared with semen without leukocytes as shown in **Supplementary Figure S8**. The corresponding female partners had *Lactobacillus iners* as the most abundant species as shown in **Supplementary Figure S9**. Bacterial metabolic functional genes that were downregulated in the semen samples with leukocytes include but not limited to hemoglobin/transferrin/lactoferrin receptor protein, MFS transporter, OPA family, sugar phosphate sensor protein UhpC as presented in **Supplementary Table S1**.

Microbiota Compositions in Couples With Positive and Negative IVF Clinical Outcome

This study compared the relative abundance of the microbiota in 12 couples with positive IVF clinical outcome and 24 couples with unsuccessful or negative IVF clinical outcome as shown in **Tables 1A**, **1B**.

Semen samples with positive IVF clinical outcome have less alpha diversity as typified by Shannon index and phylogenetic diversity (**Supplementary Figure S10**) and are significantly colonized by *Lactobacillus jensenii* group and *Faecalibacterium* and significantly less colonized by *Proteobacteria* taxa, *Prevotella*, *Bacteroidetes* taxa, and *Bacteroides* and lower *Firmicutes*/*Bacteroidetes* ratio compared with semen samples with negative IVF clinical outcome as shown in **Figure 6**.

The comparative proportion of the relative abundance at the species taxonomic level is represented in **Figure 7**.

LefSe comparison of the taxonomic microbiota biomarkers in the seminal fluid of men that had positive IVF clinical outcome and seminal fluid of those with negative IVF clinical outcome is presented in **Table 2**.

In addition, the LefSe comparison of the taxonomic microbiota biomarkers for positive IVF clinical outcome for the couples is presented in **Supplementary Table S2**.

Vaginal samples with positive IVF clinical outcome are significantly colonized by *Lactobacillus gasseri* group, higher *Firmicutes/Bacteroidetes* ratio, and significantly less colonized by *Bacteroides*, *Bacteroidetes* taxa, *Lactobacillus reuteri* group, *Lactobacillus iners*, and *Lactobacillus crispatus* as represented in **Figure 8**.

LefSe comparison of the taxonomic microbiota biomarkers in the vaginal samples of women that had positive IVF clinical outcome and vaginal samples of those with negative IVF clinical outcome is presented in **Table 3**.

The results from PICRUSt indicated that some bacterial metabolic functional genes were upregulated in the semen of men who had positive IVF clinical outcome. For example, bacterial metabolic functional gene orthologs for phenylalanyl-tRNA synthetase beta chain was significantly upregulated (P=0.0231) with LDA effect size of 2.0847, when compared with bacterial metabolic genes from seminal fluid of men with negative IVF clinical outcome. Other several bacterial metabolic functional gene orthologs that were significantly upregulated include but not limited to methionyl aminopeptidase (P=0.0231), peptide/nickel transport system permease protein (P=0.0132), chaperonin GroEL (P=0.0286), glucose-6-phosphate 1-dehydrogenase (P=0.0374), mycothione

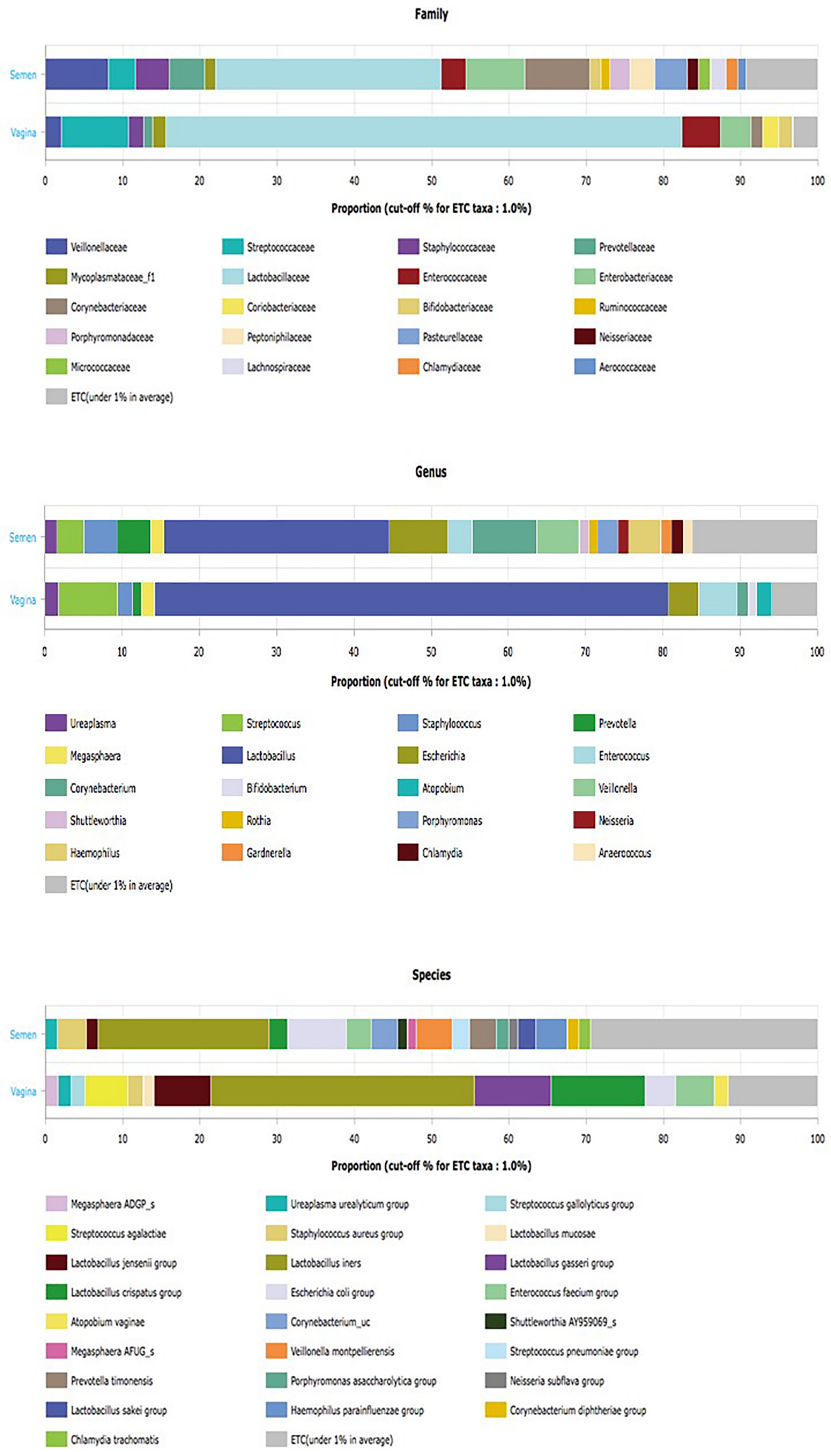

FIGURE 2 | EzBiocloud Microbiome Taxonomic Profile (MTP) pipeline showing distinct taxonomic categories (Family, Genus, and Species) at 1% cutoff between semen and vagina.

reductase (P=0.0318), multicomponent Na+:H+ antiporter subunits D, E, G, A, F, and C (**Supplementary Table S3**).

Similarly, bacterial metabolic functional genes in the vagina of women with positive IVF clinical outcome were upregulated. Notably, iron/zinc/manganese/copper transport system permease protein (P=0.0326) and diphosphoinositol-polyphosphate diphosphatase (P=0.0369) had a twofold increase with positive IVF clinical outcome when compared with negative IVF clinical outcome (**Table 4**).

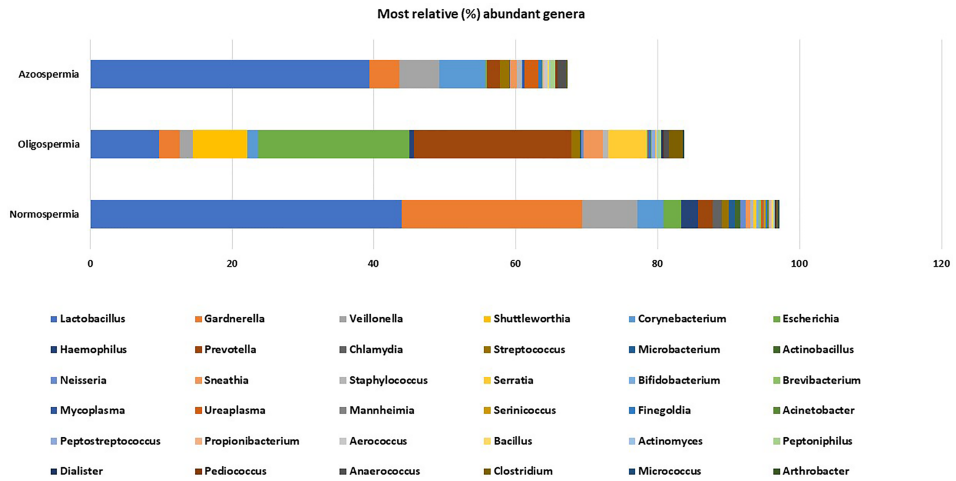

FIGURE 3 | Most relative (%) genera abundance between the semen categories.

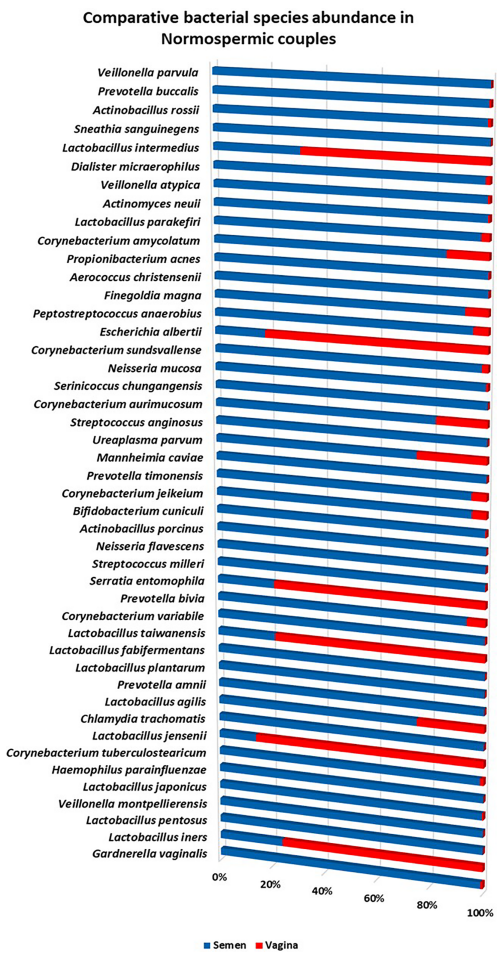

FIGURE 4 | Comparative relative (%) species abundance in normospermia couples.

DISCUSSION

To our knowledge, this is the first study from Nigeria that utilized next-generation sequencing (NGS) technology to determine the microbiota compositions of the seminal fluids and vaginal swabs from couples seeking assisted reproductive health care. This study showed a higher percentage of primary infertility (69.0%) when compared with subjects who had secondary infertility (31.0%). The high rate of primary infertility in this study was in agreement with the previous results of Ikechebelu et al. (Ikechebelu, 2003; Ikechebelu et al., 2003).

Clinical pregnancy determined with ultrasound scan showed 33% (12/36) positive IVF outcomes, which led to 91.6% (11/12) live delivery, and only 8.3% (1/12) clinical pregnancy loss (miscarriage) occurred. This clinical pregnancy rate (33%) is similar to our previous study (Ikechebelu et al., 2016) and very close to the overall pregnancy rate (36%) as reported by Haahr et al. (2019). Among couples diagnosed with secondary infertility, only 11% (4/36) had positive clinical IVF outcome, compared with 22% (8/36) of couples with primary infertility. The 16S rRNA results, when taken together, revealed that the semen microbiota is highly polymicrobial, typified by alpha diversity indexes such as Shannon index and phylogenetic diversity, but low in species concentrations, as shown in a similar study by Mandar et al. (2015). This finding was consistent with our previous pilot study of seminal fluids in a tertiary hospital that revealed varying bacterial diversities that are unique in each sample in contrast to culture-dependent methods (Ndiokwere et al., 2019). The origin of these diverse bacteria in seminal fluids is still not fully determined, but interestingly, most of the bacterial species are closely related to human vaginal microbes (Ravel et al., 2010; Anukam et al., 2019; Okoli et al., 2019), urine microbiota (Nelson et al., 2010), and urethra (Riemersma et al., 2003). In contrast, the vaginal microbiota had higher species concentrations with less

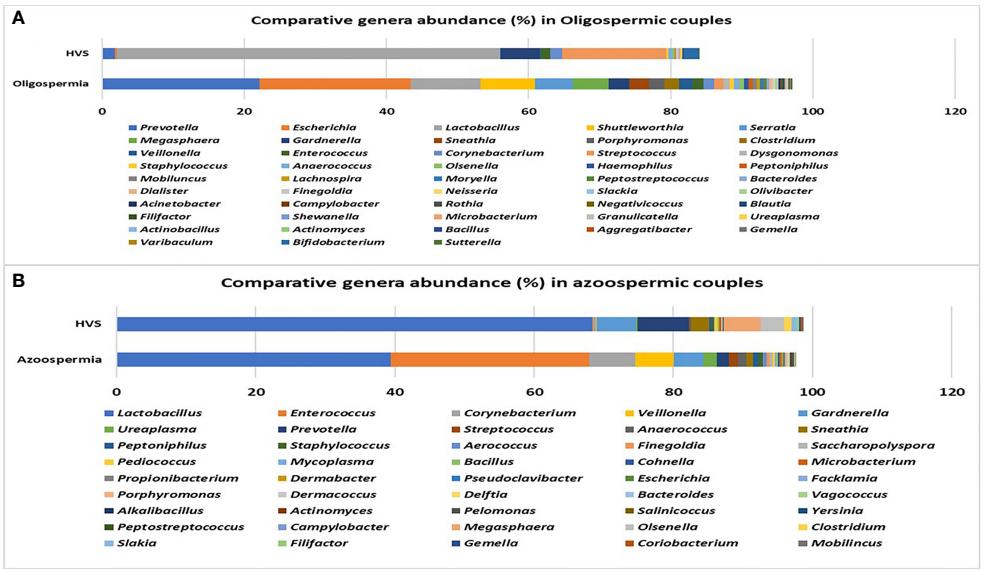

FIGURE 5 | **(A, B)** Comparative relative (%) genera abundance in oligospermia and azoospermia couples.

bacterial diversity. The family taxa *Lactobacillaceae* and the *Lactobacillus* genus were significantly higher in the vagina than in the semen (61.74 *vs* 43.86%), which is consistent with several studies showing that the healthy vagina is colonized by *Lactobacilli* that help to fend off pathogenic microbes by increasing the pH and preventing urogenital infections (Amabebe and Anumba, 2018). However, previous studies have shown that *Lactobacillus* is part of a normal microbiota of the seminal fluid in healthy subjects (Ivanov et al., 2009). It is noteworthy that couples shared many of the predominant genera (56%) and some species (41%) in their reproductive tracts, such as *Gardnerella vaginalis*, *Lactobacillus iners*, *Lactobacillus japonicus*, *Lactobacillus jensenii*, and *Lactobacillus agilis*, which suggests that a healthy vagina or vagina with dysbiosis could influence the reproductive tract microbiota composition of the sexual partner, and *vice versa*. Similar observations have been documented on the skin microbiome of cohabiting couples (Ross et al., 2017). Another interesting finding showed that couples have the same genera but different species, though this is not surprising as the physiological condition of the vagina is acidic while semen is alkaline. This study shows that *Gardnerella* (25.45 *vs* 5.86%) and *Veillonella* (7.78 *vs* 0.04%) were more in abundance in the seminal fluids with normospermia than the corresponding vagina microbiota, although *Gardnerella vaginalis* has severally been associated with bacterial vaginosis (Anukam et al., 2019). The physiological role of *Gardnerella vaginalis* in normozoospermic healthy subjects is yet to be determined as Weng et al. (2014) found that *Lactobacillus*, *Gardnerella*, *Propionibacterium*, and *Atopobium* were relatively more in abundance and significantly present in the normal semen samples. In this study, we found that relative signatures of bacterial communities could be used to disentangle semen categories. Men seeking reproductive health care in the tested population, though found to be normozoospermic, tend to have more *Lactobacillus* >*Gardnerella* >*Veillonella* >*Corynebacterium*, while the female partners have more *Lactobacillus* >*Prevotella* >*Gardnerella* >*Streptococcus*. This suggests that the source of their infertility could probably be more than altered bacterial communities. A similar finding was reported by Hou *et al.* (Hou et al., 2013) showing that infertile subjects did not have altered or unusual semen bacterial communities compared to normal sperm donors. The men categorized as oligospermia tend to have more *Prevotella* >*Escherichia* > *Lactobacillus* >*Shuttleworthia* >*Serratia* >*Megasphaera* >*Gardnerella* >*Sneathia*, and their female partners tend to have more *Lactobacillus* >*Streptococcus* >*Gardnerella* >*Lactococcus* >*Bifidobacterium* >*Prevotella*. It appears that oligospermia men may be under the influence of these pathogens that overwhelm *Lactobacillus*'s protective activities. The factors responsible for these microbial community differences observed in oligospermic couples are yet to be delineated. In contrast, azoospermia men in these cohorts were observed to have more *Lactobacillus* >*Enterococcus* >*Corynebacterium* >*Veillonella* >*Gardnerella* >*Ureaplasma* >*Prevotella*, while their female partners have more *Lactobacillus* >*Prevotella* >*Gardnerella* >*Megasphaera* >*Olsenella* >*Sneathia* >*Peptoniphillus*, and other BV-associated bacteria. Previous studies have reported that several bacteria, including *Lactobacillus iners*, *Gardnerella vaginalis*, *Escherichia faecalis*, *E. coli*, and *Staphylococcus aureus*, are associated with male infertility (Franasiak and Scott, 2015; Javurek et al., 2016). These bacterial taxa are common to both semen and vagina. The caveat is that they occur at different proportions in the semen and vaginal niche. The differences in these bacterial communities in these partners may be due to sexual intercourse, episodes of receptive oral sex, and anal sex before

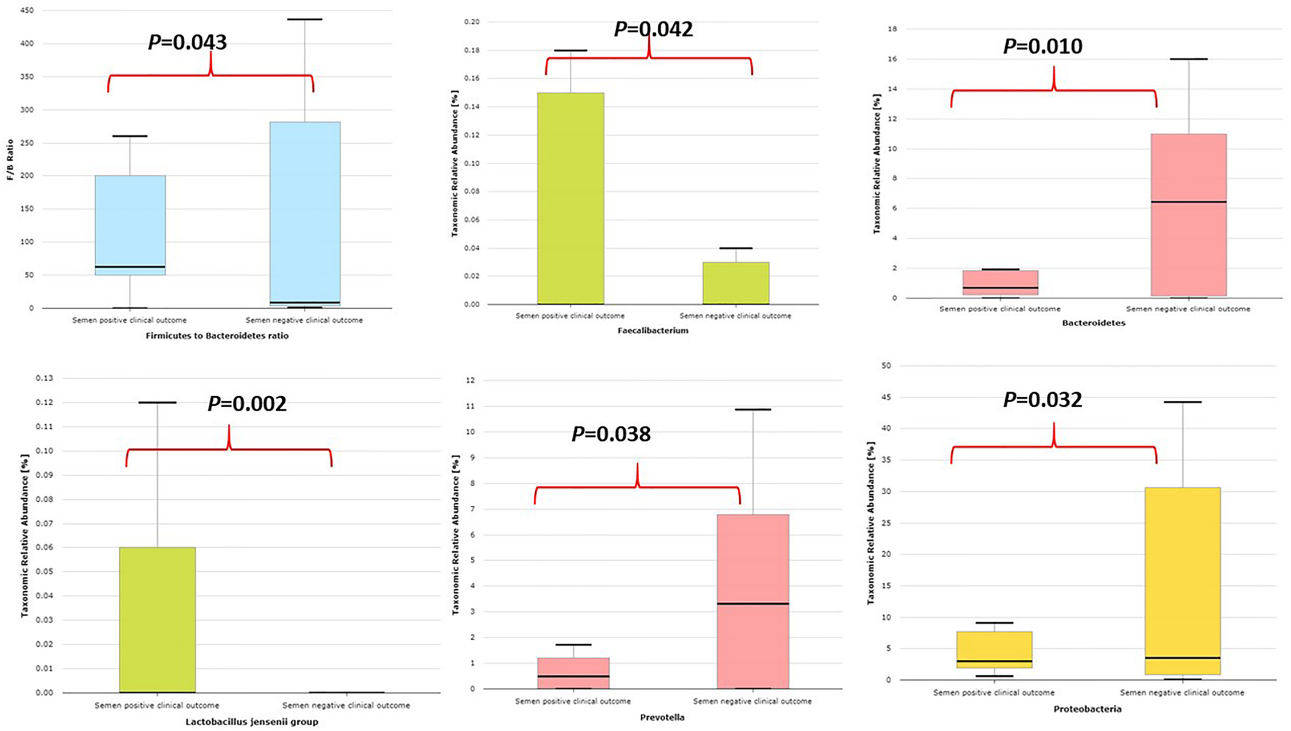

FIGURE 6 | Comparative relative abundance of some selected taxa showing significant difference between semen samples with positive IVF clinical outcome and samples with negative IVF clinical outcome.

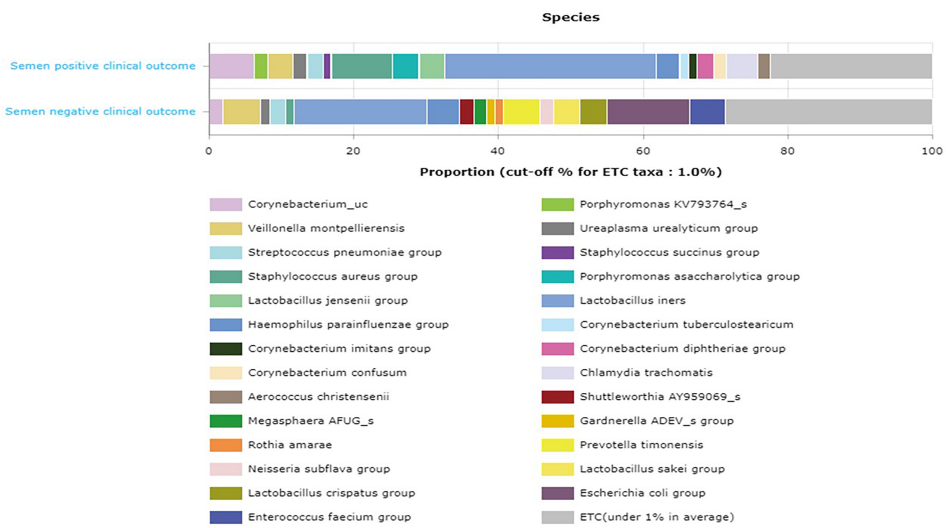

FIGURE 7 | Comparative proportion (cutoff 1.0%) of the relative abundance at the species taxonomic level between semen samples with positive IVF clinical outcome and samples with negative IVF clinical outcome.

vaginal intercourse, which has been reported to influence vaginal and genital tract microbiota in infertile couples (Beigi et al., 2005). The presence of leukocytospermia or pyospermia may have been triggered by the presence of *Gardnerella*, *Prevotella*, and other BV-associated bacteria, which leads to alteration in the partner's vaginal microbiota, and BV has been associated with a 40% increase in the risk of preterm birth (Hillier et al., 1995) and infertility (Al-Awadhi et al., 2013). The occurrence of BV-related microbiota in semen

TABLE 2 | LefSe comparison of the taxonomic biomarkers in the seminal fluid of men that had positive IVF clinical outcome and seminal fluid of those with negative IVF clinical outcome.

Taxon name	LDA effect size	p-value	p-value (FDR)	Semen positive IVF clinical outcome	Semen negative IVF clinical outcome
Cutibacterium	3.50568	0.00034	0.00034	0.76044	0.12494
Cutibacterium acnes group	3.45684	0.00067	0.00067	0.66262	0.09221
Propionibacteriales	3.48056	0.00068	0.00068	0.82203	0.23874
Propionibacteriaceae	3.49112	0.00068	0.00068	0.80718	0.21312
Salinifilum	3.70772	0.01579	0.01584	1.02006	0.00000
Leptotrichiaceae	3.12386	0.02997	0.03009	0.00000	0.26525
Facklamia hominis group	2.60266	0.02997	0.03011	0.00000	0.07949
*Lactobacillus*_uc	2.72540	0.03372	0.03390	0.01318	0.11892
*Actinobacteria*_c	4.76455	0.03899	0.03923	22.04688	10.41718
Micrococcales	2.99379	0.04751	0.04783	3.00235	2.80542
Streptomycetales	2.92289	0.04825	0.04861	0.16874	0.00170
Streptomycetaceae	2.92289	0.04825	0.04865	0.16874	0.00170
Streptomyces	2.92289	0.04825	0.04868	0.16874	0.00170
Cutibacterium avidum	2.44565	0.04825	0.04871	0.05682	0.00568
*Saccharibacteria*_TM7	2.20252	0.04825	0.04874	0.00000	0.03151
*Saccharimonas*_c	2.20252	0.04825	0.04878	0.00000	0.03151
*Saccharimonas*_o	2.20252	0.04825	0.04881	0.00000	0.03151
*Saccharimonas*_f	2.20252	0.04825	0.04885	0.00000	0.03151
*Prevotella*_uc	2.60734	0.04825	0.04888	0.00000	0.08053
Actinomyces europaeus group	2.45905	0.04825	0.04891	0.00000	0.05622
*Streptococcus*_uc	2.02046	0.04825	0.04895	0.00000	0.01467

FDR, False Discovery Rate; LDA, Linear Discriminant Analysis.

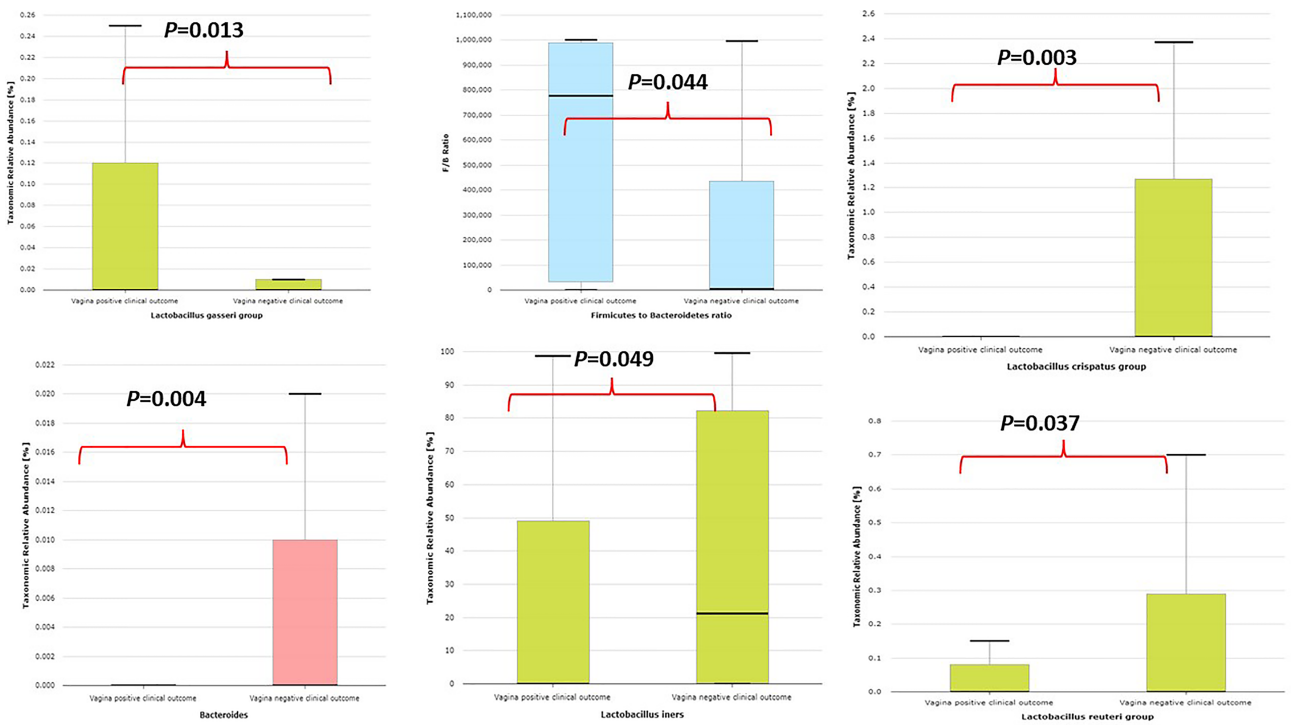

FIGURE 8 | Comparative relative abundance of some selected taxa showing significant difference between vaginal samples with positive IVF clinical outcome and vaginal samples with negative IVF clinical outcome.

suggests a possible reservoir and supports the concept of sexual transmission of BV, besides semen's alkaline properties, which may alter the acidic pH of the vagina, leading to BV (Gallo et al., 2011). An earlier study examined adherence, biofilm formation, and cytotoxicity *in vitro* for *G. vaginalis* strains isolated from women with BV as well as other BV-associated bacteria, including *Atopobium*, *Prevotella*, and *Mobiluncus* (Patterson et al., 2010). In terms of the impact of BV on IVF, several authors have observed

TABLE 3 | LefSe comparison of the taxonomic biomarkers in the vaginal swabs of women that had positive IVF clinical outcome and vaginal swabs of women with negative IVF clinical outcome.

Taxon name	LDA effect size	*p*-value	*p*-value (FDR)	Vagina positive IVF clinical outcome	Vagina negative IVF clinical outcome
Bacteroidetes	3.95843	0.00783	0.00783	0.01044	1.82702
Bacteroidia	3.95832	0.00889	0.00890	0.00978	1.82624
Bacteroidales	3.95832	0.00889	0.00891	0.00978	1.82624
Prevotellaceae	3.95382	0.01060	0.01064	0.00267	1.80030
Prevotella	3.95366	0.01060	0.01066	0.00267	1.79968
Citrobacter	2.17062	0.01579	0.01589	0.02942	0.00000
Citrobacter koseri	2.17062	0.01579	0.01591	0.02942	0.00000
***Cutibacterium acnes* group**	3.04732	0.01822	0.01839	0.00000	0.22235
Enterobacterales_uc	2.34217	0.01864	0.01884	0.04385	0.00008
Prevotella timonensis	3.79064	0.01866	0.01888	0.00224	1.23701
Cutibacterium	3.06412	0.02560	0.02594	0.00038	0.23154
Streptococcus agalactiae	4.38488	0.03698	0.03767	8.66950	3.81779
Veillonella	2.28915	0.04785	0.04881	0.02708	0.06579
Fusobacteria	2.40327	0.04825	0.04928	0.00000	0.05037
***Fusobacteria*_c**	2.40327	0.04825	0.04935	0.00000	0.05037
Fusobacteriales	2.40327	0.04825	0.04942	0.00000	0.05037
Mobiluncus	2.52442	0.04825	0.04962	0.00000	0.06573
***Mobiluncus curtisii* group**	2.51681	0.04825	0.04968	0.00000	0.06468
Veillonella dispar	2.37510	0.04825	0.04975	0.00000	0.04684

FDR, False Discovery Rate; LDA, Linear Discriminant Analysis.

TABLE 4 | Bacterial metabolic functional gene orthologs in the vagina of women with positive IVF clinical outcome and women with negative IVF clinical outcome.

Gene Ortholog	Definition	LDA effect size	p-value	p-value (FDR)	Vagina positive IVF clinical outcome	Vagina negative IVF clinical outcome
K21572	starch-binding outer membrane protein, SusD/RagB family	2.053646	0.006891	0.006898	0.000183145	0.022612675
K21573	TonB-dependent starch-binding outer membrane protein SusC	1.836336	0.006891	0.006899	0.000123226	0.013643597
K01277	dipeptidyl-peptidase III	1.106637	0.007833	0.007843	1.51541E-05	0.00237178
K00895	diphosphate-dependent phosphofructokinase	0.934877	0.010441	0.010463	5.83556E-05	0.001579854
K16363	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase/3-hydroxyacyl-[acyl-carrier-protein] dehydratase	0.903303	0.024279	0.024405	1.06454E-05	0.001411429
K11705	iron/zinc/manganese/copper transport system permease protein	1.112538	0.032613	0.032839	0.004644898	0.002253295
K07766	diphosphoinositol-polyphosphate diphosphatase	1.112288	0.036982	0.037275	0.004642652	0.002252543

FDR, False Discovery Rate; LDA, Linear Discriminant Analysis.

high rates of BV on women with reproductive IVF failure and adverse pregnancy outcome (Spandorfer et al., 2001; Wilson et al., 2002; Wittemer et al., 2004), which corroborates with the findings in this study. Surprisingly, in this study, *Lactobacillus crispatus* was found to be less colonized in women with positive IVF clinical pregnancy outcome in contrast to the work of Haahr et al. (2019). Instead, *Lactobacillus gasseri* was significantly associated with positive IVF clinical outcome. The negative IVF clinical outcome observed in these cohorts of men and women may have been due to the induction of inflammatory response that inhibited the sperms from fertilizing the ovum. Although, Vergaro et al. (2019) showed that vaginal microbiota profile at the time of embryo transfer does not affect the live birth rate in IVF cycles, however, the study has been challenged as it was ladened with poor diagnosis and flawed conclusions (Haahr et al., 2019). The microbiota biomarkers identified in women with negative IVF clinical outcomes in this study point towards an infectious perturbation of the IVF process, which corroborates the findings of Haahr et al. (2016). For example, in this study, the significant increase of *Bacteroidales*, *Prevotellaceae*, *Mobiluncus curtisii*, and *Cutibacterium acnes* in the vagina of those with negative IVF clinical outcome lends credence to previous observations on causes of IVF failure (Onemu and Ibeh, 2001; Al-Awadhi et al., 2013). *Cutibacterium acnes* are involved in the inflammation of the skin by secreting lipase enzymes, which metabolize sebum into free fatty acids, but its activity in the vagina of women with negative IVF clinical outcomes is yet to be determined. A recent study has demonstrated that IVF does not occur in a sterile environment, and the presence of *Staphylococcus* sp. and *Alphaproteobacteria* is associated with clinical indicators such as sperm and embryo quality (Štšepetova et al., 2020).

Transferrin/lactoferrin receptor proteins were significantly upregulated in the bacterial metabolic genes found in semen samples without leukocytes and with positive IVF clinical outcomes when compared with those that had negative IVF clinical outcomes. It should be noted that lactoferrin is a member of the iron-binding transferrin proteins known to have antimicrobial properties and plays a significant role in the mucosal immune response. In addition, lactoferrin aids neutrophils by regulating hydroxyl radical production and participates in the secretion of IgA antibodies (Spik and Montreuil, 1983).

It is very interesting to observe that some bacterial metabolic functional genes were upregulated in the vagina of women with positive IVF outcomes. For example, metal ions transport system permease protein such as iron/zinc/manganese/copper were upregulated. These permease systems are required in many biological processes as components of metalloproteins and serve as cofactors or structural elements for enzymes. It should be noted that some bacteria employ a variety of metal uptake and export mechanisms to regulate metal homeostasis by numerous transcriptional regulators (Porcheron et al., 2013).

CONCLUSIONS

The fact that semen samples of men with positive IVF clinical outcomes were significantly colonized by *Lactobacillus jensenii* group and *Faecalibacterium* and significantly less colonized by *Proteobacteria* taxa, *Prevotella*, *Bacteroidetes* taxa suggests an association between semen and vaginal microbiota correlation. *Lactobacillus gasseri* was significantly associated with positive IVF clinical outcome in women. In addition, this study has opened a window of the possibility of using clinically tested probiotics therapy for men and women before IVF treatment. The need for implementing this approach has been advocated (Verstraelen and Senok, 2005; Garcia-Velasco et al., 2017).

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: the 72 raw sequence reads (FASTQ files) have been deposited on the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) with Project Accession Number PRJNA762524 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA762524).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Review Committee on Human Research from Nnamdi Azikiwe University Teaching Hospital (Ref. # NAUTH/CS/66/VOL11/175/2018/111). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KCA designed the study and sourced for funding; SIO and KCA were responsible for taxonomic data organization, initial analysis and manuscript drafting; KCA was responsible for bioinformatic analysis; JII and SIO were involved in clinical evaluation and the collection of samples; KCA, JII and NRA were the principal investigators and participated in the final design of the study, coordination and drafting the manuscript. All authors contributed to the interpretation of data and made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.709372/full#supplementary-material

Supplementary Figure 1 | Principal Coordinate Analysis (PCoA) with the Bray-Curtis clustering in bacterial community diversities between semen and vagina.

Supplementary Figure 2 | The rarefaction curve showing the number of reads between semen samples and vaginal samples.

Supplementary Figure 3 | Venn diagram showing number of taxa exclusive to normospermia, oligospermia, azoospermia, and taxa that were common to the semen categories.

Supplementary Figure 4 | Comparative stacked relative (%) abundance of taxonomic phyla between semen and vagina in normospermia.

Supplementary Figure 5 | Comparative stacked relative (%) abundance of taxonomic genera between semen and vagina in normospermia.

Supplementary Figure 6 | Comparative relative abundance of some selected taxa showing significant difference between semen samples without leukocytes and semen samples leukoocytes.

Supplementary Figure 7 | Relative (%) abundance of taxonomic genera in the vaginal samples of the corresponding pyospermia samples.

Supplementary Figure 8 | Comparative relative (%) abundance of some species taxa between semen samples with leukocytes and semen without leukocytes.

Supplementary Figure 9 | Relative (%) abundance of taxonomic species in the vaginal samples of the corresponding pyospermia samples.

Supplementary Figure 10 | Comparative alpha diversities between semen samples with positive IVF clinical outcome and semen samples with negative IVF clinical outcome.

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Changes in the Vaginal Microbiome and Associated Toxicities Following Radiation Therapy for Gynecologic Cancers

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Postmenopausal women often suffer from vaginal symptoms associated with atrophic vaginitis. Additionally, gynecologic cancer survivors may live for decades with additional, clinically significant, persistent vaginal toxicities caused by cancer therapies, including pain, dyspareunia, and sexual dysfunction. The vaginal microbiome (VM) has been previously linked with vaginal symptoms related to menopause (*i.e.* dryness). Our previous work showed that gynecologic cancer patients exhibit distinct VM profiles from healthy women, with low abundance of lactobacilli and prevalence of multiple opportunistic pathogenic bacteria. Here we explore the association between the dynamics and structure of the vaginal microbiome with the manifestation and persistence of vaginal symptoms, during one year after completion of cancer therapies, while controlling for clinical and sociodemographic factors. We compared cross-sectionally the vaginal microbiome in 134 women, 64 gynecologic patients treated with radiotherapy and 68 healthy controls, and we longitudinally followed a subset of 52 women quarterly (4 times in a year: pre-radiation therapy, 2, 6 and 12 months post-therapy). Differences among the VM profiles of cancer and healthy women were more pronounced with the progression of time. Cancer patients had higher diversity VMs and a variety of vaginal community types (CTs) that are not dominated by *Lactobacilli*, with extensive VM variation between individuals. Additionally, cancer patients exhibit highly unstable VMs (based on Bray-Curtis distances) compared to healthy controls. Vaginal symptoms prevalent in cancer patients included vaginal pain (40%), hemorrhage (35%), vaginismus (28%) and inflammation (20%), while symptoms such as dryness (45%), lack of lubrication (33%) and dyspareunia (32%) were equally or more prominent in healthy women at baseline. However, 24% of cancer patients experienced persistent symptoms at all time points, as opposed to 12% of healthy women. Symptom persistence was strongly inversely correlated with VM stability; for example, patients with persistent dryness or abnormally high pH have the most unstable

microbiomes. Associations were identified between vaginal symptoms and individual bacterial taxa, including: *Prevotella* with vaginal dryness, *Delftia* with pain following vaginal intercourse, and *Gemillaceaea* with low levels of lubrication during intercourse. Taken together our results indicate that gynecologic cancer therapy is associated with reduced vaginal microbiome stability and vaginal symptom persistence.

Keywords: vaginal microbiome, postmenopausal women, gynecologic cancer, radiation toxicities, longitudinal dynamics, vaginal microbial community

INTRODUCTION

Vaginal symptoms that are highly pervasive (reported by 40%-60%) in postmenopausal women (Huang et al., 2010; Palacios et al., 2018) include dryness, burning, itching, vaginal discomfort, pain and burning when urinating, dyspareunia, recurrent urinary tract infections and spotting during intercourse. Several of those symptoms are further exacerbated in gynecologic cancer patients (which are typically already menopausal), as cancer therapies pose a significant disturbance in the vaginal environment. Two-thirds of women who receive effective cancer treatments report significant treatment-related toxicities including vaginitis, fibrosis, spotting/bleeding on exam or sex, dyspareunia, and sexual dysfunction (Dandapani et al., 2015; Campbell et al., 2019). Additionally, recurrent gynecologic cancer affects around 10-74% of patients within the first 2 years following treatment (Peiretti et al., 2012; Byun et al., 2018), depending on cancer stage and various risk factors, which introduces additional rounds of therapy and further complicates treatment-related toxicities (Campbell et al., 2019). Surgery (abdominal hysterectomy +/salpingo-oophorectomy) and chemotherapy are associated with pelvic toxicities and a variety of patient-reported symptoms, most of which are acute effects diminishing over time. In contrast, radiation therapy, the most common treatment modality for cervical and endometrial cancers, continues to exert biological effects years after treatment (Dandapani et al., 2015; Smits et al., 2017). Radiation therapy is associated with vaginal toxicities including loss of lubrication, dyspareunia, vaginal itching, discharge, and cystitis, all of which may lead to increased vaginal discomfort, infection, diminished sexual activity and decreased quality of life (Leroy et al., 2012; Klopp et al., 2014; Karabuga et al., 2015).

Recent studies suggest a key role of the vaginal microbiome (VM) in symptom manifestation. Typically in asymptomatic women the VM is dominated by *Lactobacilli* and acts as the first line of defense against vaginal infections such as bacterial vaginosis, sexually transmitted infections (STIs), and urinary tract infections (Stapleton, 2016; Chehoud et al., 2017; Fuochi et al., 2017; Campisciano et al., 2018; Łaniewski et al., 2020). The VM in asymptomatic healthy women of reproductive age has two major characteristics: (a) low bacterial diversity with typical dominance of a few lactobacilli species and (b) high temporal stability (Nunn and Forney, 2016; Greenbaum et al., 2019; Berman et al., 2020). In contrast to other human organs like the gut, where high diversity is linked to healthy states, vaginal microbial communities of low diversity are associated with stability and resilience. Stability of microbial communities is a key factor in ecosystem functioning and can be quantified within two dimensions: resilience, which is the ability of community to return to the baseline state after a perturbation causes shifts (i.e. therapy interventions) and resistance, which is the capacity of the community to remain stable upon perturbations without significant shifts (Hickey et al., 2012).

In the vaginal environment, high diversity is typically associated with instability and dysbiotic states. It is believed that estrogen is the major factor contributing to this phenomenon (Romero et al., 2014; Greenbaum et al., 2019): high estrogen levels promote glycogen production from the epithelial cells, which in turn grants advantage to lactobacilli to dominate the microbial community (Ayre, 1951; Patton et al., 2000; Mirmonsef et al., 2016). When estrogen is depleted, as it is postmenopause, the equilibrium state of the stable and low diversity community is disrupted and eventually can lead to a dysbiotic state. However, this explanation fails to account for two observations. Firstly, albeit less frequent, many postmenopausal women have been observed to maintain low diversity *Lactobacillus* dominant communities (Hummelen et al., 2011; Brotman et al., 2014). Second, some groups of both reproductive age and postmenopausal women have been observed to lack lactobacilli dominant communities, while being asymptomatic, indicating that a different equilibrium state can exist, and may vary by race/ethnicity (Wells et al., 2020). In addition, gaps in the literature related to the VM and associations with symptoms in postmenopausal women remain. For example, one review of postmenopausal vaginal communities determined they were poor in *Lactobacillus* but rich in anaerobic taxa (e.g., *Bacteroides*, *Mobiluncus*. *G. vaginalis)* (Greenbaum et al., 2019). Conversely, others have observed that in more than 50% of postmenopausal women the dominating bacteria is *Lactobacillus*, irrespective of symptoms such as vaginal dryness or vulvovaginal atrophy (Hummelen et al., 2011; Brotman et al., 2014). Nevertheless, in both later studies, a low abundance of *Lactobacillus* was still associated with vaginal symptoms.

Additionally the VM is believed to be involved in gynecologic cancers and cancer treatments. Recent studies are investigating whether or not cancer changes the microbiome or whether changes in the microbiome promote cancer (Chase et al., 2015; Contreras et al., 2016; Champer et al., 2018). In any case, the microbiome can act as an inducer of DNA damage, regulating cell growth and death and modulating host immune responses (Wang et al., 2017). In addition, and as our own work suggests (Tsementzi et al., 2020), cancer therapies may be associated with significant

changes in the VM (Bhatt et al., 2017; Muls et al., 2017). Based on such evidence, it is hypothesized that the VM might be a critical contributor to the manifestation of pathologic states in the vaginal environment, particularly in women with gynecologic cancer who experience cancer treatment interventions.

In our previous preliminary work, we examined the VM of gynecologic cancer patients from baseline pre -radiation therapy to 2-4 months post-radiation therapy as compared to healthy controls and found that significant differences exist between the two groups (Tsementzi et al., 2020). Cancer patients have higher diversity microbiomes with multiple anaerobic, and potentially pathogenic, species compared to healthy women of similar age, self-reported race and body mass index (BMI). Here we expand on this work to investigate the temporal dynamics of the VM in a time series up to 1 year post-RT, and their associations with vaginal toxicities and symptoms. We hypothesized that that differences in species composition may correlate with how vaginal communities respond to disturbances, and the magnitude of that response (stability) might be associated with manifestations of symptoms, indicating a link between stability and ecosystem functioning. We examine the shifts of VM communities during and up to a year after the completion of cancer treatments, and we compare those changes with those identified in healthy women without apparent interventions. Finally, we aim to identify VM characteristics that may be associated with vaginal symptoms, while accounting for sociodemographic, clinical and behavioral data that potentially affect the VM.

MATERIALS AND METHODS

Study Design

The study was approved by Emory University's Institutional Review Board, and informed consent was obtained from each participant. For the cancer cohort, postmenopausal women (naturally or due to surgical menopause, i.e., radical hysterectomy/removal of ovaries) with Stage IB-IIIC endometrial or cervical cancer that had been scheduled to receive radiation therapy with curative intent (external beam radiation therapy and/or brachytherapy) were invited to participate in the study. Healthy postmenopausal women without gynecologic cancers were invited to participate during their routine annual gynecologic visit. The healthy cohort was selected to match the cancer cohort in terms of the age, BMI and self-reported race/ethnicity. Exclusion criteria included history of metastatic or other primary cancer and comorbidities that may cause severe vaginal toxicities (e.g., HIV, cystic fibrosis, Type I diabetes or poorly controlled Type II diabetes, autoimmune disease, current STIs, HSV, hepatitis C, and use of interferon or immunosuppressive therapies) and the use of antibiotics or corticosteroids within 4 weeks prior to baseline assessment. Study participants were recruited within a period of 3 years (6/2016-3/2019) and all sample collection was completed by 3/2020.

A total of 134 women were recruited in the study and had samples taken at baseline (T0): 64 gynecologic cancer patients and 68 healthy participants. Among them, 52 participants (25 gynecologic cancer patients and 27 healthy women) agreed to follow up assessments and had their samples taken at an additional 3 time points past the baseline, in order to quantify long term dynamics in the vaginal microbiome. For cancer patients, the first sample was collected after cancer diagnosis and at least 4 weeks after surgery (for those patients for which surgery was prescribed) and prior to the start of radiation therapy (T0). Subsequently samples were taken 2 to 3.5 months past baseline, at which time all radiation therapy sessions had been completed (T1). The post-radiation samples were typically collected 2.5-3 weeks after the last radiotherapy session. Additionally, samples were collected at 6 months (T2) and 1 year (T3) past the baseline. Follow up samplings and evaluations were performed at the clinics for cancer patients during regularly scheduled follow-ups with their radiation oncologist. Healthy subjects were also sampled using the same time frame to account for maturation effects or other changes of the vaginal microbiome during the course of the year. For healthy participants, since they do not typically return to the clinic after their annual evaluation, follow up samplings were performed using self-collection kits that were sent to the participants' homes. Detailed written and pictorial instructions on how to use the vaginal swabs, how to complete patient reported questionnaires, and how to store and return samples in the pre-paid shipping boxes were included in the self-collection kits. Baseline VM samples and have been previously reported (Tsementzi et al., 2020).

Assessment and Evaluation of Clinical, Demographic, and Behavioral Factors

Clinical information (cancer type and stage, treatment type, BMI, age, vaginal pH), sociodemographic factors (self-identified race), and lifestyle habits (smoking, alcohol consumption) were recorded from the recruited volunteers and/or from their medical history during the baseline sampling. Vaginal pH was measured for all participants at each sampling, using sterile polyester swabs, which were rolled onto pH strips (Merck, Darmstadt, Germany) and scored from 4.0 - 7.7 according to the manufacturer instructions. Ph below 5 was categorized as "normal" and above 5 as "high".

Typical vaginal practices and other behaviors that might affect the vaginal microbiome were evaluated at baseline and at subsequent sampling points by asking all participants for their use of any of the following during the past 4 weeks: antibiotics, corticosteroids, topical estrogen and hormonal replacement therapy, vaginal douching, lubricants/moisturizers, vaginal probiotics and sexual intercourse.

Vaginal toxicities commonly caused by radiation therapy were evaluated using the clinician-reported Common Terminology Criteria for Adverse Event (CTCAE) Reporting System criteria (v5.0) items for vaginal toxicities. The NCI CTCAE is a grading (severity) scale for adverse events (AE), with each item scored on a scale from 0 (no discomfort) to 3 (severe discomfort). *Six vaginal-related toxicities* were rated by clinicians: *dyspareunia, vaginal pain, vaginal dryness, hemorrhage, inflammation, and vaginismus*. For the vast majority of participants, the clinician-rated CTCAE scores were only rated from 0 (no discomfort) to 1 (mild) and 2 (moderate discomfort), thus the scoring system was transformed from

ordinary to categorical variables (presence [score of 1-2] or absence [score of 0]) to achieve higher statistical resolution due to the small sample size available. CTCAE based toxicities were reported for all subjects at baseline, and only for cancer patients during their follow-ups at the clinics (healthy subjects did not require additional physician visits within one year and thus performed self-collections at follow-ups). The term toxicities is used throughout the manuscript to describe the aforementioned symptoms which have been scored by clinicians.

Vaginal symptoms commonly reported by postmenopausal women were evaluated at baseline and follow up assessments for all participants, using patient-reported questionnaires. Using a format modeled on the severity items of the Patient Reported Outcomes version of the CTCAE, the PRO-CTCAE (Dueck et al., 2015), each participant was asked if they experienced any of the following *four vaginal symptoms*, at the time of sampling, rated on a scale of 0 (none) to 4 (very severe): *dryness, potential yeast infection (itching), discharge or bleeding*. Finally, two additional symptom were quantified for all sexually active participants, which were asked to complete the Female Sexual Function Index (FSFI) questionnaire for evaluating sexual dysfunction (Rosen et al., 2000; Meston, 2003). The FSFI questionnaire contains 19 validated items that comprise six domains including sexual desire, arousal, lubrication, orgasm, satisfaction, and pain. Each domain is rated on a scale of 0 (no dysfunction) to 6 (severe dysfunction), and the composite FSFI score is an additive of all domains ranging from 0 to 36. A cutoff of 26 has been previously validated to differentiate between subjects with and without sexual dysfunction. In this study, the items pertaining to *lack of lubrication* and *vaginal pain during intercourse* were used as proxies of vaginal health and treated as ordinal variables in subsequent analysis.

For the longitudinal analysis of the subset of women for which time series samples were taken (n=52), frequency of symptoms was calculated based on the individual patient reports from the four time points. Patients' symptom persistence was scored for each symptom as follows: 0 (never experienced symptom), once out of four times (occasional), two or three times out of the four (frequent), and at all-time points (constantly). Additionally, behavioral data (use of corticosteroids, antibiotics, topical estrogen or HRT, vaginal lubricants or moisturizers, douching and sexual activity) were treated in a similar manner, and participants were categorized on the described four categories based on the frequency of the reported behaviors.

Vaginal Microbiome Sampling and Processing

Vaginal swab samples were collected from mid-vagina as previously described (Tsementzi et al., 2020). Once collected, all swabs were stored in the Qiagen DNeasy PowerSoil Kit buffer-containing tubes and frozen upright until they could be transported to the laboratory, where they were stored at −80 °C. Additionally, eight sampling blanks were collected at the clinics and four extraction blanks were also included during the processing steps. DNA was extracted with the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's recommendations, with minor modifications to improve DNA yield and quality: mechanical lysis by bead beating was only limited to three 5 sec vortex pulses during a 10 min 75°C incubation of the swabs with the C1 lysis buffer and with the addition of proteinase K at 50ug/ml final concentration. DNA was quantified with a qubit fluorometer, and further concentrated when needed with ethanol precipitations to achieve 5ng/ul concentrations. The V4 region of the 16S rRNA gene was amplified with the F515 and R806 primers using the previously described 2 step PCR and dual index protocol for Nextera XT (Kozich et al., 2013) and sequenced using the MiSeQ Reagent Kit v2 (Illumina). All samples were sequenced in the same run, and blanks were included in the sequencing to represent (a) sampling blanks, collected at the clinics, (b) DNA extraction blanks, one for each batch of DNA extraction and (c) PCR amplification blanks.

16S rRNA Gene Amplicon Sequencing and Processing

The 16S rRNA gene sequences were processed as previously described to exclude adaptor and primers sequences using cutadapt (Tsementzi et al., 2020). Trimming of low quality and chimeric sequences was done with the dada2 wrapper pipeline implemented in QIIME v2 (Bolyen et al., 2019), trimming sequences where the median quality dropped below q20. After the denoising step, reads were clustered into ASVs (Amplicon sequence Variants) with dada2. Sample coverage was calculated using the Turing Good and Chao estimator (R package vegan) (Oksanen et al., 2013). Taxonomy was assigned with the RDP classifier, trained with the Silva database (silva-132) trained for the V4 region using the classify-sklearn option in QIIME2 with a 0.8 confidence cutoff. The OTU table constructed to represent bacterial genera was normalized for sequencing depth using the cumulative sum scaling transformation (metagenomeSeq package) (Paulson et al., 2013).

Diversity Estimates

α-diversity was estimated using four complimentary metrics implemented in the R package vegan: Chao-1 index to estimate OTU richness (number of total OTUs present in the sample), Pielou index to estimate OTU evenness (similarity of abundances across OTUs), Shannon index (evenness and richness composite), and Faith's (PD) index to account for phylogenetic diversity. α-diversity values distribution was tested for normality with the Kolmogorov-Smirnov, and values were compared between gynecologic cancer and healthy subjects using the Kruskal-Wallis test (independent samples) and between time points using the Wilcoxon signed-rank test (dependent samples). β-diversity distances were estimated using Bray-Curtis dissimilarities (abundance weighted distance) using the R package vegan.

Definition of Vaginal Community Types

Vaginal community types were identified as previously described (Ravel et al., 2011). Similarities in the composition of the vaginal bacterial communities were assessed by hierarchical clustering of the OTU table, which was first filtered to maintain genera with at least 0.1% abundance in at least one sample. Ward's linkage

hierarchical clustering was computed on the β-diversity distances using the hclust r function. The resulting dendrogram reflects the degree of dissimilarity among samples in terms of relative microbial species abundances. Additionally, Spearman's correlation coefficient profile between communities were calculated, and clustering was done with the use of complete linkage in which the maximum distance between two clusters is computed as previously done (Brotman et al., 2014). Clusters were extracted using the cuttree function in R. Associations between metadata and vaginal community types were performed in R using ANOVA for continuous variables and Fisher's exact test for categorical variables with Bonferroni corrections for multiple testing.

Vaginal Microbial Community Stability Metrics

For the 52 participants for whom longitudinal data were available, we evaluated shifts in the microbial community composition using 3 metrics of microbiome stability. The overall stability for a vaginal microbiome community was estimated using the average of the Bray-Curtis (ABC) dissimilarity index between all samples taken from the same subject (four time points collected). Maximum Bray-Curtis distances were also evaluated and resulted in similar patterns as the average distances. Bray-Curtis distances between T0 (baseline) and the final assessment after one year (T3) were calculated as a proxy of community resilience (long-term effects in the composition). Finally, shifts between baseline and T1 (completion of therapy for cancer population) were estimated using Bray-Curtis distances and used as a proxy for community *resistance* (short-term changes after a disturbance, which in this case was assumed to be the radiation treatment for the cancer cohort).

We evaluated the relationships of the VM stability with the available metadata, as well as the prevalence and persistence of the clinician-reported vaginal toxicities and patient-reported vaginal symptoms. For this analysis, we examined three types of variables (a) constant throughout the year (i.e. age, BMI, race, cohort, cancer type, etc.), (b) one-time events at baseline (i.e. reported symptoms or behaviors at baseline), and (c) longitudinal data obtained during four sampling points (i.e., symptom persistence, persistence in behaviors such as topical estrogen use and antibiotics).

For each of the three metrics of stability, we categorized the vaginal microbiomes into three groups (high, intermediate and low stability ranging from 0-0.5, 0.5-0.75, 0.75-1 in any of the Bray-Curtis stability metrics accordingly). Associations between the stability levels and metadata were initially tested with unadjusted Fisher's exact tests and Kruskal-Wallis tests for comparing the values between cancer and healthy groups. Subsequently, we used a forward selection of the linear model in order to identify the best explanatory variables for the distribution of each of the stability metrics. For this analysis, we only used metadata with no missing values. We used a generalized linear model as implement in the glm function of R. The order of the variables was selected by the Akaike information criterion with forward model selection, as implemented in the "step" function in R. Finally, an ANOVA was run on the glm model using the anova.glm function in R.

Reference Vaginal Microbiome Datasets From Reproductive Age Women

Vaginal microbiome longitudinal data from reproductive age women were used as a reference to assess differences in the magnitude of temporal shifts in the VM in this sample of postmenopausal women. We were not able to identify longitudinal datasets that extend one year, thus we used two previously published datasets that sampled vaginal microbiomes for 3 and 4 months. In both studies the VM was sampled and sequenced with similar protocols to the ones used in the current study. The first study longitudinally evaluated the effect of menses on the composition of the vaginal microbiome, by sampling eight healthy reproductive age women 15 times during a period of 3 months (Hickey et al., 2013). The second study evaluated the temporal microbiome dynamics of 32 women and obtained 32 samples from each individual during a period of four months (Gajer et al., 2012). The raw sequence data were downloaded from the SRA archive and processed using the same pipeline as the postmenopausal vaginal microbiome data obtained here, in order to construct taxonomic distributions and evaluate metrics of stability for the vaginal microbial communities.

Statistical Analysis

Distance matrices based on the Bray-Curtis dissimilarity index were used to conduct permutational univariate and multivariate nonparametric analysis of dissimilarities (ADONIS2) using the R package vegan and P-values were adjusted for multiple testing using the Bonferroni correction (Oksanen et al., 2013). This analysis was repeated using Jaccard, and Mahallanobis distances, and all yielded consistent results unless otherwise notyed. Frequency of vaginal symptoms and personal practices were tested for associations with either (a) the vaginal microbiome structure at baseline, using either β- diversity distances or vaginal community state types and (b) with the stability metrics of the vaginal microbiome. Student's t-test was used to assess the statistical significance of differences in baseline characteristics and α -diversity metrics for which the data were normally distributed. Kruskal-Wallis tests were used for non-normal distributed metadata.

RESULTS

Study Population Characteristics

A total of 65 gynecologic cancer patients and 69 healthy postmenopausal women were recruited in the study (**Table 1**). All patients had vaginal microbiome samples taken at baseline, as previously described (Tsementzi et al., 2020). Gynecologic cancer patients and control groups had no significant differences in age, BMI, or racial distributions (**Table 1**). At baseline, 57% of cancer patients had already completed surgery (abdominal hysterectomy +/salpingo-oophorectomy) at least 4 weeks prior to the first sampling. Additionally, 33% of cancer patients received chemotherapy, whether prior or concomitant with radiation therapy. All treatment modules were completed by the second time point (T1). The most common chemotherapy regimens were 6 cycles of Carbo/Taxol,

followed by EBRT, or 5 weeks of concurrent chemoRT with Cisplatin. Healthy controls were recruited during their annual gynecologic exams, and they had no major interventions pertaining to vaginal issues recorded in their medical history.

Vaginal Toxicities and Symptoms in Gynecologic Cancer and Healthy Postmenopausal Women

Vaginal symptomatology reported by clinicians included *dyspareunia, vaginal pain, dryness, vaginismus, hemorrhage, and inflammation* (**Figure 1**). Patient reported symptoms included *dryness* and *yeast infection* (itching and/or discharge), and for participants who were sexually active (58% of healthy women and 46% of gynecologic cancer patients), *lack of lubrication during sexual intercourse* and *vaginal pain during sexual intercourse (*aka patient reported dyspareunia, FSFI item*)* were also reported.

Clinician-Reported Vaginal Toxicities

At baseline, clinicians reported that cancer patients had significantly higher instances of vaginal pain (p=0.002, adjusted z-test), bleeding (p=0.004), vaginismus (p=0.0001), and vaginal inflammation (p=0.009). All four clinician-reported toxicities were consistently reported at similar, albeit slightly lower levels at the subsequent time points and up to one year (**Figure 1A**) following treatment. In healthy controls, the most commonly reported symptom was vaginal dryness, which affected 45% of healthy controls. In contrast, cancer patients reported dryness at significantly lower levels (16%, p=0.008). However, persistence of dryness seemed to significantly decline for the healthy cohort after 3 (T1), 6 (T2) and 12 (T3) months from baseline, while it remained at the same or higher levels for those cancer patients who were experiencing dryness at baseline (**Figure 1B**).

Patient-Reported Vaginal Symptoms

Vaginal dryness, as reported by the participants, was found to be more prominent in healthy controls at baseline (**Figure 1**). However, persistence of dryness seemed to significantly decline for the healthy cohort after 3 (T1), 6 (T2) and 12 (T3) months from baseline, while it remained at the same or higher levels for those cancer patients who were experiencing dryness at baseline (**Figure 1B**). Gynecologic cancer patients self-reported more vaginal pain and lack of lubrication during sexual intercourse at subsequent time points compared to baseline. A symptom was reported as present on those subjects who scored lower than 4.2 out of six in the corresponding FSFI domain score (SI **Figure 1**). Vaginal pain was prominent in about 80% of cancer patients after the completion of radiotherapy at T1, in contrast with 20% of healthy subjects (p=0.009). Lack of lubrication was reported by ~30% of all subjects at baseline but affected 50% of cancer

TABLE 1 | Clinical and demographic information of cancer patients and healthy controls.

	Cancer T: n=65 S: n= 25	Healthy T: n=69 S: n=27	Total T: n=134 S: n=52	P-value (cancer/healthy)
Age years, mean [SD]	T: 56.1 [13.4]	T: 59.3 [7.8]	T: 57.9 [11]	T: 0.08 (Unpaired T-test)
	S: 57.5 [9.9]	S: 60.1 [9.9]	S: 58.5 [10.0]	S: 0.09 (Unpaired T-test)
BMI, mean [SD]	T: 31.4 [7.6]	T: 28.7 [7.9]	T: 30.1 [7.8]	T: 0.02 (Kruskal-Wallis)
	S: 29.1 [7.7]	S: 29.4 [7.4]	S: 29.1 [7.6]	S: 0.02 (Kruskal-Wallis)
Self-reported race, n (%)				
Caucasian	T: 29 (44.6%)	T: 31 (44.9%)	T: 60 (44.8%)	T: 0.51 (Fisher-exact)
	T: 14 (56.0%)	T: 17 (63.0%)	T: 31 (59.6%)	S: 0.57 (Fisher-exact)
African American	T: 34 (52.3%)	T: 33 (47.8%)	T: 67 (50.0%)	
	T: 10 (40.0%)	T: 9 (33.3%)	T: 19 (36.5%)	
Asian	T: 2 (3.1%)	T: 5 (7.2%)	T: 7 (5.2%)	
	T: 1 (4.0%)	T: 1 (3.7%)	T: 2 (3.8%)	
Diagnosis, n (%)				
Endometrial	T: 36 (55.4%)			
	S: 14 (56.0%)			
Cervical	T: 29 (44.6%)			
	S: 11 (44.0%)			
Type of Treatment at T0	**T: n=65**	**| S: n=25**		
Surgery	T: 30 (46%)	| T: 13 (52%)		
Surgery + Chemotherapy	T: 7 (11%)	| T: 3 (12%)		
None	T: 18 (27%)	| T: 9 (36%)		
Type of Treatment at T1		**S: n=25**		
Radiation		11 (44%)		
Radiation + Chemotherapy		14 (66%)		
None		0 (0%)		

Table depicts all subjects included in the study. After quality assurance, a total of six datasets were excluded due to low quality, thus 124 subjects had VM at T0. For a subset of those subjects (n=52), longitudinal data were obtained (depicted in grey color). After excluding low quality datasets, 42 subjects (22 cancer and 20 healthy) had longitudinal VM data at four time points.

Summary statistics are given for the total population recruited in the study and had baseline data analyzed (T) as well as for the subset of patients that had longitudinal data available (S).

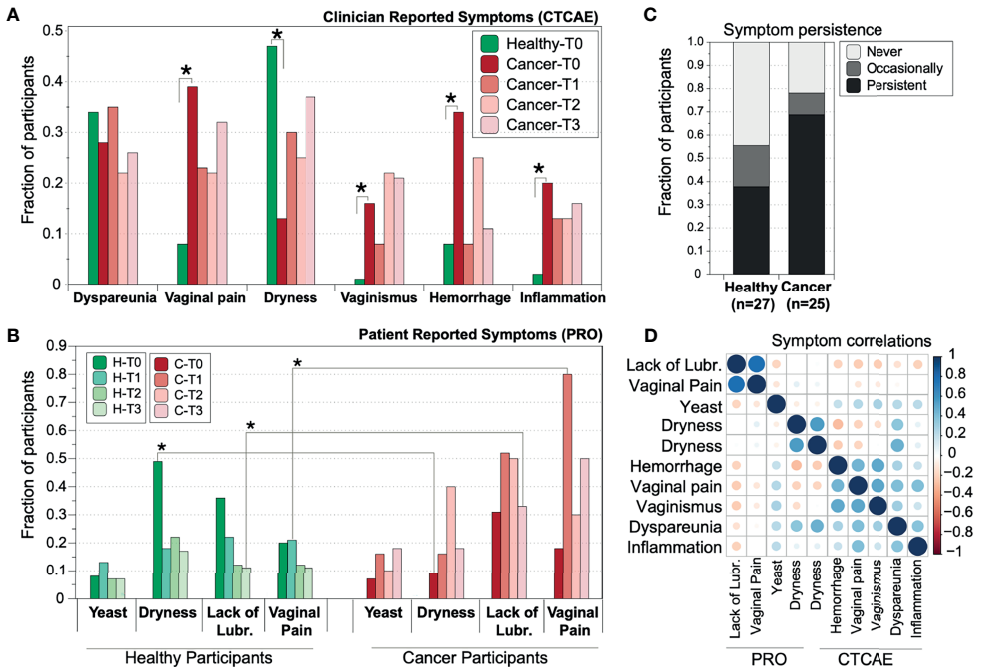

FIGURE 1 | Prevalence of reported vaginal symptoms among gynecologic cancer and healthy postmenopausal women at the different time points of the study. **(A)** Prevalence of clinician-reported toxicities **(B)** Prevalence of patient-reported outcomes (PROs)/symptoms. T0=baseline (n=134); T1 = 2.5-3mos after completion of radiation therapy (n=52), T2 = 6mos (n=52), T3 = 12mos post baseline (n=52). Star symbols indicate statistical significance (p=<0.01, z-test with correction for multiple testing) of the different proportion between gynecologic cancer and healthy cohort. **(C)** Vaginal symptoms persistence in gynecologic cancer and healthy controls. Percentage of healthy and cancer participants which reported at least one of the four patient reported symptoms: Never, Occasionally (once or twice in the year), Persistently (three or four out of four times). **(D)** Correlation of PRO symptoms. The correlations were tested after transforming all data to categorical variables (presence or absence of symptom).

subjects at the end of the year, while healthy women reported similar levels throughout the year (**Figure 1B** and **SI Figure 1**).

Among cancer patients, 20% self-reported at least one symptom consistently in all their evaluations, as opposed to only 12% of healthy women (**Figure 1C**). For the subset of people for which longitudinal data were available, persistence of symptoms was indeed more prominent in the cancer cohort, while 8% of healthy women only occasionally (i.e., 1 or 2 times out of the four) reported vaginal symptoms, 12% exhibit persistent symptomatology and around 20% never reported a symptom. In contrast, around 30% of cancer subjects report frequent or consistent symptoms, and only 7% never reported a symptom during the study duration.

Clinician Versus Patient Reports

From the symptoms investigated, dryness was scored independently by both clinicians and patients, and while discrepancies between the two reports are expected (Deshpande et al., 2011), we observed a relatively high correlation (r=0.68). Vaginal pain (reported by both clinicians and patients) and vaginismus in the same subjects were moderately correlated with a coefficient of 0.54, while all other symptoms and toxicities were not well correlated (**Figure 1D**). A slight correlation was observed between the two reported FSFI symptoms of lack of lubrication and vaginal pain during intercourse (r=0.45). This weak association might be partially explained by the small sample size, since only a subset of women was sexually active and reported FSFI scores.

Vaginal Microbiome Dataset Quality

On average, 63% of the 16S rRNA gene amplicon reads per dataset passed the quality trimming (stdev 12.8%), after removing low quality sequences, chimeras, and duplicates (**SI Figure 2B**). In addition, we included 15 blank samples in the same sequencing run to represent sampling, extraction, and amplification negative controls. For 8 out of the 15 blanks, an average 34% of the reads passed the quality thresholds which resulted in blank datasets with <3.2K good quality reads in total (**SI Figure 2A**). For comparison, the median number of sequences for all vaginal samples was 35.2K reads. Only 10 VM datasets yielded <3.2K reads (including all blanks) and were removed from subsequent analysis, resulting in 267 high quality datasets. Among all available datasets, we identified 4164 unique features (ASVs), which corresponded to 700 bacterial genera. Out of 700 genera, 154 were found in the blanks (**SI Figure 2B**), however most of them (113) were found in only 1 out of 8 samples, and 50% of the genera were never observed in any other sample. We subsequently compared each blank sample with the corresponding vaginal swab sample, which was collected at the same time, and confirmed that blanks have a significantly different taxonomic distribution from the vaginal swabs, which doesn't seem to be structured (**SI Figure 2D**). On

close inspection, the most common genera that were found in the blanks were previously known contaminants, including *Escherichia*, *Strenotrophomans*, *Ralstonia* and *Corynebacterium* (Salter et al., 2014; Glassing et al., 2016; Eisenhofer et al., 2019; Weyrich et al., 2019). Thus, removing all datasets with a similarly low yield as the blanks, eliminated contamination issues that might bias the taxonomic distributions.

The estimated sample coverage for the 267 high quality vaginal microbiome datasets, i.e., the probability for a species of the community to be observed in the actual sequence dataset obtained, was nearly complete, with an average of 99.97%.

α-Diversity of Vaginal Microbiome Communities

As shown in our previous report, cancer VMs exhibit significantly higher α-diversity in comparison with healthy controls at baseline, and this difference is further exacerbated after radiation therapy. Cancer VMs had significantly higher Shannon and Phylogenetic diversity compared to healthy VMs at baseline (p=0.003 and p=0.01 rspectively) and at T1 (p=0.005 and p=0.008) (**Figure 2**). Thus, both cancer and radiation therapy have a detectable, statistically significant effect on α-diversity. However, this effect seems to be reduced in subsequent time points, in which α -diversity is reduced for cancer patients and returns to similar levels as the healthy controls (**Figure 2**). At T2 and T3, there were no statistically significant differences in the VM between cancer patients and healthy controls. There is a clear reduction trend in α-diversity with the passing of time for cancer patients in contrast to healthy individuals who maintained similar levels of α -diversity throughout the year. Consistent with our previous observations, we didn't identify any α-diversity differences when comparing the VMs of endometrial cancer patients with the VMs of cervical cancer patients or when the comparisons were performed among Caucasian and African American women, at either baseline or subsequent sampling points.

VM Community Structure in Cancer and Healthy Vaginal Samples

We identified 7 distinct vaginal community clusters or types (CTs) among all postmenopausal women in this study (**Figure 3**). The most prominent type, CT-A, was dominated by *Lactobacillus* species (i.e. >95% abundance in 80% of the CT-A type microbiomes) and was identified in 28.6% of our study cohort (**Table 2**). Two community state types (CT-F and CT-G) were characterized by the prevalence of the *Prevotella* genus and were found in about 26% of the women. The two types were differentiated based on the presence and dominance of *Atopobium*, *Sneathia*, *Veillonella* and *Gardnerella* species for CT-F, and *Porhyromonas*, *Peptoniphillus, Fusobacterium* and *Annaerococcus* species for the CT-G type. About 11% of women were categorized within CT-B, a state type characterized by high abundance of *Gardnerella* and *Atopobium*, but with the absence of *Prevotella*. Types CT-C and CT-D were the rarest ones, with each being found in only about 6% of the women. The CT-C was characterized by high abundance of *Streptococcus*, dominating >40% of the total microbial community. Type CT-D was characterized by dominance of *Bifidobacterium* species, found in 25% abundance over the total vaginal community. Finally, the most diverse type, CT-E was found in 15% of women and included vaginal microbiomes with various diverse anaerobes, without any single taxon dominating; these VMs were clustered together due to high diversity and lack of resemblance with the other types. Notably, high diversity VMs with diverse anaerobes are typically linked with dysbiotic states (Nunn and Forney, 2016).

Among all types, the high diversity CT-E, and the *Prevotella* and *Porphyromonas* dominated CT-G type were found more commonly found among the cancer cohort (80% and 60% respectively) compared to the healthy controls (**Table 2**). All other types seemed to be equally distributed between cancer and healthy subjects. Additionally, no differences were observed in the distribution of cervicotypes among endometrial and cervical cancer, or different treatment modules.

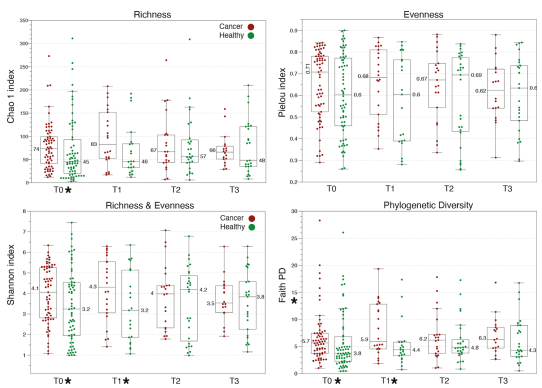

FIGURE 2 | Comparison of α-diversity metrics among healthy and gynecologic cancer vaginal microbiome communities. Cancer patients exhibit higher α-diversity than healthy controls at baseline, which further increases at T1, upon completion of therapy. Star symbols indicate a p-value <0.01 in Kruskal-Wallis test between cancer and healthy.

Effects of Clinical and Demographics Factors on the VM

In our previous publication, we concluded that the most significant predictors of the VM structure include the subject (intra-person variation), cohort (cancer vs health), abnormally high pH, and age (Tsementzi et al., 2020). In this study, we included additional behavioral data and identified that both sexual intercourse and use of topical estrogen during the 4 weeks prior to sampling were significantly associated with the differences observed on the VMs at baseline.

Using the VM community state types, we aimed to identify factors that influence the VM structure (i.e. distribution of vaginal community types). Consistent with previous results, pH and age were significantly associated with the community types (**Table 3**), while BMI and race/ethnicity had no effect. The *Lactobacillus* dominant CT-A type was typically associated with the use of topical estrogen, younger ages, and lower pH levels (**Figure 3**). On the other hand, the high diversity types CT-F, G and E were associated with sexual intercourse and abnormally high pH levels.

Vaginal Microbiome and Relationship With Reported Vaginal Symptoms

Using data from baseline, we evaluated associations between the eight different vaginal symptoms/toxicities reported (**Figure 1**) with identified vaginal community state. Dryness was the only statistically significant symptom identified to highly correlate with VM at the community type level. In particular, CT-A was inversely correlated with dryness (p=0.001) (**Table 3**).

We subsequently performed multivariate analysis of variance (MaAslin), using the individual identified OTUs, and sought to investigate associations with reported symptoms. We identified six key species that showed high associations with reported symptoms among all postmenopausal women and were independent of the cohort (**SI Table 1**). *Lactobacillus* sp. correlated with low pH (p=0.003) and *Prevotella intermedia* and *Fusobacteriales* species correlated with high pH (p=0.0001). Low abundance of *Lactobacillus* sp. is associated with increased severity of vaginal dryness regardless of age, cancer status, or race (n=61, p=0.003). *Prevotella intermedia* (and to a lesser extent other *Prevotella* sp.) strongly associated with vaginal dryness (clinician-reported CTCAE) (p=0.0004). *Delftia* sp. associated with high discomfort or pain following vaginal intercourse (p=0.0006), and unclassified members of the *Gemillaceaea* family correlated with low levels of lubrication during intercourse (p=0.0002) (patient-reported FSFI).

Longitudinal Dynamics of VM Stability in Postmenopausal Women

The magnitude of shifts in the microbial community composition was quantified for all vaginal microbiomes for

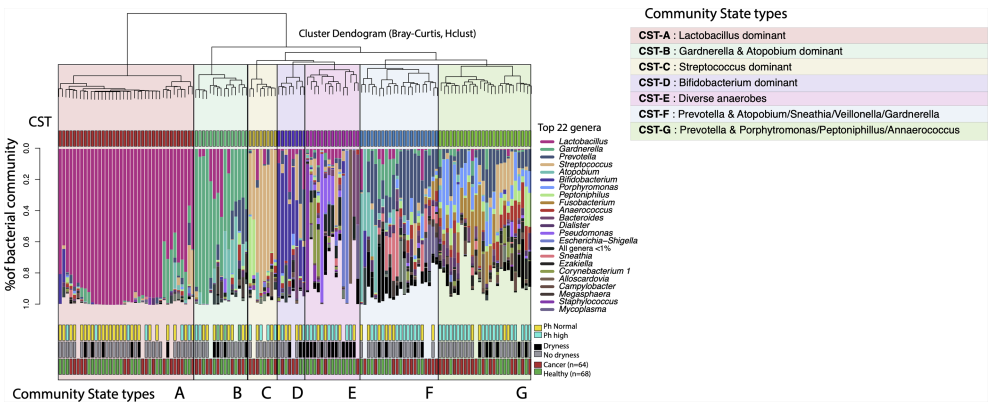

FIGURE 3 | Vaginal microbiome community state types (CST-A) identified in gynecologic cancer and healthy postmenopausal women. The dendrogram represents the hierarchical clustering of the VM communities based on Bray-Curtis dissimilarity distances. The taxonomic profiles for each VM are presented in stacked barplots for each subject, where only the most abundant 22 bacterial genera are shown (for aiding visualization). Certain CSTs are highly correlated with toxicities/symptoms, for example CST-E shows high correlation with persistent vaginal dryness (Fisher's exact test and Bonferroni correction, p=0.001).

TABLE 2 | Vaginal microbiome community types (CT) identified in healthy and gynecologic cancer postmenopausal women.

VM Community State Type	Total subjects n=124 # (% over total)	Healthy n=20 # (% of CT)	Cancer N=22 # (% of CT)	P-value Fisher's test
A	38 (28.6%)	24 (63.2%)	14 (36.8%)	0.112
B	15 (11.3%)	7 (46.7%)	8 (53.3%)	0.362
C	8 (6%)	3 (37.5%)	5 (62.5%)	0.486
D	8 (6%)	4 (50%)	4 (50%)	1
E	15 (11.3%)	3 (20%)	12 (80%)	0.0125
F	23 (17.3%)	11 (47.8%)	12 (52.2%)	0.653
G	26 (19.5%)	9 (34.6%)	17 (65.4%)	0.0789

TABLE 3 | Associations of vaginal symptoms and clinical data with identified vaginal community state types in cancer and healthy postmenopausal women.

Evaluation of associations	p-value
Anova test	
Age	0.017*
BMI	0.462
Fisher's test	
Race	0.378
HRT use	0.047*
Smoking	0.473
Ph category (normal *vs* high)	0.0004*
Sexual intercourse in the past 4w	0.029*
Symptoms (Yes/No)	
Dyspareunia	0.234
Vaginal Pain	0.23
Vaginal Dryness	0.08
Vaginismus	0.68
Vaginal Inflammation	0.11
Yeast Infection	0.059
Dryness (PRO)	0.0001*
Discharge	0.21
Bleeding	0.87

**denotes P < 0.01.*

which longitudinal data were available. We established three metrics of stability (overall stability, resistance, and resilience) for the microbial communities by leveraging the Bray-Curtis dissimilarity distances between samples from the same subject.

First, we estimated the overall stability of a VM as the average Bray-Curtis distance between all four time points and compared this metric among groups and a reference set from reproductive age women. Postmenopausal women in this study exhibited significantly more unstable VM communities compared to reproductive age women in the literature, and gynecologic cancer patients had the most unstable microbiomes in this comparison (**Figure 4**).

Second, we defined a metric for the resistance of the community as the distances observed between two consecutive time points (i.e. from baseline to T1). Consistent with our expectations, cancer patients show significantly lower resistance that healthy controls, as revealed by the much higher values of Bray-Curtis dissimilarities between T0 and T1 (**SI Figure 3A**). This observation could be explained by the fact that all participants in the cancer cohort were treated with radiation therapy during this time, as opposed to healthy subjects. Other potential disturbances associated to the effects of cancer could also be involved, compounded by the RT disturbance. However given that the RT is the most significant and impactful disturbance contrasting cancer and healthy subjects, it is the most parsimonious explanation that RT affects the VM stability.

Third, we sought to quantify the overall microbial community resilience, defined as the ability of the community to return to the initial stage after an intervention. We used the Bray-Curtis dissimilarity distances between T0 and T3 as a proxy for resilience. We observed similar distributions between the cancer and healthy individuals, indicating that after one year, the shifts observed in their VMs are of equal magnitude. Taken together with the low stability and resistance of the VMs from the cancer cohort, the above observations indicate that cancer patients experience significantly larger changes in their VM during the first year after cancer treatment, and their vaginal communities change to a larger extend compared to healthy subjects after one year (beta-diversity), despite the similar levels of α-diversity observed at the one year mark.

Metadata Correlations With the Stability of the VM

In order to identify the most significant contributors on the VM stability, we first used forward selection ANOVA on complete metadata (without missing values) to construct a generalized linear model using the stability metrics as continuous dependent variables. According to the model, overall stability is strongly influenced by the cohort, and cancer patients exhibit low stability microbiomes (**Table 4**). The cohort (cancer vs. healthy) explained about 9% of the variation according to the model (p=0.001). Additionally, the frequency of sexual intercourse (recorded as having had sexual intercourse in the past 4 weeks prior to sampling, for each time point) had a statistically significant effect (p=0.059) and explained another 6.5% of the variation observed in the microbiome stability. Frequency of sexual intercourse, as well as the use of antibiotics 4 weeks prior to sampling, was also positively correlated with low stability values, and together those parameters accounted for ~10% of the variations between T0 and T1 microbiomes (resilience metric). Finally, the frequency of use of topical estrogenwas found to be positively correlated with low resistance values and could explain 8.6% of the observed variation.

In order to take advantage of all available metadata (even if incomplete), we proceeded with univariate associations while adjusting for multiple testing for additional parameters. For this analysis, and for each of the three metrics, stability, resilience and resistance, we categorized participants in three groups: low, intermediate, and high. We then performed Fisher's and Kruskal-Wallis tests to identify significant effectors on the metrics' distributions (**SI Tables 3–5**).

Among the parameters tested, we found that the overall *stability* of the vaginal microbiome was strongly correlated with cohort (cancer *vs* healthy, p=0.045), pH level (normal or basic, p=0.044), chemotherapy intervention at T0, and with persistent dryness (p=0.0471) (**SI Table 2**). Additionally, the metric of *resilience* (differences between T0 and T3) was strongly correlated with cohort (p=0.043), chemotherapy intervention at T0, and the frequency of use of topical estrogen (0.0273) (**SI Table 3**). In other words, cancer patients, and especially those who were treated with chemotherapy before baseline (pre-radiotherapy) sampling, tend to have the most unstable microbiomes. As expected, use of topical estrogen associates with smaller microbiome changes from baseline to one year later. Finally, the short-term stability metric of *resistance* (T0-T1 comparisons) was found to highly correlate with pH levels (0.039), and persistence in the symptoms of dryness (0.0471) and yeast infection (0.032) (**SI Table 4**). This observation demonstrates that specific taxa and VM community states, which are often linked with persistent symptoms (independent of cancer), seem to show the highest variations in the short-term dynamics (i.e. T0-T1). Taken together, those results indicate that (a) cancer therapy effects might be more prominent in the long

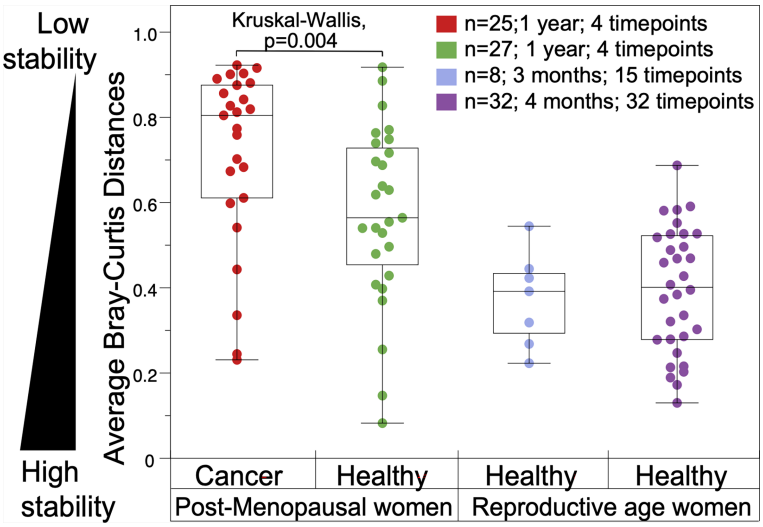

FIGURE 4 | Comparison of vaginal microbiome stability in gynecologic cancer and healthy postmenopausal subjects throughout a year (on the left). (On the right), vaginal microbiomes from reproductive age women from published literature: eight healthy reproductive age women sampled over 3 months (R. J. Hickey et al., 2013); and 32 women sampled over four months (Gajer et al., 2012).

term, instead of short time scales and (b) vaginal symptoms are associated with short term instabilities in the VM.

DISCUSSION

Vaginal Toxicities/Symptoms in Postmenopausal Women With Gynecologic Cancers

Postmenopausal women diagnosed with gynecologic cancers presented a different symptomatology and symptom persistence. At least 70% of women with cancer reported at least one symptom as being persistent during the time of the study compared to 38% of health women. Dryness and lack of lubrication were the only two symptoms that seemed to be reduced with time in the healthy cohort, presumably due to interventions such as use of topical estrogen or use of moisturizers and lubricants. With the exception of dryness, dyspareunia, and yeast infection, all other symptoms evaluated were significantly more prominent in women with gynecologic cancer at baseline. Among those symptoms, vaginismus, hemorrhage, and inflammation are known toxicities caused by cancer therapies and were observed at similar levels at each time point for the duration of one year after cancer therapies. Additionally, for the sexually active cancer participants, lack of lubrication and vaginal pain significantly increased over baseline then slightly improved with time, although these symptoms were still reported at much higher levels than in healthy controls. These observations are consistent with previous reports that show that radiation therapy significantly deteriorates sexual function, as measured by the FSFI scoring system (Bai et al., 2021). Additionally, while there is some gradual improvement, cancer patients are disproportionately affected by sexual dysfunction even one year after the completion of radiation, when compared to healthy women.

Among the vaginal toxicities/symptoms reported, only dryness seemed to be highly correlated with VM community types. Specifically in this study, vaginal dryness was inversely related to the lactobacilli dominant CST-A VMs, which is also consistent with the literature (Hummelen et al., 2011; Brotman et al., 2014; Shen et al., 2016). Only weak correlations were observed among the VM and toxicities/symptoms of hemorrhage, vaginismus, and pain; these weak associations could be related to the relatively small sample size used in our study.

While community types are not directly correlated with specific symptoms, we were able to identify individual OTUs that show strong associations with symptoms reported. For example, presence of *Delftia* sp. was associated with significantly higher pain scores following vaginal intercourse, and members of the *Gemillaceaea* family (unclassified species) correlated with low levels of lubrication during intercourse. Since none of those phylotypes are particularly abundant in the VMs, it is possible that the classification into community types will overlook those associations. On the other hand, *Lactobacillus* species are typically the dominant taxa when present in the VM, such as in the case of CST-A. As expected, lactobacilli abundance was found to inversely correlate with severity and presence of dryness as well as high vaginal pH. These observations indicate that vaginal symptoms might present different associations with the VM in each individual. Dryness is typically (but not always) is correlated with the lack of *Lactobacillus*, and it can be hypothesized that both dryness and lack of *Lactobacillus* are correlated with low estrogen levels.

Further, it is plausible that the presence of vaginal symptoms is not always correlated with a specific community structure, but rather with some equilibrium states that are maintained in an otherwise healthy vagina and varies among individuals, as our data suggests.

TABLE 4 | Forward selection ANOVA generalized linear model on factors associated with the vaginal microbiome stability metrics.

A. stability

Average BC distances	Type of variable	Df	Deviance	Pr(>Chi)	Variation Explained (%)
Cohort	constant	1	2.2243	0.02929	8.77
Sexual_Interc.	longitudinal	1	2.0644	0.05949	6.55
Race	constant	3	1.9185	0.35597	5.98
Antibiotics.4W	one-time event (TO)	1	1.8722	0.31085	1.84
HRT.	longitudinal	1	1.7631	0.33485	1.71
Antibiotics.	longitudinal	1	1.7426	0.49951	0.84
BMI	constant	1	1.7273	0.56041	0.62
Age	constant	1	1.7111	0.548	0.66
Residuals	constant	38			73.03

B. Resistance

BC TO-T1	Type of variable	Df	Deviance	Pr(>Chi)	Variation Explained (%)
Sexual_Interc.	longitudinal	1	3.9387	0.08597	5.55
Cohort	constant	1	3.8318	0.24309	2.56
Race	constant	2	3.5701	0.34451	4.01
Antibiotics.4W	one-time event (T1)	1	3.178	0.06049	6.6
Age	constant	1	3.1172	0.37862	1.4
HRT.4W	one-time event (T1)	1	3.0716	0.44598	1.09
BMI	constant	1	3.062	0.72626	0.2
Residuals					21.41

C. Resilience

BC TO-T3	Type of variable	Df	Deviance	Pr(>Chi)	Variation Explained (%)
HRT.	longitudinal	1	2.962	0.04758	8.61
Sexual_Interc.	longitudinal	1	2.79	0.12001	5.3
Age	constant	1	2.614	0.11572	5.43
Antibiotics.	longitudinal	1	2.5602	0.38447	1.65
Cohort	constant	1	2.5144	0.42237	1.41
Race	constant	3	2.3301	0.45901	5.68
Antibiotics.4W	one-time event (TO)	1	2.2762	0.78661	0.16
BMI	constant	1	2.2762	0.9812	0
Residuals					71.76

Vaginal Microbiome Structure in Postmenopausal Women

It has been well documented that the vaginal microbiome is drastically changing after menopause for the majority of women, driven by the reduction of estrogen and subsequent depletion of lactobacilli (Muhleisen and Herbst-Kralovetz, 2016; Nunn and Forney, 2016). This effect typically leads to higher diversity vaginal communities, in which the lack of a dominant species (lactobacilli) allows for the establishment of multiple lower abundance species. While the α-diversity of a community is only a proxy for a community's status, unexpectedly high values in vaginal communities are quite often implicated with a lack of equilibrium, and often with dysbiotic states (Nunn and Forney, 2016; Brooks et al., 2017; Greenbaum et al., 2019). It is noteworthy that cancer patients show significantly higher α-diversity, which seems to be reduced, at least to levels observed in healthy women, within one year following treatment.

Both α-diversity and the taxonomic distributions of the vaginal communities also showed extremely high variability among women. Less than 30% of postmenopausal women had a low diversity vaginal microbiome dominated by *Lactobacillus* spp. species (**Figure 3** and **Table 2**). The majority of women presented high diversity and lactobacilli deficient communities, which were categorized into six distinct vaginal community types (**Figure 3**), including two types with high abundance of *Prevotella* (CST-F and G), and one type with dominance of *Gardnerella* and *Atopobium* species (CST-B). Finally, about 15% of women were classified in community type CST-E (**Figure 3**), in which no single genus was prevalent, but rather a collection of multiple diverse anaerobic genera were found with high evenness. Typically, such diverse communities are linked to pathologic states, and consistent with this expectation, vaginal microbiomes of CST-E type had abnormally highly pH values (**Figure 3**). Among the parameters recorded, we found younger ages and use of topical estrogen to be more prominent among the lactobacillus-dominant CST-A. This result is not surprising as it has been shown that the loss of estrogen is gradual when entering menopause, and younger women are expected to resemble more reproductive age VMs (Brotman et al., 2014; Muhleisen and Herbst-Kralovetz, 2016). Additionally, it has been previously reported that the use of topical estrogen can, in some cases, restore VM communities to lactobacilli dominance (Shen et al., 2016). It is important to note that while the use of estrogen and age can explain the prevalence of lactobacilli for some cases, many women belonging to the CST-A type are significantly older and report no use of estrogen. Thus, the abundance of lactobacilli in such cases points to other factors that have not been documented here. No other parameters tested were found to associate with the distribution of community state types, including the BMI, which could result in high levels of systemic estrogen in obese women.

All seven of the identified community types were found in both cancer and healthy subjects, however, the high diversity and typically low acidity type CST-E, as well as CST-G, dominated by *Prevotella* and *Porphyromonas*, were more prominent among the cancer cohort. While it is not expected that gynecologic cancer and/or therapies will cause a divergence of the VMs into a single type, it is still interesting that cancer patients more commonly exhibit VM types that might be considered dysbiotic, such as CST-E. In our previous work, we showed that cancer VMs differ from healthy controls in community structure, and we were able to identify at least 15 phylotypes that discriminate the two groups. The vast majority of those phylotypes are typically low abundance species, a characteristic of the CST-E, which have been previously associated with dysbiotic states in the vaginal environment (Tsementzi et al., 2020).

Vaginal Microbiome Stability and Associations With Clinical and Behavioral Data

It has been previously reported that the vaginal microbiomes of reproductive age women are highly stable with small temporal variations, and those variations are typically associated with the menstrual cycle (Gajer et al., 2012; Brooks et al., 2017; Greenbaum et al., 2019). Here, we report for the first time the changes in the VMs in women treated for gynecologic cancer up to one year post therapy compared to healthy postmenopausal controls. We observed that the stability of the vaginal communities is significantly reduced in cancer versus controls, as well as when compared to reproductive age women (**Figure 4**). Gynecologic cancer patients exhibit the most unstable microbiomes, as quantified by average Bray-Curtis distances over the four time points studied, in comparison to healthy controls. Using three metrics of VM stability, we demonstrated that the overall stability (average of 4 time points) is significantly higher in healthy women compared to cancer cases. Similarly, the resilience of maintaining low diversity VM communities, quantified as the changes from baseline to one year later (T0-T3), is significantly higher in healthy women compared to cancer patients. Apart from cancer, which was negatively correlated with resilience, only the use of topical estrogen was found to be strongly positively correlated with resilience of the VM, and participants that consistently used topical estrogen during all 4 time points of the study tended to have the most stable vaginal communities (higher resilience).

Among the other sociodemographic and clinical factors, including sexual intercourse, race, BMI or age, no strong associations were found with the overall stability and resilience of the VM. Sexual intercourse and use of antibiotics were found to be associated with shifts in the VM when evaluating short-term dynamics. In other words, when we compared the resistance of the VM, estimated by the changes that occur from baseline to 2-3 months later, we found that antibiotics and sexual intercourse were positively correlated with low resistance. Yet, there was no significant difference in terms of microbiome resistance between cancer and healthy cohorts, an observation consistent with our previous analysis of a smaller set of cross-sectional samples (Tsementzi et al., 2020). Taken together, these results indicate that the longitudinal variability is similar between healthy and cancer subjects, when comparing the VMs 3 months after the first cancer therapy. However, cancer patients show significantly larger shifts at later time points including 6 and 12 months after radiotherapy has been completed. Additionally, the use chemotherapy in cancer patients was also found to be associated with lower stability metrics. It is plausible that the effects of radiation are manifested on a longer time scale, rather than immediately after the intervention. Alternatively, cancer treatments, which are completed within a 3-month period on average, might have a synergistic and long-term effect on the VM, explaining the observed lack of stability after a year from the baseline.

We subsequently evaluated the association between reported symptoms and VM stability. Overall, higher stability of the VM was inversely associated with the persistence of dryness and abnormally high pH. Among the eight different symptoms reported, we showed that dryness is also associated with lower resistance in the VM, when comparing baseline with T1. Dryness is overrepresented among high diversity, lactobacilli deficient VMs at baseline, and such VMs tend to have the largest changes when evaluated 3 months later, both in women treated for gynecologic cancer and in healthy controls. Moreover, high pH was also correlated with low resistance, an indication that a low acidity, dysbiotic VM is prone to larger shifts in community states and lacking in equilibrium.

Our results highlight the importance of overall stability in the individual subject VM in staving off symptoms, rather than a specific community structure, with the exception of the typical lactobacilli dominant community types. In other words, asymptomatic women might express a variety of taxonomic distributions in their VMs, and stability over time tends to associate with the lack of persistent symptoms.

There are several limitations to this study that should be noted: (a) low statistical resolution due to the small sample size and heterogeneity of the population in terms of types of neoplasm and treatment modules (b) functional redundancy of the vaginal microbiome which overwhelm the taxonomic distributions, and yet unknown functional differences of subspecies or strains within the same species, not readily resolved with 16S rRNA gene amplicon sequences. Larger samples and metagenomic functional analyses are required to overcome these limitations in the future.

Clinical Significance and Future Directions

There is a high prevalence of vaginal toxicities and symptoms associated with changes in the vaginal environment in both women treated for gynecologic malignancies and otherwise healthy postmenopausal women. However, the changes are more prevalent and persistent in women with gynecologic cancers. Larger longitudinal studies would help us better understand what constitutes a 'healthy' vaginal microbiota, as well as understanding the fluctuations that commonly occur in the postmenopausal state over time compared to changes in the vagina after cancer therapies. The community states and specific taxa found to be associated toxicities/symptoms by our study provide candidates and hypotheses to target for functional metagenomics in the future. These insights will lay the

groundwork for novel and targeted interventional approaches, like probiotics, to alleviate the morbidity and mortality associated with vaginal dysbiosis in gynecologic cancer patients.

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/, PRJNA448161.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB and KK conceived and designed the study, acquired resources and funding, and edited the original draft. RM, TE, JS, MD, JA, NK, PP, and IS consented and recruited patients. DT performed the formal analysis, data curation, DNA extraction, library preparation for sequencing and wrote the original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.680038/full#supplementary-material

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The Effect of Exogenous Sex Steroids on the Vaginal Microbiota: A Systematic Review

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Background: Exogenous sex steroids within hormonal contraception and menopausal hormone therapy (MHT) have been used for family planning and management of menopausal symptoms, without consideration of their effects on the vaginal microbiota. This is largely because their use predates our understanding of the importance of the vaginal microbiome on human health. We conducted a systematic review (PROSPERO: CRD42018107730) to determine the influence of exogenous sex steroids, stratified by oestrogen-containing or progestin-only types of contraception, and MHT on the vaginal microbiome, as measured by molecular methods.

Methods: Embase, PubMed and Medline were searched for relevant literature published through to December 1st 2020. Eligible studies reported on the effect of specific exogenous sex steroids on the vaginal microbiome using a molecular method. Data regarding the 'positive', 'negative' or 'neutral' effect of each type of contraceptive or MHT on the vaginal microbiome was extracted and summarised. A positive effect reflected sex steroid exposure that was associated with increased abundance of lactobacilli, a change to, or maintenance of, an optimal vaginal microbiota composition, or a decrease in bacterial diversity (specifically reflecting a low-diversity optimal microbiota state), relative to the control group. An exogenous sex steroid was designated as having a negative effect on the vaginal microbiome if it resulted in opposing effects (i.e. loss of lactobacilli, a non-optimal microbiota state). When no significant change was found, this was considered neutral/inconclusive.

Results: We identified 29 manuscripts reporting on the effect of exogenous sex steroids on the vaginal microbiome; 25 investigating hormonal contraceptives, and 4 investigating

MHT. Oestrogen-containing contraception, particularly reflecting the combined oestrogen and progestin-containing contraceptive pill, had a positive effect on the composition of the vaginal microbiota. Progestin-only contraception, particularly reflecting depo-medroxyprogesterone acetate, had mixed effects on the microbiota. Among post-menopausal women using MHT, exogenous oestrogen applied topically was associated with increased prevalence of lactobacilli.

Conclusion: Our findings suggest that oestrogen-containing compounds may promote an optimal vaginal microbiota, which could have clinical applications. The impact of progestin-only contraceptives on the vaginal microbiota is less clear; more data is needed to determine how progestin-only contraceptives contribute to adverse reproductive and sexual health outcomes.

Keywords: vaginal microbiota, hormonal contraceptives, menopausal hormone therapy, lactobacillus, Gardnerella vaginalis, oestrogen, progesterone, progestin

INTRODUCTION

Hormonal contraceptives have been in use since the 1960s and until recently, have not been examined for their influence on the vaginal microbiota. A vaginal microbiota associated with optimal reproductive and sexual health outcomes is characterised by *Lactobacillus* spp., although microbiome composition varies across geographical locations and specific populations (Fredricks et al., 2005; Ravel et al., 2011; Aldunate et al., 2013). Conversely, a non-optimal vaginal microbiota is typically characterised by reduced abundance of *Lactobacillus* spp. and is associated with negative sexual and reproductive health outcomes such as preterm delivery, miscarriage, low birthweight, pelvic inflammatory disease, and STI and HIV acquisition (Koumans et al., 1999; Wiesenfeld et al., 2002; Brotman et al., 2010; Chetwin et al., 2019; McKinnon et al., 2019). A non-optimal vaginal microbiota abundant in *Gardnerella* spp., including *G. vaginalis, Atopobium vaginae* and other facultative and strict anaerobes, is associated with the most common vaginal dysbiosis, bacterial vaginosis (BV) (Fredricks et al., 2005; Ravel et al., 2011; McKinnon et al., 2019). Prior systematic reviews and meta-analysis of observational data have shown that exogenous sex steroids, delivered as either oestrogen-containing and progestin-only contraceptives may exert a positive effect on the microbiota, with a decrease in BV (van de Wijgert et al., 2013; Vodstrcil et al., 2013). However, some progestin-only contraceptives (i.e. depo-medroxyprogesterone acetate [DMPA]), have been scrutinised for a suspected link between their use and an increased risk of HIV acquisition and transmission (Polis and Curtis, 2013; Deese et al., 2015; Dabee et al., 2021). Among post-menopausal women, the composition of the vaginal microbiota is implicated in vaginitis, which is a common condition in this population (Diem et al., 2018; The 2020 Genitourinary Syndrome of Menopause Position Statement of The North American Menopause Society, 2020). Vaginitis (formerly atrophic vaginitis) and other peri-and post-menopausal symptoms are managed with exogenous oestrogen within menopausal hormone therapy (MHT, also known as hormonal replacement therapy) in some cases (Castelo-Branco et al., 2005; Chollet et al., 2009). However, less is known about what constitutes an optimal vaginal microbiota in this population (Mitchell et al., 2017).

The use of hormonal contraceptives and MHT is rising globally (Contraceptive Use by Method 2019: Data Booklet. 2019) and clearly, these compounds need to be examined for their largely unappreciated effects on the vaginal microbiota. Advancements in molecular technology have allowed for more accurate and high-throughput studies examining the vaginal microbiota to be conducted. Given the uncertainty about the impact of specific hormonal contraceptives on the vaginal microbiota, this timely systematic review summarises the effect of specific oestrogen-containing or progestin-only contraceptives as well as MHT on the vaginal microbiota, and critically evaluates the strength of the findings.

METHODS

Protocol and Registration

This systematic review was conducted in line with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement (Page et al., 2021) and was prospectively registered with PROSPERO on the 5/09/2018 (International Prospective Register of Systematic Reviews; CRD42018107730).

Search Process, Eligibility Criteria

We searched the electronic databases Embase, PubMed and Medline for relevant literature published through to December 1st 2020 using the following strings: [(vagina* AND bacter* OR vagina*) AND micro* OR vagina*] AND flora AND [(hormon* AND contracept* OR oral) AND contracept* OR estrogen OR oestrogen OR progest*]. Studies were uploaded to Covidence (Veritas Health Innovation, Melbourne, Australia, www.covidence.org) and screened by two authors (L.K.R and E.L.P); with any disputes resolved by a third author (L.A.V). Conference abstracts were included for screening; only English language

studies were considered and only data from human participants was examined.

To be eligible for inclusion, studies were required to report on the most commonly used sex steroids (i.e. oestrogen and/or progestin delivered as hormonal contraceptives or MHT) and provide a measure of the composition, stability and/or diversity of the vaginal microbiota by molecular methods (**Supplementary Table 1**). Molecular methods included i) quantitative polymerase chain reaction (qPCR), ii) 16S microarray, iii) next-generation sequencing and/or iv) Sanger sequencing. Studies were excluded if they 1) did not report on sex steroid use; 2) only measured the vaginal microbiota by non-molecular methods (such as culture and microscopy); 3) were qPCR studies that did not include at least one *Lactobacillus* target or one BV-associated bacteria; 4) only included pregnant women or women undergoing *in vitro* fertilisation; or 5) were reviews. Studies were not excluded based on trial design and could include cross-sectional studies, cohorts, and randomised trials. Where two studies presented data on the same population, only the most recent study was retained, unless one presented baseline cross-sectional data and the second presented longitudinal data, in which case both were included. Where studies measured the microbiota with molecular methods and reported sex steroid use but did not present findings on the effect of sex steroids on the microbiota, the study authors were contacted for additional details. Authors from seven studies were contacted *via* email, all responded and two provided additional data for three studies.

Study Population and Interventions Assessed

We deliberately did not specify a population age, which resulted in two distinct study populations identified in the literature search; reproductive-aged women using hormonal contraception and post-menopausal women using MHT, which were analysed separately. In the reproductive-aged population, any kind of hormonal contraception type was included as a suitable intervention, however these predominantly reflected oestrogen-containing or progestin-only methods of hormonal contraception, as these are the most commonly used formulations. Where more than one sex steroid use was reported in the study population, all types were recorded. In the post-menopausal population, topical conjugated oestrogens were used. The comparator group varied between studies and included 1) baseline specimens prior to sex steroid initiation, women not using contraception, or MHT, or women using a non-hormonal contraceptive [i.e. copper IUD (Cu-IUD), condom use].

Data Extraction

Data was extracted by two authors (L.R. and E.P.). Any discrepancies were resolved through discussion with a third author (L.V.). The following data were extracted: i) study location, ii) population characteristics (e.g. HIV status, sex-worker status, age, and ethnicity), iii) frequency of sampling, iv) molecular technique applied, v) molecular outcomes reported on, vi) comparator group used, vii) sex steroid source, viii) molecular findings and ix) summarised effect of sex steroid exposure (positive, neutral or negative).

Data Analysis

For studies using next generation sequencing, the impact of exogenous sex steroids on vaginal microbiota was reported as relative abundance, bacterial diversity, community state type (CST)/vaginal microbiota cluster or group assignment, and change in these measurements over time during sex steroid exposure. All results were stratified by sex steroid type. Microarray and qPCR study findings were dependent on the panel targets tested. Most commonly, biologically significant *Lactobacillus* spp. (i.e. *L. crispatus, L. iners, L. gasseri, L. jensenii, L. johnsonii* and *L. vaginalis*) as well as BV-associated bacteria [i.e. *G. vaginalis, A. vaginae, Megasphaera, Leptotrichia, Sneathia, Prevotella*, *Lachnovaginosum* genomospecies (formerly *BVAB1*), *BVAB2*, and *Mycoplasma*] were investigated. For these studies, the impact of exogenous sex steroids on the vaginal microbiota was reported as presence/absence, prevalence, and bacterial load (as mean target copies and log concentration) of key bacteria and change in these measurements stratified by sex steroid exposure over time. Studies that measured the vaginal microbiota with denaturing gradient gel electrophoresis (DGGE) and Sanger sequencing reported types and number of bacterial species present, but not relative abundance.

Definitions of Outcomes

Findings were classified as positive, negative, or neutral and further qualified as significant or non-significant. Positive findings generally reflected an increase in *Lactobacillus* spp. among women with sex-steroid exposure. Despite controversy in the field surrounding the role of *L. iners* (Petrova et al., 2017), *L. iners* was grouped with other *Lactobacillus* spp. for the purposes of this review as it is still considered preferable over BV-associated bacteria. An exogenous sex steroid was designated to have a positive effect on the vaginal microbiota if they reported any of the following i) an increase in abundance and/or prevalence of *Lactobacillus* spp. (or decrease in BV-associated bacteria) following initiation of sex steroid use and/or relative to the control group (i.e. either baseline sample or no hormonal contraceptive-use group), ii) a change to a compositional state reflecting an optimal vaginal microbiota [defined as a vaginal microbiota dominated by lactobacilli such as CST-I, CST-II, CST-III (Ravel et al., 2011)], iii) maintenance of an optimal vaginal microbiota state over time, or iv) a decrease in bacterial diversity, specifically reflecting a low-diversity optimal vaginal microbiota state relative to the control group for that study. An exogenous sex steroid was designated as having a negative effect on the microbiota if it was associated with i) a loss of *L. crispatus* or any other *Lactobacillus* spp. relative to the control group, ii) an increase in prevalence and/or abundance of any BV-associated bacteria, iii) a change in compositional state reflecting a non-optimal state (e.g. one dominated/characterised by BV-associated bacteria such as CST-IV, CST-V), and iv) an increase in diversity associated with a higher diversity non-optimal state and/or instability of the vaginal microbiota, relative to the control group used in the study. When there was no significant change (or no difference between exposure and control group) in vaginal microbiota state, stability, or prevalence/abundance of bacterial

species reported, an exogenous sex steroid was considered to have a neutral or inconclusive effect.

Assessment of Bias and Quality

Two authors (L.K.R. and E.L.P.) assessed the risk of bias within studies and reported on i) representation of the general population, ii) intervention allocation, iii) sample size, iv) comparator group/s, v) stratification by intervention, vi) analysis adjusted for confounding variables and vii) methodology, as further defined in **Supplementary Table 2**. Studies were not excluded based on the risk of bias assessment.

RESULTS

Study Selection

Literature searches identified 5315 records from Embase, Ovid Medline and PubMed; and two records were found through other sources. Duplicate references (n=2668) were removed, generating 2649 unique references (**Figure 1**). Of these, 2384 were excluded by title and abstract review. Of the remaining 265 articles, 236 were excluded during full-text screening. The reasons for excluding full text were: molecular methods not used (n=119), review article (n=32), article not available in English (n=20), insufficient bacterial targets (i.e. qPCR did not target BV-associated bacteria or *Lactobacillus* spp.) (n=23), no sex steroid outcomes reported (n=28), study not in humans (n=8) and datasets already represented in another publication (n=6). Twenty-nine eligible studies were identified and data extracted; with 25 studies reporting on the effect of hormonal contraception among reproductive-aged women (**Table 1**) and four on the effect of MHT among post-menopausal women (**Table 2**). Two studies reported on the same cohort; however, these were treated as separate studies as one reported on baseline data (Tuddenham et al., 2019a) and the second presented longitudinal data (Tuddenham et al., 2019b).

Studies Reporting on the Effect of Hormonal Contraceptives on the Vaginal Microbiota

General Characteristics

A summary of the populations included in the studies identified is provided in **Supplementary Table 3**. Of the 25 studies addressing the effect of hormonal contraception on the vaginal

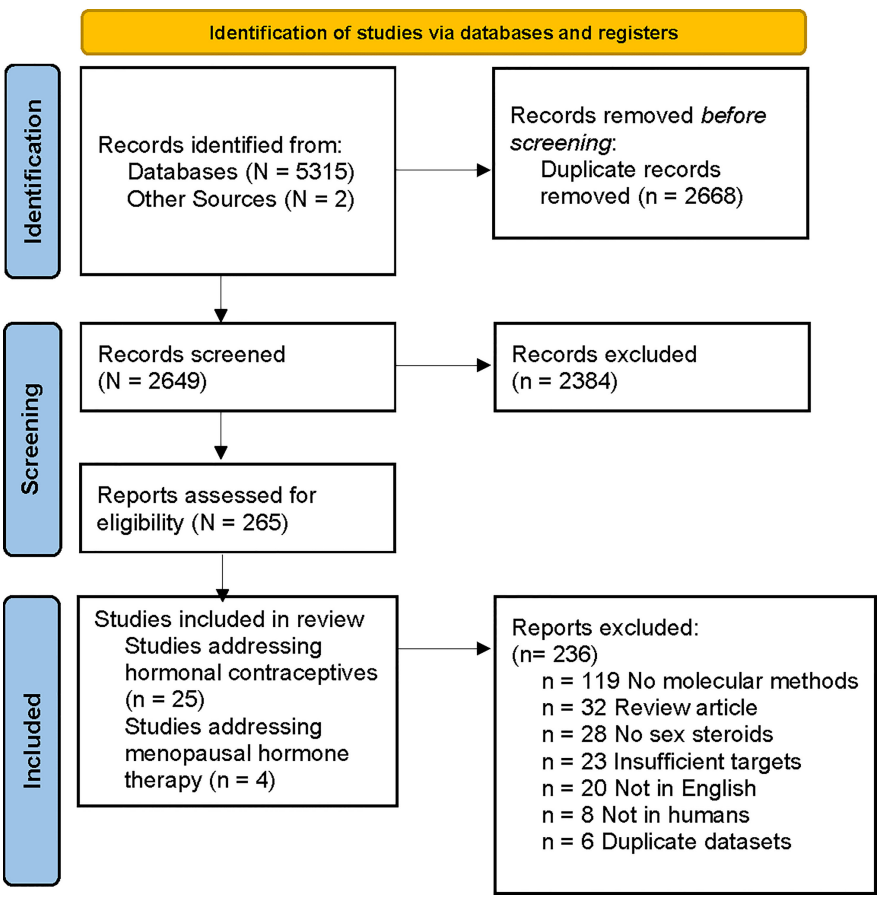

FIGURE 1 | PRISMA flow diagram of literature search and article selection. Flow chart adapted from Page et al. (2021).

TABLE 1 | Result summary of hormonal contraceptive studies.

First Author [ref]	Year	Location	Population and age if specified	Sample Size	Frequency of sampling	Molecular method used	Outcome Measure	Comparator Group	Hormone Source	Findings	Effect of exogenous sex steroids
Achilles et al. (2018)	2018	Harare, Zimbabwe	Women 18-35yo	266 women; 1047 samples	4x over 6 months	qPCR: 8 targets[a]	Prevalence and mean difference in quantity (expressed as gene copies/swab)	Baseline specimen[b] (n=218)	Oestrogen-containing: ethinyl oestradiol injectable (n=40)	No changes in prevalence or log concentration after exposure to ethinyl oestradiol	Neutral effect of ethinyl oestradiol
									Progestin-only: DMPA (n=41), Net-En (n=44), LNG-implant (n=45), ENG-implant (n=48)	Initiation of DMPA associated with a decrease in log concentration of *L.iners*	Negative effect of DMPA
Balle et al. (2020)	2020	South Africa	Women willing to initiate or change HC, HIV negative 15-19yo	130 women and girls; 329 samples	3x over 32 weeks	MiSeq 16S V4 region	CST, Shannon diversity, differential abundance, community composition	Baseline specimen[b] (n=130); alternate study arm	Oestrogen-containing: COCP (n=40), CVR (n=45)	COCP exposure associated with decreased abundance of *Prevotella*, *Mycoplasma*, *Sneathia* and *Parvimonas*; increased abundance of *L. iners*; lower Shannon diversity and likely to be CST-III compared to baseline.	Positive effect of COCP
										CVR-use no effect compared to baseline. CVR-use associated with high abundance of *Prevotella*, *Mycoplasma*, and *Parvimonas*, higher Shannon diversity and more likely to be CST-IV compared to COCP.	Neutral effect of CVR vs baseline
									Progestin-only: Net-En (n=45)	Net-En associated with higher abundance of *Prevotella*, *Sneathia*, and *Parvimonas*; more likely to be CST-IV compared to the COCP	Negative effect of Net-En
Bassis et al. (2015)	2015	Missouri, USA	Women at risk of unplanned pregnancy ≥18yo	76 women; 209 samples	3x over 12 months	MiSeq 16S V4 region	Distance metric (composition stability)	Baseline specimen[b] (n=40) and Cu-IUD (n=36)	Progestin-only: LNG-IUS (n=40)	No changes to microbial composition over 12 months compared to baseline specimen and women using Cu-IUD	Neutral effect of LNG-IUS
Borgdorff et al. (2015)	2015	Kigali, Rwanda	Non-pregnant sex-workers	174 women; 196 samples	2x over 18 months	16S Microarray	Vaginal microbiota clusters and normalised relative abundance	Women using condoms (n=96)	Oestrogen-containing: OCP[c] (n=49)	COCP use was not associated with changes in vaginal microbiota, non-significant decrease in *Sneathia* and *Prevotella* concentrations	Neutral effect of COCP
									Progestin-only: injectables[d] (n=97)	Injectable use was not associated with any changes in vaginal microbiota	Neutral effect of DMPA
Borgdorff et al. (2017)	2017	Amsterdam, Netherlands	Non-pregnant women 18-34yo	610 women; 610 samples	N/A	MiSeq 16S V3-V4 regions	Richness (number of OTUs) and diversity (Shannon Diversity Index), vaginal microbiota groups (e.g. CSTs based on dominant taxa)	Women using condoms (n=439)	Oestrogen-containing: COCP & CVR (n=241[e])	COCP and CVR use associated a non-significant decrease in *G.vaginalis*, but was overwhelmed by other factors in adjusted analyses	Non-significant positive effect of COCP
									Progestin-only: implant, hormonal patch, or injectable (n=241[e])	Not investigated	N/A
Brooks et al. (2017)	2017	Virginia, USA	Non-pregnant women using condoms, COCP, DMPA or LNG-IUS 18-44yo	682 women; 682 samples	N/A	454 Pyro-sequencing 16S V1-V3 regions	Predominant taxon, abundance (mean %), alpha diversity (inverse Simpsons), Associations between species and contraceptives by LEfSE	Women using condoms (n=186)	Oestrogen-containing: COCP (n=206)	COCP use was associated with increased abundance of *Lactobacillus spp*, favouring an *L. crispatus* dominated CST and disfavouring a high-diversity CST, and decreased abundance of BV associated bacteria compared to condoms and progestin-only HCs	Positive effect of COCP
									Progestin-only: DMPA (n=94) and LNG-IUS (n=196)	DMPA and LNG-IUS were associated with a non-significant increase in BV associated bacteria compared to COCP	Neutral effect of DMPA and LNG-IUS

(Continued)

TABLE 1 | Continued

First Author [ref]	Year	Location	Population and age if specified	Sample Size	Frequency of sampling	Molecular method used	Outcome Measure	Comparator Group	Hormone Source	Findings	Effect of exogenous sex steroids
Crucitti et al. (2018)	2018	Rwanda	Non-pregnant, HIV negative sexually active women 18-35yo	120 women; 413 samples	5x over 3 months	qPCR: 7 targets[a]	Genome equivalents per ml of targets	Baseline specimen[b] (n=120)	Oestrogen-containing: vaginal ring (n=120)	CVR use associated with *Lactobacillus* dominated vaginal microbiota cluster, increased prevalence of *L. crispatus, L. iners, L. jensenii, L. vaginalis* as well as decreased prevalence of *G.vaginalis* and *A.vaginae* compared to baseline	Positive effect of CVR
Dabee et al. (2019)	2019	Cape Town & Soweto	HIV negative women 16-22yo	59 women; 59 samples	N/A	16S MiSeq V4 region	*Lactobacillus spp* abundance vs other[f] bacteria	Women not using contraceptives (n=5); condoms only (n=28)	Oestrogen-containing: COCP (n=4), CVR (n=1)	Not investigated	N/A
									Progestin-containing: DMPA (n=14) Nur-isterate (n=67) Net-En (n=37)	DMPA and Net-En use associated with loss of *Lactobacillus spp* compared to women not using progestin-containing contraceptives	Negative effect of DMPA and Net-En
Gautam et al. (2015)	2015	Kenya, Rwanda, Tanzania, South Africa	Non-pregnant, HIV negative women 16-35yo	230 women; 313 samples	enrolment & 81 follow-up samples	16S Microarray	Vaginal microbiota clusters and normalised abundance	Women using condoms, Cu-IUD or no contraceptives (n=103)	Oestrogen-containing: COCP (n=31) Progestin-only: DMPA (n=60)	Grouped HC-use[f] not associated with any CST	Neutral effect of HCs[f]
Jacobson et al. (2014)	2014	USA	Caucasian women 21-33yo	11 women; 406 samples	9x over 12 weeks	454 Pyro-sequencing 16S V1-V3 region	Relative abundance (proportion) and prevalence	Baseline specimen[b] (n=11)	Progestin-only: LNG-IUS (n=11)	LNG-IUS was associated with a non-significant increase in *L.crispatus* compared to pre-IUS sample	Non-significant positive effect of LNG-IUS
Lennard et al. (2018)	2018	Cape Town & Johannesburg, South Africa	Black, non-pregnant, HIV negative women 16-22yo	185 women; 185 samples	N/A	16S MiSeq V4 region	Bray-Curtis diversity, relative abundance, Microbiota compositional and functional subtypes	Women using condoms (n=71)	Oestrogen-containing: OCP[c] (n=10), VR (n=1)	Not investigated	N/A
									Progestin-only: DMPA (n=24), Net-En (n=70), Injectables (n=2)[d], Implanon (n=7)	There was no significant difference in the proportion of DMPA and Net-En users assigned to each vaginal microbiota cluster compared to non HC users and other types of HC	Neutral effect of DMPA and Net-En
Marconi et al. (2020)	2020	Brazil	Non-pregnant, HIV-negative women 18-50yo	609 women; 609 samples	N/A	16S MiSeq V3-V4 region	CST	Women using condoms, women not using contraceptives (n=366)	Oestrogen-containing: OCP_1 (n=192) Progestin-only: Injectables[d] (n=51)	Grouped HC[f] was associated with a decreased risk of CST-IV	Positive effect of HC[f]
Onywera et al. (2019)	2019	Cape Town, South Africa	Heterosexual non-pregnant women with HIV or HIV/HPV 18-44yo	62 women; 62 samples	N/A	16S Ion Torrent V4 region	Diversity, community state types	Women using condoms, or not using contraception (n=37)	Oestrogen-containing: OCP[c] (n=2)	OCP[c] use was associated with non-significant lower diversity	Positive effect of OCP[c]
									Progestin-only: DMPA (n=18) and Net-En (n=5)	DMPA and Net-En use was associated with lower diversity and assignment to CST-I, CST-II and CST-III compared to non-users	Positive effect of DMPA and Net-En
Pyra et al. (2016)	2016	USA and Kenya	Non-pregnant HIV negative women 18-45yo	107 women; 107 samples	N/A	qPCR: 11 targets[a]	log load concentration	Women using condoms, Cu-IUD or no	Oestrogen-containing: OCP[c,g]	Not investigated	N/A
											Negative effect of injectables

(Continued)

TABLE 1 | Continued

First Author [ref]	Year	Location	Population and age if specified	Sample Size	Frequency of sampling	Molecular method used	Outcome Measure	Comparator Group	Hormone Source	Findings	Effect of exogenous sex steroids
								contraception[g]	Progestin-only: Injectable[d, g] and Implanon	Injectables were associated with lower prevalence of *L. iners* compared to women not using HC, but not compared to Cu-IUD users	
Ratten et al. (2021)	2021	Australia	Women with BV 18-45yo	75 women; 430 samples	8x over 6 months	16S MiSeq V3-V4 region	Vaginal microbiota group type (lactobacillus vs non-lactobacillus dominated)	Women using condoms (n=39)	Oestrogen-containing: COCP (n=37)	COCP use did not significantly affect the vaginal microbiota compared to condom use	Non-significant positive effect of COCP
Scott et al. (2019)	2019	Los Angeles, California, USA	Pre-menopausal women	23 women; 276 samples	12 weeks	16S[h] unknown region	Relative abundance, diversity, stability	Women not using HC[f]	COCP grouped HC[f,g]	HC[f] use was associated with stability of the vaginal microbiota	Non-significant positive effect of HC[f]
Song et al. (2020)	2020	Wellesley, Massachusetts, USA	Female students 18-22yo	26 women; ~1100 samples[j]	2x a day for 10 weeks	16S MiSeq V3-V4 region	CST type, Lactobacillus abundance	Women using condoms, or not using contraception (n=16)	Oestrogen-containing: COCP and systemic[j] contraceptives (n=9)	Oestrogen-containing contraceptives were associated with increased abundance of *Lactobacillus*	Non-significant positive effect of COCP and 'C-Systemic' contraceptives
									Progestin-only: local contraceptive[j] (n=6)	Local release contraceptive use was associated with decreased abundance of *Lactobacillus* compared to oestrogen-containing contraceptives	Non-significant negative effect of local release progestin-only contraceptives
Thurman et al. (2019)	2019	USA & Dominican Republic	Women, BMI <30 kg/m2 18-45yo	51 women; 101 samples	2x over 7 visits	16S HiSeq V3-V4 region	CST, abundance and diversity	Baseline, pre tenofovir-LNG-CVR insertion sample	Progestin-only: tenofovir-LNG-CVR (n=20)	The proportion of women with an optimal vaginal microbiota (CST-I, CST-II and CST-V) increased following tenofovir-LNG-CVR insertion. There was a decrease in the proportion of women with a non-optimal vaginal microbiota (CST IV) following tenofovir-LNG-CVR insertion.	Positive effect of LNG-CVR
Tuddenham et al. (2019a)	2019	Baltimore, MD, USA	Reproductive aged women changing HC status	104 women; 104 samples	baseline only	16S HiSeq 2500 V3-V4 region	CST classification	Women not using HC (n=54)	Grouped HC[f] (n=50)	HC[f] use associated with assignment to CST-I, CST-II and CST-III	Positive effect of HC[f]
Tuddenham et al. (2019b)	2019	Baltimore, MD, USA	Reproductive aged women changing HC status	105 women; 4,185 samples	2x week for 2 weeks & 7x over 2 years	16S HiSeq 2500 V3-V4 region	CST classification	Women not using HC[f] (n=32)	Grouped HC[f] (n=73)	HC[f] was associated with increased stability of the vaginal microbiota after >3 months of use and assignment to CST-I	Positive effect of HC[f]
van de Wijgert et al. (2020)	2020	Johannesburg, South Africa	Women with HIV and HPV	448 women; 847 samples	2x over 16 months	16S HiSeq 2500 V3-V4 region	Vaginal microbiota group classification relative abundance	Women using condoms/not having sex (n=333)	Oestrogen-containing: OCP[c] (n=23)	There was no significant difference in the proportion of COCP users assigned to each vaginal microbiota compared to non-HC users	Neutral effect of COCP
									Progestin-only: DMPA (n=82)	There was no significant difference in the proportion of DMPA users assigned to each vaginal microbiota compared to non-HC users	Neutral effect of DMPA
Van Der Veer et al. (2019)	2019	Amsterdam, the Netherlands	Non-pregnant STI negative women 18-36yo	25 women; 1,061 samples	Daily for one menstrual cycle; every other day for two menstrual cycles	16S MiSeq V3-V4 region	Vaginal microbiota clusters	Women using condoms (n=10)	Oestrogen-containing: COCP (n=15)	COCP use was not associated with changes in the microbiota	Neutral effect of COCP

(Continued)

TABLE 1 | Continued

First Author [ref]	Year	Location	Population and age if specified	Sample Size	Frequency of sampling	Molecular method used	Outcome Measure	Comparator Group	Hormone Source	Findings	Effect of exogenous sex steroids
Wessels et al. (2019)	2019	Kenya	Sex workers with <5yrs sex work, pre-menopausal, STI and BV negative, non-pregnant or breastfeeding women ≥18yo	58 women; 58 samples	N/A	16S MiSeq V3 region	Alpha and Beta diversity, relative abundance	Women using condoms (n=22)	Oestrogen-containing: COCP (n=14) Progestin-only: DMPA (n=22)	COCP use was associated with an increase of >98% vaginal microbiota dominance by *Lactobacillus* compared to DMPA use DMPA users had increased bacterial diversity by Shannon diversity index compared to COCP and condom use	Positive effect of COCP Negative effect of DMPA
Whitney et al. (2020)	2020	Nairobi, Kenya	HIV negative breast-feeding women, 6–14 weeks postpartum seeking contraception	54 women; 98 samples	2x; baseline & month 3	qPCR: 7 targets[a]	Concentration of BVAB & pathogenic species	non-HC methods (condoms lactational amenorrhea, rhythm) (n=21)	Progestin-only: DMPA (n=33)	DMPA use was not associated with a significant difference prevalence or concentration of any taxa	Neutral effect of DMPA-IM
Yang et al. (2019)	2019	New Jersey, USA	Non-pregnant women with no HC exposure >20 months 18-35yo	25 women; 67 samples	3x over 3 months	16S MiSeq V3-V4 region	Alpha diversity. Differences in abundance and prevalence of BV associated bacteria vs *Lactobacillus spp* after starting DMPA.	Baseline specimen[b] (n=25)	Progestin-only: DMPA (n=25)	DMPA was associated with increased diversity and lower *Lactobacillus* spp. abundance in black women and higher abundance of *Lactobacillus* spp. in white women compared to baseline specimen	Mixed effect of DMPA

NB. Contraceptives and acronyms are reported as per the original manuscript; where available, the variable region targeted in 16S rRNA sequencing is indicated (i.e. V3-V4, V1-V2); vaginal microbiota groups and clusters are as defined by authors. AV, atrophic vaginitis; BV, bacterial vaginosis; COC, combined oral contraceptives; COCP, combined oestrogen-containing oral contraceptive pill; Cu-IUD, copper intrauterine device; CST, community state type; CVR, contraceptive vaginal ring; DGGE, denaturing gradient gel electrophoresis; DMPA, depo-medroxyprogesterone acetate; ENG, etonogestrel (implant); HC, hormonal contraceptive; LNG, levonorgestrel; LNG-IUS, levonorgestrel-releasing intrauterine system; MPA, medroxyprogesterone acetate; Net-En, norethisterone enanthatel; OCP, oral contraceptive pill; OTU, operation taxonomic units; qPCR, quantitative polymerase chain reaction; yo = years old.

[a]qPCR targets varied between studies and were reported as follows: Achilles (Achilles et al., 2018) L.crispatus, L.jensenii, L.gasseri/johnsonii, L.vaginalis, L.iners, G.vaginalis, A.vaginae and Megasphaera phylotype 1; Crucitti et al. (2018) L.iners, L.crispatus, L.jensenii, L.gasseri, L.vaginalis, A.vaginae & G.vaginalis; Pyra et al. (Pyra et al., 2016) Lachnovaginosum genomospecies [BVAB1], BVAB2, M.indolicus, A.vaginae, Megasphaera spp, Leptotrichia/Sneathia, G.vaginalis, L.crispatus, L.jensenii, and L.iners; and Whitney (Whitney et al., 2020) G.vaginalis, M.hominis, Sneathia spp, G.asaccharolytica, Eggerthella spp, Megasphaera spp and Parvimonas spp.;

[b]Baseline specimen is a specimen collected prior to HC-initiation;

[c]OCP not stratified by oestrogen-containing or progestin-only;

[d]Injectable, type not specified;

[e]number of samples (n) not stratified by HC-type within each sex steroid type;

[f]HC not stratified by oestrogen-containing or progestin-only;

[g]n not provided;

[h]Platform not specified;

[i]oestrogen and progestin combined, 'systemic release' contraceptives, not specified to maintain patient anonymity;

[j]progestin-only, 'local release' contraceptives, not specified to maintain patient anonymity.

TABLE 2 | Summary of included menopausal hormone therapy studies.

Authors	Year	Location	Population	Sample Size	Sampling frequency	Method	Outcome Measure	Comparator Group	Hormone Source	Findings	Effect of exogenous sex steroids
Dahn et al. (2008)	2008	Ontario, Canada	Post-menopausal women using Permarin® MHT for >2 months with age matched controls	20 women; 20 samples	Single specimen collected from each participant	DGEE and Microarray	Predominant lactobacillus and prevalence of species	Age matched women not on MHT (n=10)	conjugated oestrogen (n=10)	MHT was association with restoration of *Lactobacillus*	Positive effect of MHT
Devillard et al. (2004)	2004	Ontario, Canada	Post-menopausal women with no urogenital infections aged 41-66yo	19 women; 75 samples	4x over 90 days	DGGE and Sanger Sequencing	Prevalence (presence/absence) and no. of species detected (diversity)	Postmenopausal women not on MHT (n=20)[a]	conjugated oestrogen (n=19)	All women receiving MHT had *Lactobacillus* detected	Positive effect of MHT
Heinemann and Reid (2005)	2005	Canada	Postmenopausal women aged 41-82yo	60 women; 60 samples	4x over 3 months	DGGE and Sanger Sequencing	Prevalence of specific species	Women not using MHT (n=20)	conjugated oestrogen (n=40)	MHT increased the prevalence of *Lactobacillus*, stability of the vaginal microbiota and decrease of diversity	Positive effect of MHT
Shen et al. (2016)	2016	Shanghai, China	Post-menopausal women with genital symptoms, BMI between 18-35yo, non-smokers without *Trichomonas* or candida	59 women; 177 samples	3x over 4 weeks	16S MiSeq V1-V3	Phylotype abundance	Women not on MHT, without AV (n=29)	conjugated oestrogen (n=30)	MHT was associated with an increase of *Lactobacillus* abundance and decrease of diversity	Positive effect of MHT

NB. All studies investigated a conjugated-oestrogen formulation (topical Premarin®); where available, the variable region targeted in 16S rRNA sequencing is indicated (i.e. V1-V3). AV, atrophic vaginitis; BMI, body mass index; DGGE, denaturing gradient gel electrophoresis; MHT, menopausal hormone therapy; yo = years old.
[a]*The comparison group is post-menopausal women not on MHT who participated in another study.*

microbiota, 20 of these were in HIV negative, non-sex workers without BV, two were in women with HIV and human papillomavirus (HPV), two were in sex workers with no other risk factors specified, and one was among women with symptomatic BV. Nine studies were cross-sectional and 16 were longitudinal cohort studies; the median follow-up time was 31 weeks (range 7 days to 24 months). Nineteen of the studies investigated the vaginal microbiota using next-generation sequencing technology, four used qPCR and two used microarray. Twelve studies were conducted in sub-Saharan Africa, eight were conducted in North America, two in Europe/Central Asia, two were in Latin America/Caribbean and one was conducted in the East-Asian and Pacific region; there were no studies in South Asia or the Middle East/North Africa. These studies represent approximately 14,000 specimens from approximately 4,400 women; of which 1,048 women were exposed to combined oestrogen- and progestin-containing contraceptives (termed oestrogen-containing contraceptives) and 968 were exposed to progestin-only contraceptives (**Table 1**). The combined-oestrogen containing contraceptive-types reported on included i) oral contraceptive pill (OCP), ii) contraceptive vaginal ring (CVR) and iii) ethinyl oestradiol-containing injectable. The progestin-only contraceptive types included i) injectable depo-medroxyprogesterone acetate (DMPA), ii) injectable norethisterone enanthate (Net-En), and iii) levonorgestrel (LNG) contained with an intrauterine system (LNG-IUS) or CVR (LNG-CVR). Other progestin-only contraceptives, including subdermal contraceptive implants and patch-use, were identified, but there was no or limited data reported and so they were not included in the review. The remaining women were either not exposed to contraceptives/or used non-hormonal contraceptive methods (including condoms or the Cu-IUD). Most of the studies (15/25) identified investigated more than one type of hormonal contraception, and we have stratified the findings by sex steroid type where possible (**Table 1**).

The Effect of Oestrogen-Containing Hormonal Contraceptive Types on the Vaginal Microbiome

Fourteen studies investigated the effect of combined oestrogen- and progestin-containing contraceptives, which included the combined oestrogen-containing OCP, an oestrogen-containing CVR and the ethinyl oestradiol-containing injectable. When grouped together, 8/14 (57%) identified a positive effect of oestrogen-containing hormonal contraception on the vaginal microbiota, 5/14 (36%) found neutral or no effect, and one study found a negative effect (7%; **Table 3**).

Oestrogen-Containing Oral Contraceptive Pills

Ten studies investigated the effect of the combined oestrogen-containing OCP on the vaginal microbiome (Borgdorff et al., 2015; Borgdorff et al., 2017; Brooks et al., 2017; Onywera et al., 2019; Van Der Veer et al., 2019; Wessels et al., 2019; Balle et al., 2020; Song et al., 2020; van de Wijgert et al., 2020; Ratten et al., 2021); six reported a positive effect on the vaginal microbiota, and four reported neutral findings (**Tables 1** and **3**). The OCPs were specified as oestrogen-containing by the authors in all studies, except for two (Borgdorff et al., 2015; Onywera et al., 2019). However, for the purpose of this review, these two studies were assigned as studies of oestrogen-containing OCP, and this was confirmed by the authors to be the most common formulation. Four studies found OCP-use had a positive effect on *Lactobacillus* spp. (**Tables 1** and **3**); three studies reported that OCP-use was associated with a higher relative abundance of *Lactobacillus* spp. compared to women not using the OCP (Brooks et al., 2017; Song et al., 2020), or women's baseline (i.e. pre-OCP initiation) specimens (Balle et al., 2020), and one reported an increase in the number of women with a vaginal microbiota dominated by *Lactobacillus* spp. (i.e. >98% relative abundance of *Lactobacillus* spp.) compared to women using condoms (Wessels et al., 2019). Balle et al. specifically identified that OCP-use increased the relative abundance of *L. iners* compared to participants' baseline specimens (Balle et al., 2020). Onywera et al. (2019) and Balle et al. (2020) both reported that OCP-use decreased bacterial diversity (measured by the Shannon diversity index), compared to non-OCP users and baseline specimens, respectively. Balle et al. (2020) also found that OCP-use decreased the relative abundance of several BV-associated bacteria, specifically *Prevotella, Sneathia* and *Parvimonas* relative to baseline and Net-En users, while Borgdorff et al. (2017) found a non-significant decrease in the relative abundance of *G. vaginalis* in OCP-users *vs* non-OCP users. Four studies found no effect of the OCP on the vaginal microbiota compared to women using condoms (Borgdorff et al., 2015; Van Der Veer et al., 2019; van de Wijgert et al., 2020; Ratten et al., 2021).

Oestrogen-Containing Contraceptive Vaginal Ring

Three studies investigated the effect of the oestrogen-containing CVR (Borgdorff et al., 2017; Crucitti et al., 2018; Balle et al., 2020). Compared to baseline specimens, Borgdorff et al. (2017) found that CVR-use was associated with a non-significant decrease in the relative abundance of *G. vaginalis* and Crucitti et al. (2018) found CVR-use was associated with a significant decrease in the prevalence *G.vaginalis* and *A.vaginae* (**Table 1**). Crucitti et al. (2018) also found that CVR-use was associated with an increase in the prevalence and load of *Lactobacillus* spp. in the vaginal microbiota, specifically *L. crispatus, L. iners, L. jensenii* and *L. vaginalis*. Conversely, Balle et al. (2020) found that oestrogen-containing CVR use had no impact on bacterial diversity compared to baseline. However, compared to OCP users, CVR-use was associated with a higher relative abundance of *Prevotella, Mycoplasma*, and *Parvimonas*, higher

TABLE 3 | Summary of beneficial, neutral/inconclusive and detrimental effects of exogenous sex steroids on the vaginal microbiota.

	Overall effect on the vaginal microbiota (n = number of studies)		
Exogenous steroid delivery method	**Positive**	**Neutral/Inconclusive**	**Negative**
Reproductive-aged women using HC			
All oestrogen-containing HC (n=14)	8	5	1
Oestrogen-containing OCP[a] (n=10)	6	4	0
CVR (n=3)	2	1	0
Ethinyl oestradio injectable (n=1)	0	1	0
All progestin-only HC (n=20)	4	8	8
DMPA (n=10)	1	6[b]	3
Net-En (n=4)	1	1	2
LNG-IUS and LNG-CVR[c] (n=4)	2	1	1
Unspecified injectables (n=1)	0	0	1
Grouped 'local release' progestin-only contraceptives (n=1)	0	0	1
Grouped unspecified HC[d] (n=5)	4	1	0
Post-menopausal women using MHT			
Conjugated-oestrogen formulation	4	0	0

[a]*A small proportion of oral contraceptive pill users may be using progestin-only pills, but this information was not available or authors suggested this was unlikely.*
[b]*Includes one paper with both positive and negative findings.*
[c]*The LNG-CVR investigated also contained tenofovir.*
[d]*includes studies that reported on hormonal contraceptives, but where the exogenous sex steroids are unspecified.*
CVR, contraceptive vaginal ring; DMPA, Depo-medroxyprogesterone acetate; IUS, Intrauterine system; HC, hormonal contraception; LNG, levonorgestrel; MHT, menopausal hormone therapy; Net-En, norethisterone enanthatel; OCP, oral contraceptive pill.

bacterial diversity and increased likelihood of having a vaginal microbiota composition designated to CST-IV (Balle et al., 2020).

Ethinyl Oestradiol Containing Injectable

Only one study investigated the effect of the ethinyl oestradiol-containing injectable among 40 women in Zimbabwe (Achilles et al., 2018). There were no significant changes to the composition of the vaginal microbiota following ethinyl oestrodial-containing injectable use relative to baseline specimens.

The Effect of Progestin-Only Contraceptives on the Vaginal Microbiotas

Twenty studies investigated the effect of progestin-only contraceptives, representing DMPA, Net-En and the levonorgestrel CVR and IUS. When looked at together, four studies identified a positive effect of progestin-only contraceptives on the vaginal microbiota, nine described a negative effect on the microbiota, one study reported different effects across patient sub-populations, and seven reported neutral or inconclusive effects (**Tables 1** and **3**).

Depot-Medroxyprogesterone Acetate Injectable

Of the ten studies that investigated DMPA (Borgdorff et al., 2015; Brooks et al., 2017; Achilles et al., 2018; Lennard et al., 2018; Dabee et al., 2019; Onywera et al., 2019; Wessels et al., 2019; Yang et al., 2019; van de Wijgert et al., 2020; Whitney et al., 2020), three reported a negative effect of DMPA on the vaginal microbiota, one reported a positive effect, five reported neutral findings and one reported mixed findings. DMPA-use was associated with a decrease in the relative abundance of *Lactobacillus* spp. in two studies (additional data provided by K. Lennard) (Dabee et al., 2019; Yang et al., 2019). In the study by Yang et al, DMPA-use had a different effect in the two study populations; it was associated with a decreased abundance of *Lactobacillus* spp. among African American/black women and an increase in relative abundance of *Lactobacillus* spp. among Caucasian/white women (Yang et al., 2019). Achilles et al. (2018) also reported a loss of *Lactobacillus* spp., specifically a decrease in log concentration of *L. iners* over six months of DMPA-use. Brooks et al. (2017) described a non-significant increase in the relative abundance of BV-associated bacteria associated with DMPA-use, which was classified as an inconclusive finding. Wessels et al. (2019) found that DMPA users had an increase in bacterial diversity (measured by Shannon diversity index). Conversely, Onywera et al. (2019) found that DMPA-use was associated with lower bacterial diversity (measured by Shannon diversity index) and a vaginal microbiota assigned to *Lactobacillus* spp. dominated CSTs (CST-I, CST-II and CST-III) relative to women using condoms or no contraceptives. One study found that DMPA had no effect on the prevalence and concentrations of BV-associated bacteria *Sneathia, M. hominis* and *Parvimonas* species type 1 *vs* women who did not use contraception/used non-hormonal contraceptives (Whitney et al., 2020). However, this study did not specifically look at the effect of DMPA on lactobacilli. Three studies found that there was no significant difference in the proportion of DMPA-users assigned to specific vaginal microbiota groups associated with optimal or non-optimal compositions compared to non-hormonal contraceptive users and/or users of other hormonal contraceptive types (additional data provided by [Lennard et al. (2018), van de Wijgert et al. (2020)] (Borgdorff et al., 2015; Lennard et al., 2018; van de Wijgert et al., 2020). In addition, Pyra et al. (2016) investigated the effect of injectable contraceptives but did not specify the type used. They defined injectable contraceptives as having a negative effect, as their use was associated with a lower prevalence of *L. iners*. Overall, DMPA had mixed effects with nearly equal numbers of studies reporting positive or neutral effects and negative effects on the vaginal microbiota.

Norethisterone Enanthate Injectable

Four studies investigated the effect of Net-En on the vaginal microbiota (Lennard et al., 2018; Dabee et al., 2019; Onywera et al., 2019; Balle et al., 2020), two of which had negative findings, one had positive findings and one had neutral findings. Balle et al. (2020) compared the effects of Net-En with participants' baseline samples on the vaginal microbiota and found no effect of Net-En initiation. However, Net-En users were also more likely to be assigned to CST-IV than participants using the combined oestrogen-containing OCP (Balle et al., 2020). Dabee et al. (2019) found use of Net-En was associated with lower relative abundance of *Lactobacillus* spp. compared to women not using any hormonal contraception. In contrast, Onywera et al. (2019) found that Net-En use was associated with lower bacterial diversity (measured by Shannon diversity index) and assignment to *Lactobacillus* dominated CSTs compared to women not using hormonal contraception, however this represented a small proportion of Net-En users captured in this review (n=5). Lennard et al. (2018), had the most Net-En users (n=70) in their study and found that Net-En use was not associated with any specific vaginal microbiota composition when compared to women not using hormonal contraception, or women using other types of hormonal contraception (additional data provided by K. Lennard).

Levonorgestrel-Containing Contraceptive Rings and Intra-Uterine Systems

Four studies investigated the effect of levonorgestrel-containing systems on the vaginal microbiota (Jacobson et al., 2014; Bassis et al., 2015; Brooks et al., 2017; Thurman et al., 2019), two of which had positive findings, one had negative findings and the other had neutral findings. Thurman et al. (2019) found that the proportion of women with an optimal vaginal microbiota (CST I, II and V) increased following tenofovir-LNG-CVR insertion. There was a decrease in the proportion of women with a non-optimal vaginal microbiota (CST IV) following tenofovir-LNG-CVR insertion (Thurman et al., 2019). Three studies investigated the LNG-IUS with discordant findings; Jacobson et al. (2014) found that following insertion of LNG-IUS, the relative abundance of *L. crispatus* increased. Conversely, Brooks et al. (2017) compared LNG-IUS use to OCP-use and found a non-significant change in the relative abundance of BV-associated bacteria, while Bassis et al. (2015) found no difference in the

vaginal microbiota composition between women using LNG-IUS and those using a Cu-IUD.

Song et al. (2020) grouped contraceptives as either oestrogen-containing or combined progestin-only "local release" contraceptives due to the small sample size (n=7, n=4 respectively). Compared to women not using hormonal contraception, oestrogen-containing contraceptives were associated with a non-significant increase in *Lactobacillus* spp. and use of progestin-only contraceptives was associated with a non-significant decrease in on the abundance of *Lactobacillus* spp (Song et al., 2020).

Studies That Grouped All Hormonal Contraceptives Together and Did Not Specify Type

Five studies did not stratify findings by hormonal contraception type (**Tables 1** and **3**; four reported a positive effect of grouped hormonal contraception-use on the vaginal microbiota and one reported a neutral effect. Scott et al. (2019) and Tuddenham et al. (2019b) found that grouped hormonal contraception-use was associated with stability of the vaginal microbiota. Both the cross-sectional and longitudinal studies by Tuddenham et al. (Tuddenham et al., 2019a; Tuddenham et al., 2019b) found that hormonal contraception-use was associated with a vaginal microbiota defined by optimal CSTs, and Marconi et al. (2020) found that hormonal contraception-use was associated with a decreased risk of having CST-IV. Gautam et al. (2015) reported that hormonal contraception-use did not change the vaginal microbiota.

Studies Reporting on the Effect of Menopausal Hormone Therapy on the Vaginal Microbiota

General Characteristics

Four studies that investigated the effect of MHT on the vaginal microbiota were identified (**Tables 2** and **3**) (Devillard et al., 2004; Heinemann and Reid, 2005; Dahn et al., 2008; Shen et al., 2016). These studies represent 332 samples from 158 women, and all four assessed the effect of a topical conjugated-oestrogen containing formulation (topical Premarin®). All studies were in post-menopausal women, with three studies performed in Canada (Devillard et al., 2004; Heinemann and Reid, 2005; Dahn et al., 2008) and one in China (Shen et al., 2016) (**Supplementary Table 3**). One study was cross-sectional (Dahn et al., 2008) and the other three were longitudinal (Devillard et al., 2004; Heinemann and Reid, 2005; Shen et al., 2016); the median follow up time was 10 weeks (range 4-13 weeks). Three studies used DGGE based methods (Devillard et al., 2004; Heinemann and Reid, 2005; Dahn et al., 2008); two were in combination with Sanger sequencing (Devillard et al., 2004; Heinemann and Reid, 2005) one used microarray. Shen et al. (2016) was the only study to use next-generation sequencing (**Supplementary Table 3**).

The Effect of Menopausal Hormone Therapy on the Vaginal Microbiota

All four studies found that use of topical conjugated-oestrogens increased the prevalence of *Lactobacillus* spp. compared to women not using menopausal hormone therapy (Devillard et al., 2004; Heinemann and Reid, 2005; Dahn et al., 2008; Shen et al., 2016) (**Tables 2** and **3**). Shen et al. (2016) and Heinemann and Reid (2005) also found that conjugated-oestrogen use decreased the bacterial diversity of the vaginal microbiota and in addition, Heinemann and Reid (2005) reported that its use increased the stability of the vaginal microbiota over time.

Assessment of Study Bias

We assessed each of the studies for Selection Bias, Sample size and Measurement Bias across 6 domains, with a summary score for risk of bias generated (**Supplementary Table 2**). Studies with the lowest summary score were considered to have the lowest risk of study bias, however none of the included studies had a low risk of bias across all criteria assessed (**Table 4**).

Studies Reporting on the Effect of Hormonal Contraceptives on the Vaginal Microbiota Among Reproductive-Aged Women

When looking at selection bias across the 25 studies that investigated hormonal contraception, the study population was not described for only one study (Scott et al., 2019). One study was conducted on samples from a randomised control trial (Ratten et al., 2021) and in one, study participants were assigned treatment sequentially (Yang et al., 2019). For the remaining 22 studies, the participants self-selected their intervention. Eleven studies included a sample size of <100 participants total, which we assessed as high risk of bias (Jacobson et al., 2014; Dabee et al., 2019; Onywera et al., 2019; Scott et al., 2019; Van Der Veer et al., 2019; Yang et al., 2019; Song et al., 2020; Ratten et al., 2021). Eighteen studies used age-matched women not using hormonal contraceptives as the comparator group (e.g. no contraceptives or condom-use) (low risk), and seven used the baseline specimen prior to contraception initiation as the comparator group (low/medium risk) (Jacobson et al., 2014; Bassis et al., 2015; Achilles et al., 2018; Crucitti et al., 2018; Tuddenham et al., 2019b; Yang et al., 2019; Balle et al., 2020). Five studies did not stratify hormonal contraceptive exposure by oestrogen-containing or progestin-only (high risk) (Gautam et al., 2015; Scott et al., 2019; Tuddenham et al., 2019a; Tuddenham et al., 2019b; Marconi et al., 2020). Nine studies did not adjust analyses for confounding variables such as sex and douching practices (high risk) (Bassis et al., 2015; Gautam et al., 2015; Achilles et al., 2018; Crucitti et al., 2018; Tuddenham et al., 2019a; Tuddenham et al., 2019b; Onywera et al., 2019; Wessels et al., 2019; Song et al., 2020).

Studies Reporting on the Effect of Menopausal Hormone Therapy on the Vaginal Microbiota

Of the four MHT studies, one study did not define the study population (unknown risk) (Dahn et al., 2008), one was conducted in healthy women (classified as low risk or bias) (Devillard et al., 2004), and two were conducted in women with urogenital symptoms or infection (medium risk) (Heinemann and Reid, 2005; Shen et al., 2016). Women self-selected their therapy in all four studies, and all studies had fewer than 100 women and did not demonstrate sample size calculations for the effect of MHT use on the vaginal microbiota (high risk).

TABLE 4 | Risk of Bias Summary Table.

First author [ref]	Year	Location/ Country	Selection Bias		Sample Size	Measurement Bias			*Summary of the overall risk of study bias*
			Representative of the general population?	Randomly allocated?	Adequate sample size?	Comparator Group?	Stratified by hormone?	Adjusted?	
Studies of the effect of Hormonal Contraceptives									
Achilles et al. (2018)	2018	Zimbabwe	0	2	0	1	0	1	4
Balle et al. (2020)	2020	South Africa	1	0	0	1	0	0	2
Bassis et al. (2015)	2015	USA	1	2	0	1	0	1	5
Borgdorff et al. (2015)	2015	Rwanda	1	2	0	0	0	0	3
Borgdorff et al. (2017)	2017	Netherlands	0	2	0	0	0	0	2
Brooks et al. (2017)	2017	selected from VaHMP	0	2	0	0	0	0	2
Crucitti et al. (2018)	2018	Rwanda	0	2	0	1	0	1	2
Dabee et al. (2019)	2019	Cape Town & Soweto	0	2	1	0	0	0	3
Gautam et al. (2015)	2015	Africa	1	2	0	0	1	1	5
Jacobson et al. (2014)	2014	USA	0	2	1	1	0	0	4
Lennard et al. (2018)	2018	South Africa	1	2	0	0	0	0	3
Marconi et al. (2020)	2020	Brazil (multiple sites)	0	2	0	0	1	0	3
Onywera et al. (2019)	2019	Cape Town, South Africa	1	2	1	0	1	1	6
Pyra et al. (2016)	2016	Multiple countries	0	2	0	0	0	0	2
Ratten et al. (2021)	2020	Australia	1	0	1	0	0	0	2
Scott et al. (2019)	2019	USA	?*	2	1	0	1	0	4
Song et al. (2020)	2020	Wellesley, Massachusetts, USA	1	2	1	0	0	1	5
Thurman et al. (2019)	2019	USA and Dominican Republic	0	0	1	0	0	0	1
Tuddenham et al. (2019a)	2019	Baltimore, MD, USA	0	2	0	0	1	1	4
Tuddenham et al. (2019b)	2019	Baltimore, MD, USA	0	2	0	1	1	1	5
van de Wijgert et al. (2020)	2019	Johannesburg, South Africa	1	2	0	0	0	0	3
Van Der Veer et al. (2019)	2019	Amsterdam, the Netherlands	0	2	1	0	0	0	3
Wessels et al. (2019)	2019	Kenya	1	2	1	0	0	1	5
Whitney et al. (2020)	2020	Nairobi, Kenya	1	2	1	0	0	0	4
Yang et al. (2019)	2019	New Jersey, USA	0	1	1	1	0	0	3
Studies of the effect of Menopausal Hormone Therapy									
Dahn et al. (2008)	2008	Canada	?*	2	1	0	0	1	4
Devillard et al. (2004)	2004	Multiple countries	0	2	1	2	0	1	6

(Continued)

TABLE 4 | Continued

First author [ref]	Year	Location/ Country	Selection Bias		Sample Size	Measurement Bias			*Summary of the overall risk of study bias*
			Representative of the general population?	Randomly allocated?	Adequate sample size?	Comparator Group?	Stratified by hormone?	Adjusted?	
Heinemann and Reid (2005)	2005	Canada	1	2	1	0	0	1	5
Shen et al. (2016)	2016	China	1	2	1	2	0	1	7

*Patient population characteristics not described.

Two studies had age-matched MHT-free women as comparators (low risk) (Dahn et al., 2008; Shen et al., 2016). In one study the comparator group was women who were not using MHT who had participated in another study (high risk) (Devillard et al., 2004). The fourth study compared findings to a group of women not using MHT, but these women had different symptoms to the MHT-exposed group (high risk) (Shen et al., 2016). All studies were stratified by hormone source (low risk), but no study adjusted for confounding variables (high risk).

DISCUSSION

Exogenous sex steroids contained within hormonal contraceptives and menopausal hormone therapy have been used principally for family planning and management of menopausal symptoms, without consideration for potential effects on the vaginal microbiota. This is because their use in healthcare predates our current understanding of the importance of the vaginal microbiota in human health, largely due to the fact that they were adopted before the technology existed to assess the vaginal microbiota. Prior systematic reviews and a meta-analysis of observational data found that oestrogen-containing contraceptives in particular have a beneficial effect on the vaginal microbiota, as measured by non-molecular methods (van de Wijgert et al., 2013; Vodstrcil et al., 2013). In this systematic review, we aimed to determine the effects of exogenous sex steroid use on the vaginal microbiota, as measured using modern, molecular-based methods such as high-throughput sequencing. We found that oestrogen-containing contraceptives, particularly the combined oestrogen and progestin-containing OCP, had a positive effect on the composition of the vaginal microbiota. Among post-menopausal women using MHT, exogenous oestrogen also appeared to positively influence the vaginal microbiota. However, the significance of an optimal vaginal microbiota as defined in reproductive-aged women is not as well understood in the post-menopausal population. In contrast, contraceptives containing progestin alone had mixed effects on the vaginal microbiota of reproductive-aged women. Overall, our systematic review shows that oestrogen may play a role in supporting an optimal vaginal microbiota in both reproductive aged and peri/post-menopausal women. However, further well-powered studies with appropriate control groups are required to explore the specific effects of different oestrogen-containing and progestin-only contraceptives.

The Impact of Exogenous Oestrogen on the Vaginal Microbiota

We found that exogenous oestrogen, which predominantly reflected the use of the combined oestrogen and progestin-containing OCP, had a positive impact on the vaginal microbiota composition. Specifically, there was an increase in the prevalence and abundance of *Lactobacillus* spp. observed following oestrogen-exposure. *Lactobacillus* spp., particularly *L. crispatus*, characterise a vaginal microbiota associated with optimal reproductive and sexual health outcomes (Petrova et al., 2015; Anahtar et al., 2018). The positive effect of lactobacilli is proposed to be due to the production of lactic acid, which lowers the vaginal pH in addition to conferring antimicrobial and immunomodulatory benefits, and inhibiting the growth of anaerobic bacteria (Aldunate et al., 2015; Hearps et al., 2017; Tachedjian et al., 2017; Tyssen et al., 2018; Delgado-Diaz et al., 2020; Plummer et al., 2021). Different lactobacilli can produce two different lactic acid isomers, the L-isomer and D-isomer. The D-isomer is hypothesised to offer more protection against some bacterial upper genital tract infections such as chlamydia, however the L-isomer is more potent in inactivating HIV compared to the D-isomer at threshold concentrations *in vitro* (O'Hanlon et al., 2011; Aldunate et al., 2013; O'Hanlon et al., 2013; Witkin et al., 2013; Witkin, 2018; Edwards et al., 2019). In reproductive-aged women, endogenous oestrogen stimulates glycogen production by epithelial cells, which is then metabolised by *Lactobacillus* spp. as an energy source resulting in production of lactic acid (Boskey et al., 2001; O'Hanlon et al., 2011; O'Hanlon et al., 2013; van der Veer et al., 2019; Clabaut et al., 2021). Among women taking exogenous oestrogen, the amount of free glycogen available may increase or be more consistent throughout the menstrual cycle, and in turn indirectly increase lactic acid production (Mirmonsef and Spear, 2014; Nunn and Forney, 2016). Exogenous oestrogen could therefore have a therapeutic role among reproductive-aged women with a paucity of *Lactobacillus* spp. in their vaginal microbiota, such as in women with BV and vaginitis. In the one pilot randomised controlled trial to randomise women with BV receiving antibiotic treatment to adjunctive combined oestrogen-containing OCP-use or no OCP-use, there was no significant effect of sex steroid-exposure on BV recurrence rates (Vodstrcil et al., 2019). However, the findings of this study were impacted by the small sample size and attrition, and larger studies may be required to help us determine whether the use of oestrogen-

containing contraceptives adjunctively or alone may positively influence the vaginal microbiome of women with BV.

The oestrogen-containing CVR had mixed impacts on the vaginal microbiota. Oestrogen-containing CVR-use was associated with an increase in several *Lactobacillus* spp. including *L. crispatus, L. iners, L. vaginalis* (Crucitti et al., 2018) and decrease in BV-associated bacteria such as *G. vaginalis* (Borgdorff et al., 2017; Crucitti et al., 2018). However, oestrogen-containing CVR-use was also shown to have no effect (Balle et al., 2020). Similarly, among women using progestin-only CVRs and intrauterine systems, the composition of the vaginal microbiota varied (Jacobson et al., 2014; Bassis et al., 2015; Brooks et al., 2017; Thurman et al., 2019). The high variability between these studies may be because of the limited number of studies, the different control groups used and/or the timing of the post-insertion specimens relative to when the contraceptive system was inserted. More studies are needed to understand the impact of CVR-use and IUS-use on vaginal microbiota composition.

Topical conjugated oestrogens in post-menopausal women was also associated with an increase in the abundance and prevalence of *Lactobacillus* spp. While there is abundant evidence to support the use of oestrogen to relieve post-menopausal symptoms such as vaginal dryness and discomfort (Hickey et al., 2016), we identified only four studies investigating menopausal hormone therapy on the vaginal microbiota. As there is limited information about the role of the vaginal microbiota in post-menopausal women, we do not know if there are benefits associated with re-establishing a *Lactobacillus* dominant vaginal microbiota in post-menopausal women.

The Effect of Progestin on the Vaginal Microbiota in Reproductive-Aged Women

There is global interest in how progestin-only hormonal contraception, especially DMPA, might impact the vaginal microbiota and may increase the risk of STI and/or HIV acquisition. Our systematic review found that the effect of progestin-only contraceptives on the vaginal microbiota was mixed. Of note, half (n=11) of these papers examined DMPA-use. In contrast to oestrogen-containing contraceptives, progestin-only contraceptives had an inconsistent effect on the abundance and prevalence of *Lactobacillus* spp. as well as other metrics such as bacterial diversity and prevalence/abundance of BV-associated bacteria. The inconsistent findings of the effect of progestin-only contraceptives on the vaginal microbiota within and/or between sub-populations may be explained by host genetics and gene polymorphisms (Dabee et al., 2021), however further research is needed. The effect of DMPA on the vaginal microbiota is of particular interest due to its high rate of use in sub-Saharan Africa, and concerns it may enhance HIV transmission/acquisition (Galvin and Cohen, 2004; Curtis et al., 2020; Smith et al., 2020). This was reflected in our study screening process, which identified most progestin-only studies investigated the impact of DMPA on the vaginal microbiota. These studies were predominantly of women in sub-Saharan Africa, where DMPA usage coincides with high rates of HIV. Other possible detrimental effects following initiation of DMPA include epithelium thinning in the vagina, tissue inflammation and altered cell-mediated immune responses. In fact all of these effects increase a woman's susceptibility to BV, HIV and other STIs (van de Wijgert et al., 2013; Murphy et al., 2014). Using previously published microbiome data from the CAPRISA-004 trial (Klatt et al., 2017), women with a *Lactobacillus*-dominant vaginal microbiota (primarily reflecting *L. iners*) who were using DMPA had a 3-fold increased risk of HIV acquisition (relative to women using Net-En or OCP) (Noël-Romas et al., 2020). In addition, higher serum-MPA concentrations were associated with evidence of inflammation in the vaginal mucosal fluid of *Lactobacillus*-dominated women (Noël-Romas et al., 2020). Interestingly, these effects of DMPA and serum-MPA were not observed in non-*Lactobacillus*-dominant women. The authors concluded that there is an interaction between the microbiome, hormonal contraceptives, and HIV susceptibility, demonstrating the importance of the both the vaginal microbiota and hormonal contraceptives in assessing HIV risk. Indeed, specific BV-associated bacteria themselves have been shown to enhance inflammatory pathways, which further complicates our understanding of these relationships (Dabee et al., 2021). The Evidence for Contraceptive Options in HIV Outcomes (ECHO) trial went on to assess if the risk of HIV differed with the use of three contraceptive methods – DMPA, LNG implant and Cu-IUD - but found no substantial difference in risk of HIV acquisition by contraceptive method used (Ahmed et al., 2019). The results from subsequent molecular analyses from the ECHO trial are pending and will be of great interest due to the concern regarding the possible impact of contraceptive practices on the risk of HIV acquisition and transmission. Clearly the relationship between progestin-only contraceptive use and the vaginal microbiota are more complex than for oestrogen-containing contraceptives, and require further investigation in the context of different populations and settings, as observed in the study by Yang et al. (2020).

Strengths and Limitations

Our systematic review identified a significant number of studies that reported on hormonal contraception-use on the vaginal microbiota in reproductive aged women (n=25). We restricted our inclusion criteria to studies that measured the vaginal microbiota by molecular methods to reduce the significant bias which can be introduced by culture-dependant methods and microscopy. These methods only identify organisms that are able to be cultivated or are limited by taxonomic resolution. Despite the differences in methods used, next-generation sequencing was the most common measurement recorded in this review (n=20). However, all molecular methods have different biases, from DNA extraction, to data analysis, which should be considered. For example, different approaches to the steps used in 16S rRNA gene sequencing pipelines (i.e. DNA extraction method, primer selection, variable region selection, 16S rRNA gene reference database etc) can result in different findings when applied to the same samples (Pollock et al., 2018; Balkus et al., 2019). Additionally, microarray and qPCR results are dependent on the panel of targets tested. These factors further limited our ability to compare findings across studies. This study had a number of additional limitations. In the reproductive-aged

cohort, most studies were conducted in sub-Saharan Africa (n=12) and North America (n=8) and therefore the effects of hormonal contraceptives may not be generalizable to other populations. This was even more apparent in the post-menopausal cohort where three of the four studies included were conducted in Canada, with the fourth from China. The small number of studied population groups, and the diverse findings across studies limited further comparison of our findings. Our analysis of bias also identified several other limitations specific to each of the included studies (**Table 4**). There was a high degree of variation in how the studies were conducted. For example, the contraceptive types assessed, how long contraceptives were used for, if there was a wash-out period from previous exogenous steroid exposure, the sample size, and how the data were analysed. The comparator groups also varied and included the participants' baseline specimens or specimens from patients using non-hormonal contraceptives such as condoms or a Cu-IUD. The use of a Cu-IUD as the control group may have overestimated the benefit of the hormonal contraceptive assessed, as Cu-IUD use itself has been associated with dysbiosis (Erol et al., 2014; Achilles et al., 2018). Notably, many studies had small sample sizes, and, in most cases, patients self-selected their exposure and self-reported adherence to use. Furthermore, the effects of exogenous sex steroid exposure were not adjusted for behavioural practices. Despite contacting authors to retrieve more information, several studies were unable to specify which hormonal contraception-types were included in their hormonal contraception-use group/s, which prevented our ability to include the studies in analyses stratified by sex steroid-type. Regardless, of the five studies which included unspecified hormonal contraceptive-types, four found a positive influence of their use on the vaginal microbiota. Finally, there are newer formulations of hormonal contraceptives and menopausal hormone therapy that were not captured in this review.

Conclusions

Access to contraceptives is an invaluable part of modern women's healthcare, but until recently there was little consideration as to how this might affect the vaginal microbiota. With increasing use of hormonal contraceptives and menopausal hormone therapy globally, and improved understanding of how the microbiota may contribute to negative reproductive and gynaecological outcomes, it is important we understand the immediate and long-term impact of specific exogenous sex steroid use on the vaginal microbiota. Advances in high throughput DNA sequencing has made molecular analyses of the vaginal microbiota accessible and allowed us to gain more insight into the effects exogenous sex steroids may have on specific organisms, as well as its overall composition.

Our findings suggest that oestrogen-containing contraceptives and MHT may promote an optimal vaginal microbiota in some populations, which could have clinical applications, such as adjunctive therapy to improve management of BV and vaginitis (atrophic). While research is needed to support the use of exogenous-oestrogen as an adjunctive therapy, our data suggests it does not impact the composition of the vaginal microbiota in a detrimental way. The impact of progestin-only hormonal contraceptives was less consistent as there was equal evidence that they have either a negative or neutral impact on the vaginal microbiota. Clearly, more data is needed in order to confirm that DMPA and other progestin-only contraceptives are not contributing to adverse reproductive and sexual health outcomes. The molecular results from the ECHO trial are greatly anticipated and may provide some answers. Additional prospective studies from a wider range of populations that identify the underlying mechanisms by which progestin-only contraceptives alter the vaginal microbiota are also needed.

In summary, this review highlights the complex nature of the relationship between progestin-only contraceptives and the vaginal microbiome, and confirms the potential benefits of exogenous oestrogen in conferring a vaginal microbiota associated with optimal health outcomes for women.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LR, LV, and CB conceived and designed the study. LR and EP conducted the formal analysis, extracted all data, and interpreted the data, with support from LV and CB. GM provided advice around molecular technologies used. LM, GT, and DB provided additional data interpretation. CB, LV, GM, CF, and SG provided supervision. LR drafted the initial manuscript with supervision from LV, and EP, CB, CF, GM, SG, DB, GT, and LM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.732423/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Vaginal Microbiota, Genital Inflammation and Extracellular Matrix Remodelling Collagenase: MMP-9 in Pregnant Women With HIV, a Potential Preterm Birth Mechanism Warranting Further Exploration

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Background: Pregnant women living with HIV infection (PWLWH) have elevated rates of preterm birth (PTB) in which HIV and cART are implicated. PWLWH also have a high prevalence of adverse vaginal microbiota, which associate with genital tract inflammation. The mechanism underlying PTB in PWLWH is unknown. We present the first data in PWLWH on genital-tract matrix-metalloproteinase-9(MMP-9), an important collagenase implicated in labour onset, and tissue inhibitor of metalloproteinases-1(TIMP-1) and explore correlations with local inflammation and vaginal bacteria.

Material and Methods: Cervical vaginal fluid (CVF) collected by a soft cup and high vaginal swabs (HVS) were obtained from PWLWH and HIV uninfected pregnant women (HUPW) at three antenatal time points. Maternal characteristics, combination antiretroviral therapy (cART) exposure, and pregnancy outcome were recorded. Concentrations of MMP-9, TIMP-1 and ten cytokines were measured by immunoassays. Vaginal microbiota composition was determined through 16S rRNA amplicon sequencing. MMP-9, TIMP-1 and cytokine concentrations were compared by HIV status, cART, and prematurity and in PWLWH correlations with polymorphonuclear leucocytes, cytokines and bacterial genera were explored.

Results: CVF was available for 50 PWLWH (108 samples) and 12 HUPW (20 samples) between gestation weeks 14-38. Thirty-six PWLWH conceived on cART and 14 initiated post-conception. There were five and one PTB outcomes in PWLWH and HUPW respectively. PWLWH had higher mean CVF concentrations of MMP-9 ($p<0.001$) and TIMP-1 ($p=0.035$) in the second trimester compared with HUPW with a similar trend in the

third trimester. PWLWH also had higher CVF values of cytokines: IL-1β, IL-8, IL-12 and TNF-α in both trimesters compared to HUPW ($p \leq 0.003$). In PWLWH, MMP-9 positively correlated with TIMP-1 (r=0.31, p=0.002) and CVF polymorphonuclear leucocytes (r=0.57, p=0.02). Correlations were observed between MMP-9 and three cytokines: IL-1β (r=0.61), IL-8 (r=0.57) and TNF-α (r=0.64), p<0.001, similarly for TIMP-1. Abundance of anaerobic pathobionts correlated with MMP-9: *Gardnerella* (r=0.44, p<0.001), *Atopobium* (r=0.33, p=0.005), and *Prevotella* genera (r=0.39, p<0.001). Conversely proportion of *Lactobacillus* genera negatively correlated with MMP-9 (rho=-0.46, p<0.001). MMP-9/TIMP-1 ratio increased with gestational age at sampling in PWLWH, but this was no longer significant after adjusting for confounders and no difference by prematurity was observed in this sub-study.

Conclusions: Here we show strong correlations of MMP-9 to genital tract inflammation and sub-optimal bacterial genera in PWLWH indicating the ascending genital tract infection pathway may be a contributory mechanism to the high risk of PTB.

Keywords: HIV - human immunodeficiency virus, preterm (birth), metalloproteinase, microbiome and dysbiosis, *Gardnerella* species

INTRODUCTION

Pregnant women living with HIV infection (PWLWH) are disproportionately affected by PTB outcomes, with some cohorts experiencing two to four-fold the risk observed in the general population (Thorne et al., 2004; Short and Taylor, 2014; Wedi et al., 2016). The aetiology of this phenomenon is unclear but the successful use of combination antiretroviral therapy (cART) to prevent mother to child transmission of HIV (PMTCT) has not had the same impact on high rates of PTB and may increase risk of this complication (Thorne et al., 2004).

Literature around this obstetric complication in PWLWH suggest that it is the result of a complex interplay of high background risk factors for PTB e.g. African and Caribbean ethnicity (Tuomala et al., 2005; Li et al., 2019), anaemia (Chen et al., 2012), low body mass index (BMI) (Young et al., 2012), hypertension (Chen et al., 2012) and pre-eclampsia (Wimalasundera et al., 2002), cART exposure (class of third drug e.g. protease inhibitors and timing in relation to conception) (C Short and Taylor, 2014) and infection factors. The latter include advanced HIV disease (Slyker et al., 2014; Wedi et al., 2016; Favarato et al., 2018), intercurrent co-infections with bacterial vaginosis (Taha, 1999; Slyker et al., 2014), sexual transmitted infections (Slyker et al., 2014; Shava et al., 2019), other pathogens e.g. cytomegalovirus, malaria (McDonald et al., 2019; Moraka et al., 2019), and higher risk and severity of chorioamnionitis (Goldenberg et al., 2006; Ategeka et al., 2019; Obimbo et al., 2019).

Preterm birth in the general population is an umbrella syndrome with multiple aetiologies (Goldenberg et al., 2008). Ascending genital tract infection is thought to be a significant factor in premature rupture of membranes (Brown et al., 2018) and spontaneous preterm labour (Elovitz et al., 2019) and potentially impair placentation, all effected through changes in maternal immune surveillance and tolerance (Mor et al., 2017). The pathologic mechanism of PTB in PWLWH is unclear. We hypothesize the elevated risk of PTB is the result of an inflammatory process driven by alternations in: the balance of cytokines at the maternal foetal interface (Fiore et al., 2006; Short and Taylor, 2014; Short et al., 2021) and dysregulation of immune activation of maternal T cells (Short et al., 2017) to promote local immune cell infiltration in the female reproductive tract; both of which are influenced by local vaginal microbiota composition (Short et al., 2013; Anahtar et al., 2015; Short et al., 2021).

We and others have described that PWLWH in European and African settings have a microbiota either dominated by *Lactobacillus iners* or with a diverse anaerobic bacteria community structure (Price et al., 2019; Gudza-Mugabe et al., 2020; Short et al., 2021). *Lactobacillus* single species dominance in pregnancy, particularly *L. crispatus*, is associated with term birth outcomes whereas *L. iners* species dominance, the least stable *Lactobacillus* community structure, and mixed anaerobes are associated with PTB (Kindinger et al., 2017; Brown et al., 2018; Elovitz et al., 2019; Fettweis et al., 2019). Within our London HIV PTB study cohort all PTB occurred in women with these adverse vaginal microbiota groups (Short et al., 2021). How these bacteria exert their effects on downstream pathways to trigger labour is not fully elucidated. Pilot data from our group exploring differentially expressed cervicovaginal immune proteins by HIV status using proteomic profiler arrays indicated that matrix metalloproteinase 9 (MMP-9), extracellular matrix metalloproteinase protein inducer (EMMPRIN) and neutrophil gelatinase B-associated lipocalin (NGAL) were significantly upregulated compared to HIV uninfected pregnant women (HUPW) (Short, 2019).

MMP-9 (also known as gelatinase B) is a zinc dependent endopeptidase involved in extracellular matrix (ECM) remodelling of type IV collagen (and to lesser extent type V) and elastin that is part of several physiological processes including uteroplacental re-modelling in reproduction, cell migration (particularly neutrophils), angiogenesis and wound

healing(Juanjuan Chen and Khalil, 2017). It is known to be upregulated in membrane rupture, placental detachment and myometrial contractility in term and preterm labour (Weiss et al., 2007; Sundrani et al., 2012; Ulrich et al., 2019). MMP-9 expression occurs in multiple cell types, many of which are found at the maternal-foetal interface although the source of MMP-9 in the events leading to labour is not fully understood. Its expression is thought to be regulated by cytokines: IL-1β, IL-8, IL-10 and TNF-α, prostaglandins (Peltier, 2003; Padron et al., 2020), and tissue inhibitor metalloproteinase-1 (TIMP-1) (Juanjuan Chen and Khalil, 2017). Much of the available data on MMP-9 in pregnancy are derived from plasma and amniotic fluid samples, tissue explants, cell lines and rat models with minimal data on the cervical and lower genital compartments (Choi et al., 2009; Becher et al., 2010). Human data exist on the relationship between MMP-9 and intra-amniotic infection but the direct relationship with vaginal microbiota as the source are scant (Locksmith et al., 2001; Kindinger et al., 2016; Padron et al., 2020) and non-existent in PWLWH.

Here we explore MMP-9, TIMP-1 and cytokine concentrations, and local polymorphonuclear leucocytes in a subgroup of PWLWH from the London HIV PTB study for whom cervicovaginal fluid (CVF) and high vaginal bacterial metataxonomic data were available and examine the association with lower genital tract microbiota composition. The full characterization of the vaginal microbiota of PWLWH from the London HIV Preterm birth study has been described previously (Short et al., 2021). These data have been used for correlation analyses with CVF MMP-9, TIMP-1 and cytokine concentrations.

METHODS

Study Design and Setting

The London HIV PTB study has been described previously (Short et al., 2021). PWLWH and HUPW were prospectively recruited between 2013-2017 at eleven tertiary care hospitals across London. The study was approved by the NHS Health Research Authority National Research Ethics Service (NRES) (Ref 13/LO/0107). The London HIV PTB study is an exploratory hypothesis generating pilot study and thus no sample size calculation has been performed. This is a sub-analysis of the main study of the group of women who provided lower female genital tract (FGT) samples.

Participants and Sample Collection

Exclusion criteria were: <18 years old, inability to give written informed consent, current injecting drug use, multiple gestation pregnancy and *in vitro* fertilization. All women not previously known to be living with HIV infection underwent routine antenatal screening with fourth generation combined HIV antibody and p24 antigen tests and syphilis serology in the first trimester. For PWLWH screening for gonorrhea and chlamydial infection was routinely offered as per national guidelines. Gestational age was determined by obstetric ultrasound at 10-14 weeks. Maternal demographics were recorded and for PWLWH: ART regimen, timing of cART exposure in relation to conception and immunovirological parameters were also documented. Gestational age at delivery, mode of delivery and any obstetric complications were recorded. Preterm birth was defined as delivery <37 completed weeks gestation.

Participants were invited to undergo lower FGT sampling at three time points across the second and third trimesters: 14.0-21.9 weeks; 22.0-27.9 weeks; 28.0-38.0 weeks. Clinician or self-taken high vaginal swab [HVS (BBL ™ CultureSwab™ MaxV Liquid amies swab)] and paired menstrual soft cup (Instead®, Evofem Ltd™) samples were obtained. Soft cups were retained for a minimum of five minutes prior to removal. All FGT samples were kept on ice and stored at -80°C within two hours of collection until further processing.

Quantification of CVF MMP-9, TIMP-1, Cytokines and Polymorphonuclear Leucocytes

CVF was thawed and extracted from the soft cup as previously described (Short et al., 2018) and diluted in an extraction buffer containing a protease inhibitor cocktail (Castle et al., 2004) in a dynamic range to fall within the standard curve of the intended assay. ELISA assays (Human Quantikine®, R and D Systems™) were used to measure concentrations of MMP-9 and TIMP-1 according to manufacturer's instructions (dilution range 40-800-fold). Multiplex chemiluminescent assays [V-plex Human Pro-inflammatory cytokine panel, Meso Scale Discovery™ (MSD)] were used to measure concentrations of ten cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, and TNF-α according to the manufacturer's instructions (dilution range 4-100-fold). All samples were run in duplicate. The inter-plate coefficient of variance (CV) value provided by the manufacturer was 8% for MMP-9, 4% for TIMP-1 (R and D Systems™) and for the cytokine multiplex plates the CV is typically <10% (MSD™). An intra-plate CV cut off ≤15% was used as the limit of acceptable variation between duplicates for these analyses.

Light microscopy was performed on 19 samples from PWLWH for whom a paired dry HVS was available to grade polymorphonuclear leucocyte count by ordinal scale: 0, 1–5, 6–10, 11–20, 21–30, and 31+ per high-powered field over an average of 3 fields.

DNA Extraction and 16S rRNA Gene Sequencing (Metataxonomics)

For PWLWH bacterial DNA was extracted from the HVS using a combination of enzymatic digestion and mechanical disruption of cell membranes and the QIAamp® DNA Mini kit (Qiagen™), as previously described (MacIntyre et al., 2015). The V1-V2 hypervariable regions of the 16S rRNA gene were amplified with a fusion primer set that includes four different 28F primers chosen to improve detection of *Bifidobacteriales* and a 388R primer(24). The 28F-YM forward primer (5′-GAGTTTGATCNTGGCTCAG-3′) was mixed in a ratio of 4:1:1:1 with 28F *Borrellia* (5'-GAGTTT GATCCTGGCTTAG-3'), 28F *Chloroflex* (5′-GAATTTGA TCTTGGTTCAG-3') and 28F *Bifido* (5'-GGGTTCGATTCTGG CTCAG-3') (RTL Genomics Amplicon Diversity Assay List). The forward primers included an Illumina i5 adapter (5′-AATGAT ACGGCGACCACCGAGATCTACAC-3′), an 8-base-pair (bp)

bar code and primer pad (forward, 5′-TATGGTAATT-3′). The 388R reverse primer (5′-TGCTGCCTCCCGTAGGAGT-3′) was constructed with an Illumina i7 adapter (5′-CAAGCAGAAGAC GGCATACGAGAT-3′), an 8-bp bar code, a primer pad (reverse, 5′-AGTCAGTCAG- 3′). The pair end multiplex sequencing was performed on an Illumina MiSeq™ platform (Illumina® Inc.) at Research and Testing Laboratory (Lubbock, TX, USA).

Sequence Analysis

The MiSeq SOP pipeline and software package Mothur were used to analyse RNA sequence data. Highly similar amplicons were clustered into operational taxonomic units (OTUs) using the kmer searching method and the Silva bacterial database (www.arb-silva.de/). All OTUs had a taxonomic cut-off of ≥97%. Classification was performed using the Ribosomal Database Project (RDP) reference sequence files and the Wang method (Wang et al., 2007). The RDP MultiClassifier script was used for determination of OTUs (phylum to genus) and species level taxonomies were determined using USEARCH (Edgar, 2010). To account for potential bias introduced by differences in sequence depth, samples were rarefied to the smallest OTU read count (n= 7738) and proportion of total reads calculated for each sample to generate species level abundance.

Statistical Analyses

Cross-Sectional 2nd and 3rd Trimester Comparison by HIV Status

CVF from 50 PWLWH and 12 HUPW were available for cross-sectional analyses, each participant contributed one sample per trimester group. Maternal demographics were compared by Welch, Mann-Whitney U and Fisher Exact tests according to variable type, data distribution, variance, and group size. Mean MMP-9, TIMP-1, MMP-9/TIMP-1 ratio and cytokine concentrations were compared in a cross-section of second trimester (PWLWH n=47, HUPW n=10) and third trimester samples (PWLWH n=33, HUPW n= 9) by HIV status and cART exposure using Welch tests and ANOVA.

Longitudinal Analyses in PWLWH

Using longitudinal data from PWLWH alone (108 samples) associations of polymorphonuclear leucocyte count, cytokine concentrations and bacterial genera abundance with MMP-9, TIMP-1 concentrations and their ratio were explored initially by Spearman's correlation and then partial correlation, adjusting for maternal age, race and BMI. To identify potential indirect effects between MMP-9, TIMP-1 and bacterial genera abundance, key cytokines were then included in partial correlation as covariates to test if the associations changed. A Bonferroni adjustment of p=0.01 was used as the threshold of significance to account for multiple comparison. To retain sensitivity p-values > 0.01 < 0.05 were considered as a trend.

Significant correlations were then characterised by hierarchical multiple linear regression adjusting for confounders, with patient ID included as a random effect and predictors inputted as second factors. Predicted models of the inter-relationship between MMP-9, TIMP-1 with key bacterial species and cytokines were postulated from regression results.

Mediation analyses were used to investigate whether the predicted relationship between *Gardnerella* and *Lactobacillus* genera and MMP-9 was mediated by indirect effects of key inflammatory cytokines or TIMP-1 [PROCESS macro, Model 4, version 4.0 (Preacher and Hayes, 2008)]. The 95% bootstrapped confidence interval for indirect effects is based on 1000 samples and considered significant if the bootstrapped confidence intervals did not cross zero. All analyses were performed in statistical software package, SPSS (version 27; IBM, Armonk NY, USA).

RESULTS

Study Participants Characteristics

PWLWH and HUPW were of similar age (median 35 and 33 years respectively), see **Table 1**. The predominant race of PWLWH was Black (82%) whereas Caucasian race was the most common in HUPW (58%). PWLWH had higher median body-mass index (BMI) and lower CD4 cell counts than HUPW. There was no difference in gestational age at delivery and proportion of women delivering prematurely between PWLWH and HUPW in this sub-analysis (5 (10%) and 1 (8%), p=0.68), see **Table 1**.

cART Exposure in PWLWH

Thirty-six (72%) PWLWH conceived on cART and 14 initiated cART post conception (27%). cART comprised a backbone of two nucleoside analogue reverse transcriptase inhibitors and one of the following third agents: a Protease Inhibitor (PI) n = 17 (34%); a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) n = 20 (40%); an Integrase Strand Transfer Inhibitor (INSTI) n = 10 (20%) and a third NRTI n = 3 (6%).

Cross-Sectional Analyses of CVF MMP-9, TIMP-1, and Cytokines in the Second and Third Trimester by HIV Status

CVF MMP-9, TIMP-1 and Pro-Inflammatory Cytokine Concentrations Are Higher in PWLWH Than HUPW

PWLWH had a higher mean concentration of MMP-9 in the second and third trimesters, although this did not reach significance in the third trimester (4 fold higher, p<0.001 and 1.5 fold, p=0.213 respectively), see **Table 2**. PWLWH had a higher mean concentration of TIMP-1 in the second and third trimesters (12 fold higher, p=0.035 and 3 fold higher, p=0.044 respectively). There was no statistical difference observed in MMP-9/TIMP-1 ratio by HIV status in the second and third trimester but a non-statistical trend towards an increase in ratio between second and third trimesters for PWLWH (1.7 fold higher, p=0.276) but not HUPW was observed, see **Table 2**.

PWLWH had higher concentrations of most measured CVF cytokines in the cross-section of second and third trimester samples, see **Supplementary Figures 1A, B**. The CVF cytokines with the most marked difference by HIV status in both trimesters were: IL-1β (19-22 fold higher, 2nd p=0.002, 3rd p=0.003); IL-8 (13-16-fold higher, 2nd p<0.001, 3rd p<0.001); IL-

TABLE 1 | Maternal Demographics by HIV status.

Characteristic	PWLWH n=50	HUPW =12	P value
Age/years			
[Median (range)]	35 (21-45)	33 (20-43)	0.205
Ethnicity [n (%)]			<0.001
Caucasian	4 (10)	7 (58)	
Black	41 (82)	3 (25)	
Other	5 (8)	2 (18)	
BMI [Median (IQR)]	25 (22-30)	23 (20-25)	0.032
Baseline CD4 +/cells/mm^3 [Median (IQR)]	620 (433-724)	970 (900-1390)	<0.001
Gestational age at delivery/weeks [Median (IQR)]	39 (38-40)	40 (38-41)	0.512
Birth outcome [n (%)]			
Term	42	27	0.682
Preterm	5	1	
Missing	3	1	
Birth weight/grams [Median (IQR)]	3190 (2908-3375)	3100 (2800-3200)	0.456

12 (8-9 fold higher, 2nd p<0.001, 3rd p<0.001) and TNF-α (15-27 fold higher, 2nd p<0.001, 3rd p=0.002), see **Table 3**.

CVF MMP-9, TIMP-1 and Pro-Inflammatory Cytokine Concentrations in PWLWH Do Not Differ by cART Exposure or Pre-Term Birth

In PWLWH no statistical differences in CVF MMP-9, TIMP-1 concentration or MMP-9/TIMP-1 ratio were observed by cART timing or class of the third drug, see **Supplementary Figure 2**. CVF cytokines: IL-1β, IL-8, IL-12 and TNF-α concentrations in PWLWH did not differ by cART timing or class of third ART drug, see **Supplementary Figures 3A, B**. There was no difference in MMP-9, TIMP-1 or CVF cytokines: IL-1β, IL-8, IL-12 and TNF-α by prematurity in either second or third trimesters, see **Supplementary Tables 1, 2**.

Longitudinal Analyses in PWLWH

CVF MMP-9 Positively Correlates With TIMP-1, Polymorphonuclear Leucocytes, Pro-Inflammatory Cytokines and Vaginal Pathobiont Abundance in PWLWH

In PWLWH CVF MMP-9 positively correlated with polymorphonuclear leucocyte count (r=0.57, n=19, p=0.02), see **Figure 1A**, and TIMP-1 concentration (r=0.31, n=108, p=0.002), after adjusting for maternal age, BMI and ethnicity, see **Figure 1B**. There was no statistical correlation of MMP-9 with gestational age at sampling (r=0.008, n=108, p=0.935).

MMP-9 positively correlated with IL-1β (r=0.61, n=91, p<0.001), IL-8 (r=0.57, n-91, p<0.001) and TNF-α (r=0.64, n=91, p<0.001), after adjusting for maternal age, BMI and ethnicity, see **Figure 2A**. Mean abundance of adverse anaerobic pathobionts also correlated positively with MMP-9 (p ≤ 0.005): *Gardnerella* (r=0.44, n=77, p<0.001), *Atopobium* (r=0.33, n=77, p=0.005), and *Prevotella* genera (r=0.39, n=77, p<0.001), see **Figure 2B**. Conversely mean proportion of *Lactobacillus* genera negatively correlated with MMP-9 (r= -0.46, n=77, p<0.001).

When partial correlation was controlled for cytokine IL-1β the associations between MMP-9 and key bacterial genera abundance were no longer significant (*Gardnerella:* r=0.10, p=0.433; *Atopobium*: r=0.15, p=0.225; *Prevotella*: r=-0.11, p=0.358 and *Lactobacillus*: r=-0.13, p=0.285). When partial correlation was controlled for IL-8 there was a lowering of the correlation efficient with *Gardnerella* (r=0.37, p=0.002) and *Lactobacillus* genera (r=-0.36, p<0.001) but the associations retained significance, the association with *Atopobium* (r=0.29, p=0.016) or *Prevotella* (r=0.23, p=0.056) were lost. When partial correlation was controlled for TNF-α there was a reduction of the correlation efficient with *Lactobacillus* (r=-0.26, p=0.034), *Gardnerella* (r=0.20, p=0.106), *Atopobium* (r=0.21, p=0.088) and *Prevotella* genera (r=0.23, p=0.057).

CVF TIMP-1 Positively Correlates With Pro-Inflammatory Cytokines and Vaginal Pathobiont Abundance in PWLWH

In PWLWH TIMP-1 positively correlated with IL-1β (r=0.59, n=91, p<0.001), IL-6 (r=0.31, n=91, p=0.004), IL-8 (r=0.30, n=91, p=0.005) and TNF-α (r=0.42, n=91, p<0.001) after controlling for maternal age, BMI and ethnicity, see **Figure 3A**. Mean abundance of adverse anaerobic pathobionts correlated positively with TIMP-1 (p<0.02): *Gardnerella* (r=0.51, n=77, p<0.001), *Atopobium* (r=0.56, n=77, p<0.001) and *Prevotella* (r=0.29, n=77, p=0.015), after controlling for

TABLE 2 | Mean MMP-9, TIMP-1 and MMP-9/TIMP-1 ratio in second and third trimesters by HIV status.

Group	2nd trim MMP-9 ng/mL	3rd trim MMP-9 ng/mL	2nd trim TIMP-1 ng/mL	3rd trim TIMP-1 ng/mL	2nd trim MMP-9/TIMP-1 ratio	3rd trim MMP-9/TIMP-1 ratio
PWLWH	2823 (1740-3906)	3289 (2074-4490)	230 (36-425)	135 (68-172)	114 (45-183)	437 (-172-1046
HUPW	767 (308-1225)	2241 (980-3502)	20 (2-37)	54 (6-102)	201 (-117-519)	190 (-49-429)
P value	<0.001	0.207	0.035	0.044	0.561	0.439

Second trimester analyses: PWLWH n=47, HUPW=10; Third trimester analyses: PWLWH n=33, HUPW= 9. Protein concentrations are given as geometric mean (95%CI).

TABLE 3 | Mean IL-1β, IL-8, IL-12 and TNF-α in second and third trimesters by HIV status.

Group	2nd trim IL-1β pg/mL	3rd trim IL-1β pg/mL	2nd trim IL-8 pg/mL	3rd trim IL-8 pg/mL	2nd trim IL-12 pg/mL	3rd trim IL-12 pg/mL	2nd trim TNF-α pg/mL	3rd trim TNF-α pg/mL
PWLWH	10161 (4202-16118)	15930 (6519-25341)	95513 (65149-125877)	89231 (60837-117625)	63 (43-83)	124 (82-165)	291 (175-406)	493 (205-781)
HUPW	458 (81-836)	846 (62-1630)	6065 (-82-12213)	6819 (523-13115)	7 (-7-21)	15 (-16-47)	19 (-19-57)	18 (-15-51)
P value	0.002	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	0.002

Second trimester analyses: PWLWH n=47, HUPW=10; Third trimester analyses: PWLWH n=33, HUPW= 9. Protein concentrations are given as geometric mean (95%CI).

maternal age, BMI and ethnicity, see **Figure 3B**. Conversely, mean proportion of *Lactobacillus* genera negatively correlated with TIMP-1 (r=-0.61, n=77, p<0.001). There was no statistical correlation of TIMP-1 with polymorphonuclear leucocytes (r=-0.23, n=19, p=0.400) or gestational age at sampling (r=-0.15, n=108, p=0.146).

When partial correlation between TIMP-1 and key bacterial genera abundance was controlled for cytokine IL-1β, there was a substantial reduction in associations (*Gardnerella:* r=0.23, p=0.058; *Atopobium*: r=0.46, p<0.001; *Prevotella*: r=-0.03, p=0.829 and *Lactobacillus*: r=-0.39, p=0.001). When partial correlation was controlled for IL-8 there was minimal change of the correlation efficient with *Gardnerella* (r=0.47, p<0.001), *Atopobium* (r=0.54, p<0.001) and *Lactobacillus* genera (r=-0.57, p<0.001) but the association with *Prevotella* was lost (r=0.20, p=0.109). When partial correlation was controlled for TNF-α there was a moderate reduction of the correlation efficient with *Gardnerella* (r=0.39, p=0.001), *Atopobium* (r=0.51, p<0.001), *Prevotella* genera (r=0.16, p=0.187) and *Lactobacillus* (r=-0.52, p<0.001).

When TIMP-1 was included in partial correlation between MMP-9 and bacterial abundance, the strength of the correlation co-efficients were lowered, some of which retained significance (*Gardnerella:* r=0.35, p=0.003; *Atopobium*: r=0.20, p=0.096; *Prevotella*: r=0.34, p=004 and *Lactobacillus*: r=-0.36, p=0.002).

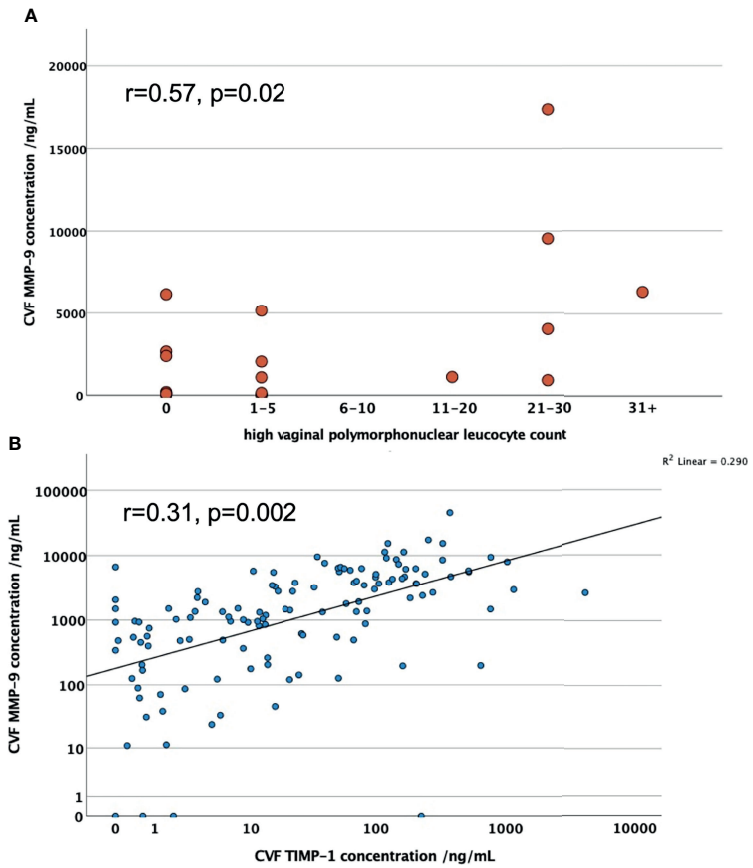

FIGURE 1 | **(A)** Scatter graphs to explore correlation of CVF MMP-9 concentrations with high vaginal polymorphonuclear leucocyte counts in PWLWH; **(B)** Scatter graph to explore correlation between CVF MMP-9 and TIMP-1 concentrations in PWLWH. r = correlation co-efficient, controlled for maternal age, BMI and ethnicity.

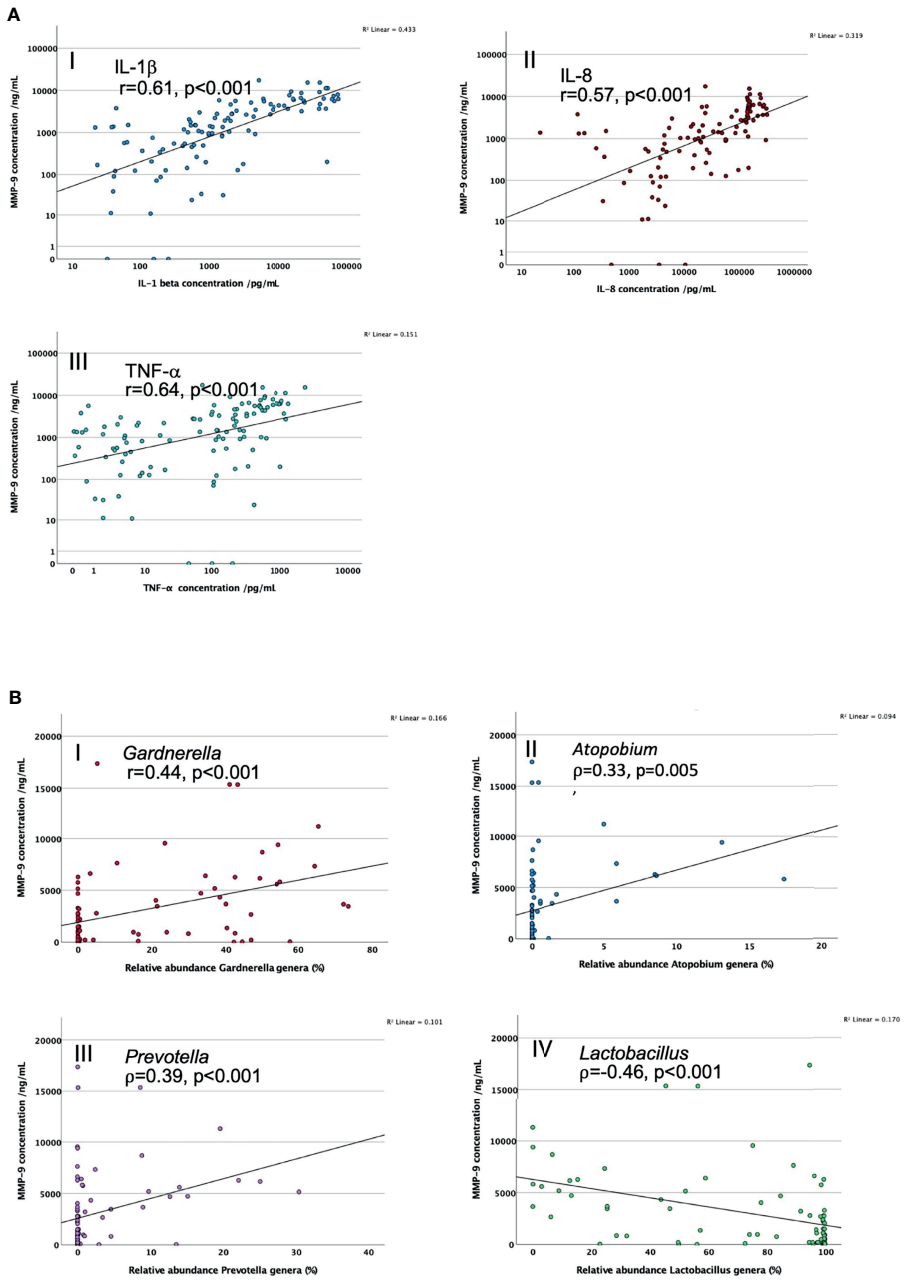

FIGURE 2 | **(A)** Scatter graphs to explore correlation of CVF MMP-9 concentrations with key CVF cytokine concentrations. I. IL-1β; II. IL-8; III. TNF-α. **(B)** Scatter graphs to explore correlation of CVF MMP-9 concentrations with proportions of key vaginal bacterial genera. I. *Gardnerella;* II. *Atopobium;* III. *Prevotella;* IV. *Lactobacillus.* r = correlation co-efficient, controlled for maternal age, BMI and ethnicity.

CVF MMP-9/TIMP-1 Ratio Positively Correlates With Abundance of Vaginal *Lactobacillus* Genera and Negatively Correlates With *Gardnerella* Genera and Pro-Inflammatory Cytokines in PWLWH

There was a negative association between MMP-9/TIMP-1 ratio and IL-1β (r=-0.22, n=91, p=0.044) and TNF-α (r=-0.20, n=91, p=0.076), after controlling for maternal age, BMI and ethnicity in PWLWH, see **Figure 4A**. There was also a trend towards a negative correlation between MMP-9/TIMP-1 ratio with relative abundance of *Gardnerella* genera (r=-0.28, p=0.02), and a positive association with *Lactobacillus* genera (r=0.31, p=0.01), see **Figure 4B**.

Spearman's correlation showed MMP-9/TIMP-1 ratio was positively associated with polymorphonuclear leucocyte count (rho=0.49, n=19 p=0.02) and gestational age at sampling (rho=0.27, n=108, p=0.004), see **Figure 5A, B**. The significance

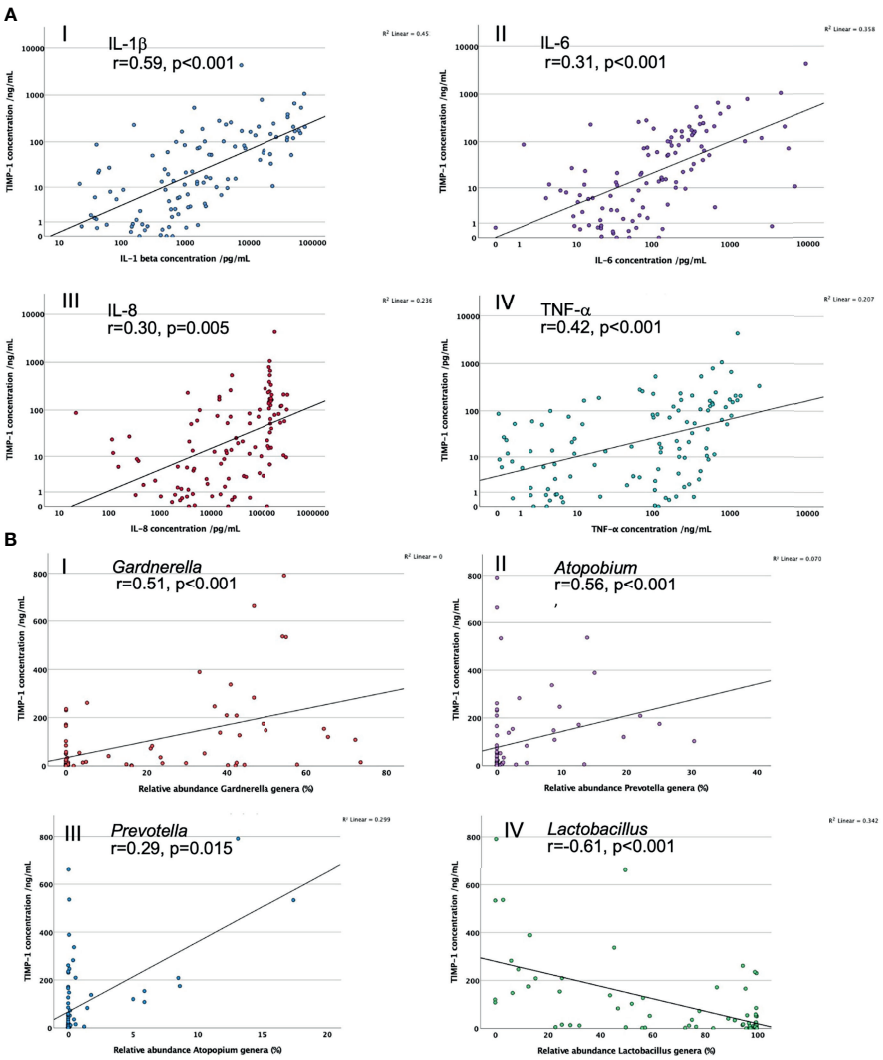

FIGURE 3 | **(A)** Scatter graphs to explore correlation of CVF MMP-9 concentrations with key CVF cytokine concentrations. I. IL-1β; II. IL-6; III. IL-8; IV.TNF-α. **(B)** Scatter graphs to explore correlation of CVF MMP-9 concentrations with proportions of key vaginal bacterial genera. I. *Gardnerella;* II. *Atopobium;* III. *Prevotella;* IV. *Lactobacillus.* r = correlation co-efficient, controlled for maternal age, BMI and ethnicity.

of these correlations were lost after controlling for maternal age, BMI and ethnicity: polymorphonuclear leucocyte count (r=0.36, n=19, p=0.184) and gestational age at sampling (r=0.005, n=108, p=0.603).

Modelling of the Relationship Between Vaginal Bacteria, Cytokines, TIMP-1 and MMP-9 in PWLWH

To explore the relationship between MMP-9, TIMP-1, bacterial genera and IL-1β, hierarchical multiple linear regression was performed. The first step of the model was adjusted for potential confounders: maternal age, ethnicity and BMI, with patient ID included as a random effect and MMP-9, TIMP-1, IL-1β, *Gardnerella* and *Lactobacillus* genera abundance inputted as a second factors. In step one maternal age, ethnicity and BMI explained 5% of the variance in CVF MMP-9 concentration. After entry of predictor variables the total variance of the model as a whole was 47%, F (8, 64) =7.02, p<0.001. In the final model, only two measures were statistically significant with IL-1β recording a higher beta value (b=0.68, p<0.001) than TIMP-1 (b=-0.29, p=0.039).

Based on the significant associations identified in the preceding analyses, four models of mediation between vaginal bacterial genera and MMP-9 with indirect effect of IL-1β mediation and TIMP-1 mediation were proposed, see **Figure 6** and **7**. There was a significant indirect effect of vaginal *Gardnerella* abundance on CVF MMP-9 concentration through IL-1β consistent with mediation, b=0.31 BCa CI [0.19-0.43], see **Figure 6**. There was a significant indirect effect of vaginal *Lactobacillus* abundance on CVF MMP-9 concentration

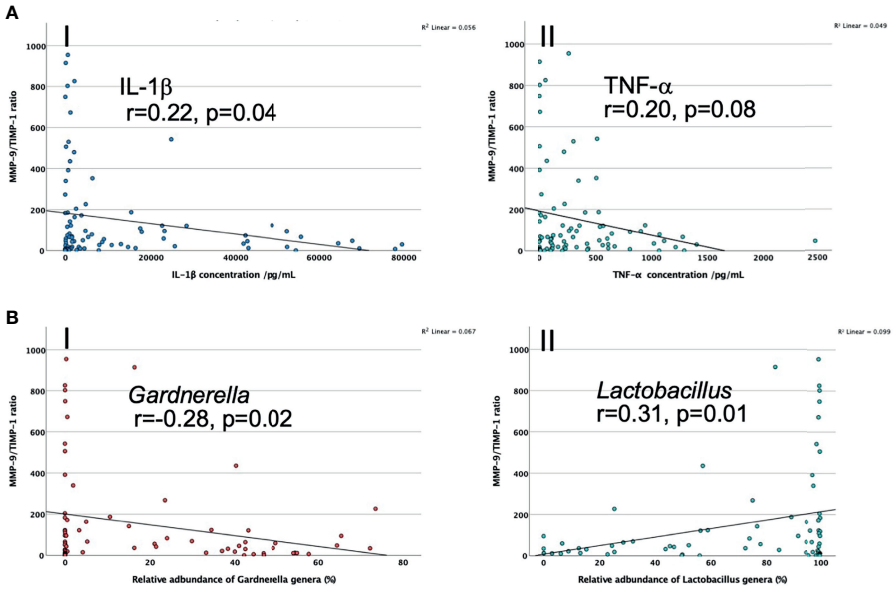

FIGURE 4 | **(A)** Scatter graphs to explore correlation of CVF MMP-9/TIMP-1 ratio with I. IL-1β; II.TNF-α in PWLWH; **(B)** Scatter graph to show the association between CVF MMP-9/TIMP-1 ratio and I. *Gardnerella* genera; II. *Lactobacillus* genera in PWLWH. r = correlation co-efficient, controlled for maternal age, BMI and ethnicity.

through IL-1β consistent with mediation, b=-0.35 BCa CI [-0.48--0.23]. When exploring mediation of TIMP-1, there was no significant indirect effect on the relationship between either *Gardnerella* or *Lactobacillus* on MMP-9 concentrations, see **Figure 7**.

DISCUSSION

The main findings of these exploratory analyses include that PWLWH have high CVF concentrations of MMP-9, TIMP-1, pro-inflammatory cytokines and MMP-9/TIMP-1 ratios in comparison to the HUPW participants. In PWLWH, MMP-9 and TIMP-1 were highly correlated and MMP-9/TIMP-1 ratio increased with gestational age at sampling. CVF MMP-9 and TIMP-1 concentrations strongly correlated with vaginal proportions of anaerobic genera: *Gardnerella; Atopobium* and *Prevotella* and key inflammatory cytokines: IL-1β, IL-8 and TNF-α. Higher vaginal abundance of *Lactobacillus* genera associated with lower MMP-9 concentrations. Both MMP-9 and MMP-9/TIMP-1 ratio positively correlated with vaginal polymorphonuclear leucocyte counts. Exploration of indirect effects between MMP-9, *Gardnerella* and *Lactobacillus* genera and IL-1β indicate full mediation suggesting the association between vaginal bacterial and this interstitial collagenase are likely to be driven by changes in expression of this key pro-inflammatory cytokine. TIMP-1 was not shown to mediate the relationship between bacteria and MMP-9 in these data. Within this sub-analysis of PWLWH and HUPW who donated CVF to the HIV PTB study, there was no difference in expression of MMP-9 or TIMP-1 by prematurity, but numbers were small and unlikely to be powered to show such a difference.

The concentration of MMP-9 observed in the lower FGT of PWLWH in both second and third trimesters was much higher than previously published values of expression in serum of HUPW (Sorokin et al., 2010; Tency et al., 2012; Chen and Khalil, 2017), amniotic fluid of labouring and non-labouring HUPW [(Maymon et al., 2000; Locksmith et al., 2001, (Maymon et al., 2000; Locksmith et al., 2001; Myntti et al., 2017)] and the cervical mucous from HUPW (Becher et al., 2010). Becher and colleagues explored the expression of MMP-9, TIMP-1 and IL-8 in the cervical mucous plug (CMP) in pregnancy and found that MMP-9 and IL-8 expression was greatest at the distal portion of the CMP closest to the vagina (Becher et al., 2010). They also demonstrated that MMP-9, IL-8 but not TIMP-1 concentrations increased from early to late pregnancy and were higher in women labouring preterm compared to term. MMP-9 and TIMP-1 concentrations in our analyses of HUPW were 200 and 2-fold higher in the second trimester than those observed in CMP by Becher and colleagues in early pregnancy, with a positive CVF MMP-9/TIMP-1 ratio compared to an inverse ratio observed in Becher's CMP samples. This is likely to be the result of an addition vaginal source of MMP-9 and differences in sample handling including the use of protease inhibitors. In addition, Becher and colleagues did not show a relationship between MMP-9 and vaginal bacteria but considered a limited number of pathobionts using less sensitive culture methods.

MMP-9 is known to be expressed by many cells at the maternal foetal interface including: neutrophils; macrophages; NK cells;

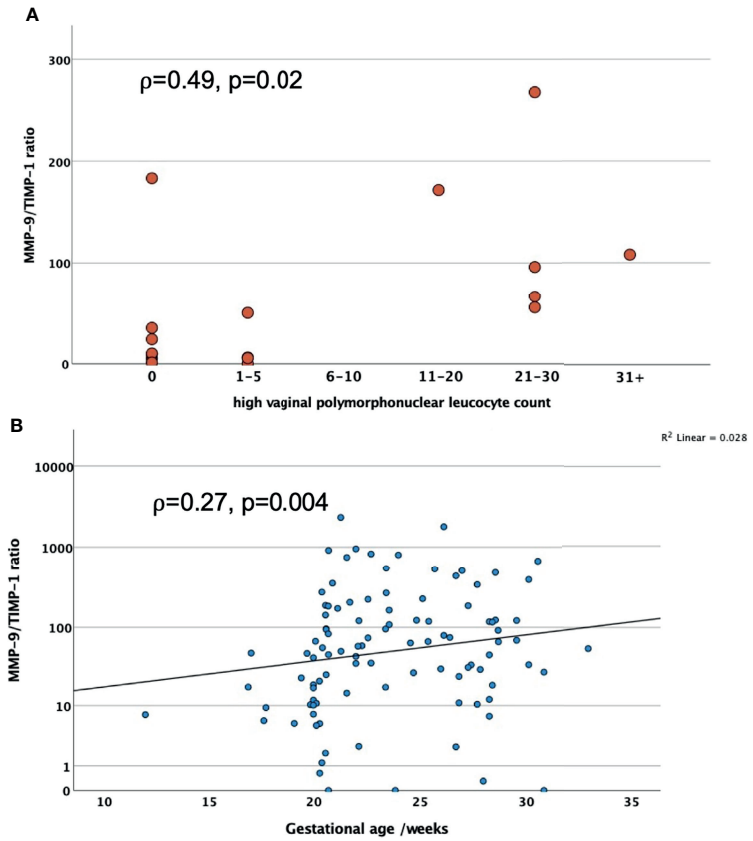

FIGURE 5 | **(A)** Scatter graphs to explore correlation of CVF MMP-9/TIMP-1 ratio with polymorphonuclear leucocyte counts in PWLWH; **(B)** Scatter graph to show the association between CVF MMP-9/TIMP-1 ratio and gestational age at sampling in PWLWH ρ = rho.

vascular endothelial cells of the decidua; cervical fibroblasts and epithelial cells (Lockwood et al., 2008; Naruse et al., 2009; Gonzalez et al., 2011; Ulrich et al., 2019). Gonzalez and colleagues demonstrated that in PTB macrophages are the main source of MMP-9 at the cervix, whereas MMP-9 production at term is non-leucocyte dependant (Gonzalez et al., 2011). We hypothesise that neutrophils and macrophages are the predominant source of MMP-9 in the lower FGT of PWLWH and MMP-9 expression in the cervical environment is elevated as a result of an inflammatory response to vaginal microbiota composition, supported by the observed associations with polymorphonuclear leucocytes. Further in-depth modelling of the relationship with local innate immune cells in this cohort was limited by the small numbers for which matched vaginal polymorphonuclear leucocyte counts were available.

MMP-9 concentrations in CVF increase with cervical ripening at term (Choi et al., 2009) and are proposed to enable cervical effacement and dilatation through remodelling of the ECM (Peltier, 2003; Challis et al., 2009). In addition to its ECM effects, MMP-9 has a role processing other molecules including inflammatory cytokines with a demonstrated role in term and preterm labour: IL-8 and IL-1β. The correlations observed in these data between key inflammatory cytokines and MMP-9 may be the result of MMP-9 cleavage of these chemokines and cytokines or as a result of upregulation by downstream signalling of the innate immune response to vaginal pathobionts or both. The reciprocal upregulation observed in TIMP-1 may be the result of positive feedback and homeostatic regulation. TIMP-1 upregulation was not directly proportional to that seen with MMP-9 and hence there was an increase in MMP-9/TIMP-1 ratio across gestation, which could result in a balance that favours collagen IV degradation in ECM.

These analyses are the first to demonstrate the association between vaginal anaerobes, inflammation, and ECM remodelling proteases in PWLWH and adds to the body of evidence for an ascending infection model of PTB with a vaginal source and associated inflammatory response at the cervix, lowering the threshold for early initiation of labour. It must be borne in mind that associations do not imply causality and HIV-associated PTB is likely multi-factorial, including a role of cART which may modulate some of these pathways. HIV Protease Inhibitors, the most implicated class of antiretrovirals, are purported to not affect extracellular proteases but could potentially affect inhibitory protein activity. No demonstrable difference in expression of MMP-9 and TIMP-1 was observed by class of third agent in this small sample which may be the result of type 2 error. cART may also

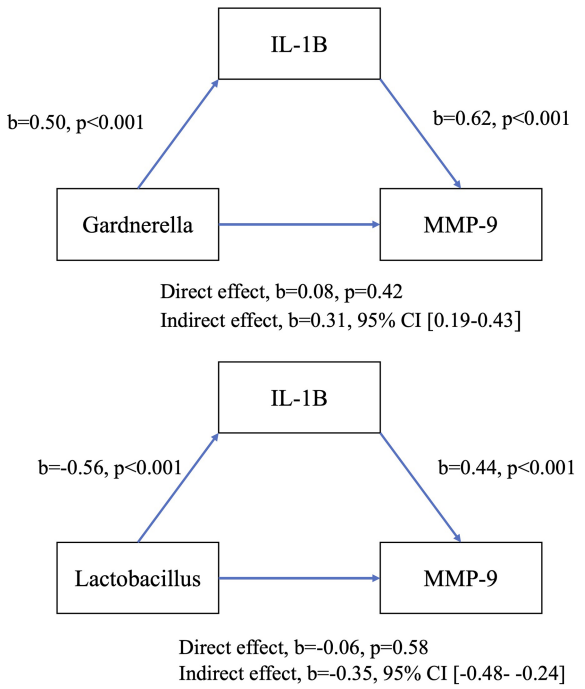

FIGURE 6 | Model of direct and indirect relationships between MMP-9, key bacterial genera and IL-1β.

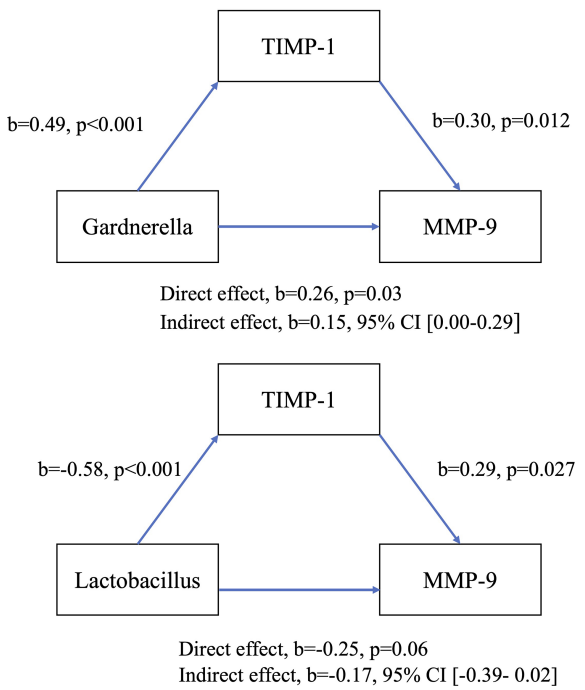

FIGURE 7 | Model of direct and indirect relationship between MMP-9, key bacterial genera and TIMP-1.

exert its effects directly on the vaginal microbiota (Ray et al., 2021). Genetic polymorphisms may also explain the elevated expression of MMP-9, 82% of whom were of Black race (Pereza et al., 2014; Pandey and Awasthi, 2020). The racial heterogeneity of this cohort may bias conclusions comparing by HIV status however analyses within PWLWH were adjusted for ethnicity and remain valid. The ethnicity of the PWLWH cohort reflect the women receiving antenatal care in the UK, many of whom are African and Caribbean migrants and may be generalisable to women in other settings, given the majority of PWLWH reside in Africa (Unaids.org, 2018). The findings of this study would ideally be replicated in a larger cohort, powered to look at differences by prematurity and include racially matched controls. The role of racial disparity in PTB risk is in itself an important question and is likely to be the result of many interplaying factors including: prejudice, genetics, socioeconomics, healthcare access and stress influencing downstream factors such as background co-morbidities, neuroendocrine, infection, microbiota, and immune mediators (Braveman et al., 2021).

Altered angiogenesis at implantation and placentation has been postulated as a mechanism underlying HIV associated PTB and low birth weight (Conroy et al., 2017). MMP-9 is a key regulator of this process in normal pregnancy and dysregulated expression has been implicated in both pre-eclampsia and IUGR (Juanjuan Merchant et al., 2004; Świerczewski et al., 2012; Chen and Khalil, 2017; Ardiani et al., 2019), the former of which was rarely seen in PWLWH in the pre cART era (Wimalasundera et al., 2002). More recently a case control study in Cameroon compared MMP-9 expression in plasma collected from the placental intervillous space by HIV status and found no difference in expression between groups suggesting any differences in MMP-9 in PWLWH are unlikely to be placental in origin (Esemu et al., 2019).

Within these sub-analyses we did not show any difference in gestational age at delivery or expression of CVF MMP-9 and TIMP-1 by prematurity. The small sample size reduces power to observe these differences in PTB rates, which were seen in the main HIV PTB study cohort (Short, 2019; Short et al., 2021). There was however a moderate association between MMP-9/TIMP-1 ratio with gestational age that warrants further exploration and validation in a larger group, ideally with matched controls and exploring other network proteins such as EMMPRIN and Lipocalin-2, the transcriptome and presence of local immune cells.

In conclusion, we have shown vaginal anaerobes and key pro-inflammatory cytokines positively correlate with ECM modifying protease MMP-9 and its inhibitory protein TIMP-1 in PWLWH, the ratio of which increase in pregnancy and may result in cervical remodelling as one mechanism underlying HIV-associated PTB.

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.ebi.ac.uk/ena, PRJEB41429; ena-STUDY-CUMICRO-18-11-2020-15:38:11:823-609.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NHS Health Research Authority National Research Ethics Service (NRES) (Ref 13/LO/0107). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-ES, PB, GT, and DM conceived and designed the study. Patient recruitment and sample collection were undertaken by C-ES and RQ. Experiments and data collection were performed by C-ES, RQ, XW, VP and YL. Data processing, analyses, and interpretation were performed by C-ES, AS, PB, GT, and DM. All figures and tables were generated by C-ES. C-ES wrote the first draft of the manuscript and all authors contributed critical revisions to the paper, interpretation of the results and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.750103/full#supplementary-material

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REVIEW
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The Microbiome as a Key Regulator of Female Genital Tract Barrier Function

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The microbiome, the collection of microbial species at a site or compartment, has been an underappreciated realm of human health up until the last decade. Mounting evidence suggests the microbiome has a critical role in regulating the female genital tract (FGT) mucosa's function as a barrier against sexually transmitted infections (STIs) and pathogens. In this review, we provide the most recent experimental systems and studies for analyzing the interplay between the microbiome and host cells and soluble factors with an influence on barrier function. Key components, such as microbial diversity, soluble factors secreted by host and microbe, as well as host immune system, all contribute to both the physical and immunologic aspects of the FGT mucosal barrier. Current gaps in what is known about the effects of the microbiome on FGT mucosal barrier function are compared and contrasted with the literature of the gut and respiratory mucosa. This review article presents evidence supporting that the vaginal microbiome, directly and indirectly, contributes to how well the FGT protects against infection.

Keywords: microbial factors, host factors, microbiome, barrier, vagina, female genital tract (FGT), tissue explant, sexually transmitted infection (STI)

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THE FEMALE GENITAL TRACT: ANATOMY AND MICROBIOME

The female genital tract (FGT) can be divided into two distinct regions: lower and upper. The lower part of the FGT is composed of the vaginal canal and ectocervix, while the upper region is defined by the endocervix and uterus proper (Carias and Hope, 2018). The epithelium of the vaginal canal is organized in a stratified squamous configuration up until and including the ectocervix, which enables a barrier with multiple layers of epithelial cells to protect against extracellular pathogens (**Figure 1**). The stratified squamous epithelium originates from a basement membrane lined with progenitor cells, which then mature into fully senescent, and then keratinized epithelium (Chung et al., 2019; Ali et al., 2020). This allows for multiple levels of tight junction formation that keep out pathogens, while allowing the layer closest to the lumen to still be permissive to transudate from the blood and slough off to allow for mucosal shedding. This stratified squamous layer transitions to a simple columnar epithelial pattern at a point known as the 'transformation zone', which defines the area where the ectocervix becomes the endocervix. The simple columnar epithelial barrier continues into the uterine endometrium and beyond, and, as a result, the upper FGT has historically been thought to be more vulnerable to pathogens (Shattock and Moore, 2003). This is relevant in cases of

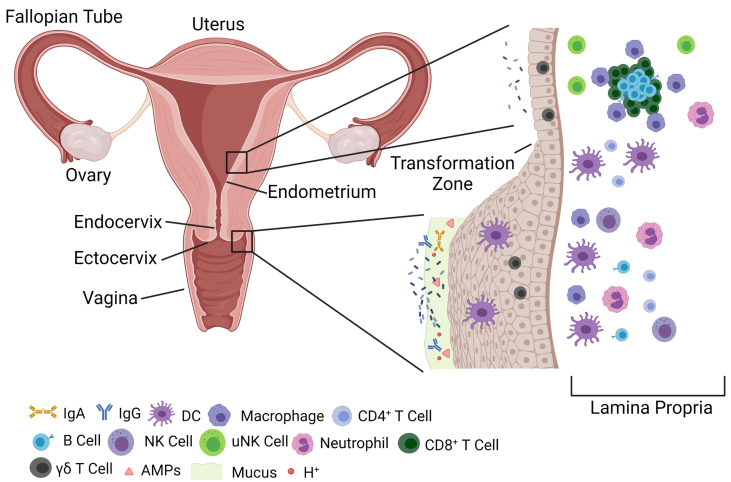

FIGURE 1 | Overview of FGT anatomy and components of barrier function. The FGT is divided generally into an upper (endometrium and endocervix) and a lower (vagina and ectocervix) tract. The lower FGT is considered the primary point of contact for pathogens, and harbors multiple innate immune mechanisms, such as AMPs, mucus, and immunoglobulins that prevent transmission across the epithelial barrier. The epithelium itself is composed of a stratified squamous epithelium that arises from a basement membrane and terminates in fully keratinized, senescent, cells. Microbiota, primarily *Lactobacillus*, can be found in high abundance within the lower FGT, but have also been shown to be resident within the upper FGT in lower numbers. As the epithelium progresses to the upper FGT it changes to a single columnar epithelium at a junction known as the 'transformation zone' between the ectocervix and endocervix. The upper FGT harbors unique uNK populations, as well as lymphoid aggregates consisting of B cells surrounded by $CD8^+$ T cells and macrophage. These immune gatekeepers balance immune surveillance and response with the need to maintain a fertile environment for pregnancy. FGT, Female genital tract; IgA, Immunoglobulin A; IgG, Immunoglobulin G; DC, Dendritic cell; NK, Natural killer; uNK, Uterine natural killer; AMP, Antimicrobial peptide.

cervical ectopy, more common among women who are young, pregnant, or on oral contraceptives, where the endocervix protrudes into the vaginal canal (Loudon et al., 1978; Wright et al., 2014). This protruding endocervix increases the vulnerability of the upper FGT to infection during sexual intercourse, and has been associated with the acquisition of sexually transmitted infections (STIs), such as in the case of chlamydia or human immunodeficiency virus (HIV) (Lee et al., 2006; Kleppa et al., 2015). In addition to the physical barrier of the epithelium, there is a layer of cervicovaginal mucus composed of mucus secreted by goblet cells of the endocervix that combines with cellular debris, vaginal transudate, and immune components to create a matrix that traps potential pathogens and prevents them from reaching or interacting with the epithelial layer and potential target cells (Lacroix et al., 2020). These immune components include secreted proteins such as immunoglobulins, which can bind to pathogens and interact with the cervicovaginal matrix to allow clearance from the FGT by mucus flow (Jensen et al., 2019), antimicrobial peptides (AMPs), and proteases. Despite being a mucosal membrane, where IgA antibodies tend to predominate, the vaginal mucosa is primarily characterized by IgG antibodies that are thought to originate from the vaginal transudate produced from the plasma that crosses from the bloodstream into the vaginal canal (Fahrbach et al., 2013; Oh et al., 2019). In addition to physical methods, there are chemical methods of pathogen protection that rely on the maintenance of an acidic environment within the lower FGT. This low pH environment feeds into, and in turn is fed by, a *Lactobacillus* dominant microbiome (Linhares et al., 2011).

The microbiome of the FGT differs between the lower and upper portions of the tract, with the lower FGT estimated to have a bacterial load of 10^2-10^4 fold higher than that seen in the upper FGT (Chen et al., 2017; Baker et al., 2018). Microbiomes of the lower FGT, henceforth referred to as vaginal microbiomes, are divided into five separate community state types (CSTs) that capture the overall dominance of a particular bacterium within the vagina (Ravel et al., 2011; Ma and Li, 2017). While bacteria of each state are all considered commensal, or regular residents in healthy individuals, microbiomes that are non-*Lactobacillus* dominant tend to be associated with poor health outcomes and high degrees of inflammation (Gautam et al., 2015; Petrova et al., 2015; Ma and Li, 2017; De Seta et al., 2019). The vaginal mucosal microbiome is typically less diverse than other sites, with *Lactobacillus* species comprising the major microbiota of the vaginal mucosa (Ravel et al., 2011; Aldunate et al., 2015; Petrova et al., 2015; Song et al., 2020). Among the *Lactobacillus* species, *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* are the most common species identified at the human vaginal mucosa. A large-scale cross-sectional study showed that *L. crispatus, L. gasseri, L. iners, and L. jensenii* were dominant in 26.2%, 6.3%, 34.1% and 5.3% of the samples analyzed, respectively (Ma et al., 2012). The remaining 28% of samples were dominated with non-*Lactobacillus* species and displayed high diversity in microbial composition (Ma et al., 2012). Women are grouped into the five CSTs based primarily on the predominance of a given *Lactobacillus* species in the vaginal mucosa. CST-I, -II, -III, and -V are characterized by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* dominance, respectively, whereas CST-IV represents a highly diverse, or non-*Lactobacillus* dominant, microbiome (Ravel et al., 2011). CST-I, -II, and -V are

considered optimal vaginal microbiomes, characterized by low pH (pH less than 4.0–5.0), high lactic acid concentrations, and a less inflammatory state of the vaginal microenvironment (Hedges et al., 2006; Ravel et al., 2011). CST-III women who are dominated by *L. iners* differ from other *Lactobacillus* dominated CST groups in that they display higher pH ranges and relatively higher inflammatory statuses at the vaginal mucosa (Verstraelen et al., 2009). Ongoing studies found differences in the genome size and genome structure of *L. iners* compared to other *Lactobacillus* species, however additional studies are required to explain how these differences contribute to the differences in vaginal milieu (Mendes-Soares et al., 2014). CST-IV is dominated by a varied range of facultative or strictly anaerobic bacteria in the vaginal mucosa, and is considered a non-optimal vaginal microbiome. It is characterized by higher pH levels (pH >5.0), lower levels of lactic acid, and an increased inflammatory state (Hedges et al., 2006; Ravel et al., 2011; Vagios and Mitchell, 2021). Despite the differences displayed in CST-IV, when present without any symptoms it is considered a healthy state. Bacterial genera that dominate in the CST-IV group, however, such as *Atopobium*, *Gardenella*, *Mobiluncus*, *Prevotella*, *Anaerococcus*, and *Sneathia*, are often associated with a symptomatic disorder of the vaginal microbiome known as bacterial vaginosis (BV) (Schellenberg et al., 2011; Ma et al., 2012).

The microbiome is regulated through (1) the microbial species that exert their own influence on species pressure, (2) the introduction of new species through foreign introduction, and (3) host factors such as genetics, diet, and hormones that promote the growth of certain bacterial genera. There have been a great deal of association studies performed analyzing the link between the vaginal microbiome and FGT barrier function and immunity, but studies of regulatory mechanisms have been lacking. In order to examine these relationships in depth, several models have been generated in which the conditions of both the lower and upper FGT are simulated and microbes of interest can be included to characterize their role in affecting immune response, barrier permeability, and the secretion of metabolites. These models present the best opportunity to move many of the discovered relationships past association and into causal explanation.

STUDY MODELS OF FGT BARRIER FUNCTION

Most experimental models of the FGT mucosa focus primarily on the lower FGT, composed of the ectocervix and vagina, with a smaller number focusing on the upper FGT, composed of the endocervix and endometrium. Initial studies utilized monolayer cultures of cervical and vaginal epithelial cells to study barrier function, but more recently three-dimensional (3D) models using cervical and vaginal epithelial cells have been developed. Cervical and vaginal explant tissue models, and *in vivo* systems, have also been used to study the regulation of cervicovaginal barrier function. A brief description of these models, and their use in studying FGT mucosal barrier function, will be discussed in the following sections (**Figure 2** and **Table 1**).

In Vitro Models

Immortalized vaginal (Vk2), ectocervical (Ect1), and endocervical (End1) epithelial cell lines have been generated to study FGT epithelial function by transducing the primary cells isolated from cervicovaginal biopsies with human papillomavirus 16 (HPV-16) E6 and E7 genes (Fichorova et al., 1997). These epithelial cell lines have been used in generating both 2D (monolayer) and 3D models (**Figure 2**). These epithelial cells are grown on tissue culture-treated plasticware in monolayer models to study the effects of exogenous factors on epithelial function and properties. Vk2, Ect1, and End1 cells have been reported to express certain toll like receptors (TLRs) in 2D

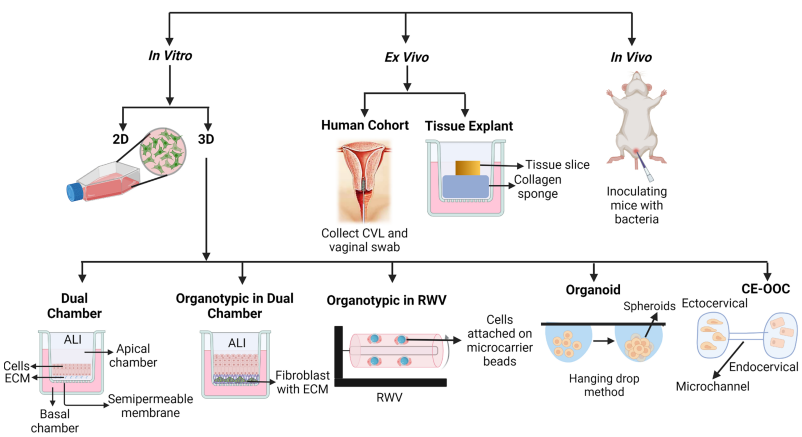

FIGURE 2 | Different types of models to study cervicovaginal mucosal barrier function. 2D, Two dimensional; 3D, Three dimensional; ECM, Extracellular matrix; ALI, Air-liquid interface; CVL, Cervicovaginal liquid; RWV, Rotating well vessel; CE-OOC, Organ-on-chip cervical epithelial.

TABLE 1 | Comparison between different models used to study cervicovaginal mucosal barrier function.

Models	Brief description	Cells used	Applications	Advantages	Limitations	Reference
In Vitro	Monolayer (2D): Immortal cell lines are grown on tissue culture-treated plasticware	Vaginal (Vk2), ectocervical (Ect1), and endocervical (End1)	Wound healing, changes in the expression of transcripts, and secreted and intracellular proteins with various stimulation conditions	Fast, easy, and cost-effective	Cells are not differentiated, less (or no) cell to cell junction formation, and some proteins/ molecules found in physiological tissues are not expressed when cells are grown in 2D	(Fichorova et al., 1997; Dusio et al., 2011)
	Dual-Chamber (3D): Cells are grown on a semipermeable membrane coated with ECM. Cells are grown using an ALI condition. Both immortal and primary cells are used in this model	Vk2 and primary genital epithelial cells (GECs)	Cell permeability, regulation of cell to cell junction formation and cell differentiation, and transmigration of pathogens or immune cells through the epithelial layer	Similar to physiological tissue in terms of multilayer formation, differentiation, and cell to cell junction formation of epithelial cells	High technical variability, large-scale experimental requirements, highly expensive, gene expression profiles are different between transformed and primary cells, mucus secretion is not reported, and it is not possible to study a mucosal anaerobic environment	(Gali et al., 2010; Kaushic et al., 2011; Lee et al., 2016)
	Organotypic model (3D) in RWV: Immortal epithelial cell lines are attached to collagen-coated microcarrier beads. Bead attached cells are transferred to the RWV bioreactor and cultured for 39-42 days	Vaginal (V191), endocervical (A2EN), and endometrial (HEC-1A)	Colonization effects of *Lactobacillus* species and BV-associated bacteria such as *Atopobium*, *Prevotella*, and *Gardenella* on the barrier function of epithelial cells	Fully differentiated epithelial cells with tight junctions, microvilli, and mucus secretion	High technical variability, large-scale experimental requirements, highly expensive, gene expression profiles are different between transformed and primary cells	(Laniewski et al., 2017; Łaniewski and Herbst-Kralovetz, 2019)
	Organotypic model (3D) in Dual Chamber: Primary epithelial cells are grown on a semipermeable membrane coated with an ECM and fibroblast cell mixture using a dual-chamber system. Cells are cultured in ALI conditions with nutrient supplements and growth factors. The model contains an apical cell layer, suprabasal layer, and basal cell layer after differentiation	Primary ectocervical cells isolated from vaginal-ectocervical (VEC) tissue	Effect of douching, feminine products, and anti-fungal creams on cervicovaginal epithelial cells	Express cytokeratins similarly to cervical tissue	High technical variability, large-scale experiment requirements, highly expensive, time-consuming, requires ethics approval for collecting primary tissue, mucus secretion is not reported, and it is not possible to study a mucosal anaerobic environment	(Aldunate et al., 2015)
	Stratified ectocervical organoid and cystic endocervical organoid models (3D): Cells are embedded into a basement membrane extract and plated in 30 µL droplets on pre-warmed 24-well suspension culture plates, then grown in culture media with growth supplements to develop spherical organoids	Primary ectocervical and endocervical cells from hysterectomy tissue	Study morphological, transcriptomic, and phenotypic differences of ectocervical and endocervical tissue, and infection studies with pathogenic microbes such as HSV2 and HPV	Possible to expand for longer passage numbers, and cryopreserved samples can be successfully used from thawed organoids		(Lõhmussaar et al., 2021)
	Organ-on-chip cervical epithelial (CE-OOC): Ectocervical and endocervical cells are grown in two different chambers which are connected with microchannels	Immortalized ectocervical and endocervical epithelial cells	Study EMT and MET processes of ectocervical and endocervical cells, and modulation of both ectocervical and endocervical cell function concomitantly in the same model in the presence and absence of infection and inflammation	Possible to study the nature and interaction of ectocervical and endocervical cells	Differentiation and tight junction formation of both ectocervical and endocervical cells, mucus secretion, and multilayer formation of ectocervical cells, are not reported, gene expression profiles are different in transformed and primary cells and it is not possible to study a mucosal anaerobic environment	(Tantengco et al., 2021)
Ex Vivo	Explant tissue: Cervical and vaginal tissue collected from human participants and cultured in ALI conditions in the dual-chamber system	Cervical or vaginal biopsy	Thickness of the epithelial layer, tissue permeability, tight junctions, cell proliferation, pathogen migration, regulation of protein and transcript expression, and the interaction between	The closest model to an *in vivo* system, since all cells in the explant model have physiological features	Highly expensive, the requirement for ethics approval, laborious, time-consuming, high biological variability, and it is not possible to study a mucosal anaerobic environment	(Phalguni et al., 2006; Mitchell et al., 2014; Goldfien et al., 2015)

(Continued)

TABLE 1 | Continued

Models	Brief description	Cells used	Applications	Advantages	Limitations	Reference
			epithelial and fibroblast cells			
	Human cohort: Establish a cross-sectional longitudinal cohort to collect CVL, epithelial cells, and/or immune cells	N/A	Study secreted proteins, secreted metabolites, mucus properties in CVL, and cellular proteins from epithelial cells	Ability to look at the impact of microbial diversity, demographic characteristics, individual behavior, etc., in affecting metabolites, mucus properties, and protein secretion and expression in relation to FGT barrier function	Highly expensive, the requirement for ethics approval, laborious, time-consuming, and challenges studying mechanism with a general restriction to observational findings	(Borgdorff et al., 2016)
In Vivo	Inoculating mouse models with commensal and BV associated bacteria present in the human FGT mucosa	N/A	Study secreted proteins, secreted metabolites, mucus properties in CVL, and cellular proteins from epithelial cells	Possible to study mucosal degradation of BV associated bacteria	Translation of findings to humans, expensive, requirement for ethics approval, laborious, and time-consuming	(Lewis et al., 2013)

ECM, Extracellular matrix; ALI, Air-liquid interface; GECs, Primary genital epithelial cells; RWV, Rotating well vessel; EMT, Epithelial-mesenchymal transition; MET, Mesenchymal-epithelial transition; CVL, Cervicovaginal lavage; FGT, Female genital tract; BV, Bacterial vaginosis; N/A, Not Applicable.

culture and have been exposed to TLR agonists to induce the production of cytokines, chemokines, and antimicrobial peptides (AMPs) (Fichorova et al., 1997; Dusio et al., 2011). Study of epithelial wound-healing properties has primarily been performed using 2D culture models (Dusio et al., 2011). The FGT mucosal metabolite low-molecular-weight hyaluronic acid (LMW-HA) has been shown to regulate barrier function using 2D culture of Vk2 cells. Treatment of Vk2 monolayers with LMW-HA induces secretion of AMPs, such as β-defensin-2, and promotes the wound-healing process by increasing migration of Vk2 cells through activation of phosphatidylinositol 3-kinase and myosin light chain kinase (Dusio et al., 2011). The 2D epithelial monolayer model is often used because of its simplicity, short-time frame, cost-effectiveness, and reproducibility of the results. The 2D monolayer model of cervicovaginal epithelium, however, differs vastly from physiological cervicovaginal tissue in terms of cellular differentiation, proliferation, protein expression, multilayer formation, and cell to cell junction formation between stratified squamous epithelial cells, as well as in the interaction between epithelial cells and the extracellular matrix (ECM) which is completely absent in the 2D model (Soares et al., 2012; Chitcholtan et al., 2013) (**Table 1**). Hence, 3D models of the cervicovaginal epithelium have been developed to overcome some of these limitations.

In 3D models, epithelial cells are grown on ECM such as collagen, matrigel, or fibronectin. When a dual-chamber culture system is used in a 3D model, transwell inserts with semipermeable membranes coated with ECM material are used (Gali et al., 2010; Lee et al., 2016; Woods et al., 2021). Primary or immortalized epithelial cells are grown on the supporting ECM in the upper chamber, and the lower chamber can be used for culturing any tissue resident cells, such as immune cells. Using this culture system, epithelial cells can be induced to differentiate and polarize in air-liquid interface (ALI) conditions (Lee et al., 2016). In ALI conditions, liquid culture media in the upper chamber (the apical side of the epithelium) is removed after the epithelial monolayer on the ECM becomes confluent (**Figure 2**), allowing the nutrients to be supplied by the culture media in the bottom chamber (the basal side of the epithelium). ALI conditions, simulating a mucosal environment where nutrients are supplied by blood and epithelium is exposed to air, trigger cellular differentiation and formation of cell-cell junctions (Lee et al., 2016). This model is suitable for studying epithelial integrity, regulation of tight junction and adhesion junction formation, and transmigration of pathogens through the epithelium. Trans-epithelial electrical resistance (TEER) and fluorophore-conjugated dextran diffusion assay are commonly used to assess epithelial integrity in the dual-chamber model systems (Gali et al., 2010). Vk2 and Ect1 cells in particular have been grown in dual-chamber 3D culture model systems to study epithelial immune and barrier function (Gali et al., 2010; Lee et al., 2016) (**Table 1**). The ALI model of Vk2 cells exhibits both multilayer and tight junction formation, and has been used to show that stimulation of Vk2 cells with the hormone progesterone increases their susceptibility to herpes simplex virus 2 (HSV2) infection (Lee et al., 2016). Primary genital epithelial cells (GECs), particularly endocervical and endometrial cells, have been isolated from hysterectomy tissue, cultured in the dual-chamber system, and shown to form tight junctions as well (Kaushic et al., 2011; Ferreira et al., 2013). Following exposure to HIV-1, increased GEC permeability was observed in this model system. Co-culture of HIV-exposed GECs with the probiotic bacteria *Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1, as well as the female sex hormone estrogen, was shown to restore GEC barrier function and tight junction formation (Dizzell et al., 2019). The major limitation of this 3D model includes high technical variability, variations between cell lines and primary cells, and, most of all, its deviation from physiological conditions, for example mucus-secretion and an anaerobic environment (Gali et al., 2010; Lee

et al., 2016). As a consequence, this is not an ideal system to study interactions between the microbiome and epithelium.

The organotypic vaginal-ectocervical (VEC) tissue model also uses a dual-chamber system. In the VEC tissue model, primary ectocervical cells are isolated from the VEC tissue obtained from hysterectomy and cultured on transwell inserts using ALI conditions to induce differentiation and polarization (Aldunate et al., 2015) (**Figure 2**). Before culturing the ectocervical epithelial cells on the transwell inserts, the inserts are seeded with a fibroblast and collagen mixture to create a basal membrane. Fibroblast cells are isolated from the same donor used to obtain ectocervical cells to avoid allogeneic immune effects. Ectocervical cells are then cultured on top of the fibroblast and collagen gel matrix, which creates a VEC tissue model containing an apical cell layer, suprabasal layer, and basal cell layer post-differentiation, along with a similar type of cytokeratin expression compared to actual VEC tissue (Ayehunie et al., 2006). This model shows more resistance against washings, lubricants, and anti-fungal creams compared to non-organotypic epithelial models, and represents a more relevant physiological scenario (Aldunate et al., 2015) (**Table 1**). Although the 3D and VEC tissue dual-chamber models are advantageous for cell differentiation, tight junction formation, and cytokeratin expression, they still lack many aspects of the FGT mucosa. For example, studying the barrier properties of mucus at the FGT mucosa is not possible with these models, since mucus secretion has yet to be reported using VEC tissue models.

Another 3D epithelial tissue model using a rotating wall vessel (RWV) bioreactor and immortal vaginal (V191), endocervical (A2EN), and endometrial (HEC-1A) cell lines has been developed by Dr. M. Herbst-Kralovetz's group. The epithelial tissue in this model has been shown to secrete mucus, and exhibit proper cytokeratin expression, microvilli, and tight junction formation (**Figure 2** and **Table 1**) (Hjelm et al., 2010; Radtke et al., 2012; Doerflinger et al., 2014; Laniewski et al., 2017; Łaniewski and Herbst-Kralovetz, 2019; Ilhan et al., 2020). To start, epithelial cells are grown on collagen-coated dextran microcarrier beads. The cell and bead suspensions are then transferred to the RWV bioreactor to be cultured for 39-42 days to generate fully differentiated 3D tissue models (**Figure 2**). When used in studying the interaction between epithelium and microbes, colonization of this vaginal RWV-3D tissue model with the anaerobic bacterium *Atopobium vaginae* resulted in increased secretion of cell-associated mucins, proinflammatory cytokines and chemokines, and AMPs as compared to colonization with *Lactobacillus* species (Doerflinger et al., 2014). Although the RWV-3D tissue model allows the evaluation of the role of mucus and anaerobic microbes in regulating epithelial barrier function, the challenges of reproducibility and the differences in gene expression between cell lines and primary tissue have not been resolved.

The organoid models, such as the stratified ectocervical organoid and cystic endocervical organoid models, use primary ectocervical and endocervical cells isolated from cervical tissue obtained during hysterectomy procedures (**Table 1**) (Lõhmussaar et al., 2021). Primary cells are embedded into a basement membrane extract and plated in 30 μL droplets on pre-warmed 24-well suspension culture plates, then grown in culture media with growth supplements to develop spherical organoids (**Figure 2**) (Lõhmussaar et al., 2021). Both organoid models have been used to study cellular susceptibility to HSV-2 infection, but have yet to be used to study epithelial barrier function (Lõhmussaar et al., 2021).

The most current *in vitro* cervical tissue model is the organ-on-chip cervical epithelial (CE-OOC) model. The CE-OOC model contains two chambers to co-culture immortalized ectocervical and endocervical epithelial cells, and the two chambers are connected by micro-channels that allow the epithelial cells to migrate (**Figure 2**) (Tantengco et al., 2021). The phenomena of epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET) have been studied using the CE-OOC model (**Table 1**). In that, ectocervical cells have been reported to form fibroblastoid morphology, and endocervical cells form pseudopods while migrating through micro-channels from one chamber to the other, in monoculture systems (Tantengco et al., 2021). This model has been used to study normal and pathogenic cellular remodeling of the cervix, and the interaction between ectocervical and endocervical cells during infection and inflammation (Tantengco et al., 2021). Although *in vitro* 3D models of epithelium provide a close resemblance to physiological tissue, they still present challenges, such as high technical variability, large-scale experimental requirements, high costs, and being time intensive. Moreover, these 3D models cannot be used to study epithelial barrier properties in the presence of non-epithelial cells such as stromal and sub-epithelial immune cells (**Table 1**).

Ex Vivo Models

Cervical tissue explants, either collected from cervical tissue after hysterectomy or cervical punch biopsies, have been previously used for HIV transmission studies (Phalguni et al., 2006; Grivel and Margolis, 2009; Mitchell et al., 2014; Trifonova et al., 2018). Ectocervical, endocervical, and transformation zone physiology in particular have been studied using this model (**Table 1**) (Goldfien et al., 2015). The cervical explant tissue model is the closest model to an *in vivo* model, since the explant tissue is composed of epithelial cells, subepithelial immune cells, and fibroblasts. This model allows for studying the interaction between epithelial, immune, and fibroblast cells during infection as well. Fresh and cryopreserved cervical tissue explants provide similar results in infection studies, indicating the ability to use frozen tissue explants as an alternative to fresh tissue explants. This allows researchers to circumvent one of the major potential drawbacks of using cervical tissue explants—sample preservation (Phalguni et al., 2006; Fox et al., 2017). The cervical and endometrial tissue explant model has been used to observe the effect of hormonal contraceptives in changing barrier properties and infection susceptibility as well. Explant tissue models are used to study hormonal contraceptive effects on epithelial layer thickness, cell proliferation, alteration of tight junction and secretory proteins, and gene transcription

(**Table 1**). The explant tissue model is not without challenges. It still lacks an anaerobic environment, and can be highly variable due to biological differences between tissue donors.

Besides isolating explant tissue, cervicovaginal lavage (CVL) and vaginal swabs from women have also provided crucial insights in uncovering the regulation of cervicovaginal barrier function (**Figure 2**). Proteomic studies with CVL using high throughput mass spectrophotometry have reported that an increase in vaginal microbiome diversity is associated with a decrease in a protein expression profile that supports barrier function (Borgdorff et al., 2016). The limitations that come with this model are challenges achieving the proper ethics and appropriate number of clinical participants, along with longer time frames and higher expense (**Table 1**).

In Vivo Models

Although not perfect, the use of animal models allows the investigation of how vaginal bacterial composition, and other local factors, regulate cervicovaginal barrier function in a complete living system (Lewis et al., 2013). Various types of tissue, such as upper and lower FGT, and fluids, such as CVL and plasma, can be isolated from animal models for systematic analyses to obtain a more comprehensive, and unbiased, picture (**Figure 2** and **Table 1**). For example, a study using C57BL/6 mice (6–8 weeks old) colonized with the mouse-adapted strain of *G. vaginalis* showed degradation of vaginal mucus and consequent weakening of vaginal barrier properties compared to control mice (Lewis et al., 2013). To study microbes that infect only human cells, bone marrow-liver-thymus (BLT)-transplanted humanized mouse models have been used. The BLT mouse has been used to test microbicides against HIV-1 and study changes in cervicovaginal barrier properties during HIV transmission (Paul et al., 2011; Maud and Andrew, 2013; Nirk et al., 2018; Shariq et al., 2019). These BLT mouse models, in concert with non-human primate (NHP) models, are critical in studying the interaction between cervicovaginal epithelial cells and sub-epithelial immune cells in the regulation of cervicovaginal barrier function in the context of HIV infection. The microbial composition and physiological conditions of these animal models are different from that of the human, and need to be considered when translating findings to human research or treatment.

FACTORS THAT AFFECT/REGULATE EPITHELIAL BARRIER FUNCTION

Microbiome Factors

Microbial Diversity

The composition of vaginal microbial communities influences the vaginal defense system of the mucosal barrier by inhibiting the growth of disease-causing pathogenic microorganisms by either producing microbicidal compounds, or by enhancing host physical and immunological barrier function. One of the critical components of physical barrier function at the FGT mucosa is mucus. *L. crispatus* dominated vaginal microbiomes are associated with mucus that traps microbes, such as HIV, more efficiently as compared to the mucus associated with vaginal microbiomes dominated by *L. iners* or *Gardenella* species (Nunn et al., 2015). The knowledge of how vaginal microbes affect the role of mucus in defense remains scarce. Mucus can be compromised in the case of degradation by BV-associated bacteria. BV-associated bacteria, such as *Gardnerella*, *Prevotella*, and *Bacteroides*, make the sialidase enzyme that actively degrades sialic acid—a critical component of FGT mucus (Lewis et al., 2013; Vagios and Mitchell, 2021). Other mucus-degrading enzymes, including mucinases, sulfatases, galactosidases, and prolidases, damage FGT barrier integrity by contributing to the watery vaginal discharge characteristic of BV (Lewis et al., 2013; Vagios and Mitchell, 2021). Besides mucus, host proteins in vaginal secretions also play a key role in vaginal epithelial barrier function. Proteomic analysis of CVL samples collected from a cross-sectional study of 50 Rwandan female sex workers resulted in four groups with distinct compositions of dominating vaginal microbiomes. Group 1 is associated with *L. crispatus* dominant vaginal microbiomes, group 2 is associated with *L. iners* dominant vaginal microbiomes, group 3 is associated with anaerobe dominant vaginal microbiomes along with intermediate vaginal microbial diversity, and group 4 is associated with anaerobe dominated vaginal microbiomes along with high vaginal microbial diversity. The protein profiles of the CVL samples from these women further showed that increasing vaginal microbial diversity was positively associated with increased protein levels in the biological pathways of cell death, proteasome and protease activity, and pro-inflammatory cytokines. The increased vaginal microbial diversity was negatively associated with the levels of keratin protein, cornified envelop proteins, humoral immune molecules, such as IGHA1 and IGHG2, and anti-protease activity (Borgdorff et al., 2016). Together, this study showed that increasing vaginal microbial diversity is associated with increases of detrimental, and decreases of beneficial, host protein levels pertaining to epithelial vaginal barrier function. As these are associations, it remains to be shown whether changes in host barrier function permit the diversification of vaginal microbiome or whether the diversification of vaginal microbiome causes the changes in host barrier function. Moreover, the molecular mechanisms of how vaginal microbial diversity interacts with vaginal mucosal protein expression remain to be sought.

AMPs are part of the mucosal chemical barrier against the foreign environment. Vaginal levels of AMPs are also found to be closely related to vaginal microbial diversity. Increased FGT microbial diversity is associated with decreases in AMPs, such as lysozyme C (LYZ) and ubiquitin (RPS27A), and increased S100A9 (Borgdorff et al., 2016). Changes in AMP levels have also been reported during BV, with human beta defensin-2 (HBD-2), lactoferrin, and cathelicidin (LL-37) found to be higher in the CVL of women with BV (Fan et al., 2008; Gregory et al., 2011; Frew et al., 2014). *In vitro* experiments also found increased secretion of HBD-2 from epithelial cells co-cultured with BV associated bacteria, such as *A. vaginae* and *L. iners* (Doerflinger et al., 2014). However, how increases in vaginal microbial diversity, and the condition of BV, influence secretion of AMPs remains to be explored.

Microbial Byproducts

Vaginal fluid contains not only host factors, but also microbial metabolites and enzymes. This profile is largely dependent on the composition of the vaginal microbiome and the interaction between host cells and microbiota (Srinivasan et al., 2015). The D-lactic acid (D-LA) isoform is predominantly produced over L-lactic acid (L-LA) in *L. crispatus*, *L. gasseri*, and *L. jensenii*, as compared to *L. iner*s, which produces only L-LA (Steven et al., 2013; Nunn et al., 2015). It is suggested that vaginal concentrations of D-LA and L-LA may be a key determinant in vaginal microbiome diversity and vaginal health. Vaginal mucus with high concentrations of D-LA exhibits enhanced trapping of HIV-1 particles, compared to mucus with low concentrations of D-LA characteristic of *L. iners* dominant microbiomes. (Nunn et al., 2015). Both D-LA and L-LA can reduce the pH of the vaginal mucosa and prevent the growth of yeast and other microbes, as well as reduce the release of pro-inflammatory molecules by cervicovaginal epithelial cells (Hearps et al., 2017). Hearps et al. showed that *in vitro* treatment of human vaginal and cervical epithelial cell lines with LA (pH 3.9) induced production of the anti-inflammatory cytokine IL-1RA. Further, when added simultaneously or prior to stimulation, LA inhibited the TLR agonist-induced production of pro-inflammatory mediators IL-6, IL-8, TNFα, RANTES, and MIP3α from epithelial cell lines, and prevented IL-6 and IL-8 production elicited by exposure to seminal plasma (Hearps et al., 2017). Similar anti-inflammatory effects of LA hold true for primary cervicovaginal cells and when organotypic epithelial tissue models are used.

In addition, *Lactobacillus* species, except for *L. iners*, also produce hydrogen peroxide and bacteriocins as byproducts that inhibit growth of pathogenic non-indigenous bacteria (Vallor et al., 2001; Mich` et al., 2015). Alternatively, *L. iners* secretes cholesterol-dependent cytolysin (CDC) and *G. vaginalis* secretes vaginolysin, both of which are known to have cytotoxic activity against vaginal and cervical epithelial cells (Cadieux et al., 2009; Macklaim et al., 2013). In an *in vitro* study, culture supernatant of *L. iners* and *Gardenella* increased the permeability of ectocervical and endocervical cell culture by impairing the cellular adhesion molecule E-cadherin, and increased release of soluble cadherin compared to *L. crispatus* culture supernatant (Anton et al., 2018). In contrast, *L. crispatus* secreted byproducts in culture supernatant that helped to restore ectocervical and endocervival barrier integrity, previously disrupted by *Gardenella* culture supernatant (Anton et al., 2018). Unfortunately, this study did not explore the byproduct profiles in these culture supernatants. Succinate and short-chain fatty acids, such as butyrate, propionate, and acetate, commonly present in the gut mucosa, are also found at low levels in the vaginal mucosa. In the condition of BV, however, much higher levels of SCFAs have been found (Mirmonsef et al., 2011). A meta-transcriptomic study also showed the up regulation of butyrate metabolizing enzymes, butyryl-CoA-dehydrogenase, and butyrate kinase during BV, associated with increased *P. amnii* and *Megasphaera* (Macklaim et al., 2013). Usually, SCFAs exhibit a beneficial role in gut mucosa by improving gut epithelial integrity, differentiation, proliferation, and reducing the secretion of pro-inflammatory molecules from gut mucosal epithelial and immune cells (Blacher et al., 2017). Knowledge of the role of SCFAs in the FGT mucosa remains limited. An *in vitro* study found contrary results that high levels of SCFAs can induce pro-inflammatory molecule secretion from vaginal and cervical epithelial cells (Delgado-diaz et al., 2020). More studies are required to explore the impact of higher SCFA levels in changing FGT mucosal barrier function and its association with BV conditions.

Host Factors

Immune Cells/Immune Factors

The description of the FGT immunologic milieu deserves another review or book chapter by itself. This review will only summarize key immune cells and mediators that are relevant for the discussion of the interactions between FGT host cells and the microbiome.

The lower FGT is characterized by IgG present from vaginal transudate and local mucosal IgA antibodies (Fahrbach et al., 2013; Gunn et al., 2016), antimicrobial peptide secretion (Yarbrough et al., 2015), and the presence of both adaptive and innate immune cells (Zhou et al., 2018; Mei et al., 2019). T cells exist primarily below the substratum of the epithelium, and provide cytokine and chemokine responses to address invading pathogens or barrier tissue damage. Macrophage and neutrophils are present in the tissue, maintaining the homeostasis between the microbial environment and the host epithelium. In response to invasion of the FGT, macrophage and neutrophils function to remove pathogenic, and bystander commensal, bacteria and promote wound healing (Wira et al., 2005; Jennifer et al., 2021). The FGT immunologic milieu changes as the FGT transforms to the upper compartment, where the physiological emphasis changes from focusing on homeostasis and defense to focusing on the balances of both homeostasis and the readiness to maintain a potential pregnancy (Ochiel et al., 2008; Agostinis et al., 2019). The uterine cavity is home to the unique uterine natural killer (uNK) cell population, as well as a high degree of gamma delta (γδ) T cells and macrophage (Amy et al., 2012; Zhou et al., 2018; Monin et al., 2020). At the upper FGT, the profiles of secreted antimicrobial peptides and cytokines focus on the production of anti-inflammatory cytokines alongside AMPs. The uterus is able to achieve high degrees of immune surveillance due to the presence of lymphoid aggregates, dense groupings of B and $CD8^+$ T cells, which allow for targeted and controlled responses to foreign antigen in the localized immune environment of the uterus (Yeaman et al., 2001; Agostinis et al., 2019). Advances in uterine immune regulation are summarized in detail in the review articles by Anne et al, Abebe et al, and Liman et al. (Anne et al., 2014; Abebe et al., 2021; Liman et al., 2021).

The composition of the microbiome found in the lower and upper FGTs are quite distinct; however, there is a clear association between the species present within the lower FGT and the species found in the upper FGT (Baker et al., 2018; Agostinis et al., 2019). For example, *Lactobacillus* species

typically dominate the lower FGT, but comprise only approximately a quarter of the upper FGT microbiome on average, while other genera such as *Pseudomonas*, *Acinetobacter*, and *Sphingobium* make up the remaining portion. It is unknown whether these genera arise from occult introduction, or if they travel from the gut and other sites to colonize the uterus directly (Łaniewski et al., 2020). For much of the history of FGT microbiome research, the upper FGT has been thought to be a sterile environment in healthy women, but this notion has been challenged by recent advances in sequencing and sampling technology that have uncovered a potentially omnipresent, if variable, uterine microbiome.

Microbiome composition has been associated directly with health and disease (Swain and Ewald, 2018). The interplay between the microbiota and the human immune system in the gut, and to a lesser extent in the respiratory tract, have been examined extensively during the last decade to yield translational knowledge with implications for mucosal health. However, such knowledge at the FGT remains scarce. It was thought that host immune cells and immune mediators play a direct role in shaping the composition of the FGT microbiome, which consequently impacts barrier function and the ability to protect against STIs. There still remains no direct evidence for this hypothesis, however. Based on what has been learned of the gut mucosa (Yasmine and Oliver, 2017; Zheng et al., 2020), the FGT microbiota could provide critical signals for the development and function of the host immune system, and, the host immune system, in turn, could have evolved multiple means by which to maintain its symbiotic relationship with the microbiome. The microbiome of the lower and upper FGTs interact with the immune mechanisms of the host primarily through interaction with immune components, such as immunoglobulins, where they can reduce the efficacy of these molecules *via* neutralization through degradation or competitive binding. General inflammation and mucus breakdown has also been observed with the CST-IV, or BV-associated, microbiome (Lewis et al., 2013; Lacroix et al., 2020). How the host immune system is 'educated' to accommodate the commensal microbial community is critical. At the gut mucosa, such mechanisms exist to tolerate food antigens and microbes that are essential for gut function (Chistiakov et al., 2014). If such a tolerating mechanism fails, undesirable inflammation results in disease. At the FGT, the combination of liquefaction of the mucus barrier and increased recruitment of immune cells due to local pro-inflammatory cytokines and chemokines leads to increased risk of immune cell infection by pathogens such as HIV, as well as direct epithelial infection by pathogens such as HSV-2 and chlamydia (Koumans et al., 2007; Passmore et al., 2016; Bautista et al., 2017). This also creates a self-reinforcing cycle where host pro-inflammatory immunity can have non-specific and antigen-specific removal and/or inhibition of commensal microbes, and potentially further prevent the establishment of a *Lactobacillus* dominant microbiome. Disrupting the homeostasis of the commensal microbial environment may potentiate further exacerbation of inflammation at the FGT mucosa. How the uterus, and pregnancy benefit from hosting commensal microbes, and how the uterine/endometrial immune system tolerates its associated microbiota to maintain a homeostatic microenvironment, urgently require further studies.

Hormones and Age

Hormones have a direct influence on both the immune system and the microbial community present within the FGT. Together, all three play crucial roles in regulating FGT barrier function. During pregnancy, and at puberty, increases in the female sex hormone estrogen promote the proliferation and differentiation of vaginal epithelial cells. Glycogen from exfoliated and lysed epithelial cells is converted by α-amylase, and the product is then metabolized to lactic acid by *Lactobacillus* species in the vaginal lumen. A glycogen-rich environment directly promotes the growth and dominance of *Lactobacillus* species (Amabebe and Anumba, 2018). This is further reinforced through the production of hydrogen peroxide, bacteriocins, and biosurfactants from *Lactobacillus* species. Elevated estrogen levels are also associated with a thicker epithelial lining with greater elasticity, perhaps *via* promoting the proliferation and differentiation of FGT epithelial cells. The synergistic effect of estrogen-high and *Lactobacillus* dominant environments associates most strongly with improved barrier function, vaginal health metrics, and protection from STIs and cancer.

In contrast, progesterone, the other commonly considered female sex hormone, has no significant association with any CST group. Although progesterone has been shown to play a protective role in preventing inflammation-induced preterm labor during pregnancy (Dodd and Crowther, 2009), the use of the hormonal contraceptive medroxyprogesterone acetate, a progesterone analogue that has different biological effects, is associated with increased vaginal microbiome diversity that potentially modulates vaginal inflammation and increased HIV-1 susceptibility in humanized mouse models (Wessels et al., 2019). The use of depot medroxyprogesterone acetate (DMPA) is also associated with the CST IV-type microbiome in the FGT, and a higher risk for acquiring BV (Wessels et al., 2019). The reduction in robust epithelial barrier support, with concomitant increased risk of inflammatory and dysbiotic states, is thought to be partially responsible for explaining DMPA's association with increased HIV risk and proteome signatures of epithelial barrier disruption (Birse et al., 2017). Several studies also reported the association of elevated progesterone with a more inflammatory environment and a less robust barrier at the vaginal epithelium *via* unknown mechanisms (Tjernlund et al., 2015; Vitali et al., 2017; Bradley et al., 2018). While the mechanism requires further investigation, DMPA has also been reported to have neutral or even detrimental effects to the elasticity of the vaginal membrane leading to increased chances of vaginal dryness and epithelial tearing (Irvin and Herold, 2015; Birse et al., 2017; Zalenskaya et al., 2018). With the cyclic nature of the human menstrual cycle, FGT barrier function may change throughout a given cycle depending on the balance of estrogen and progesterone. Indeed, during menses there is often a complete loss of a woman's characteristic CST, which is often restored shortly at the end of menses (Gajer et al., 2012). In

general, the human FGT microbiome appears to remain stable despite the shifts in hormones throughout the menstrual cycle. While it is clear that highly elevated estrogen and DMPA levels have direct effects on vaginal microbiome composition, it is unknown if the FGT microbiome has its own impacts on hormone production, or activity, locally within the FGT.

Outside of female sex hormones, oxytocin, a hormone and neuropeptide normally produced in the hypothalamus and released by the posterior pituitary, has been explored as an alternative to estrogen-based therapies for vaginal dryness. It has been shown to be effective clinically as a vaginal gel in alleviating menopausal vaginal symptoms and *in vitro* in promoting proliferation of Vk2 vaginal epithelial cell lines (Kallak and Uvnäs-Moberg, 2017; Torky et al., 2018). Tremendous work remains to validate oxytocin's role in the interplay between the immune system, the microbiome, and its effects on FGT barrier function.

Age, inextricably linked with hormone levels, is a core determinant of the FGT microbiome. Data across ages for FGT microbiomes only exists for the lower FGT, with microbiome signatures for the upper FGT only existing for women of reproductive age (Gupta et al., 2019; O'Callaghan et al., 2020). In early infancy the lower FGT is colonized by *Lactobacillus* species due to the prenatally inherited high estrogen levels from the mother's blood. Approximately six weeks post-birth, vaginal microbiomes progress to a microbiome more reminiscent of skin with a complete loss of *Lactobacillus* dominance. This persists up until puberty, when production of estrogen increases, and menstruation begins, and *Lactobacillus* species dominate the lower FGT once again. This is notably true in those of Caucasian and Asian descent, but this pattern is less prevalent in those of African ancestry where vaginal microbiomes may develop into polymicrobial communities with greater frequency (Ravel et al., 2011; Fettweis et al., 2014). It remains unknown whether this is the influence of genetic or cultural and epigenetic factors. Regardless, the higher prevalence of polymicrobial vaginal microbiomes is considered a risk factor for HIV acquisition and BV development in this population (Kyongo et al., 2015; Alcendor, 2016; Dabee et al., 2019). Along with changes to the microbiome, pH levels begin to decrease from 7.0 in pre-puberty to the 4.0–5.0 range typical of normal, healthy, vaginas (Gupta et al., 2019). This is mostly due to the dominance of *Lactobacillus* species and the production of lactic acid and hydrogen peroxide at the vaginal mucosa. These influences persist throughout a woman's sexual lifetime, even after the onset of menopause, where, due to a decline in estrogen levels the microbiome transitions back to a phenotype similar to the pre-pubertal state, with a coinciding increase in pH and loss of *Lactobacillus* dominance. This puts postmenopausal women at increased risk of barrier disruption and STIs due to the combined effects of a less robust epithelial barrier, a lack of *Lactobacillus*-mediated protection at the FGT, and increased pH levels (Brotman et al., 2018; Gliniewicz et al., 2019; Murphy et al., 2019).

Host Metabolites

It is not always possible to distinguish host vs microbial metabolites, and, indeed, both can be responsible for the production of the same molecule. Lactic acid, for example, is produced by both vaginal epithelial cells and by *Lactobacillus* species as a result of glycogen metabolism and is thought to be one of the main drivers behind the reduction in vaginal pH seen with increased *Lactobacillus* dominance (Amabebe and Anumba, 2018; Ratter et al., 2018; Song et al., 2020). There is evidence that in heightened states of inflammation, the metabolic profile of the FGT changes to one consistent with greater cellular leakage and cellular breakdown. In addition, variation in the metabolic profile of the FGT between the subsets of women with or without BV has been observed (Srinivasan et al., 2015; Parolin et al., 2018; Ceccarani et al., 2019). Lower levels of lactate, amino acids, and dipeptide, and higher amounts of bioactive metabolites such as signaling eicosanoid 12-hydroxyeicosatetraenoic acid (12-HETE), were found in the vaginal mucosa of women with BV (Srinivasan et al., 2015; Parolin et al., 2018). Lower amounts of sialic acid and higher levels of mannose epitopes were also found in the CVL of women with BV as compared to those without BV (Linlin et al., 2015). Here, decreased levels of vaginal sialic acid during BV may be explained by the increased activity of the sialidase enzyme produced by BV-associated bacteria (Lewis et al., 2013; Vagios and Mitchell, 2021). How changes in host metabolites impact microbial composition and host cell function, and how the microbiome, in turn, influences the metabolic expression of host cells remains to be characterized. It is still unclear to what degree host-derived metabolites originate from the blood, and enter the lower FGT *via* plasma transudate, and to what degree they are produced locally by resident epithelial and immune cells.

GAPS IN KNOWLEDGE

Our knowledge regarding the microbiome and host factors in regulating cervicovaginal mucosal barrier properties lags far behind what is known at other mucosal sites, such as the gut and respiratory mucosa. Although higher diversity of the vaginal microbiome is associated with reduced FGT barrier properties, the mechanism behind the feature of *Lactobacillus* species in preventing growth and biofilm formation of pathogenic microbes at the FGT remains a particularly interesting point of research (Parolin et al., 2021). Similarly, the interaction of host cells, including immune cells and non-immune cells, with bacterial communities in different CST groups and BV-associated microbes needs to be investigated in depth. Recently, studies showed the difference in the metabolomic profile of the vaginal mucosa in healthy and BV states (Srinivasan et al., 2015; Parolin et al., 2018; Ceccarani et al., 2019). However, the effect of specific bacteria in regulating host metabolic pathways, and the role of those differentially abundant metabolites in regulating barrier properties and disease susceptibility, has not yet been explored. Furthermore, tryptophan levels were reported to be higher in women's vaginal mucosa during dysbiosis, or BV, as compared to healthy women. The effect of gut bacteria-derived tryptophan metabolites, such as indole-3-ethanol, indole-3-pyruvate, and the receptor of these metabolites, aryl hydrocarbon receptor (AHR), in regulating gut epithelial and skin keratinocyte barrier function implores us to investigate their role in vaginal epithelial barrier function

(Scott et al., 2020; Uberoi et al., 2021). Metatranscriptomic, metaproteomic, and metabolomic approaches are necessary to identify reproductively and metabolically active bacteria, secreted bioactive metabolites from those bacteria, and metabolic proteins or enzymes involved in generating those metabolites. Altogether this can be used to determine the role of those bacteria and bioactive metabolites in regulating the ecology of the FGT mucosal microenvironment and host barrier properties (Macklaim et al., 2013). Besides having gaps in which factors affect FGT barrier function, using both *in vitro* and *ex vivo* models to test microbiome effects on FGT barrier function presents its own challenges, especially with regards to representing the physiological condition of the FGT mucosa properly. For example, studying the composition of microbes, microaerophilic and low pH conditions of the mucosa, and the shedding nature of the outer epithelial layer are all insufficient with current models. *In vivo* models provide alternative approaches, but the translation of the findings from animal models to human application is challenging given the discrepancy in microbial compositions.

SUMMARY

Mucosal barriers are critical gatekeepers in allowing nutrients and beneficial molecules to, and preventing pathogenic and infectious agents from, entering the body. The health of the FGT is guarded by its mucosal barriers and the integrity of FGT mucosal barriers is governed by the interplay between host immune system, hormones, and the FGT microbiota. However, our knowledge of how FGT homeostasis is maintained is still lacking. With this knowledge, an understanding of risk factors for STI acquisition, and a broader understanding of vaginal and uterine health, can be implemented in future research questions as well as care and policy decisions for sexual health, sexual quality of life, and reproductive interventions.

AUTHOR CONTRIBUTIONS

AP participated in the researching and reviewing of research articles, defining the hypothesis, writing and revising the manuscript, and designing **Figure 1**. ABS participated in the researching and reviewing of research articles, defining the hypothesis, writing and revising the manuscript, and designing **Figure 2** and **Table 1**. RCS participated in the researching and reviewing of research articles, defining the hypothesis, writing, reviewing, and revising the manuscript, and reviewing the figures and table. AP and ABS contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

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Immunometabolic Analysis of *Mobiluncus mulieris* and *Eggerthella* sp. Reveals Novel Insights Into Their Pathogenic Contributions to the Hallmarks of Bacterial Vaginosis

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The cervicovaginal microbiome plays an important role in protecting women from dysbiosis and infection caused by pathogenic microorganisms. In healthy reproductive-age women the cervicovaginal microbiome is predominantly colonized by protective *Lactobacillus* spp. The loss of these protective bacteria leads to colonization of the cervicovaginal microenvironment by pathogenic microorganisms resulting in dysbiosis and bacterial vaginosis (BV). *Mobiluncus mulieris* and *Eggerthella* sp. are two of the many anaerobes that can contribute to BV, a condition associated with multiple adverse obstetric and gynecological outcomes. *M. mulieris* has been linked to high Nugent scores (relating to BV morphotypes) and preterm birth (PTB), whilst some bacterial members of the *Eggerthellaceae* family are highly prevalent in BV, and identified in ~85-95% of cases. The functional impact of *M. mulieris* and *Eggerthella* sp. in BV is still poorly understood. To determine the individual immunometabolic contributions of *Eggerthella* sp. and *M. mulieris* within the cervicovaginal microenvironment, we utilized our well-characterized human three-dimensional (3-D) cervical epithelial cell model in combination with multiplex immunoassays and global untargeted metabolomics approaches to identify key immune mediators and metabolites related to *M. mulieris* and *Eggerthella* sp. infections. We found that infection with *M. mulieris* significantly elevated multiple proinflammatory markers (IL-6, IL-8, TNF-α and MCP-1) and altered metabolites related to energy metabolism (nicotinamide and succinate) and oxidative stress (cysteinylglycine, cysteinylglycine disulfide and 2-hydroxygluatrate). *Eggerthella* sp. infection significantly elevated multiple sphingolipids and glycerolipids related to epithelial barrier function, and biogenic amines (putrescine and cadaverine) associated with elevated vaginal pH, vaginal

amine odor and vaginal discharge. Our study elucidated that *M. mulieris* elevated multiple proinflammatory markers relating to PTB and STI acquisition, as well as altered energy metabolism and oxidative stress, whilst *Eggerthella* sp. upregulated multiple biogenic amines associated with the clinical diagnostic criteria of BV. Future studies are needed to evaluate how these bacteria interact with other BV-associated bacteria within the cervicovaginal microenvironment.

Keywords: vaginal microbiome, vaginal dysbiosis, organotypic 3D culture, biogenic amines (BAs), global metabolic and regulatory networks, women's health, cervical epithelial barrier, genital inflammation

INTRODUCTION

In healthy reproductive age women, the cervicovaginal microbiome is generally dominated by *Lactobacillus* spp. These beneficial bacteria acidify the cervicovaginal microenvironment *via* lactic acid production, which contributes to protection against infections by pathogenic and opportunistic microorganisms (O'hanlon et al., 2011). The depletion of *Lactobacillus* spp. leads to the colonization of the lower female reproductive tract (FRT) by a diverse consortium of facultative and obligate anaerobic bacteria, a disorder is referred to as bacterial vaginosis (BV) (Workowski and Bolan, 2015; Muzny et al., 2020). Importantly, BV is associated with a range of adverse gynecologic and obstetric outcomes including an increased risk of sexually transmitted infections (STI) and preterm birth (PTB). Microbiologically, BV is characterized by the presence of a polymicrobial biofilm covering the surface of cervicovaginal epithelium (Muzny et al., 2019). *Mobiluncus mulieris* and *Eggerthella* sp. are two of the many anaerobes that may contribute to the biofilm formation, yet their mechanistic contributions to BV and related adverse gynecologic and obstetric outcomes are still poorly understood (Danielsson et al., 2011; Machado and Cerca, 2015).

Mobiluncus spp. are motile, curved rod-shaped bacteria that are isolated from vaginal secretions from women with BV and are associated with high Nugent scores (a method to diagnose BV) (Sprott et al., 1983; Roberts et al., 1985; Hallén et al., 1987; Teo et al., 1987; Vetere et al., 1987; Moi et al., 1991; Gatti, 2000; Srinivasan and Fredricks, 2008). In addition, *M. mulieris* has been isolated from extragenital sites, such as breast and umbilical abscesses (Glupczynski et al., 1984). In previous epidemiological studies, *M. mulieris* have been also linked to PTB (Holst et al., 1994; Hillier et al., 1995; Meis et al., 1995). Genital inflammation has been implicated in PTB and *M. mulieris* has been hypothesized as a microbial driver in such inflammatory states (Dude et al., 2020). The flagella of *M. mulieris* has been previously demonstrated to stimulate Toll-like receptor 5 (TLR5) activation, which links to the elevation of key inflammatory markers (IL-6, IL-8, and TNF-α) and PTB (Anahtar et al., 2015; Onderdonk et al., 2016; Dude et al., 2020; Dela Cruz et al., 2021). In addition, *M. mulieris* has been shown to exert sialidase activity (Culhane et al., 2006). Notably, this bacterial enzyme cleaves sialic acid from highly glycosylated proteins present in the cervical mucus plug and its activity is associated with BV (Smayevsky et al., 2001), PTB and chorioamnionitis (Critchfield et al., 2013; Racicot et al., 2013; Smith-Dupont et al., 2017).

Some bacterial members of the *Eggerthellaceae* family are highly prevalent in BV, identified in ~85-95% of cases (Fredricks et al., 2005; Fredricks et al., 2007; Srinivasan et al., 2012; Shipitsyna et al., 2013). Interestingly, one member of the *Eggerthellaceae* family have also been linked to all four of the Amsel criteria (vaginal pH, vaginal odor, vaginal discharge and the presence of clue cells) used to diagnose BV in clinical settings (Srinivasan et al., 2015). *Eggerthella* spp. [previously classified as *Eubacterium* (Kageyama et al., 1999)] are non-motile anaerobic coccobacilli that are part of the healthy human gut microbiome (Finegold et al., 1983; Schwiertz et al., 2000). The taxonomy of the *Eggerthellaceae* family requires further investigation to classify them into their appropriate genus and species. However, *Eggerthella* spp. can also cause bacteremia and sepsis with high mortality rates (Lau et al., 2004a; Lau et al., 2004b; Thota et al., 2011; Lee et al., 2012). This suggests that in the FRT, *Eggerthella* spp. might play a role in the pathophysiological processes that manifest as adverse obstetric and gynecologic outcomes.

To determine the individual immunometabolic contributions of *Eggerthella* sp. and *M. mulieris* within the cervical microenvironment, we utilized our well-characterized human three-dimensional (3-D) cervical epithelial cell model that recapitulates several physiologically relevant features of *in vivo* tissue, including TLR expression, microvilli, intercellular junctional complexes and secretory material. We combined this advanced bioreactor-derived 3-D cell culture model with multiplex immunoassay and global untargeted metabolomics approaches to identify key immune mediators and metabolites related to *M. mulieris* and *Eggerthella* sp. infections of the lower FRT. We chose the 3-D cervical model since the cervix is a critical area impacted by cervicovaginal microbiota that, when disrupted, can lead to PTB, increased STI acquisition and other gynecological sequalae associated with BV.

METHODS

Human Cervical Epithelial Cell Culture and Generation of the 3-D Cervical Model

Human cervical epithelial cells (A2EN) were generously provided by Dr. Alison Quayle at Louisiana State University Health Sciences Center (Herbst-Kralovetz et al., 2008; Buckner et al., 2011) and were routinely maintained in keratinocyte serum-free media (KSFM) (Fisher Scientific) supplemented with epidermal growth factor (5 ng/ml), bovine pituitary extract (50 μg/ml), $CaCl_2$ (Gibco) and primocin (100 μg/ml; *In vivo*Gen) at 37°C in a 5% carbon dioxide (CO_2) humidified atmosphere. Short tandem repeat DNA profiling confirmed that cells were not contaminated with other cell lines

found in available databases. For downstream experiments, we used cervical epithelial cells (passage ~50–60) cultured as monolayers or 3-D cervical cell models. Monolayer cultures were seeded at ~2 × 10^5 cells/ml into tissue culture-treated 24-well plates. Prior to seeding, cells were enumerated by trypan blue exclusion. The 3-D cervical cell models were generated as previously described (Radtke and Herbst-Kralovetz, 2012; Radtke et al., 2012; Jackson et al., 2020). Briefly, cervical epithelial cell monolayers were trypsinized and counted using a Countess automated cell counter (Invitrogen). The single cell suspension (~1 × 10^7) was combined with 300 mg of hydrated Cytodex-3 collagen-coated dextran microcarrier beads (Sigma-Aldrich) suspended in pre-warmed KFSM-primocin medium. The mixture was transferred to a rotating-wall vessel (RWV) bioreactor (Synthecon). Bioreactors were incubated at 37°C for 28-days at 20 rpm, with daily medium changes. After 28-days the 3-D cervical cell models were harvested, washed and resuspended in antibiotic-free KFSM medium, enumerated, and distributed into 24-well plates at a density of ~5 x 10^5 cells/well for downstream experiments.

Bacterial Strains and Growth Conditions

All bacterial strains used in this study were obtained from the Biodefense and Emerging Infections (BEI) Research Repository (NIAID, NIH as a part of the Human Microbiome Project). *M. mulieris* strain UPII-28I and *Eggerthella* sp. strain MVA1 were cultured on tryptic soy agar (TSA) (Becton Dickinson) supplemented with 5% defibrinated sheep blood (Quad Five) at 37°C under anaerobic conditions generated using anaerobic environment chambers and AnaeroPacks (Thermo Scientific). Due to large taxonomic restructuring of vaginal species over the last five years we decided to confirm our strains taxonomic classification. Although not much genomic information is available yet for *Eggerthella* sp. strain MVA1 there is a sequence read SRX655730 in the NCBI Sequence Read Archive. This read in the SRA reports that *Eggerthella* sp. MVA1 has 86.46% sequence identity with the *Eggerthellaceae* family and 83.23% identity with the *Eggerthella* genus using their Sequence Taxonomic Analysis Tool (STAT) (Katz et al., 2021). There is also a 16S rRNA sequence (JX103988) available that has 99% sequence identity with *Eggerthella lenta*. Future comparative genomic analyses are needed to designate a species for *Eggerthella* sp. strain MVA1.

Bacterial Infections

M. mulieris UPII-28I and *Eggerthella* sp. MVA1 were cultured on TSA agar with sheep's blood for 16-18 hours prior to infection. Bacterial strains were harvested and resuspended in sterile Dulbecco's phosphate-buffered saline (PBS) and adjusted to an optical density at 600 nm (OD_{600}) for infection assays. The OD_{600} 0.5 reflected the CFU/ml range of 1 x 10^8 – 1 x 10^9, likely due to bacterial cell clumping as observed on the SEM. Monolayers were infected with adjusted bacterial suspensions (20 µl of bacterial suspension adjusted to OD_{600} of 0.05, 0.5 and 5.0 per 1 x 10^5 cells and incubated for 24 hours under anaerobic conditions at 37°C for use in cytotoxicity assays. The 3-D cervical cell aggregates were infected with adjusted bacterial suspensions (20 µl of bacterial suspension adjusted to OD_{600} of 0.5 per 1 x 10^5 cells and incubated under anaerobic conditions at 37°C for 24-hours. In a preliminary experiment the bacterial recovery 24 hours after the infection of the 3-D cervical cell model with both *M. mulieris* UPII-28I and *Eggerthella* sp. MVA1 was within 0.5 of a log of the initial infection dose. PBS-treated cells served as mock-infected controls. Culture supernatants were immediately used for cytotoxicity assays or stored at -80°C for downstream immunoproteomic and metabolomic analyses.

Lactate Dehydrogenase Assay (LDH)

Culture supernatants from cervical epithelial monolayer cell infections were used to assess cytotoxicity using the CyQUANT LDH assay (Thermo Fisher Scientific) according to the manufacturer's protocol. LDH activity was measured by recording absorbance values at 490 nm and 680 nm and the percentage LDH activity was calculated according to the equation: $\frac{\text{sample LDH activity}}{\text{lysed control LDH activity}} \times 100$.The assay was performed using three independent biological replicates.

Scanning Electron Microscopy

Human 3-D cervical cell models were infected with *M. mulieris* UPII-28I and *Eggerthella* sp. MVA1 for four hours under anaerobic conditions at 37°C. Samples were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) and prepared for scanning electron microscopy (SEM) as described previously (Hjelm et al., 2010; Mcgowin et al., 2013). Infected 3-D cervical cell aggregates were imaged with a JSM-6300 JEOL scanning electron microscope and IXRF model 500 digital processor (IXRF systems) at the Electron Microscopy Core at Arizona State University. Representative images collected for each bacterium were selected for inclusion in the figure. Pseudo-coloring of the SEM images was performed using Adobe Photoshop CS6 v13.

Multiplex Immunoassays

Cell culture supernatants from 3-D cervical cell models infected with *M. mulieris* UPII-28I and *Eggerthella* sp. MVA1 were collected from three independent experiments. The levels of five cytokines: (interleukin (IL)-1α, IL-1β, IL-1RA, IL-6, tumor necrosis factor-α (TNF)-α), seven chemokines: fractalkine, IL-8, interferon γ-induced protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein-1β (MIP-1β), regulation on activation, normal T-cell expressed and secreted (RANTES) and three growth factors: platelet derived growth factor-AA (PDGF-AA), transforming growth factor-α (TGF-α), vascular endothelial growth factor (VEGF) were measured using customized MILLIPLEX® multianalyte profiling (MAP) Human Cytokine/Chemokine Panel 1 array (Millipore) and compared to PBS mock infections. Data was collected using a Bio-Plex® 200 (Bio-Rad) platform and evaluated using Manager (5.0) software (Bio-Rad). A five-parameter logistic regression curve fit was used to determine the concentration. All samples were analyzed in biological triplicate, each containing two technical replicates.

Untargeted Metabolomics Analysis

Cell culture supernatants from 3-D cervical cell models infected with *M. mulieris* UPII-28I and *Eggerthella* sp. MVA1 from three independent experiments were sent to Metabolon Inc. (Durham, NC) for untargeted global metabolomics analysis. Metabolites were resolved using ultra-performance liquid chromatography with mass

spectrometry (UPLC-MS) as described previously (Ilhan et al., 2020; Salliss et al., 2021). The sample extracts were dried then reconstituted in solvents compatible to four different methods. Sample aliquots were analyzed using: acidic positive ion conditions that were chromatographically optimized for more hydrophilic or hydrophobic compounds, basic negative ion optimized conditions and negative ionization conditions. The MS analysis used dynamic exclusion with a scan range covering 70-1000 m/z. The Laboratory Information Management System (LIMS) was used for data extraction and peak-identification, QC and compound identification.

Statistical Analysis

All assays and infections were performed as at least three biological replicates. Statistical differences between the mean protein concentrations among groups were determined by one-way ANOVA with Bonferroni *post-hoc* test using Prism v9.1.1 software (GraphPad). ClustVis (Metsalu and Vilo, 2015) was used to perform hierarchical clustering analysis (HCA) on the Bio-Plex data (ln-transformed and Pareto scaled, Euclidean distance measures and average linkage clustering). Metabolomics data analyses, including HCA, Spearman's correlation analysis, principal component analysis (PCA) and metabolite enrichment pathway analysis, were performed with MetaboAnalyst 5.0 (Pang et al., 2021). Prior to analysis the metabolomics data was log-transformed, and Pareto scaled. Relative abundance is the normalized values from the area under the curve of the metabolite peaks collected that are rescaled to set the median equal to 1, before inputting any missing values as the minimum. To determine the significance between the mean relative abundances of metabolites among groups (infection vs. PBS control), two-tailed paired Student's t-tests was performed using the rstatix R package. To correct for multiple comparisons, *p*-values were adjusted using false discovery rate (FDR) and *q*-values were reported. *p*-values below 0.05 were considered significant.

RESULTS

Eggerthella sp. and *Mobiluncus mulieris* Do Not Induce Significant Cytotoxicity in Colonized 3-D Cervical Epithelial Cell Models

First, we assessed whether *Eggerthella* sp. and *M. mulieris* infections induced cytotoxicity in cervical epithelial cell monolayers at three doses which corresponded with the final OD_{600} of 0.1, 0.01 and 0.001 of $1x10^5$ cervical cells/ml. Using LDH cytotoxicity assays, we found that there was no significant cytotoxicity induced following infection with *Eggerthella* sp. and *M. mulieris* at any dose tested (**Supplementary Figure 1**).

We confirmed colonization of 3-D cervical cell models with *Eggerthella* sp. and *M. mulieris* by SEM (**Figure 1**). Both *Eggerthella* sp. and *M. mulieris* formed clusters and interacted simultaneously with multiple cells in some areas. *Eggerthella* sp. colonized the 3-D cervical cell models in smaller clusters and longer chains

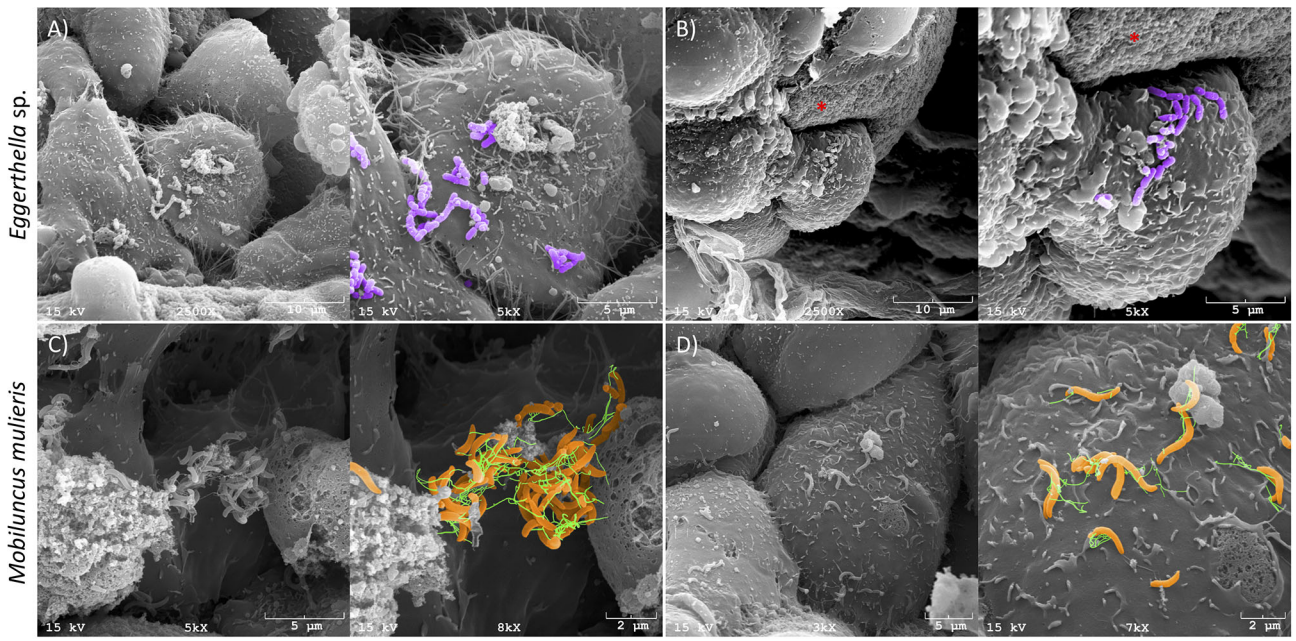

FIGURE 1 | *Eggerthella* sp. and *M. mulieris* colonize 3-D human cervical epithelial cell models. Pseudo-colored scanning electron microscopy (SEM) images of **(A)** and **(B)**, *Eggerthella* sp. MVA1 and **(C, D)**, *M. mulieris* UPII-28I showing colonization of 3-D cervical epithelial cells. *Eggerthella* sp. MVA1 exhibit a coccobacilli morphology and colonized 3-D cervical cells in small clusters or short chains. *M. mulieris* UPII-28I cells exhibit a curved rod-shaped morphology and were pseudo-colored orange with flagella-like structures pseudo-colored green. The * indicates the collagen-coated microcarrier beads used to generate the 3-D cervical epithelial cell models.

(**Figures 1A, B**). *M. mulieris* exhibited flagella-like structures which appeared to interact with other bacterial cells (**Figure 1C**) and epithelial cell surfaces (**Figure 1D**).

Infection of 3-D Cervical Aggregates With *M. mulieris* Upregulated Levels of Several Key Proinflammatory Cytokines and Chemokines, Whereas Infection With *Eggerthella* sp. Elevated IL-1α Secretion

To investigate the host immune response to *M. mulieris* and *Eggerthella* sp., we infected 3-D cervical cell models with each bacterium for 24 hours and measured levels of secreted cytokines (IL-1α, IL-1β, IL-1RA, IL-6, TNF-α), chemokines (fractalkine, IL-8, IP-10, MCP-1, MCP-3, MIP-1β, RANTES) and growth factors (PDGF-AA, TGF-α, VEGF). Data from the infectious conditions were compared to PBS mock-infected controls.

We performed hierarchical clustering analysis (HCA) to visualize patterns of immune mediator expression by 3-D cervical cell models in response to bacterial infection (**Figure 2A**). HCA demonstrated distinct immune mediator profiles of *M. mulieris* and *Eggerthella* sp. as each condition clustered separately from the PBS mock-infected controls. Using the multiplex assays, we found that infection of 3-D cervical cell models with *M. mulieris* significantly upregulated expression of IL-6 ($p<0.0001$), IL-8 ($p<0.0001$), MCP-1 ($p<0.01$) and TNF-α ($p<0.01$) whereas infection with *Eggerthella* sp. significantly upregulated only IL-1α ($p=0.01$) (**Figure 2B** and **Supplementary Figure 2**). IL-6, IL-8, TNF-α and IL-1α are all proteins linked to increased genital inflammation (Hannun and Obeid, 2018; Łaniewski et al., 2018). This data indicated that *M. mulieris* promoted a proinflammatory response in 3-D cervical cell models to a greater extent than *Eggerthella* sp.

Eggerthella sp. and *M. mulieris* Infections Distinctly Altered Extracellular Metabolomes Corresponding to Amino Acid and Lipid Superpathways in 3-D Cervical Epithelial Cell Models

To discern the effect of *Eggerthella* sp. and *M. mulieris* infections on the cervicovaginal extracellular metabolome, we performed untargeted global metabolomics analysis using supernatants collected from 3-D model experiments. The metabolomics analysis identified 314 known metabolites. To compare global metabolic profiles of *Eggerthella* sp. and *M. mulieris*, principal component analysis (PCA) and Spearman's correlation analysis (**Figure 3**) were employed. Biological replicates from each bacterial infection and PBS mock-infected controls clustered together and showed distinct separation of each condition by PCA (**Figure 3A**). Principal component 1 (PC1) explained 42% of variance and was significantly different ($p<0.001$) between *Eggerthella* sp. and mock-infected controls; principal component

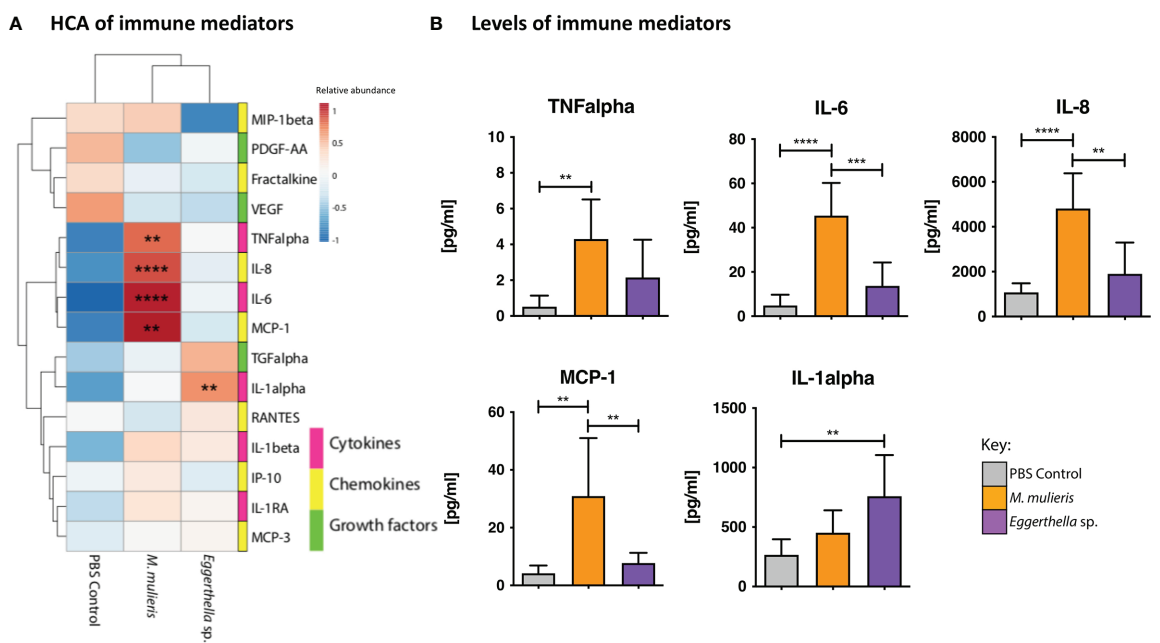

FIGURE 2 | *M. mulieris* significantly elevated production of inflammatory cytokines and chemokines compared to *Eggerthella* sp. and PBS mock-infected controls in 3-D cervical aggregates. **(A)** Hierarchical clustering analysis (HCA) of cytokine, chemokine, and growth factor profiles secreted by 3-D cervical cells in response to infection with *Eggerthella* sp. MVA1 or *M. mulieris* UPII-28I. The data was log-transformed and Pareto-scaled prior to clustering. HCA was performed using Euclidean distance measures and average linkage clustering algorithms. **(B)** Bio-Plex analysis of cytokines, chemokines and growth factors secreted by 3-D human cervical cells infected with *M. mulieris* UPII-28I and *Eggerthella* sp. MVA1 for 24h in anaerobic conditions. TNF-α, IL-6, IL-8 and MCP-1 were all significantly elevated by *M. mulieris* UPII-28I, whilst *Eggerthella* sp. MVA1 significantly increased expression of IL-1α. Statistical significance was determined by one-way ANOVA and Bonferroni *post-hoc* multiple comparisons. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

2 (PC2) explained 21.2% of the variance scores and contributed to separation of *M. mulieris* and *Eggerthella* sp. from the mock-infected controls ($p<0.05$). Spearman's correlation analysis showed each bacterial infection and the PBS mock-infected controls clustered distinctly from one another with each of the biological replicates grouped together (**Figure 3B**), therefore showing good replicability, and supporting the PCA analysis.

Overall, infection with *Eggerthella* sp. and *M. mulieris* significantly ($p<0.05$) altered the abundance of 68 and 44 metabolites, respectively, compared to mock-infected controls (**Supplementary Figure 3**). Of these differentially abundant metabolites, *Eggerthella* sp. and *M. mulieris* shared 17 significantly altered metabolites (**Figure 3C**). Next, we grouped significantly altered metabolites by superpathway and compared superpathway profiles between the two bacterial infections. Metabolites representing the amino acid superpathway (57.4% and 31.8% respectively) and the lipid superpathway (22.1% and 18.2% respectively) were profoundly influenced by infection with *Eggerthella* sp. and *M. mulieris* (**Figure 3D**). The overall composition of the superpathways between *Eggerthella* sp. and *M. mulieris* was significantly different ($p=0.0147$).

Next, we conducted metabolic pathway enrichment analysis on the metabolomics data sets to identify metabolic pathways significantly enriched by each bacterial infection (**Figure 4**). *Eggerthella* sp. infection significantly ($p<0.05$) enriched 23 subpathways, mostly associated with the amino acid superpathway (**Figure 4A**) while *M. mulieris* infection significantly enriched 24 subpathways and the most significant were from the lipid superpathway (**Figure 4B**). We also compared and contrasted these subpathways between *Eggerthella* sp. and *M. mulieris* (**Figures 4C, D**). Following comparisons of amino acid and lipid subpathways, we observed that *Eggerthella* sp. enriched a vast number of amino acid subpathways, twice that of *M. mulieris*. The majority of subpathways enriched by *M. mulieris* were also enriched by *Eggerthella* sp. Conversely, we noted that *M. mulieris* enriched twice the number of lipid subpathways than *Eggerthella* sp., with only sphingolipid metabolism being unique to *Eggerthella* sp. infection.

Eggerthella sp. Infection Significantly Altered Levels of Sphingolipids and *M. mulieris* Infections Significantly Altered Levels of Long-Chain Fatty Acids

Since both *M. mulieris* and *Eggerthella* sp. infections significantly modulated lipid metabolic pathways in culture supernatants, we

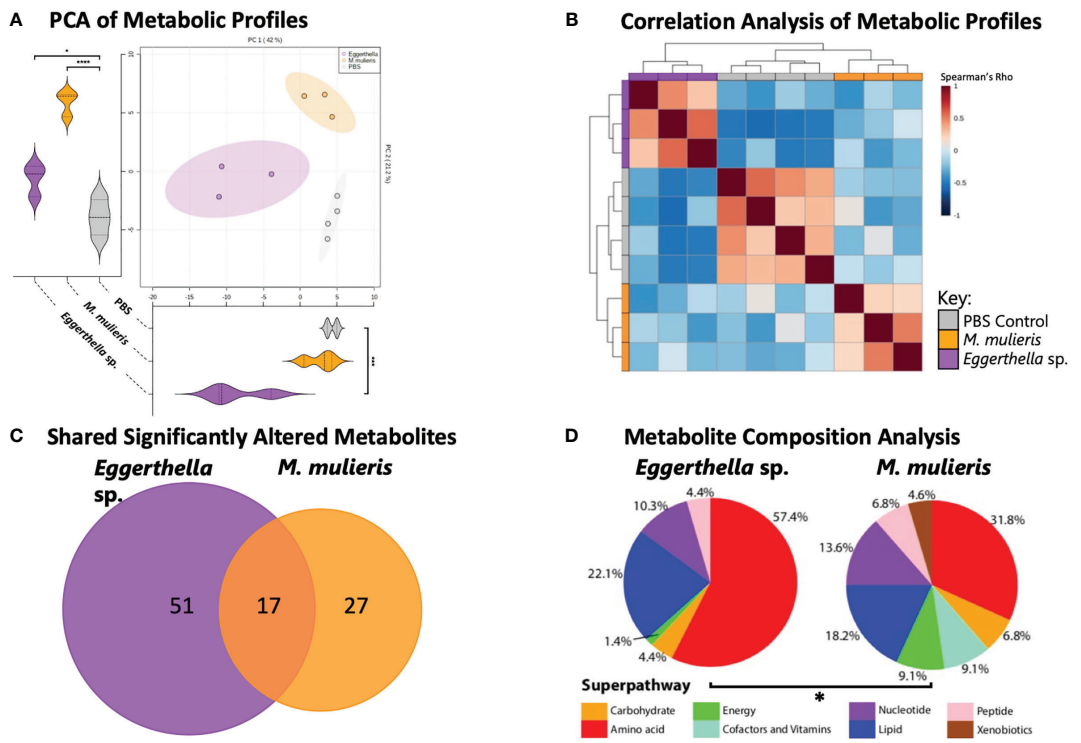

FIGURE 3 | *M. mulieris* and *Eggerthella* sp. infections of 3-D cervical cell models resulted in distinct metabolic profiles. **(A)** Principal component analysis (PCA) shows distinct clustering between each bacterial metabolic profiles and the PBS mock-infected controls. PC1 and PC2 score significance was determined using one-way ANOVA with Bonferroni *post-hoc* tests. *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$. **(B)** Spearman's correlation heatmap of metabolic profiles demonstrating clustering of biological replicates for each infection. **(C)** Venn diagram indicating the unique or overlapping metabolites that were significantly altered ($p<0.05$) between the two bacterial infections. The significant differences in metabolite abundances among infections were determined using Student's t-tests with Welch's correction and compared to PBS mock-infected controls. **(D)** Pie charts showing the percentage of significantly ($p<0.05$) altered metabolites grouped by superpathway compared to PBS mock-infected controls (total number of significantly changed metabolites for *Eggerthella* sp. MVA1 and *M. mulieris* UPII-28I were 68 and 44 respectively). The significant difference between composition of superpathways was determined with chi-squared (χ^2) test (*, $p<0.05$).

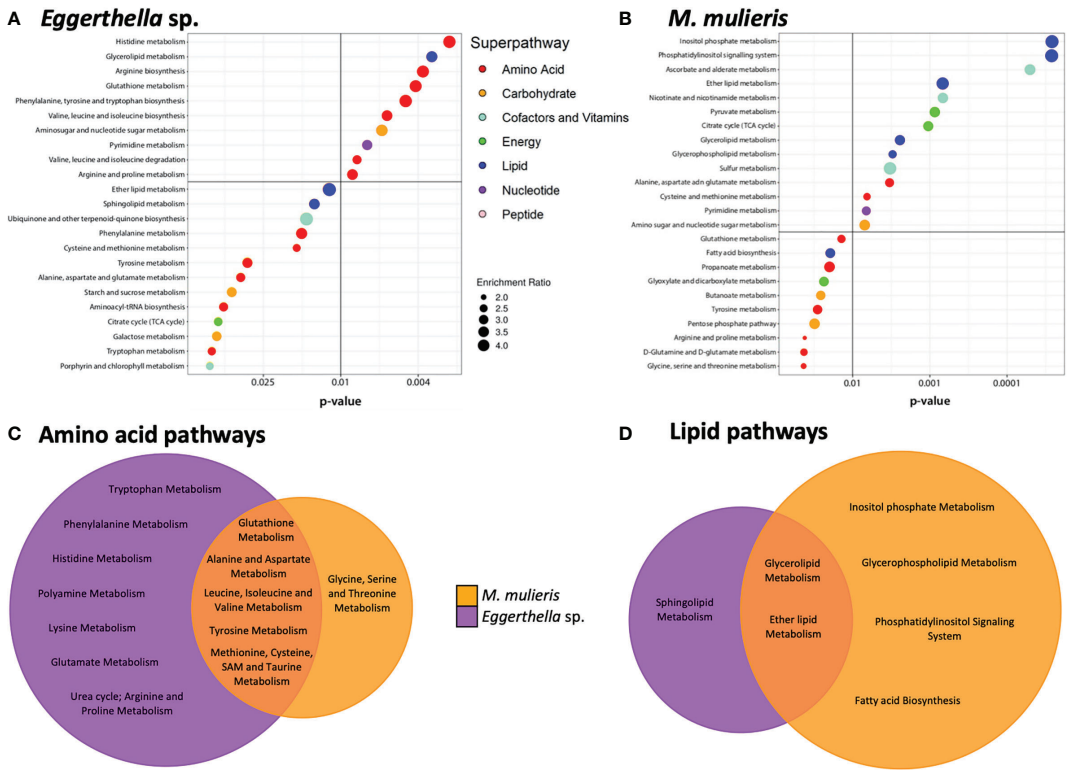

FIGURE 4 | *Eggerthella* sp. primarily altered amino acid subpathways whilst *M. mulieris* significantly altered lipid-related subpathways. Metabolic pathway enrichment analysis for **(A)** *Eggerthella* sp. MVA1 and **(B)** *M. mulieris* UPII-28I infections of 3-D cervical cell models. All subpathways shown were significantly enriched ($p<0.05$) using metabolite set enrichment analysis (MSEA). Colored circles next to the subpathways indicate which superpathway each subpathway belongs to. Venn diagrams comparing the significantly altered ($p<0.05$) **(C)** amino acid and **(D)** lipid subpathways by *Eggerthella* sp. MVA1 and *M. mulieris* UPII-28I infections.

identified the specific lipids with differential abundance ($p<0.05$) between bacterial infections compared to PBS mock-infected controls. We found 21 significantly altered lipids between both bacterial infections (**Figure 5A** and **Supplementary Figure 4**). These lipids can be classified into three categories of metabolism: sphingolipid metabolism, glycerolipid metabolism and inositol phosphate metabolism. Overall, *Eggerthella* sp. induced differential abundance of more lipids (16) than *M. mulieris* (7) and both significantly depleted the levels of glycerol ($p=0.024$ and $p=0.0433$, respectively) and glycerophosphorylcholine (GPC) $p=0.0389$ and $p=0.00931$, respectively) (**Figure 5C**). *Eggerthella* sp. infection mainly resulted in accumulation of glycerolipids and sphingolipids in contrast to *M. mulieris* which predominantly depleted long chain fatty acids; arachidate ($p=0.0368$), margarate ($p=0.000919$) and stearate ($p=0.00504$) (**Figure 5B**). Interestingly, the sphingolipids that were significantly altered by *Eggerthella* sp. were also elevated following *M. mulieris* infections but did not reach significance following infection with the latter species (**Figure 5D** and **Supplementary Figure 4**). Sphingolipids are closely linked to epithelial barrier function and inflammation (Hannun and Obeid, 2018; Harrison et al., 2018).

Eggerthella sp. Infection Significantly Elevated Biogenic Amines and Other Metabolites Associated With BV Symptoms and Diagnosis, Whereas *M. mulieris* Infection Modulated Metabolites Related to Energy Metabolism and Oxidative Stress

In clinical settings, BV is often diagnosed using the Amsel criteria (Amsel et al., 1983) which are based on the main symptoms of BV (vaginal pH, vaginal odor, vaginal discharge and the presence of clue cells). Thus, we determined whether infection with *Eggerthella* sp. or *M. mulieris* induced differential abundance of metabolites related to BV diagnosis in our 3-D human cervical cell models (**Figure 6**). We also evaluated the metabolites previously identified in cervicovaginal lavages collected from women with BV (Srinivasan et al., 2015). It is well established that biogenic amines are strongly associated with BV (Nelson et al., 2015) and linked to vaginal odor and elevated pH (Srinivasan et al., 2015; Borgogna et al., 2021). Cell culture supernatants from the 3-D cervical cell models infected with *Eggerthella* sp. significantly accumulated the biogenic amines cadaverine ($p=0.0373$) and putrescine ($p=0.0101$) and their

precursors citrulline (p=0.00598) and ornithine (p=0.00669), as well as several other BV-related metabolites (**Figure 6A**, **Supplementary Figure 5**). In contrast, *M. mulieris* infections did not result in accumulation of any biogenic amines detected in our samples. *M. mulieris* infection significantly influenced metabolites related to energy metabolism: nicotinamide (p=0.00429) and succinate (p=0.0335); and oxidative stress: 2-hydroxyglutarate (p=0.0365) and cysteinylglycine (p=0.00103) (**Figure 6B** and **Supplementary Figure 5**). Intriguingly, relative abundance of one BV-related metabolite, N-acetylneuraminate (sialic acid), was significantly and differentially altered by both bacteria. Sialic acid was significantly increased by *Eggerthella* sp. (p=0.0376) and significantly decreased by *M. mulieris* (p=0.00886), which suggested that both bacteria possess sialidase activity. A potential reason why *M. mulieris* significantly decreased sialic acid could be due to it being able to catabolize sialic acid. Phenyllactate is a relatively understudied metabolite that was significantly upregulated by *Eggerthella* sp. infection (p=0.0028) and exhibited the largest fold change out of any metabolites detected in our data set (~1,150 fold). Overall, *Eggerthella* sp. infections significantly altered multiple metabolites related to BV symptoms, particularly biogenic amines and those linked to epithelial barrier function, such as sphingolipids and glycerolipids (Hannun and Obeid, 2008; Bittman, 2013; Jernigan et al., 2015). In contrast *M. mulieris* infections significantly increased metabolites related to energy metabolism and oxidative stress (**Figure 7**).

DISCUSSION

BV is characterized by colonization of the cervicovaginal epithelium by a diverse community of anaerobic bacteria. *M. mulieris* and bacterial species from the family *Eggerthellaceae* are relatively understudied bacteria compared to many of the other BV-associated microorganisms. In this study we aimed to examine how two vaginal isolates: *Eggerthella* sp. strain MVA1 and *M. mulieris* strain UPII-28I, influence the immunometabolic landscape in the context of the lower FRT, as well as the potential pathophysiological contributions of these species to BV.

In recent years, there has been a reclassification of members belonging to the *Eggerthellaceae* family leading to questions related to the contributions of specific family members to the BV state. Although genomic information is limited for *Eggerthella* sp. strain MVA1, there is a sequence read SRX655730 available in the NCBI Sequence Read Archive. The

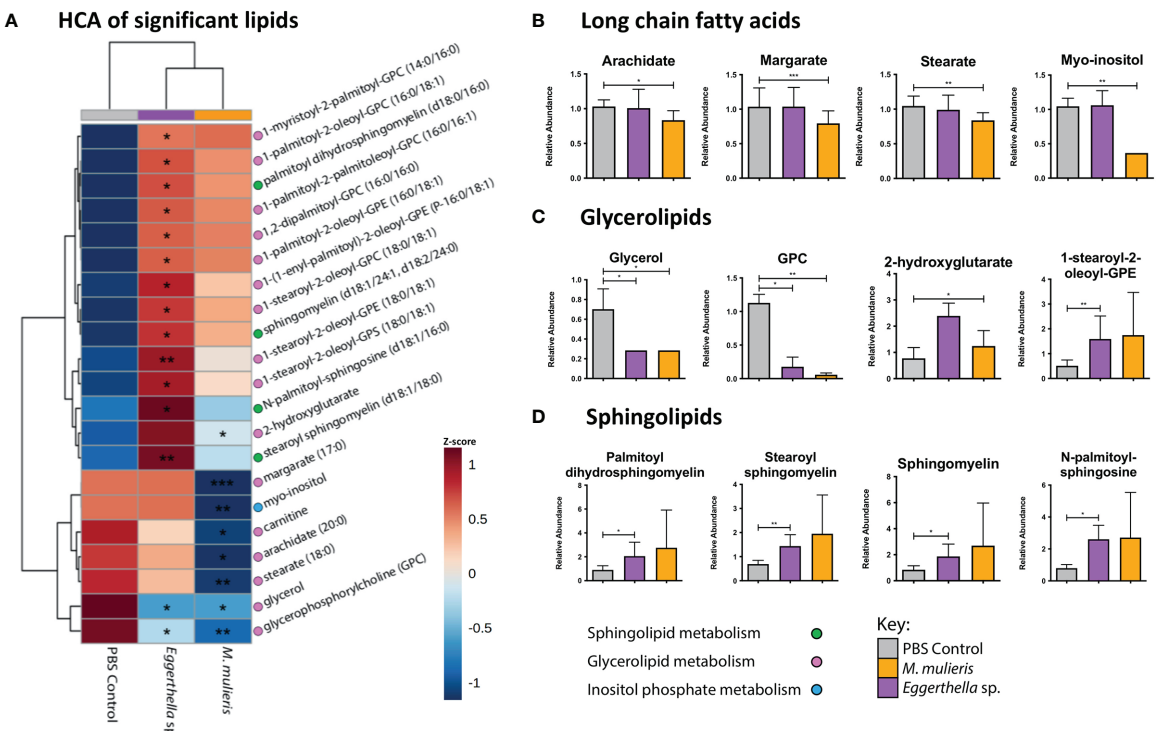

FIGURE 5 | *Eggerthella* sp. significantly altered the abundance of more lipids than *M. mulieris*. **(A)** Hierarchical clustering analysis (HCA) of differentially abundant lipids (p<0.05) determined by Students t-tests with Welch's correction of *Eggerthella* sp. MVA1 and *M. mulieris* UPII-28I infection compared to the PBS mock-infected control. HCA was performed using Euclidean distance measures and average linkage clustering algorithms. Relative abundance graphs of significant lipids classified into long-chain fatty acids **(B)**, glycerolipids **(C)** and sphingolipids **(D)**. The brackets after the lipids indicate how many carbons and how many double bonds there are in the structure of the lipid. The slash between numbers separates the information about the two hydrocarbon chains of the lipid, whilst the P- prefix indicates a neutral plasmalogen species and the d for sphingomyelins indicates a 1,3 dihydroxy chain. Significant differences between the bacteria and PBS mock-infected controls. *, p<0.05; **, p<0.01; ***, p<0.001.

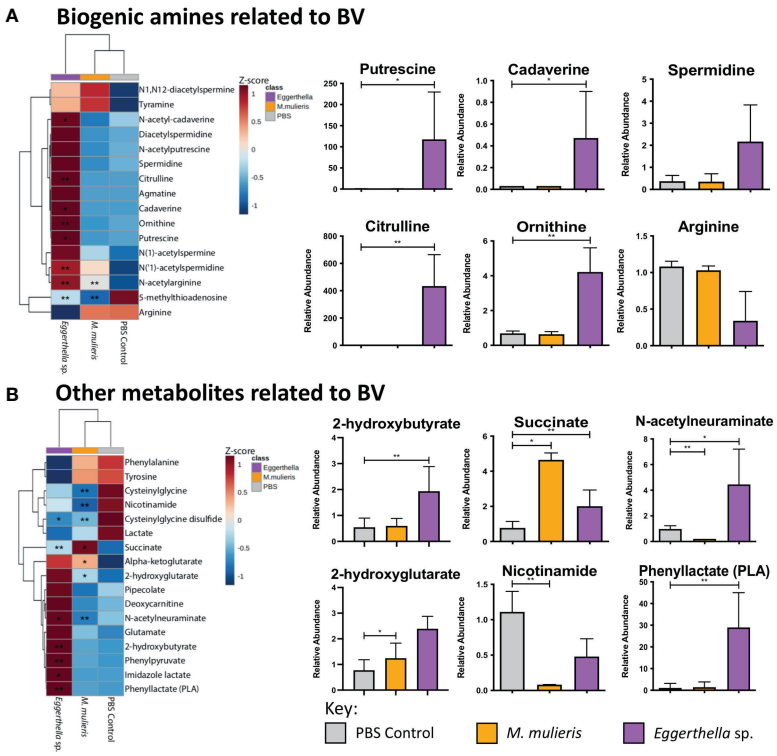

FIGURE 6 | *Eggerthella* sp. significantly elevated the production of metabolites associated with biogenic amines and *M. mulieris* significantly altered metabolites related to energy metabolism. Hierarchical clustering analysis (HCA) and relative abundance of the BV-associated metabolites of *Eggerthella* sp. MVA1 and *M. mulieris* UPII-28I infection compared to the PBS mock-infected control: biogenic amines **(A)** and other metabolites related to BV symptoms **(B)**. HCA was performed using Euclidean distance measures and average linkage clustering algorithms. Statistical significance was calculated using Student's t-tests with Welch's correction compared to PBS mock-infected control; *, $p<0.05$; **, $p<0.01$.

16S rRNA gene was sequenced from isolate *Eggerthella* sp. MVA1 (JX103988) and shares 99% sequence identity with *Eggerthella lenta*, however the species was not assigned by the BEI repository. There is very little information about *E. lenta* in the FRT and the vaginal microbiome since it is predominantly a gut microbe. *E. lenta* is found at low abundance in the FRT and may be transferred to the vagina from the gastrointestinal tract (Priputnevich et al., 2021). Unfortunately, until the comparative genomic analysis is performed, we cannot classify a species for the strain *Eggerthella* sp. MVA1. The family *Eggerthellaceae* also contains *Coriobacteriales* bacterium DNF00809, previously classified as *Eggerthella* sp. type 1 by Srinivasan et al. (Srinivasan et al., 2016) before culture and whole genome sequencing of the species. Previous studies showed that this bacterium was present in 85-95% of women with BV compared to those without BV (Fredricks et al., 2005; Fredricks et al., 2007; Srinivasan et al., 2012; Shipitsyna et al., 2013). These previous studies have also shown that *E. lenta* is much less prevalent in the FRT than *Coriobacteriales* bacterium DNF00809, which is no longer classified as an *Eggerthella* species. Although beyond the scope of this study, an in-depth analysis of taxonomic classification of vaginal bacteria belonging to the family *Eggerthellaceae* should be further investigated and clear taxonomic nomenclature should be referenced for this family to reflect the complexity of its lineage and putative role in BV and the cervicovaginal environment. Our study provides data related to the immunometabolic contributions with one of these understudied vaginal strains from the family *Eggerthelleacae*.

It is important to investigate BV-associated bacteria in the cervical epithelium since disruption of the microbiota at this mucosal site can lead to PTB, increased STI acquisition and other gynecological sequalae (Brunham and Paavonen, 2020). BV can also lead to ascension of pathogenic bacteria to the upper FRT, therefore promoting endometritis and pelvic inflammatory disease (Eckert et al., 2002). To determine the individual immunometabolic contributions of *Eggerthella* sp. and *M. mulieris* within the cervical microenvironment, we utilized a bioreactor-derived 3-D cervical epithelial cell model (Barrila et al., 2010; Hjelm et al., 2010; Gardner and Herbst-Kralovetz, 2016) in combination with multiplex immunoassays and global untargeted metabolomic approaches to identify key metabolites and immune mediators, respectively, related to *M. mulieris* and *Eggerthella* sp. infections.

Organotypic 3-D human cervical epithelial cell models recapitulate many features of parental tissue that are not observed in monolayer cell culture models. Our advanced 3-D cell culture model exhibits physiologically relevant features, such as TLR expression, microvilli, intercellular junctional complexes

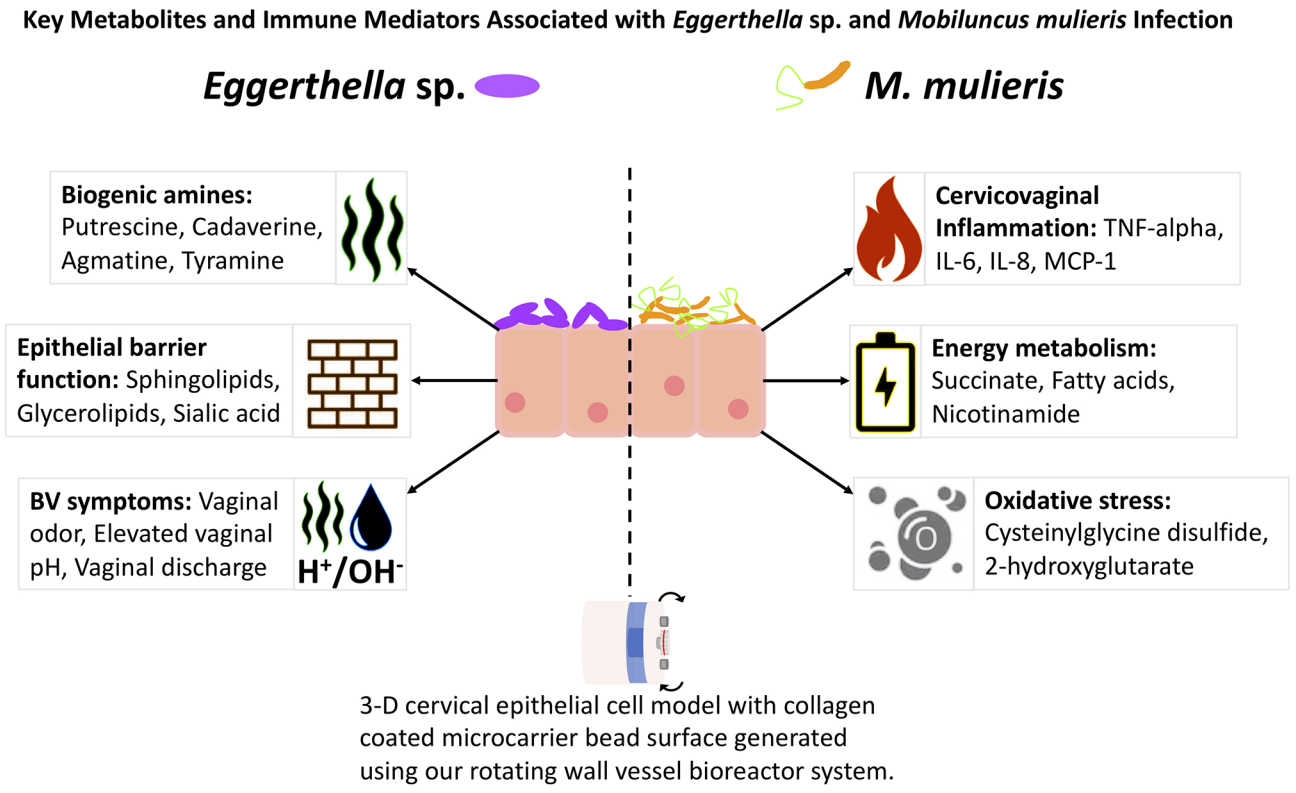

FIGURE 7 | Comparisons of *Eggerthella* sp. and *M. mulieris* infections of 3-D cervical epithelial cell models. Supernatants from infections of 3-D cervical cell models with *Eggerthella* sp. MVA1 and *M. mulieris* UPII-28I were compared to PBS mock-infected controls in Bio-Plex and global metabolomics analyses. *M. mulieris* UPII-28I significantly increased inflammatory markers: IL-6 ($p<0.0001$), IL-8 ($p<0.0001$), MCP-1 ($p<0.01$) and TNF-α ($p<0.01$). Metabolites related to energy metabolism, such as nicotinamide ($p=0.00429$) and succinate ($p=0.0335$) were increased following *M. mulieris* UPII-28I infection but are relatively understudied in relation to BV. Furthermore, metabolites linked to oxidative stress, such as 2-hydroxyglutarate ($p=0.0365$), cysteinylglycine ($p=0.00103$) and cysteinylglycine disulfide ($p=0.00238$) were significantly altered by *M. mulieris* UPII-28I and are associated with inflammation. *Eggerthella* sp. MVA1 on the other hand significantly altered levels of the biogenic amines cadaverine ($p=0.0373$) and putrescine ($p=0.0101$) and elevated levels of sphingolipids, glycerolipids and sialic acid ($p=0.0376$), each of which are metabolites relating to the epithelial barrier function.

and secretory material that could influence how BV-associated bacteria can colonize the model in a way similar to *in vivo* tissues (Gardner et al., 2020; Łaniewski and Herbst-Kralovetz, 2021; Salliss et al., 2021) These features provide a more accurate representation of the *in vivo* state which is more amenable to translational research efforts and studying host-microbe interactions; however, each model system has its strengths, weaknesses and utility (Hjelm et al., 2010; Radtke and Herbst-Kralovetz, 2012; Radtke et al., 2012; Herbst-Kralovetz et al., 2016). The close relation of 3-D human cervical epithelial cells to cervical tissue allows us to investigate how these pathogens can cause pathophysiological changes to the cervicovaginal microenvironment and epithelia. Using SEM, we demonstrated colonization of the 3-D cervical epithelial cell model by *Eggerthella* sp. and *M. mulieris* (**Figure 1**). We evaluated the cytotoxicity of each bacterium and determined that neither *Eggerthella* sp. nor *M. mulieris* infections induced significant cytotoxicity in cervical cells.

Inflammation is a driver of many disease processes including BV where it has been previously associated with PTB (Meis et al., 1995; Goldenberg et al., 2000; Romero et al., 2007). Our immune mediator analysis revealed that both *M. mulieris* and *Eggerthella* sp. induced a proinflammatory response in 3-D cervical epithelial cell models. *Eggerthella* sp. significantly increased IL-1α while *M. mulieris* significantly elevated IL-6, IL-8, MCP-1 and TNFα. *M. mulieris* has been linked to the significant elevation of IL-6, IL-8 and TNFα *in vitro* (Anahtar et al., 2015; Dude et al., 2020). Flagella, such as those expressed by *M. mulieris*, have been linked to activation of TLR5 (Hayashi et al., 2001; Dela Cruz et al., 2021) which leads to the stimulation of the NF-κB signaling pathway and, in consequence, upregulation of IL-6, IL-8 and TNFα (Nasu and Narahara, 2010). Intriguingly, upregulation of IL-6 and IL-8 as well as the NF-κB signaling pathway have been connected to PTB (Romero et al., 2014). Clinical studies showed that IL-6, IL-8 and TNFα are some of the most common cytokines associated with PTB (Murtha et al., 1998; Coleman et al., 2001; Romero et al., 2014; Ville and Rozenberg, 2018). However, there is no clear diagnostic marker for PTB and proinflammatory cytokine and chemokine profiles differing among women who deliver pre-term (Wei et al.,

2010; Fettweis et al., 2019). In addition, elevation of proinflammatory markers including IL-6 and IL-8 are associated with HIV infection risk (Mlisana et al., 2012; Rodriguez Garcia et al., 2015). Genital inflammation can lead to an increased risk of STI acquisition, with the infected epithelia being damaged, allowing the pathogens that cause STIs access to deeper tissues (Passmore et al., 2016). Inflammation of cervical and vaginal tissues induces recruitment of immune cells to the lower FRT which facilitates the spread of HIV and other STIs (Masson et al., 2014; Anahtar et al., 2015; Masson et al., 2015; Fichorova et al., 2020).

Oxidative stress has been closely linked to inflammation (Reuter et al., 2010). Oxidative stress becomes damaging when there are a disproportional amount of reactive oxygen species (ROS), that overwhelm the antioxidants capacity of glutathione (Schafer and Buettner, 2001). High levels of ROS can induce cellular damage and promote many inflammatory states including cancer (Perwez Hussain and Harris, 2007; Reuter et al., 2010; Burton and Jauniaux, 2011) by recruiting inflammatory markers such as cytokines and chemokines and stimulating NF-κB signaling (Reuter et al., 2010). In this study, we found that *M. mulieris* significantly altered multiple metabolites associated with oxidative stress, including cysteinylglycine, cysteinylglycine disulfide and 2-hydroxyglutarate, whilst *Eggerthella* sp. significantly altered 2-hydroxybutyrate and cysteinylglycine disulfide. Depletion of cysteinylglycine and cysteinylglycine disulfide, two intermediates in the glutathione synthesis pathway, could either indicate increased glutathione biosynthesis or signify an increase in the levels of ROS. Disruption of redox balance due to the elevated levels of ROS has been linked to activation of cell signaling pathways including those responsible for the regulation of inflammatory cytokines and PTB (Schieber and Chandel, 2014; Moore et al., 2018). Notably, increase of ROS can lead to lipid peroxidation which can free lipids from cell membranes (Burton and Jauniaux, 2011; Moore et al., 2018). The lipid peroxidation and membrane damage by ROS might be a mechanistic link behind the increased concentrations of lipids following infections of 3-D cervical models with *Eggerthella* sp. and *M. mulieris*.

Through our global untargeted metabolomics analyses, we found that *Eggerthella* sp. significantly altered twice as many lipids as *M. mulieris*. Specifically, sphingolipids were significantly elevated exclusively by *Eggerthella* sp. We also found similar significantly altered glycerolipids and sphingolipids as those reported by Salliss et al., 2021 that were elevated following infection with another BV-related microorganism: *Megasphaera micronuciformis* (Salliss et al., 2021). Sphingolipids are components of eukaryotic cell membranes and have been related to proinflammatory signaling pathways and apoptosis (Kolter and Sandhoff, 2006; Hannun and Obeid, 2008; Hannun and Obeid, 2018). Our results demonstrate that *M. mulieris* induced higher abundance of sphingolipids and most glycerolipids relative to *Eggerthella* sp. and PBS mock-infected controls, although the levels did not reach significance. We hypothesize that this may be related to the observation that *M. mulieris* has been shown to increase membrane permeability in cervical epithelial cells grown on transwells inserts (Dude et al., 2020) potentially by freeing these membrane-associated lipids. Both bacteria induced extracellular accumulation of multiple lipids related to epithelial barrier function (Bittman, 2013; Jernigan et al., 2015). Considering these results, we hypothesize that *Eggerthella* sp. and *M. mulieris* may play a role in increasing membrane permeability, although significant cytotoxicity was not observed in our experiments. Unexpectedly, compared to other lipids long-chain fatty acids (LCFAs) were significantly depleted by *M. mulieris* infection. It is possible that the depletion of LCFAs could result from an ability of *M. mulieris* to catabolize these liberated LCFAs a as an energy source. Unfortunately, the genomic sequence of *M. mulieris* is not fully annotated, therefore it is unclear if this bacterial species synthesizes all proteins necessary to facilitate the catabolism of the LCFAs.

The epithelial barrier function and the physiological properties of the mucosal membranes lining the FRT are crucial in protecting the cervix from BV-associated bacteria (Rodriguez Garcia et al., 2015). One of the key pathophysiological changes during BV is disruption of the epithelial barrier function, which allows pathogenic bacteria to access deeper tissues and induce inflammation (Muzny et al., 2019). Sialic acid is the terminal sugar moiety on glycans of cell surface glycoproteins and mucins. The epithelium of the FRT is lined with highly glycosylated mucins which limit adhesion and colonization of pathogenic bacteria during BV (Linden et al., 2008; Barrila et al., 2010; Lewis and Lewis, 2012; Radtke et al., 2012). In addition, sialic acid residues can bind to pathogens and induce host cell signaling to generate an immune response (Macauley et al., 2014; Bhide and Colley, 2017). Significant alterations in the levels of sialic acid following bacterial infections indicates bacteria-mediated sialidase activity. Previous clinical studies have revealed elevated levels of sialidase and sialic acid in the cervicovaginal fluids of women with BV (Briselden et al., 1992; Moncla et al., 2015). Infection of 3-D cervical cell models with both *M. mulieris* and *Eggerthella* sp. significantly altered the levels of sialic acid, indicating that both species exert sialidase activity. As *M. mulieris* decreased the levels of extracellular sialic acid, we hypothesize that this species catabolizes sialic acid residues that are liberated from the cell surfaces (Culhane et al., 2006), similarly to other BV-associated bacterium *Gardnerella vaginalis* (Lewis et al., 2013). The sialidase activity of *M. mulieris* could play a role in PTB since the mucus plug created during pregnancy contains multiple mucins which could be degraded by sialidase, therefore allowing pathogenic bacteria to ascend to the uterus (Mcgregor et al., 1994; Lewis and Lewis, 2012; Smith-Dupont et al., 2017; Baker et al., 2018). Consequently, ascension of pathogenic bacteria into the upper FRT during pregnancy can lead to chorioamnionitis and PTB (Galinsky et al., 2013). *M. mulieris* and elevated levels of IL-8 have been previously associated with amniotic infection and PID (Hillier et al., 1988; Larsson et al., 1989; Hitti et al., 2001).

Sialidase activity has been noted as a potential diagnostic marker for BV (Briselden et al., 1992) along with several cervicovaginal metabolites, some of which are highlighted by Srinivasan et al., (2015). Amongst the metabolites

associated with BV, biogenic amines are also considered key players in many aspects of BV pathogenesis (Nelson et al., 2015; Srinivasan et al., 2015; Borgogna et al., 2021). The Amsel criteria and Nugent scores are two methods to diagnose BV (Amsel et al., 1983; Nugent et al., 1991). Putrescine and cadaverine have been linked to decreased *in vitro* growth of *Lactobacillus* spp. and high Nugent scores in women with BV (Borgogna et al., 2021). Both putrescine and cadaverine are associated with increased vaginal pH, vaginal amine odor and vaginal discharge that manifest during BV (Srinivasan et al., 2012; Yeoman et al., 2013; Nelson et al., 2015). Through our metabolomics analysis we found that *Eggerthella* sp. significantly elevated both putrescine and cadaverine in the extracellular milieu, which corresponds with observations from Srinivasan *et al.* (Srinivasan et al., 2012). In contrast, *M. mulieris* did not elevate any biogenic amines in our current study. Previously, *M. mulieris* has been linked to elevated trimethylamine (Spiegel, 1991; Africa et al., 2014), however, this polyamine was not detected in our metabolomics analysis. Phenyllactate was found to be significantly elevated for *Eggerthella* sp. with the highest fold change (1150-fold) of all the metabolites measured. Although the role of this metabolite in the cervicovaginal microenvironment is still not clear, we have previously observed its accumulation following infections with other vaginal bacteria (Łaniewski and Herbst-Kralovetz, 2021; Salliss et al., 2021). The severity of BV has been associated with increased risk of HIV and other STI acquisition (Allsworth and Peipert, 2011); thus, contribution of *Eggerthella* spp. and *M. mulieris* to clinical symptoms of BV mechanistically links these species to poor health outcomes related to BV.

As with all experiments and biological models there are limitations (Herbst-Kralovetz et al., 2016). The 3-D cell culture model we have used is a robust tool that can provide mechanistic insights into host-microbe interactions in the cervical microenvironment. Our model, as with most human *in vitro* cell culture models, requires the use of pH-buffered medium; thus, it cannot mimic the acidic pH found in healthy women *in vivo* without impacting cellular viability However, the BVAB tested in this study thrive in a more neutral pH environment, which is a characteristic of our model (Barrila et al., 2010; Hjelm et al., 2010; Gardner and Herbst-Kralovetz, 2016). We also acknowledge that the bacterial strains used in this study may not represent the other closely related strains or species. As stated previously, the taxonomy of *Eggerthella* sp. MVA1 is still incomplete; therefore, we cannot generalize our findings to the other members of the *Eggerthellaceae* family. *Mobiluncus mulieris* is closely related to *Mobiluncus curtsii*; however, the two species are unique from each other in terms of physical characteristics and enzymatic activity. *M. curtsii* is smaller in size, can hydrolyze starch and hippurate and produce citrulline, ornithine and ammonia from arginine whilst *M. mulieris* cannot (Spiegel and Roberts, 1984). Future studies utilizing additional well-characterized bacterial isolates in mono- or polymicrobial infections are needed to better understand the individual contributions of these BVAB to poor gynecologic and obstetric outcomes.

Overall, we found *Eggerthella* sp. infections significantly altered multiple metabolites related to BV symptoms. These metabolites included the biogenic amines putrescine and cadaverine, as well as their precursors, and metabolites linked to epithelial barrier function, such as sphingolipids and glycerolipids (Ghosh et al., 1997; Bittman, 2013; Jernigan et al., 2015; Hannun and Obeid, 2018; Harrison et al., 2018; Heaver et al., 2018). *M. mulieris* infections significantly elevated multiple proinflammatory markers that are linked to PTB in addition to metabolites related to energy metabolism and oxidative stress (**Figure 7**). This study sheds light into the mechanisms that *Eggerthella* sp. and *M. mulieris* may utilize to promote BV. Our data suggests that *Eggerthella* sp. plays a key role in the production of biogenic amines, which contribute to the elevated vaginal pH and the amine odor, whilst *M. mulieris* potentially impacts the membrane permeability and induce proinflammatory immune responses. The increased concentration of lipids present in *M. mulieris* infection could also link into the increased immune response (Hannun and Obeid, 2018; Albeituni and Stiban, 2019; Sukocheva et al., 2020). The link to inflammation and the altered metabolic microenvironment fits into the hypothesis of Muzny et al. (2020) that proposes early colonizers establish biofilm and evade host defense responses whereas secondary colonizers mediate inflammation, an altered metabolic microenvironment and symptoms associated with BV. Based on our data, we propose that *M. mulieris* is functioning as a secondary colonizer in this hypothetical model of BV. In contrast, *Eggerthella* sp. while not inflammatory, exhibits metabolic activity consistent with our definition of a secondary colonizer, but may also participate in the early stages of biofilm formation. However, further *in vitro* studies investigating these microorganisms in polymicrobial settings in conjunction with longitudinal clinical studies are needed to elucidate microbe-microbe interactions and determine the role of these bacteria in the context of BV biofilms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MH-K, PŁ, and JM conceived of the experimental design and interpretation of the data. JM conducted experimental infections of the 3-D cervical cell aggregates and the Bio-Plex analyses. Cell supernatants were sent to Metabolon, Inc. for global untargeted metabolomics analysis. RM carried out the cytotoxicity experiments as well as the analysis of the cytotoxicity data, Bio-Plex and metabolomics data. RM was also responsible for

writing the first draft of the manuscript, drafting and editing the figures and revising the manuscript. JM obtained SEM images of infected 3-D human cervical cell models and assisted in the statistical analysis. MH-K, JM, and PŁ provided support and advice on writing, figures and tables, and also read and revised the manuscript. MH-K and PŁ provided guidance of the experimental and writing processes. MH-K supervised the research and provided funding acquisition, project administration and resources. All authors read, revised, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.759697/full#supplementary-material

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Vaginal Dysbiotic Microbiome in Women With No Symptoms of Genital Infections

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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 *Tenericute*s, 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

pathway, CoA biosynthesis pathway I and pyrimidine nucleotide salvage; whereas BV bacteria had characteristic aromatic amino acid biosynthesis, pentose phosphate pathway, carbohydrate degradation. In conclusion, women with asymptomatic BV have vaginal microbiota significantly different than women with normal microbiota. Furthermore, the study provides insights into the vaginal microbial structure of Indian women that will enable us to explore the prospective candidates for restoring the vaginal microbiota.

Keywords: microbiome & dysbiosis, vaginal microbiota (VMB), asymptomatic BV, Lactobacillus, bacterial vaginosis (BV)

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 215/2012) 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^{0}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 ACTACHVGGGTATCTAATCC. 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 QIIME2 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 compute the distribution of the samples of studied groups, viz., BV and Normal in Principal Coordinates Analysis (PCoA). Alpha diversity was estimated using the number of observed 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		Age (yrs)		Menstrual phase		
		(mean ± SD)	P value	(mean ± SD)	P value	Follicular (n)	Luteal (n)	P value
Normal	19	3.85 ± 0.56	0.0001	30.79 ± 7.40	0.2402	11	8	0.5273
BV	20	5.46 ± 0.69		33.26 ± 5.42		9	12	
Total	39	_		_		20	20	

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 evennessindex 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* spps. (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 LefSe analysis. Discriminate analysis using LefSe 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

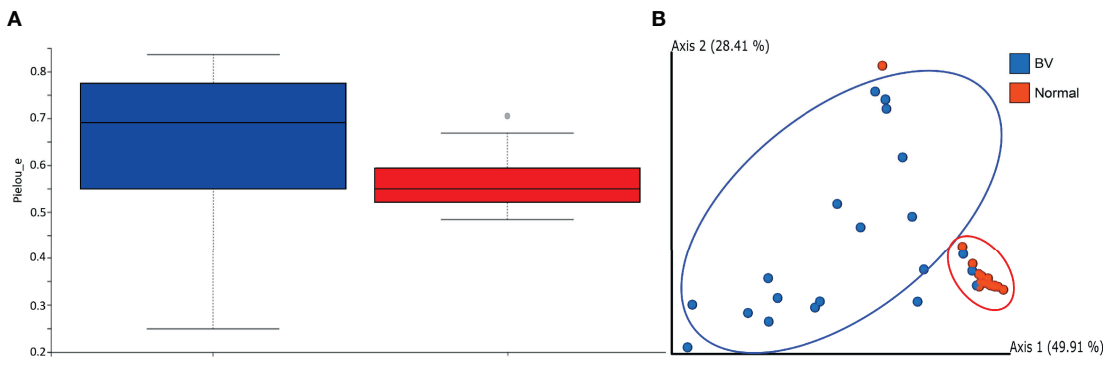

FIGURE 1 | **(A)** Alpha-diversity represented by the Pielou evenness index in the BV and normal samples indicating significant difference (p-value = 0.0165) between the groups based on the Kruskal-Wallis test. The boxes represent the distributions of the alpha diversity index and show the median for each condition. Whiskers extend to the furthest data point. **(B)** PCoA generated using weighted Unifrac distance indicates distinct clustering of samples from each group. Each point corresponds to an individual sample. For each experimental group, an ellipse around the centroid is depicted. The first two components of the variance are represented by Plotting BV(blue) vs Normal (Red) samples with significant separation between the two groups. The first two components represent 78.32 percent of variance, individually depicted in parentheses next to Axis1 and Axis2.

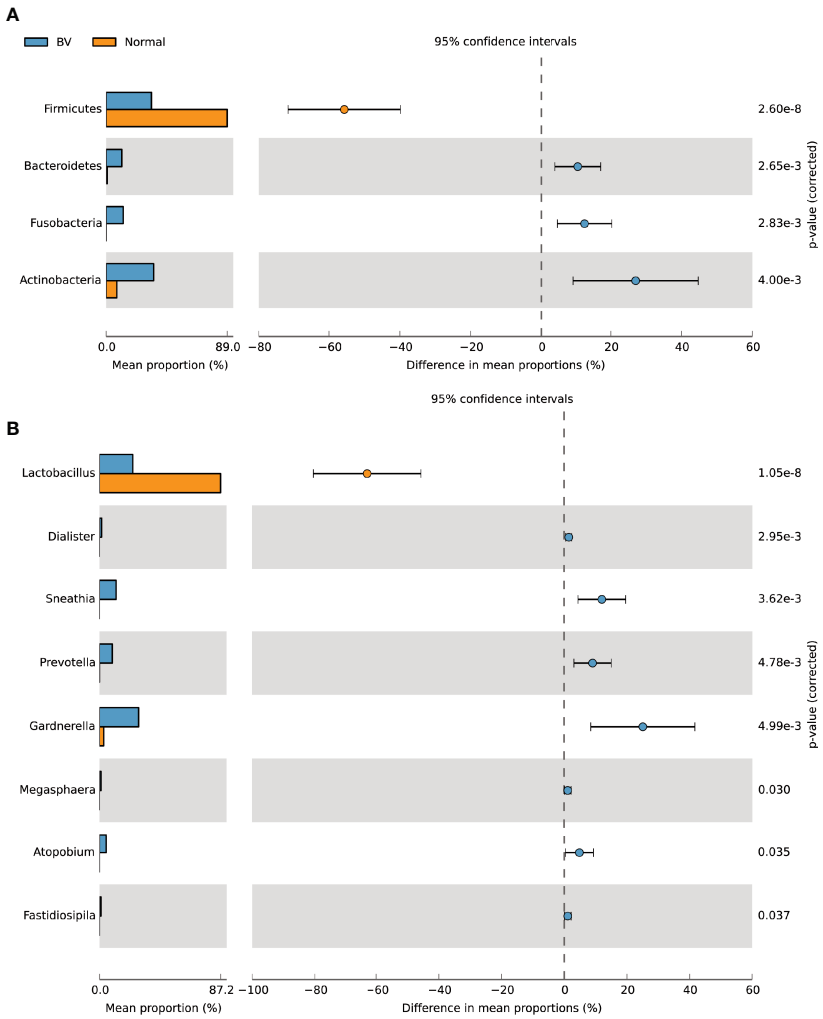

FIGURE 2 | Relative abundance of bacteria at the **(A)** phylum and **(B)** genus level in the normal and BV groups as computed by Welch's t-test using STAMP software. The middle shows the difference between proportions of abundance in the 95% confidence interval, and the value at the right is the P-value. $P < 0.05$ represents the significant difference between the two groups.

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

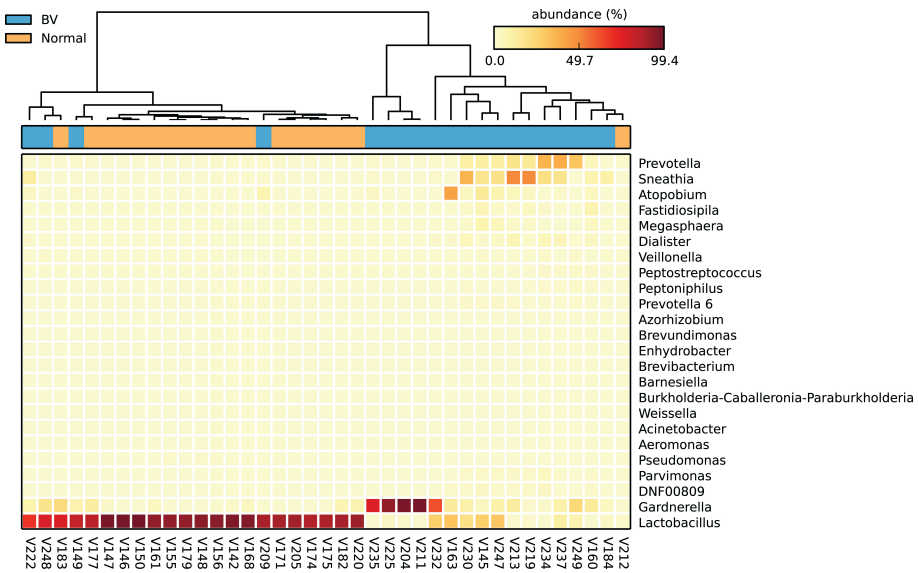

FIGURE 3 | Genus level composition of the vaginal microbiota in normal and BV samples. The heatmap depicts the relative abundances of the most abundant genera. Each column represents one sample. Orange are samples with normal microbiota and Blue are BV samples.

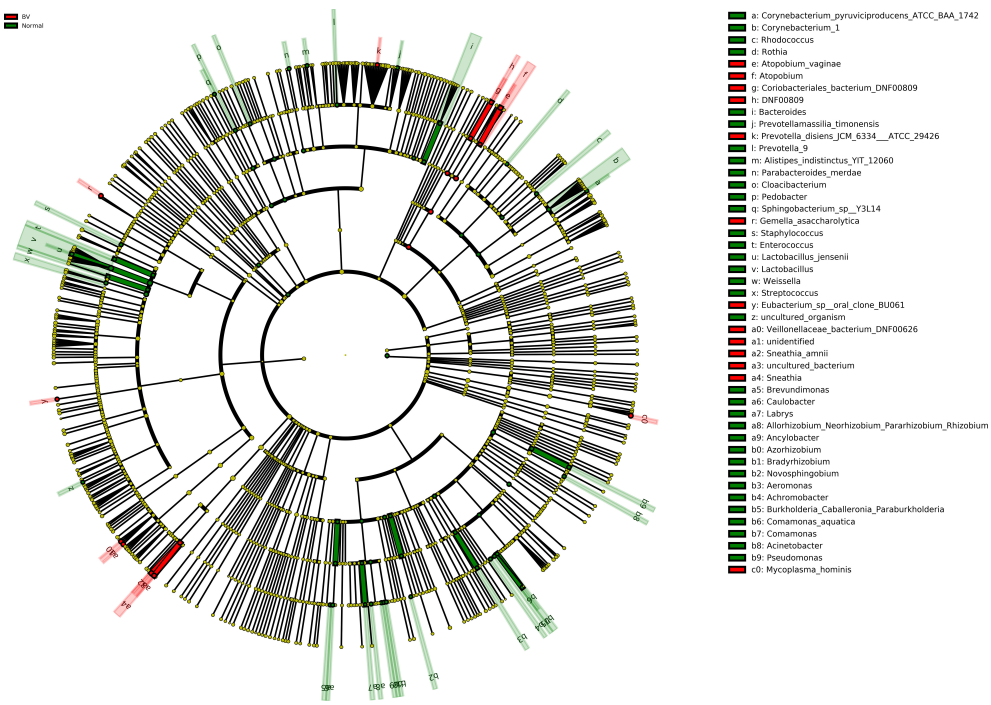

FIGURE 4 | Cladogram representation of differentially abundant bacterial families detected using LEfSe. The cladogram diagram shows the microbial species with significant differences in the two groups. Red and green, indicate BV and normal microbiota groups respectively, with the species classification 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.

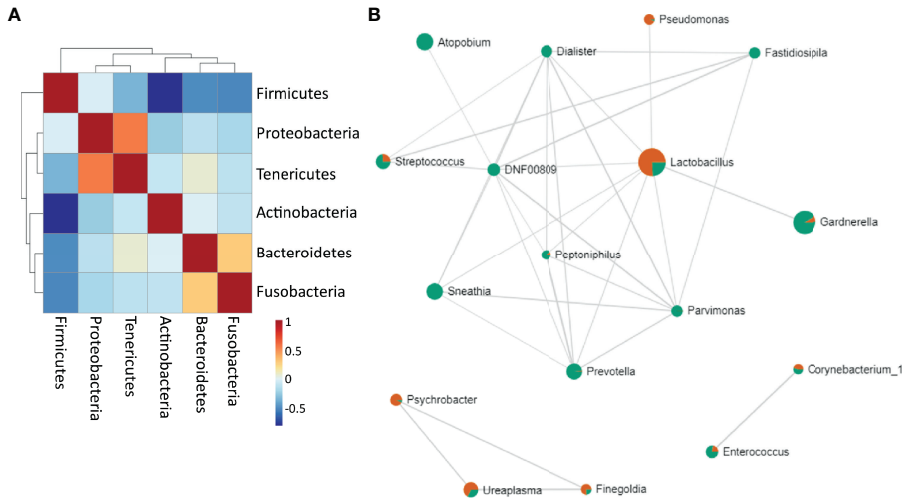

FIGURE 5 | Bacterial correlation **(A)** at the phylum level. Positive correlations are represented as red and negative correlations as blue **(B)** Network of associations between the bacteria at the genus level. Red are samples with normal microbiota and green are BV samples. The nodes represent genera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

properties (Fatmawati et al, 2020). *Weissella* is another lactic acid bacterium known to produce H_2O_2 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

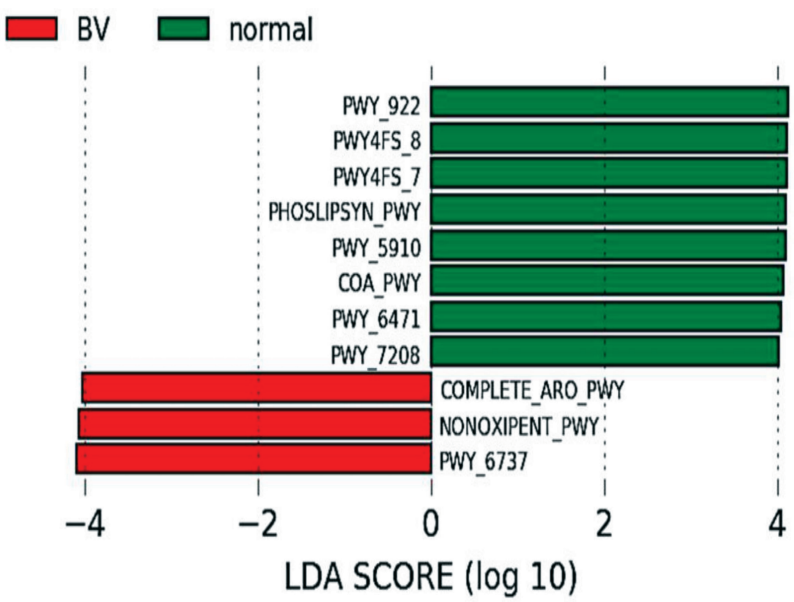

FIGURE 6 | Predicted functional profiling: Differential pathway abundance predicted by PICRUSt between normal and BV group. Pathways (BioCyc IDs) with an LDA score of 4 and above in normal (Green) and BV (Red) samples are represented.

TABLE 2 | The enriched pathways in vaginal microbiota of Indian women.

Sample	Pathway code	Pathway name
Normal samples	PWY 922	Mevalonate pathway I
	PWY4FS-8	phosphatidylglycerol biosynthesis II
	PWY4FS-7	phosphatidylglycerol biosynthesis I
	Phoslipsyn-PWY	superpathway of phospholipid biosynthesis I
	PWY-5910	Geranylgeranyl diphosphate biosynthesis I
	Coa-pwy	coenzyme A biosynthesis I
	PWY-6471	peptidoglycan biosynthesis IV
	PWY-7208	pyrimidine nucleobases salvage
BV samples	Complete ARO PWY	Amino acid biosynthesis
	Nonoxipent pwy	Pentose phosphate pathway
	PWY 6737	carbohydrate degradation

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 were enriched in asymptomatic BV women. Women who gave preterm birth had a vaginal microbiome enriched with the nonoxidative branch of the pentose phosphate pathway (Odogwu et al., 2021). Thus, functionally, many of the enriched BV pathways were also reflective of vaginal microbiota associated with preterm birth. Our findings of functionally enhanced pentose phosphate pathway in asymptomatic BV women have implications of the possibility of preterm deliveries in these women if not treated. Though the need to treat or not treat BV has been controversial our study supports that women with asymptomatic BV need to be treated to prevent adverse pregnancy outcomes.

From this study, we were able to establish the vaginal microbiome of non-pregnant Indian women for the first time. Our study shows that women with no genital symptoms of bacterial vaginosis may have a dysbiotic microbiome. The prevalence of asymptomatic BV in otherwise healthy women and functional pathways 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

funding and review of the manuscript. All authors contributed to the article and approved the submitted version.

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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

Supplementary 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

Supplementary 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.

Supplementary Table 1 | Sequence statistics of the samples after deionizing by DADA2

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

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Bacterial Vaginosis: What Do We Currently Know?

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The vaginal microbiome is a well-defined compartment of the human microbiome. It has unique conditions, characterized by the dominance of one bacterial species, the *Lactobacilli.* This microbiota manifests itself by a low degree of diversity and by a strong dynamic of change in its composition under the influence of various exogenous and endogenous factors. The increase in diversity may paradoxically be associated with dysbiosis, such as bacterial vaginosis (BV). BV is the result of a disturbance in the vaginal ecosystem; i.e., a sudden replacement of *Lactobacilli* by anaerobic bacteria such as *Gardnerella vaginalis, Atopobium vaginae, Ureaplasma urealyticum, Mycoplasma hominis*, and others. It is the most common cause of vaginal discharge in women of childbearing age, approximately 30% of all causes. The etiology of this dysbiosis remains unknown, but its health consequences are significant, including obstetrical complications, increased risk of sexually transmitted infections and urogenital infections. Its diagnosis is based on Amsel's clinical criteria and/or a gram stain based on the Nugent score. While both of these methods have been widely applied worldwide for approximately three decades, Nugent score are still considered the "gold standard" of BV diagnostic tools. Given the limitations of these tools, methods based on molecular biology have been developed as alternative rational strategies for the diagnosis of BV. The treatment of BV aims at restoring the balance of the vaginal flora to stop the proliferation of harmful microorganisms. Prescription of antibiotics such as metronidazole, clindamycin, etc. is recommended. Faced with the considerable uncertainty about the cause of BV, the high rate of recurrence, the unacceptable treatment options, and clinical management which is often insensitive and inconsistent, research on this topic is intensifying. Knowledge of its composition and its associated variations represents the key element in improving the therapeutic management of patients with the most suitable treatments possible.

Keywords: vaginal microbiome, *Lactobacillus*, dysbiosis, bacterial vaginosis, sexually transmitted infection, bacterial vaginosis-associated bacteria

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1 INTRODUCTION

The vaginal microbial community is complex and dynamic, consisting of a group of bacteria typically characterized by abundant *Lactobacilli* that evolve during the life of the woman, depending on age, hormonal estrogen levels, sexual practices and the environment (Kumar et al., 2011; Bilardi et al., 2016b). The vaginal microbiota plays a crucial role in women's health (infection, reproduction…), and that of their fetuses (Li et al., 2012).

BV is a dysbiosis of the vaginal microbiota characterized by a shift from *Lactobacilli* dominance to that of a mixture of various anaerobic bacteria (Mårdh, 1993; Hay, 2002). It is the most common vaginal disorder worldwide in women of childbearing age (Cristiano et al., 1996; Hogan et al., 2007; Trabert and Misra, 2007). BV is associated with significant adverse healthcare outcomes, including increased susceptibility to sexually transmitted infections, urogenital infections, pelvic inflammatory disease, and an increased risk of abnormal pregnancy (Marrazzo and Hillier, 2013). The etiology of BV is still unknown. Standard antibiotic therapy often fails, with an estimated relapse rate of 50% at six months follow-up (Bradshaw et al., 2006; Bretelle et al., 2015).

2 NORMAL HEALTHY VAGINAL FLORA

The vaginal ecosystem is colonized from the very first hours of the birth of a female and remains throughout her life until death (Romero et al., 2014). Women of childbearing age produce about 1 to 4 mL of vaginal fluid, containing 10^8 to 10^9 bacterial cells per mL (Danielsson et al., 2011).

2.1 Composition of Normal Vaginal Flora

The vaginal flora was first described by the German gynecologist Albert Döderlein in 1892, who reported a homogeneous vaginal flora of gram-positive bacilli in healthy women (Lepargneur and Rousseau, 2002). They were named "Döderlein's bacilli" and were later identified as members of the Lactobacillus genus by Beijerink in 1901 (Lepargneur and Rousseau, 2002). Under normal conditions, 70-90% of the vaginal bacterial species in healthy premenopausal women are *Lactobacilli* (Africa et al., 2014). As molecular techniques have advanced, our understanding of the diversity and complexity of the vaginal bacterial community has broadened (Fredricks et al., 2005). Among more than 200 *Lactobacillus* species with standing in the nomenclature, over 20 species have been found in the vaginal flora (Huang et al., 2014). Sequencing of the 16 rRNA gene revealed that the vaginal bacterial community, mainly composed of *Lactobacilli*, is classified into five groups named community state types, namely I, II, III, IV and V (Ravel et al., 2011). Four of these groups are dominated by *Lactobacillus*. The first is dominated by *L. crispatus*, the second by *L. gasseri*, the third by *L. iners*, and the fifth by *L. jensenii*, while the fourth contains a smaller proportion of *Lactobacilli* but is composed of a polymicrobial mixture of strict and facultative anaerobes (*Gardnerella, Atopobium, Mobiluncus, Prevotella…*). Although there is always a temporal transition between vaginal bacterial communities (**Figure 1**) (Gajer et al., 2012).

Thus, many other bacteria are present at lower concentrations in healthy vaginal flora, such as *Peptostreptococcus, Bacteroides, Corynebacterium, Streptococcus*, and *Peptococcus* (Kumar et al., 2011).

The composition of the vaginal microbiota evolves throughout a woman's lifespan. Various physical and hormonal changes occur in the vagina biotope during these different stages of a woman's life (Muhleisen and Herbst-Kralovetz, 2016; Nuriel-Ohayon et al., 2016) (**Figure 2**).

2.2 Variability of Vaginal Flora According to Ethnicity

Vaginal bacterial communities of women of childbearing age may vary between women from different regions, but also between women of different ethnicities living in the same geographical area (Hickey et al., 2012). In 2011, a study by Ravel et al. characterized the vaginal microbiota of asymptomatic North American women with pyrosequencing, showing that the vaginal flora of Asian and white American women was dominated by *Lactobacilli*, unlike Hispanic and African-American women, of whom only 60% had a Lactobacillus-dominated vaginal flora (Ravel et al., 2011). In addition, Caucasian and Asian women tend to have high levels of *L. crispatus* and lower levels of *L. iners* compared to African women (Green et al., 2015). In another study using 16S rRNA gene sequencing, Fettweis et al. demonstrated that the vaginal microbiota of European-ancestry women was dominated by *Lactobacilli*, as opposed to African-American women, who presented a mixed vaginal community containing, among others, *Mycoplasma hominis, Aerococcus*, *L. iners* and numerous strict anaerobes, including gram-positive anaerobic cocci, BV-associated bacteria, *Sneathia, Prevotella amnii, Megasphaera, Atopobium*, and *Gardnerella vaginalis* (Fettweis et al., 2014). The vaginal pH also differs between racial groups. African-American and Hispanic women had a vaginal pH (4.7 and 5.0, respectively) higher than what is considered the norm (<4.5) (Hickey et al., 2012).

2.3 Role of the Vaginal Microbiota in Women's Health

The vaginal flora presents one of the most important defense mechanisms for reproductive function and maintaining a healthy environment. The stability of this flora prevents the proliferation of commensal microorganisms and colonization by pathogens, thereby preventing infection (Deidda et al., 2016; Donders et al., 2017). Bacteria form a adhered monolayer on the vaginal mucosa and produce antimicrobial compounds that maintain this health equilibrium, such as hydrogen peroxide (antimicrobial product protecting against deleterious microorganisms) (Cherpes et al., 2008; Sgibnev and Kremleva, 2015), lactic acid (which maintains the normal vaginal pH between 3.5 to 4.5) (O'Hanlon et al., 2013; Tachedjian et al., 2017), bacteriocins (antibiotics that inhibit the growth of

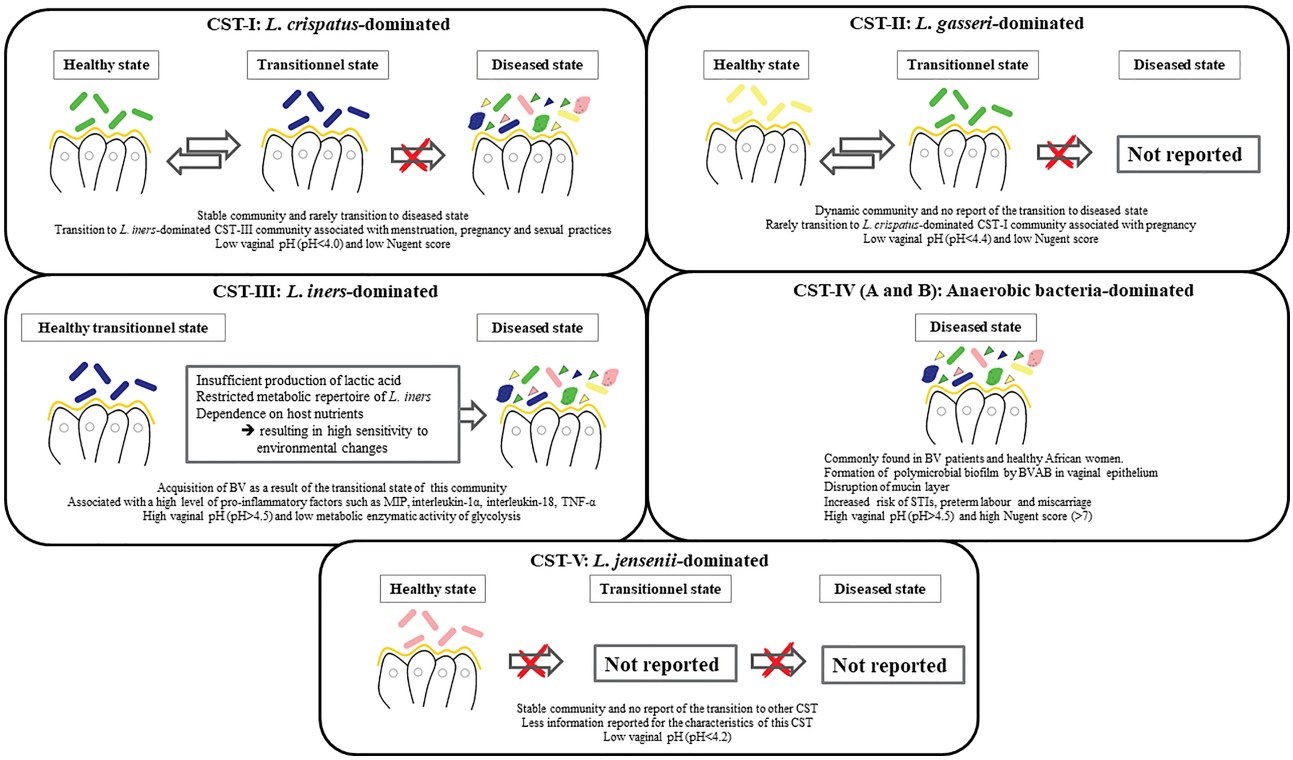

FIGURE 1 | Schema illustrating the 5 vaginal community state types (CSTs).

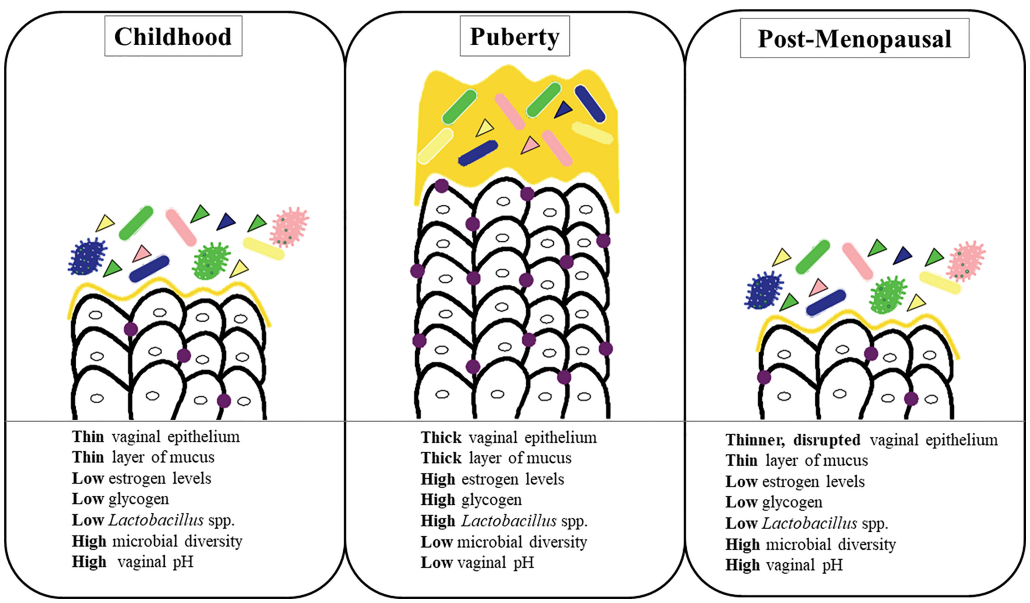

FIGURE 2 | Radical change in the vaginal microbiome over a woman's lifespan.

harmful microorganisms within the vagina) (Stoyancheva et al., 2014), and arginine deaminase enzyme (metabolizes arginine into citrulline and ammonia (NH3), depriving anaerobic pathogens of this amino acid necessary for their growth) (Rousseau et al., 2005; Makarova et al., 2006).

Notably, *L. crispatus* and *L. jensenii* may produce hydrogen peroxide, an oxidizing agent, toxic for catalase-negative bacteria and also capable *in vitro* of inhibiting HIV-1 and herpes simplex virus type 2 (Aldunate et al., 2013; Borges et al., 2014). The vaginal acids produced can, in the presence of viral RNA, stimulate the maturation of dendritic cells, activation of 17 subclasses of T helper lymphocytes, and the production of protective inflammatory cytokines and interferon-γ (Witkin, 2015).

In addition to the role of *Lactobacilli*, cervical mucus is mainly composed of mucin, which protects the vaginal mucosa and optimizes its barrier role against microbial colonization. Analyses of the composition of cervical mucus and vaginal secretions have demonstrated the presence of several proteins with antimicrobial activities that act independently of the presence of antibodies, such as lactoferrin, lysosyme, calprotectin [also known as MRP8/MRP14 ("myeloid related protein")], cathelicidin LL-37 (Nasioudis et al., 2017).

3 BACTERIAL VAGINOSIS

3.1 Background

Formerly known as non-specific vaginitis (Amsel et al., 1983), BV is characterized by a change in the vaginal flora composition, with a dramatic depletion of *Lactobacilli* due to a significant overgrowth of obligate or facultative anaerobes previously a minority in the vagina (Mårdh, 1993; Marrazzo and Hillier, 2013), such as *Gardnerella vaginalis, Atopobium vaginae, Ureaplasma urealyticum, Mycoplasma hominis, Prevotella, Peptoniphilus, Megasphaera, Mobiluncus*, and several fastidious and uncultured bacteria, including BV-associated bacteria (BVAB-1 to 3) (Romero et al., 2014; Margolis and Fredricks, 2015; Zozaya et al., 2016). The factor triggering this overgrowth of anaerobic bacteria is unknown. It is linked to an alkaline vaginal ecosystem due to an increase of vaginal pH following the loss of *Lactobacilli* protective effects (**Figure 3**).

The diversity of the vaginal flora in patients with BV was described in 1921 by Schröder (Kumar et al., 2011). In 1955, Gardner and Dukes claimed that the etiological agent of BV was *Haemophilus vaginalis* (Gardner and Dukes, 1955), a gram-negative rod later renamed *Gardnerella vaginalis* (Green et al., 2015; Mendling, 2016). The bacteria present in the microbiota of BV form a biofilm on the vaginal epithelium and secrete a cytotoxin capable of killing the epithelial cells (Huang et al., 2014). In addition, *G.vaginalis* produce proteolytic enzymes able to degrade proteins and decarboxylases that convert amino acids. Not being degraded, the amine compounds become malodorous (fishy odor: "Whiff test") thank to an increase of the vaginal pH (Gonzalez Pedraza Aviles et al., 1999). Subsequently, cytotoxicity resulting from the combination of organic acids present in the vagina during BV and bacterial polyamines leads to the production of a vaginal discharge caused by the exfoliation of vaginal epithelial cells (Sobel, 2017). Furthermore, this bacterium, cover the vaginal epithelial cells, causing the formation of "clue-cells", a specific characteristic of BV (Khan et al., 2007).

A few years later, *G. vaginalis* was found in 40% of healthy women, and its role was disputed (Machado et al., 2016). Thus, colonization of *G. vaginalis* does not always promote BV (Hickey and Forney, 2014), suggesting that this bacterium alone may be essential but not sufficient for BV development. In addition to *G. vaginalis*, some anaerobic bacteria are highly associated with BV, indicating that BV is a polymicrobial syndrome, which does not follow Koch's postulates (Kumar et al., 2011).

Recent progress in research of BV pathogenesis have determined the existence of 13 different species within the genus *Gardnerella* (Vaneechoutte et al., 2019). Although these *Gardnerella* species may be closely linked genetically, only a few of them may be implicated in disease such as BV (Vaneechoutte et al., 2019). Healthy women may be colonized by non-pathogenic *Gardnerella* species, whereas virulent strains are involved in the development of BV. Advances in technology, particularly next-generation sequencing, have clarified much of this issue. Arguably, all papers dealing with *G. vaginalis* prior to that of Vaneechoutte et al. are not specifically about *G. vaginalis* but rather about *Gardnerella* spp (Vaneechoutte et al., 2019).

Based on a recent prospective study, an updated conceptual model on the pathogenesis of BV was outlined (Alves et al., 2014; Schellenberg et al., 2016; Castro et al., 2017; Muzny et al., 2019). The potential synergistic relationship between *G. vaginalis, P. bivia, A. vaginae* was studied (Muzny et al., 2018; Gilbert

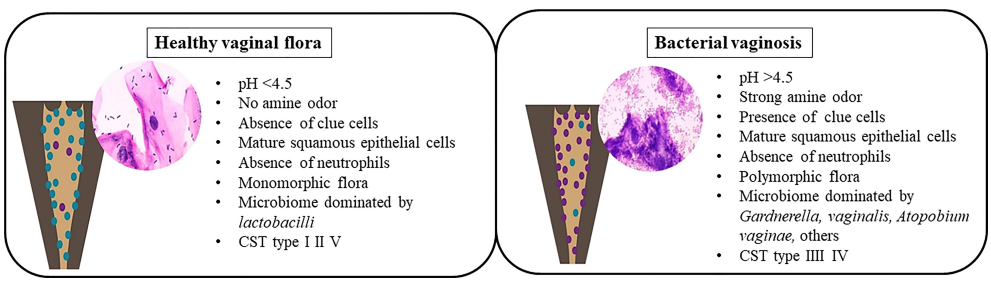

FIGURE 3 | Healthy vaginal flora versus bacterial vaginosis.

et al., 2019). After sexual exposure to virulent strains of *G. vaginalis*, these strains displace vaginal *Lactobacilli* and begin to form a BV biofilm on the vaginal epithelium (Machado and Cerca, 2015; Beebout et al., 2019). Subsequently, proteolysis by *G. vaginalis* occurs which promotes the growth of *P. bivia*. This bacterium produces an ammonia product which in turn promotes the growth of *G. vaginalis* and the biofilm develops (Gilbert et al., 2019; Castro et al., 2019). These 2 bacteria then produce sialidase that degrades the biofilm and *P. bivia* may thus degrade the mucin layer of the vaginal epithelium (Briselden et al., 1992; Gilbert et al., 2019). After the loss of the protective mucus layer, increased adhesion of other BV-associated bacteria, including *A. vaginae*, to the polymicrobial biofilm will occur (Hardy et al., 2016). The role of the other bacteria remains unknown (Muzny et al., 2019). Further research focusing on the complex interactions between bacteria during BV is needed.

3.2 Diagnosis

BV ranges from asymptomatic to an increase in vaginal discharge with or without a fishy odor (Gardner and Dukes, 1955; Majigo et al., 2021). The collection of a specimen for diagnosis can be performed using a speculum during the pelvic exam. When there is no reason for a pelvic exam as part of the clinical evaluation, a self-collected vaginal swab may also be provided (Menard et al., 2012; Camus et al., 2021).

3.2.1 Amsel Criteria and Nugent's Score

Two main categories of diagnostic strategies for BV exist: the "bedside" method introduced in 1983, mainly based on real-time clinical criteria –"Amsel's criteria" (Amsel et al., 1983), and laboratory-based testing developed in 1991, relying on the evaluation of morphotypes on gram staining – " Nugent's score" (Nugent et al., 1991). Amsel's criteria and Nugent's score are the most common diagnostic methods used for BV. Furthermore, the World Health Organization (WHO) has considered the Nugent's score as a gold standard for studies. However, the current recommended best clinical practice for diagnosing BV in women is gram staining microscopy according to the Hay-Ison criteria, as it is easier and faster to use (Sherrard et al., 2018). The Hay-Ison criteria were analogous to the Nugent's scores. Hay's grades I, II, and III were similar to Nugent's scores 0-3, 4-6, and 7-10 (Ison and Hay, 2002).

Even if Nugent's score is considered as a gold standard by the WHO, it has some pitfalls. In fact, intermediate flora is so far an uncharacterized category and is a challenge in the diagnosis of BV. In addition, the identification of morphotypes is subjective and technician-dependent, thus the diagnosis may be influenced by individual skills and experience (Menard et al., 2008; Antonucci et al., 2017).

Recently, a study made by Wang et al. provided a proof of concept for a deep learning-based model to quantify Gram staining and, consequently, automated Nugent score classification. Deep learning methods, particularly convolutional neural network (CNN) models, have demonstrated excellent performance in computer vision tasks. This model outperformed human healthcare professionals in terms of accuracy and stability for three diagnostic categories of Nugent scores. The deep learning model may offer translational applications in automating the diagnosis of BV with appropriate supporting hardware (Wang et al., 2020).

3.2.2 Molecular Diagnostic Technique

The diagnosis of BV is problematic and challenging because of its intricate polymicrobial features and a wide range of clinical features (Money, 2005). In order to overcome these diagnostic problems, alternative diagnostic strategies have been attempted, such as molecular, enzymatic and chromatographic techniques.

A molecular technique used in the diagnosis of BV is specific quantitative real-time PCR (qPCR) test. It is a quantitative, reproducible and reliable molecular biology tool that measures the presence of bacteria presents in BV, such as *Atopobium vaginae, BVAB2, Gardnerella vaginalis, Leptotrichia/Sneathia* spp., *Megasphaera* spp., and *Mobiluncus* spp.… (Breding et al., 2020). Many studies proposed objective molecular cut-off values from bacterial load to predict BV (Menard et al., 2008; Redelinghuys et al., 2017).

Several commercially molecular diagnostic assays have been reported for the diagnosis of BV in women including the NuSwab R multiplex quantitative PCR (Cartwright et al., 2012; Cartwright et al., 2018), SureSwab BV DNA real-time quantitative assay, BD Max vaginal panel (Gaydos et al., 2017) and BV multiplex assay (Hilbert et al., 2016). The quantification of these bacteria therefore makes it possible to establish a precise diagnosis of BV, with a sensitivity ranging from 90.5% to 96.7% and a specificity ranging from 85.8% to 95% compared to Amsel criteria and Nugent score (Coleman and Gaydos, 2018).

Even though these tests have a higher sensitivity and specificity than the currently available diagnostic tools, they are not point-of-care tests and are more expensive. However, Dessai et al. are the first to report on the performance of the BD AffirmTM VPIII test as a POCT in a prenatal population. But the test has been shown to be inadequate as a screening test for vaginal infections in pregnancy (Dessai et al., 2020).

Finally, POC tests for BV are not available or are simply too expensive to be used routinely. It is therefore mandatory that the development and evaluation of new diagnostic tests include a cost analysis.

3.2.3 Other Emerging Strategies

As the presence of sialidase is currently considered a key indicator of BV in the clinical examination, an enzymatic approach has been developed: The OSOM R BVBlue R test as a POC diagnostic test for BV. It is based on the qualitative detection of a high level of sialidase produced by anaerobic pathogens in vaginal fluid samples. It has been shown to be reliable compared to conventional methods such as Amsel criteria and Nugent score (Myziuk et al., 2003; Shujatullah et al., 2010; Khatoon et al., 2013; Madhivanan et al., 2014)

In addition, a recent study by Liu et al., showed the feasibility of turn-on tetravalent sialic acid-coated tetraphenylethene luminogen (TPE4S) as a powerful diagnostic tool for high-throughput fluorescence-guided diagnosis of BV. This study uses light signal intensity to detect and measure the relative concentration of sialidase in a vaginal sample. All reagents are

present in a reagent bead and sample buffer, essentially allowing for a one-step test. The test is highly sensitive and quantitative, with a sensitivity and specificity of 95.40% and 94.94%, respectively, compared to the Amsel method and 92.5% and 91.8% compared to the BVBlue diagnostic results. Notably, this method gives a more accurate classification and quantification of BV severity based on relative fluorescence intensity (I/I0). Thus this test can be a potential tool for diagnosis of BV, and risk assessment of patients with BV based on sialidase activity levels and monitoring of antibiotic therapy (Liu et al., 2018).

Another new approach based on immunodetection also targeting sialidase has been developed for the diagnosis of BV. The nanophotonic operating principle of this biodetection method allows a cheaper, faster and simpler analysis than the indirect enzyme-linked immunosorbent assay (ELISA). This nanotechnology has a high sensitivity and specificity (96,29%, respectively). This method offers an original approach to perform a very rapid diagnosis of BV (Rodríguez-Nava et al., 2021).

3.3 Epidemiology and Risk Factors

BV may appear at any reproductive age (between 15 to 44 years-old). Its prevalence rates vary considerably among the geographic regions of the world, within the same country, and even within the same population, depending on ethnic origin and socioeconomic status. Although its exact prevalence remains difficult to determine, BV occurs between 4-75%, depending on the population studied (Onderdonk et al., 2016; Bitew et al., 2017). Intermediate in the USA (29%), the prevalence of BV is estimated to be low in Europe, with a maximum (> 20%) in Poland and Norway (Kenyon et al., 2013). In Africa, the estimated prevalence tends to be high. However, BV prevalence is lowest in west Africa (6-8% in Burkina Faso, 14.2% in Nigeria) than southern and eastern Africa: 32.5% in Zimbabwe, 37% in Kenya, 38% in Botswana, and 68.3% in Mozambique (Kenyon et al., 2013; Afolabi et al., 2016; Bitew et al., 2017).

3.3.1 Sexual Practices

Although the absence of a known causal agent makes it difficult to characterize BV as a sexually transmitted infection (STI) (Morris et al., 2001; Reid, 2018), it is strongly associated with sexual activities and has some characteristics of a sexually transmitted disease not by microorganism transfer, but by mechanical or chemical interaction such as contact with highly alkaline semen (Guédou et al., 2013; Muzny et al., 2013; Lewis et al., 2017). Overall, BV is diagnosed in post-pubertal women who have never been sexually active, but at a lower prevalence than those who are sexually active (Cherpes et al., 2008). The prevalence varies with the number of sex partners. It has been evaluated at 18.8% for non-sexually active women, 22.4% for women with one lifetime partner and 43.4% and 58% for women having 2-3 lifetime sex partners and those having ≥ 4-lifetime sex partners, respectively (Koumans et al., 2007).

In this dynamic, sex workers had a higher bacterial vaginal diversity but a much lower abundance of *Lactobacillus* species than women who are not engaged in sex work (Wessels et al., 2017). Compared with male partners of healthy women, BV-related bacteria can be found in the penile skin, urethra (Zozaya et al., 2016), spermatozoa, and prostatic fluid microbiota (Gallo et al., 2011; Hou et al., 2013) of male partners of women with BV. Furthermore, biofilm fragments have been found in their urine and sperm (Swidsinski et al., 2010a; Swidsinski et al., 2010b), suggesting that male partners are a reservoir, and also that heterosexual transmission may occur. Nevertheless, there is no corresponding illness in male partners (Verstraelen et al., 2010). Use of condoms by male partners also prevents acquisition and recurrence of BV (Verstraelen et al., 2010). Also, since the preputial area of some men hosts BV-associated microorganisms, male circumcision may reduce the risk of BV (Margolis and Fredricks, 2015).

Prevalence rates also depend on the nature of the couple and their sexual practices. In fact, BV prevalence varies between 10-30% in heterosexual women, and is more frequent, 25-50%, in women who have sex with women (WSW) (Forcey et al., 2015; Bilardi et al., 2016b). The reasons for this difference in prevalence are not clear, although sexual activities involving the transmission of vaginal fluid increase the risk of BV acquisition (Marrazzo and Hillier, 2013). Several studies have indicated that certain sexual behaviors, including non-coital sexual practices such as digital and penile penetration, anal and oral intercourse followed by vaginal penetration, enhance the risk of BV (Kenyon and Osbak, 2014). In WSW, a symptomatic female sexual partner, receptive oral sex, and the use and sharing of unwashed sex toys constitute risk factors for BV (Cherpes et al., 2008). These observations have led some to consider BV as not an infection, but rather a taxonomic change in the vaginal microbiota resulting from translocation of oral (Africa et al., 2014) or fecal (Fenollar and Raoult, 2016) microbiota during non-coital sexual practices.

3.3.2 Other Bacterial Vaginosis Risk Factors

Additionally, genital hygiene can also promote disequilibrium in the vaginal microbiota. One study found that patients who did not wash their vaginal region were more susceptible to BV than those who often washed the vaginal region, a prevalence of 53.9% and 40.2%, respectively. Similarly, the prevalence of BV is higher in patients who do not change their underpants frequently compared to those who change it more frequently (57.6% versus 36.9%) (Bitew et al., 2017). In addition, other sexual sanitary habits, including vaginal douching and washing (Aslan and Bechelaghem, 2018), as well as cigarette smoking (Nelson et al., 2018), certain contraceptive methods like disposable intra-uterine devices (Achilles et al., 2018) and stress (Marrazzo and Hillier, 2013) may also enhance the risk of developing BV.

3.4 Bacterial Vaginosis Complications and Women's Health

Women with BV are vulnerable: the presence of BV-related bacteria and/or sexually transmissible microorganisms in the BV microbiota can lead to opportunistic infections (Wiesenfeld et al., 2003; Brotman et al., 2014; Rumyantseva et al., 2019; Shipitsyna et al., 2020). During this imbalance, 10-30% of pregnant women with BV give birth prematurely, a preterm delivery often accompanied by perinatal mortality, up to 70% worldwide (Svare et al., 2006; Afolabi et al.,

2016). During pregnancy, BV increases the risk of preterm labor, late miscarriage, intrauterine fetal death, preterm rupture of the membranes, amniotic fluid infections, chorioamnionitis, post-abortion and postpartum infections in these women (Wilson et al., 2011; Nelson et al., 2015a; Kairys and Garg, 2017; Brown et al., 2018).

In non-pregnant women, bacteria involved in BV can initially cause cervicitis, endometritis, salpingitis, and urinary tract infections (Georgijević et al., 2000). After damage of the cervix, bacteria can migrate from the lower to upper genital tract, reaching the uterus and fallopian tubes and causing illnesses such as pelvic inflammatory disease (Wilson et al., 2011; Sharma et al., 2014), post-hysterectomy infections (Marrazzo and Hillier, 2013), and even cervical cancer or tubal infertility (van van Oostrum et al., 2013; Babu et al., 2017). Likewise, BV is associated with significantly increased rates of acquiring herpes simplex virus (Nardis et al., 2013), human immunodeficiency virus (McClelland et al., 2018), papillomavirus (Kero et al., 2017) and transmission of the pathogens causing syphilis, chancroid, gonorrhea, trichomoniasis, and chlamydia (Brotman, 2011; Bitew et al., 2017).

3.5 Treatment and Management of Bacterial Vaginosis

Considering that clinical cure corresponds to the disappearance of all symptoms, the treatment of BV is currently focused on stopping the proliferation of BV-associated microorganisms and restoring the normal vaginal flora (Marrazzo and Hillier, 2013). Typically, clinical therapies include the use of antibiotics with broad activity against anaerobic microbes and protozoa: clindamycin and the nitroimidazoles (metronidazole and tinidazole) and/or use of probiotics (Kumar et al., 2011; Bacterial Vaginosis, 2015; Bradshaw and Sobel, 2016).

3.5.1 Antibiotic Therapies

The first line of therapy recommended by the World Health Organization (WHO) is 500 mg oral metronidazole twice daily for one week (Bacterial Vaginosis, 2015; World Health Organization, 2021). However, treatment with metronidazole may cause side effects such as gastrointestinal pain, nausea, and vomiting (Machado et al., 2016). Other proposed therapeutic regimens include 300 mg oral clindamycin twice daily for one week, 100 mg of intravaginal clindamycin ovule daily for 5 days and an application of 0.75% intravaginal metronidazole gel for 5 days or 2% of intravaginal clindamycin cream at bedtime for one week (Donders et al., 2014; World Health Organization, 2021). However, it should be noted that local application of clindamycin may damage latex-based products such as condoms and may also trigger pseudomembranous colitis (Machado et al., 2016).

In addition, the use of tinidazole, a drug similar to metronidazole, has been approved and proposed as an alternative therapy in an oral regimen (either 2 g/day for 2 days or 1g/day for 5 days) if metronidazole and clindamycin are not tolerated (Bacterial Vaginosis, 2015; Dickey et al., 2009).

Some researchers have evaluated the efficacy of other antimicrobial agents, such as azithromycin, secnidazole or ornidazole and rifaximin (Schwebke and Desmond, 2007; Thulkar et al., 2012; Laghi et al., 2014). Secnidazole has shown activity similar to that of the recommended nitroimidazoles and also spares *Lactobacilli*, a beneficial characteristic in BV treatment. Even for Rifaximin, it acts on BV by restoring *Lactobacilli* and increasing lactic acid in patients (Bagnall and Rizzolo, 2017).

Taken locally or orally, these antimicrobial agents have almost similar efficacy, with cure rates around 58% to 92% after 1 month of treatment (Donders et al., 2014). Nevertheless, these results are temporary, leading to recurrence or re-infection at rates above 50% within 6-12 months of treatment (Bilardi et al., 2016a; Bradshaw and Sobel, 2016). The reasons for this high relapse rate remain unclear. However, it appears that, with the formation of bacterial biofilms, these recommended therapies only temporarily eradicated BV-associated microorganisms, or these bacteria are reintroduced in the vagina by their sex partners (Marrazzo et al., 2012; Bradshaw and Brotman, 2015; Margolis and Fredricks, 2015). Further, the presence of some BV-associated bacteria such as *Peptoniphilus lacrimalis, Megasphaera* type 2 and BVAB-1 to 3 at the beginning of treatment is strongly related to BV recurrence, thus causing antibiotic failure (Marrazzo et al., 2008). To this end, two recent studies have examined the acceptability, tolerability, and especially the efficacy of concomitant partner treatment to improve the cure of BV (Schwebke et al., 2021; Plummer et al., 2021). The first study by Schwebke et al, showed no significant reduction in BV recurrence in female partners after treatment of the male partner with multidose metronidazole, although women whose partners adhered to multidose metronidazole were less likely to fail treatment (Schwebke et al., 2021). The second study showed that simultaneous partner treatment had a significant change in the overall composition of genital microbiota in both partners immediately after treatment (Plummer et al., 2021).

3.5.2 Non-Antibiotic Therapies

As antibiotic treatments can have a negative impact on the stability of the vaginal flora, *Lactobacillus* probiotics, an alternative and complementary therapy to antibiotic treatment, has been developed to help restore and maintain the healthy vaginal flora (Bradshaw et al., 2012). Probiotics consist of living microorganisms that confer a health benefit on the host when they are administered in an appropriate quantity (Borges et al., 2014). Nine studies from 1989 to 2014 tested the effectiveness of vaginal or oral *Lactobacillus* probiotics (Fredricsson et al., 1989; Hallén et al., 1992; Wewalka et al., 2002; Mastromarino et al., 2009; Hummelen et al., 2010; Hemalatha et al., 2012; Ling et al., 2013; Vujic et al., 2013; Vicariotto et al., 2014). The results showed that both oral and vaginal *Lactobacillus* treatments were effective in curing acute BV. On balance, the application of *Lactobacillus* in the form of vaginal capsules (containing$\geq 10^8$ CFU of *Lactobacillus* strains per dose) or a fermented milk product (containing$\geq 5\times 10^9$ CFU of *Lactobacillus* strains per dose) may be an equally effective alternative to standard antibiotic capsules. Only the strains *L. reuteri* RC-14 and *L. rhamnonus* GR-1 have positive clinical effects (Kumar et al., 2011; Ouarabi et al., 2017). Administrated orally (twice daily) or vaginally (once weekly), probiotics may restore the normal *Lactobacillus*-dominated microbiota and reduce BV recurrence (Daliri and Lee, 2015). Nevertheless, probiotics have had minimal success in African women (Margolis and Fredricks, 2015).

In the same context, a new study on the applicability of three strains of *Lactobacillus* spp. (*Lactobacillus delbrueckii* DM8909,

Lactiplantibacillus plantarum ATCC14917 and *Lactiplantibacillus plantarum* ZX27) according to their *in vitro* probiotic capacities. These three *Lactobacillus* spp. strains have shown efficacy in the treatment of BV by limiting the growth, adhesion, biofilm formation and virulence properties of *G. vaginalis* (Qian et al., 2021).

In addition, sucrose-containing products may promote recolonization, since sucrose is metabolized by *Lactobacilli*. A triple-blind randomized clinical trial compared the efficacy of a sucrose vaginal gel versus a metronidazole vaginal gel for 5 days in 70 women with diagnosed BV (Khazaeian et al., 2018). The sucrose vaginal gel was as effective as metronidazole treatment, according to this individual RCT.

Another option is to combine *Lactobacilli* with estriol. Two studies, a PC-RCT and a head-to-head RCT, were conducted on the efficacy of vaginal capsules containing *L. acidophilus* and 0.03 mg estriol (Gynofor®) in BV (Parent et al., 1996; Donders et al., 2010). Compared to placebo, the cure rate of BV was significantly higher in the treated group, but according to the comparative study, the combination of estriol and *Lactobacilli* is equivalent, but not better, results than antibiotic treatment.

In terms of method of application, vaginal suppositories deposit *Lactobacillus* strains directly on the vaginal mucosa, whereas oral probiotics survive gastrointestinal transit and increase the number of strains in the colon and feces. In turn, this may promote recolonization of the vagina due to the proximity of the rectum and vagina (Tidbury et al., 2020).

3.5.3 New Emerging Therapies

The management of BV urgently requires implementation of new therapeutic strategies. To disrupt BV-associated biofilms, studies are underway to investigate the role of novel agents such as DNases, retrocyclines, antiseptics, and plant-derived compounds in the treatment of BV (Machado et al., 2016). Dequalinium chloride, an antiseptic, has reported similar efficacy to clindamycin intravaginal cream (Weissenbacher et al., 2012). Thymol, a molecule found in thyme essential oil, has shown an inhibitory effect on biofilms *in vitro* (Braga et al., 2010). The application of acidifying agents, such as vitamin C or buffering agents (polycarbophil or boric acid), in combination with a nitroimidazole antibiotic, has been demonstrated to reduce the recurrence of BV, potentially by destructuring the vaginal biofilm (Machado et al., 2016).

In this regard, other promising therapeutic agents for the treatment of BV are under investigation. Including DNase agents that can disrupt vaginal biofilms by targeting extracellular DNA essential for their structural integrity (Hymes et al., 2013), retrocyclin 101, a synthetic cyclic antimicrobial peptide that inhibits the growth and development of *G. vaginalis in vitro* (Hooven et al., 2012), and the amphoteric tenside pessary (WO3191), which disrupts biofilms after metronidazole treatment and promotes the growth of *Lactobacillus* species (Gottschick et al., 2017; Algburi et al., 2017).

Furthermore, given the success demonstrated by fecal microbiota transplant (FMT) in the management of various intestinal disorders and diseases such as recurrent *Clostridium difficile* infection, pseudomembranous colitis, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), the use of vaginal microbiota transplantation (VMT) in BV treatment is a new therapeutic approach that modulates the vaginal microbiota in order to eradicate this scourge and reduce adverse gynecological outcomes (DeLong et al., 2019).

Given the high rates of recurrence and relapse, research is needed to identify and evaluate these novel biofilm-disrupting treatment strategies.

4 CONCLUSIONS AND PERSPECTIVES

The taxonomic composition and bacterial proportion of the vaginal microbiota are under the influence of intrinsic and external factors over the female lifespan. In the last decades, understanding of the bacterial diversity of this ecosystem has been increased by molecular methods. Dominated by *Lactobacilli* that protect against infection, the vaginal flora of healthy women is less complex than that of patients with BV, which presents a diverse microbiota containing numerous obligate anaerobic and uncultivable species. This polymicrobial condition is associated with relatively uncomplicated clinical symptoms that do not occur in all affected women, thus complicating the determination of its etiology. Treatment is usually unsuccessful, with a high rate of relapse. Future studies that thoroughly examine the vaginal bacterial community are needed to cultivate the bacteria associated with BV and its treatment failure, in order to study antibiotic resistance and to establish more effective alternative therapeutic strategies that reduce BV symptoms as well as its associated complications. Overall, unlocking the enigma of the pathogenesis of BV is key for the prevention and management of this public health problem.

AUTHOR CONTRIBUTIONS

LAC, FF, and KD have equally contributed to the preparation of this manuscript. All authors contributed to the article and approved the submitted version.

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The Effect of Gender-Affirming Medical Care on the Vaginal and Neovaginal Microbiomes of Transgender and Gender-Diverse People

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Transgender and gender diverse individuals may seek gender-affirming medical care, such as hormone therapy or surgery, to produce primary and/or secondary sex characteristics that are more congruent with their gender. Gender-affirming medical care for transmasculine individuals can include testosterone therapy, which suppresses circulating estrogen and can lead to changes in the vaginal epithelium that are reminiscent of the post-menopausal period in cisgender females. Among transfeminine individuals, gender-affirming medical care can include vaginoplasty, which is the surgical creation of a vulva and neovaginal canal, commonly using penile and scrotal skin. The effect of gender-affirming medical care on the vagina of transmasculine individuals and on the neovagina of transfeminine individuals is poorly characterized. This review summarizes what is known of the epithelium and local microbiota of the testosterone-exposed vagina and the neovagina. We focus on potential pathogens and determinants of gynecological health and identify key knowledge gaps for future research.

Keywords: vagina, neovagina, transgender, gender diverse, microbiome, bacterial vaginosis

INTRODUCTION

Transgender and gender diverse (TGD) individuals have a gender identity that is incongruent with the sex/gender they were assigned at birth. Other key terminology used in this review is listed in **Table 1**. It is estimated that 0.2-0.5% of the North American adult population is TGD (Conron et al., 2012; Scheim and Bauer, 2015; Helman et al., 2016; Meerwijk and Sevelius, 2017; Zucker, 2017; Jaffray, 2020). Many TGD individuals seek gender-affirming medical care, such as hormone therapy

TABLE 1 | Key terminology.

Gender	Social characteristics differentiating women/girls, men/boys, and gender-diverse people, including gender identity, gender expression, gender roles, and institutional gender.
Sex	Biological characteristic differentiating females, males, and intersex people, including chromosomal, anatomical, and physiological factors.
Transgender (trans)	A person whose gender identity does not align with that associated with their sex assigned at birth. *Adjective*
Cisgender (cis)	A person whose gender identity aligns with that associated with their sex assigned at birth. *Adjective*
Transmasculine (tM)	An individual assigned female at birth who identifies as male, man/boy, masculine, non-binary and/or something other than a woman/girl. *Adjective*
Transfeminine (tF)	An individual assigned male at birth who identifies as female, woman/girl, feminine, non-binary and/or something other than a man/boy. *Adjective*
Transgender and gender diverse	An inclusive term for those who have a gender identity that is incongruent with the sex/gender they were assigned at birth. This can include many different identities such as transmasculine, transfeminine, trans, non-binary, two-spirit, genderqueer, agender, and many others.
Gender dysphoria (generic)	Discomfort or distress caused by a discrepancy between an individual's experienced/expressed gender and their assigned gender and/or primary or secondary sex characteristics.
Gender dysphoria (diagnostic label)	A diagnostic label used when an individual meets the full set of DSM-5* criteria for gender dysphoria.
Gender-affirming medical care	The process through which individuals alter their primary and/or secondary sex characteristics to align with their sense of gender identity through medical treatment.
Vaginoplasty	The surgical construction of a vaginal cavity. Vaginoplasty is a gender-affirming surgery that is undergone by some transfeminine individuals.
Full-depth vaginoplasty	A vaginoplasty surgery that creates a neovagina with sufficient depth, approximately 9cm or more
Vulvaplasty/Zero-Depth Vaginoplasty	A vaginoplasty surgery that does not create a vaginal cavity.
Neovagina	A term used to refer to the vagina that has been surgically constructed through vaginoplasty.
Vaginectomy	The surgical closure of the vaginal cavity.

**DSM-5, Diagnostic and Statistical Manual of Mental Disorders. DSM–5 is the standard classification of mental disorders used by mental health professionals in the United States.*

or surgery, to produce primary and/or secondary sex characteristics that are more congruent with their gender. Gender-affirming medical care can be a critical and life-saving step for many: a meta-analysis of 28 studies reported significant improvements in gender dysphoria (80% of individuals), psychological symptoms (78%), quality of life (80%), and sexual function (72%) for those who underwent gender-affirming medical care with hormones and/or surgery (Murad et al., 2010).

Although not all TGD individuals identify as a binary gender (either a man or woman), gender-affirming medical care, including exogenous sex hormones, hormone blockers and/or surgeries, is sometimes used by TGD people to either masculinize or feminize the body. In this respect, we use the terms transfeminine (tF) for individuals assigned male at birth but who do not identify as male and may undergo feminizing gender-affirming medical care, and transmasculine (tM) for individuals who were assigned female at birth but do not identify as female and may undergo masculinizing gender-affirming care. Likewise, we use the terms cis female (cF) for individuals who were assigned female at birth and identify as female and the term cis male (cM) for individuals who were assigned male at birth and identify as male.

Hormone therapy is a common component of gender-affirming medical care for TGD individuals. The 2015 US Transgender Survey (USTS) reported that 49% of TGD individuals have received hormone therapy and a further 29% desired it (James et al., 2019). Feminizing hormone therapy usually consists of testosterone suppression, estrogen (estradiol) and occasionally progestin; these promote the development of secondary sex characteristics such as breasts, body fat redistribution, and softening of the skin, among others (T'Sjoen et al., 2019). Masculinizing hormone therapy usually consists of testosterone, which promotes secondary sex characteristics such as suppression of menstrual cycles, voice deepening, facial and body hair growth, body fat redistribution, and clitoral enlargement (T'Sjoen et al., 2019).

Genital surgery may also be a component of gender-affirming medical care. The 2015 USTS reports that 10% of tF individuals had completed vaginoplasty (the surgical creation of a neovaginal cavity) and a further 45% of respondents reported wanting to have the procedure in the future (James et al., 2019). Vaginectomy and other masculinizing gender-affirming genital surgeries are rarer, but hysterectomy is relatively common. The USTS reported 8% of tM respondents had undergone hysterectomy with a further 44% desiring to have this surgery, but only 2% had undergone metoidioplasty and/or phalloplasty (James et al., 2019). In Ontario, Canada, 2009-2010 data estimate even higher proportions, with 15% of tF Ontarians having completed vaginoplasty and 13% of tM Ontarians having undergone hysterectomy (Scheim and Bauer, 2015).

We will employ the commonly used term "neovagina" to refer to vaginas that are surgically created by vaginoplasty, and "vagina" to refer to vaginas that were present at birth. Furthermore, we will refer to the present-at-birth vaginas of those taking testosterone therapy (tM individuals) as *testosterone dominant vaginas* (TDV) and vaginas of reproductive-aged individuals who are not taking testosterone therapy (including

both cF and TGD individuals not on testosterone therapy) as *estrogen dominant vaginas* (EDV). The field of transgender medicine is relatively new, and little is known of the effects of testosterone therapy on the TDV nor of estrogen therapy on the neovagina, but it is clear that both genital microenvironments are distinct from the comparatively better studied EDV. As social acceptance increases and access to gender-affirming medical care continues to improve, the number of TGD individuals who will need tailored gynecological care is increasing. The provision of inclusive healthcare is necessary to achieve optimal health and reduce inequities experienced by TGD communities (American College of Obstetricians and Gynecologists' Committee on Gynecologic Practice and American College of Obstetricians and Gynecologists' Committee on Health Care for Underserved Women, 2021).

This review summarizes what is known of the epithelium and local microbiota of the TDV and the neovagina. We focus on potential pathogens and determinants of gynecological health and identify key knowledge gaps. Our review raises more questions than it provides answers, underscoring an urgent need for research on these distinct genital microenvironments.

MICROENVIRONMENT OF THE TESTOSTERONE DOMINANT VAGINA

Estrogen Shapes the Vaginal Microenvironment

The vaginal mucosa is a stratified squamous epithelium that undergoes continuous renewal through proliferation of basal cells, and thus newly formed epithelial cells are pushed outward towards the lumen by the subsequent cell generations. As basal cells lose contact with the basement membrane, they begin to differentiate, expressing cytokeratins K4/K13 and K1/K10 (which form intermediate filaments, and whose expression is organ-specific), to eventually reach full maturation in the superficial layers (Waseem et al., 1998). Maturation of vaginal epithelial cells is regulated by estrogen, which promotes epithelial cell proliferation and thus increases thickness of the epithelium (Ayehunie et al., 2015). Estrogen also promotes the production of glycogen, a glucose polysaccharide, by vaginal epithelial cells (Cruickshank, 1934; Anderson et al., 2014). Cell-cell junctions are lost during cellular maturation and the loosely connected, glycogen-rich cells of the superficial layer are readily shed into the vaginal lumen. Glycogen from shed epithelial cells is catabolized by both human and bacterial α-amylases in the vaginal lumen to smaller polymers that are a preferred carbon source of beneficial *Lactobacillus* spp., but also of non-desirable anaerobic bacteria (Mirmonsef et al., 2014; Spear et al., 2014; van der Veer et al., 2019; Nunn et al., 2020). Lactobacilli then metabolize glycogen-derived polymers into lactic acid, which reduces the pH of the vaginal lumen, favoring the proliferation of lactobacilli and inhibiting the growth of pathogenic organisms such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and those associated with bacterial vaginosis (BV) (Graver and Wade, 2011; O'Hanlon et al., 2011; O'Hanlon et al., 2013; Gong et al., 2014; Mirmonsef et al., 2014; Breshears et al., 2015; Mirmonsef et al., 2016; Nardini et al., 2016; Edwards et al., 2019). In addition to reducing the pH, *Lactobacillus* spp. also prevent colonization by pathogens through the production of bacteriocins and biosurfactants (Valore et al., 2002). In the absence of *Lactobacillus* spp., the EDV is colonized by a diverse set of strict and facultative gram-positive and gram-negative anaerobes (e.g., *Atopobium*, *Prevotella*, *Gardnerella*) (Ravel et al., 2013; France et al., 2020; Holm et al., 2020; Turpin et al., 2021). Communities dominated by diverse anaerobes are reminiscent of BV, which is characterized by abnormal discharge, itching, malodour, and an elevated pH (Eschenbach et al., 1988; Schwebke et al., 1996; Redelinghuys et al., 2020). Even in the absence of symptoms, vaginal microbiomes deficient in lactobacilli ("molecular BV") are associated with impaired epithelial maturation, increased mucosal inflammation, changes in epithelial barrier function, increased susceptibility to sexually transmitted infections (chlamydia, gonorrhea, HIV and HPV, among others), and reproductive risks (Leitich et al., 2003; Wiesenfeld et al., 2003; Brotman et al., 2010; Arnold et al., 2016; Zevin et al., 2016; Joag et al., 2019; Tamarelle et al., 2019; O'Hanlon et al., 2020).

Effect of Testosterone Therapy on the Vaginal Microenvironment

Testosterone therapy is highly effective in allowing TGD individuals to develop the secondary sex characteristics associated with masculinity, but the suppression of estrogen (**Table 2**) can induce epithelial thinning reminiscent of the estrogen-deprived post-menopausal cF vagina (Baldassarre et al., 2013).

In the absence of estrogen, vaginal epithelial cell proliferation slows, and the epithelium becomes thinner and more fragile, leading to dryness, irritation, and dyspareunia (pain during intercourse) (Pessina et al., 2006; Perrone et al., 2009; Baldassarre et al., 2013). Decreased estrogen in the post-

TABLE 2 | Expected Serum Hormone Ranges of Populations of Interest (Leinung et al., 2018; Greene et al., 2019; Greene et al., 2020; M. C. Laboratories, 2021).

Population	Estrogen (pg/ml)	Progesterone (ng/ml)	Testosterone (ng/dl)
Cis Women, Reproductive-Age	15 – 350	0.9 – 24	8 – 60
Cis Women, Post-Menopausal	<10	≤0.2	8 – 60
Cis Men	10 – 40	≤0.2	240 – 950
Transmasculine	29 – 51	0.3 – 0.7	320 – 630
Transfeminine	207	Not available	9 – 34

menopausal period is also associated with reduced glycogen deposition and, combined with reduced epithelial proliferation and turnover, results in a marked reduction in the availability of free glycogen in the mucosa (Mirmonsef et al., 2015). Potentially due to reduced glycogen availability, the vaginal microbiome post-menopause is significantly less likely to be dominated by lactobacilli, and instead is more likely to be colonized with a unique, diverse microbiota. This microbiota has some overlap with molecular BV in the EDV (e.g., *Prevotella*, *Gardnerella*, *Dialister*), but is clearly distinct, with higher abundance and prevalence of genera such as *Streptococcus*, *Corynebacterium*, *Finegoldia*, *Peptoniphilus*, *Anaerococcus*, and *Bifidobacterium* and lower abundance of BV-associated genera *Atopobium*, *Sneathia*, and *Megasphaera* (Brotman et al., 2014; Mirmonsef et al., 2014; Shen et al., 2016; France et al., 2020). The importance of estrogen in shaping the vaginal microbiome is underscored by the effects of local or systemic estradiol-based hormone replacement therapy post-menopause, which restores lactobacilli dominance, decreases vaginal pH, and alleviates symptoms of vaginal fragility (Brotman et al., 2014; Shen et al., 2016). In TDV, testosterone therapy has been shown to thin the epithelium, with histological evaluation revealing lowered cell proliferation, loss of the intermediate and superficial strata, and reduced glycogen deposition compared to pre-menopausal EDV (Baldassarre et al., 2013). Recently published data indicates that TDV tissue has elevated levels of inflammation, edema, collagen fibrosis, and granulation tissue (Schardein et al., 2021). Transmasculine individuals on testosterone therapy frequently experience symptoms of vaginal atrophy similar to those of the post-menopausal state, including dryness, irritation, bleeding with vaginal penetration (sex or medical examination), and dyspareunia (Peitzmeier et al., 2014; Potter et al., 2015). These symptoms can have a substantial impact on quality of life, and as such some tM individuals opt for topical estriol or estradiol administered directly to the vaginal mucosa *via* cream, a ring, or tablets (Santen, 2015). While local estrogen-based therapy to treat vaginal atrophy is included in multiple trans care guidelines, the efficacy of this approach has not been documented in tM (Deutsch, 2016; Obedin-Maliver and de Haan, 2017; Bourns, 2020).

Microbiome of the Testosterone Dominant Vagina

To our knowledge, there has been a single study describing the TDV microbiome (Winston McPherson et al., 2019). In this study, 16S rRNA gene sequencing was used to identify the proportional abundances of bacterial species in TDV swabs collected from 28 tM individuals on testosterone therapy. Only 3/28 TDV had a *Lactobacillus*-dominated microbiota; instead, the majority of TDVs had microbiota composed of a diverse set of anaerobic taxa, more like microbiota observed in post-menopause cF than molecular BV (i.e., containing *Anaerococcus*, *Corynebacterium*, *Finegoldia*, *Peptoniphilus*, *Streptococcus*, in addition to *Prevotella*, *Dialister*, *Gardnerella*, but with low abundance of *Atopobium*, *Sneathia*, *Megasphaera*). Despite sharing some similarities with the post-menopausal vagina, the microbiota observed in the TDV was also clearly distinct, with higher abundance of *Campylobacter*, *Fusobacterium*, *Parvimonas*, and *Porphyromonas*, indicating testosterone augmentation may influence the composition of the vaginal microbiota beyond that of estrogen reduction. It is notable that, of the three individuals who had a *Lactobacillus*-dominated microbiota, two were prescribed topical estradiol (a total of 4 individuals in the study had been prescribed topical estradiol to treat symptoms of vaginal atrophy). Despite limited statistical power in this relatively small study, the correlation between vaginal estrogen therapy and presence of a *Lactobacillus*-dominated microbiota was statistically significant ($p=0.045$). This study provides an important first assessment of the TDV microbiota, suggesting it is distinct from the microbiota observed post-menopause, and that *Lactobacillus*-dominated microbiota are rare.

Knowledge Gaps

Despite the progress made in recent years, several key gaps remain in the literature. Additional studies are warranted to confirm and expand on the seminal publication by McPherson et al. (Winston McPherson et al., 2019). Larger longitudinal studies, including following TGD participants through the initiation of testosterone therapy and studies of individuals who have been on testosterone for decades, would provide detailed information on the specific effects of testosterone therapy. Additionally, many TGD individuals interrupt testosterone therapy to become pregnant (Obedin-Maliver and Makadon, 2016). Molecular BV in cF individuals is associated with higher risk of serious reproductive risks (Leitich et al., 2003); it is unknown how the unique microbiota of the TDV may influence reproductive outcomes. Finally, to inform appropriate clinical treatment guidelines for the medical care of TGD individuals, new studies should focus on relating microbiota composition and function to symptomology, immune status, and local energy sources available to microbes.

Another important knowledge gap is whether locally administered vaginal estrogen therapy could be used to treat vaginal atrophy and promote *Lactobacillus* colonization in tM individuals without interfering with the masculinizing effects of testosterone. Local estrogen therapy is commonly recommended to treat vaginal atrophy post-menopause (Kaur et al., 2020; Shim et al., 2021), and, in low doses, this therapy can alleviate symptoms without substantially increasing systemic estrogen levels (Lethaby et al., 2016). The effect of local estrogen therapy on systemic levels will likely be dependent on characteristics of the vaginal microenvironment [reviewed in (Santen, 2015)], including vaginal epithelial thickness and the local microbiome. Given the potential benefits, research is warranted to assess the acceptability and efficacy of locally administered vaginal estrogen therapy in TGD individuals on testosterone.

Third, our ability to study the effect of testosterone therapy on vaginal and cervical epithelia have been hampered by lack of appropriate model systems. Monolayers of cells in submerged culture do not replicate the stratified epithelium of the vagina and do not provide an appropriate environment for the culture of vaginal bacteria, while animal models do not replicate the

relationship between the human vagina and its unique *Lactobacillus*-dominated microbiota (Couri et al., 2012; Barfod et al., 2013; Cassone and Sobel, 2016). Three-dimensional air-liquid interface cell culture allows for stratification of cultured vaginal epithelial cells and provides a more relevant environment for vaginal bacteria (Lee et al., 2016; Zhu et al., 2017). The utility of this model in delineating the impact of testosterone therapy on the cervicovaginal epithelium warrants further investigation.

THE NEOVAGINAL MICROENVIRONMENT

Vaginoplasty and the Neovaginal Epithelium

Penile inversion vaginoplasty is the gold standard surgical technique of feminizing genital surgery (Bizic et al., 2014; Horbach et al., 2015; Buncamper et al., 2016; Dreher et al., 2018; Bustos et al., 2021; Moises da Silva et al., 2021). This surgery was first introduced in the early 1900's and has undergone various permutations in search of the optimal outcome (Horbach et al., 2015). The ideal outcome of this surgery is a concordant vulvar anatomy, moist and hairless vagina with sufficient depth and width for types of penetration desired (if any), erogenous sensation, and requiring minimal maintenance (Garcia et al., 2020). This surgery requires many surgical steps; orchiectomy, clitoroplasty, penile de-gloving and resection of the corpora cavernosa, shortening and splaying of the urethra, surgical dissection of the space between the bladder and the rectum and the inversion of the flap of preserved penile tube skin and placement into this space. Frequently, the penile tube skin alone is insufficient to generate a vaginal canal with adequate depth and additional skin grafts, most commonly from scrotal skin, are used to augment length (Selvaggi et al., 2005; Goddard et al., 2007; Goddard et al., 2007; Dy et al., 2018). Hair from scrotal grafts is typically removed intraoperatively by thinning the scrotal graft and cauterizing visible follicles. The proportion of scrotal/penile skin used to line the canal is influenced by the amount of tissue present and the depth of the pelvic dissection and is not well identified in the literature.

Alternative procedures are used to create sufficient depth in the case that the penile and scrotal skin is insufficient. These techniques are generally recommended for revision surgeries because of their accompanying risks and complications. Bowel pedicle flaps (i.e., sigmoid colon, ileum, and transverse colon), regional and isolated skin flaps (i.e., thigh or lower abdomen), peritoneal flaps, and the incorporation of urethral mucosa into the inverted skin flap are all identified in the literature. Despite the multiple options available to line the neovagina, the penile scrotal flap is by far the most commonly used (Horbach et al., 2015; Buncamper et al., 2016), with the rectosigmoid colon bowel flap as the most common alternative procedure (Horbach et al., 2015).

Understanding the physiology and structure of neovaginal canals made with different tissues is essential, because features of the neovaginal epithelium are very likely to shape microbiome composition and function, and thus are expected to have a substantial impact on gynecological health and quality of life. It is well established that environment (i.e., local levels of oxygen, humidity, and environmental exposures) shapes the local microbiome, unambiguously demonstrated by the effect of penile circumcision on the composition of the coronal sulcus microbiota (Liu et al., 2013). Elimination of the foreskin increases water loss and oxygen tension on the coronal sulcus, which decreases the abundance of many strict anaerobes (including *Prevotella, Finegoldia, Peptotreptococcus, Peptoniphilus, Porphyromonas, Dialister, Murdochiella*, and *Negativococcus*) and increases aerobes and facultative anaerobes (e.g., *Corynebacterium* and *Staphylococcus*). However, in addition to the environment, characteristics of the epithelium itself can have a dramatic influence on the local microbiome. For example, during puberty estrogen induces changes in the vaginal epithelium, increasing thickness and glycogen content, which is associated with a dramatic shift in the local microbiome, from one dominated by a diverse set of strict and facultative anaerobes to *Lactobacillus* domination and an acidic pH (<4) (Schaller, 1990).

Similar to the vaginal epithelium, penile skin is a stratified squamous epithelium that is constantly renewing through proliferation of basal cells. However, the epithelial layer of penile skin is thinner than that of the pre-menopausal EDV (100 vs 300μm) (Baldassarre et al., 2013; Carias and Hope, 2019), expresses different cytokeratins (K5/K14 in intermediate layers followed by K1/10 in superficial layers), and has a soft-cornified outer layer (15-20μm thick) comprised of terminally differentiated keratinocytes that have undergone programmed cell death, lack nuclei and organelles, and are filled with keratin bundles (Stankler and Walker, 1976; Dinh et al., 2012). Fully mature skin keratinocytes limit water loss by extruding lamellar bodies to form an intercellular lipid envelope, and provide mechanical integrity through specialized cell junctions called corneodesmosomes. Desquamation of skin corneocytes is controlled by degradation of corneodesmosomes and this process frees keratin and fatty acids that are nutrient sources for bacteria and shape the microbiota (Gupta and Ramnani, 2006; Houben et al., 2008; Bragulla and Homberger, 2009; Grice and Segre, 2011). This contrasts with the outermost layer of the EDV, which has loosely connected superficial cells filled with glycogen, which when shed release glycogen and promote colonization with lactobacilli (Pask et al., 2008; Bragulla and Homberger, 2009; Menon et al., 2012; Anderson et al., 2014; Tjernlund et al., 2015). The sigmoid colon epithelium is also highly distinct from that of the EDV and the penis. It is a single-layer columnar epithelium expressing the cytokeratin pair K8/K18 and containing highly specialized epithelial cells such as goblet cells (producing mucus) and Paneth cells (producing antimicrobial peptides), among others.

Very little is known of the influence of surgical invagination and exogenous estrogen on the differentiation pattern of epithelial cells in the neovagina. It is possible that reduced water loss from surgical invagination may alter the epithelial differentiation patterns of once-penile skin, for example, through reduced lamellar body and corneodesmosome formation, resulting in an epithelial surface more similar to the EDV. However, while vaginal

epithelial cells have the ability to differentiate into corneocytes in response to hormonal or mechanical signals, potentially owing to their expression of both K4/K13 (typical of non-cornified stratified epithelia) and K1/K10 (Schaller, 1990; Schaller and Genz, 1990; Schaller et al., 1993; Bragulla and Homberger, 2009), epithelial cells derived from skin and the sigmoid colon contain low levels of glycogen and do not express K4/K13 (Menon et al., 2012). One small study has examined the microstructure of the neovaginal epithelium created from penile skin (n=9) (Dekker et al., 2007). This study observed that cornification was reduced but not lost, and no glycogen production was observed, even among the three participants who had vaginoplasty more than nine years prior and had been receiving estrogen hormone therapy for >11 years. The absence of glycogen and retention of cornification suggest that it would be difficult for the neovagina to support a *Lactobacillus*-dominated microbiota. While a neovagina constructed from entirely penile skin and one that includes sigmoid colon may have similar environmental exposures (oxygen levels, estrogen levels, etc.), factors such as residual cornification or the presence of goblet cells producing mucus may dictate what bacteria colonize the neovagina microenvironment, and what bacteria are beneficial vs. pathogenic. Therefore, different treatment courses may be required for individuals suffering from neovaginal symptoms, depending on the tissue used to create their neovaginal canal. Altogether, our knowledge of the epithelia used for vaginoplasty does not support the notion that an optimal neovaginal microenvironment would comprise *Lactobacillus* spp. and an acidic pH.

The Neovaginal Microbiome

Despite the enormous impact of the vaginal microbiome on cF sexual and reproductive health, there have been few reports of the microbiota colonizing the neovagina and there is no knowledge of what microbiota are optimal vs. associated with inflammation, symptoms, and STI risk. Until recently, data on the microbiota colonizing the neovagina were limited to case reports and small studies that used limited culture-based detection methods or targeted PCR to detect the presence of specific species of interest (classic STI pathogens or *Lactobacillus* spp.) (Bodsworth et al., 1994; Haustein, 1995; Weyers et al., 2009; Weyers et al., 2010; de Haseth et al., 2018; Radix et al., 2019). These assays fail to capture the vast majority of species present and provide no information on the composition or structure of bacterial communities in the neovagina. Recently, one study by Birse et al. (2020) used a combination of proteomics and 16S rRNA gene sequencing to examine the neovaginal microbiome in five tF individuals, four of whom had penile inversion vaginoplasty and one whom had sigmoid vaginoplasty (median 10 years post-vaginoplasty, range 4-36 years). While the small sample size limits the ability to draw conclusions, this important study is the first examination of the neovaginal microbiome and hints at interesting hypotheses (**Table 3**). Of the four tF individuals who underwent penile inversion vaginoplasty, *Lactobacillus* was detected in one individual at low abundance. Instead, highly prevalent genera in the penile-skin lined neovagina included *Prevotella* and *Peptostreptococcus* (both also prevalent in molecular BV of the EDV, the post-menopausal vagina, and the TDV); *Peptoniphilus* and *Corynebacterium* (also prevalent in the post-menopausal vagina and the TDV); and *Porphyromonas* and *Campylobacter* (also prevalent in the TDV). Interestingly, these genera are also highly abundant/prevalent within the foreskin fold of the uncircumcised penis. The skin under the foreskin fold is usually colonized with a diverse set of strict and facultative anaerobes, the most prevalent and abundant being *Prevotella*, *Porphyromonas*, and *Peptoniphilus*, while the circumcised penis is usually dominated by *Corynebacterium* (Price et al., 2010; Liu et al., 2013). The abundance of penile anaerobes is associated with inflammation and risk of STIs in uncircumcised heterosexual cM (Liu et al., 2013; Prodger et al., 2021); it remains to be investigated if the same is true in the neovagina. It is interesting to note that, based on one study of the TDV (Winston McPherson et al., 2019) and one small study of the neovagina, that the penile skin lined neovagina appears more similar in microbiota composition to the TDV and the uncircumcised penis than to the EDV or the post-menopausal vagina.

In contrast, the neovaginal microbiome of the only participant who had sigmoid vaginoplasty was clearly distinct, completely lacking *Prevotella* and instead defined by taxa common in the gut microbiota, *Bacteroidaceae* and *Enterobacteriaceae* (Birse et al., 2020). These interesting data suggest that, even many years post-vaginoplasty, the origin of the tissue used defines the colonizing microbiota. It is therefore critically important that all future research of the neovaginal microenvironment consider tissue source, and ideally be powered to afford stratification by tissue source. As a field, researchers and clinicians should be aware that different standards of care and clinical recommendations may be required depending on the tissue used for neovaginal construction.

Knowledge Gaps

There are several important knowledge gaps in our understanding of the neovaginal microbiome that urgently need to be filled to improve neovaginal healthcare. Preliminary data from Trans PULSE Canada (n=2,873), a national survey of the TGD population in Canada, show nearly half of participants with vaginoplasty experienced gynecological symptoms in the past year, including malodor, abnormal or disturbing discharge, and itching (personal communication, Trans PULSE Canada). Such symptoms are frequently associated with BV in the EDV; however, the underlying cause of these symptoms in the neovagina remains uncharacterized. Neovaginal swabs sent for clinical diagnostics frequently return the results "altered vaginal flora inconsistent with bacterial vaginosis" and treatments established for the EDV (metronidazole) are frequently ineffective (Jain and Bradbeer, 2007; van der Sluis et al., 2020). Candidiasis is another common cause of vaginal itching in the EDV; there has been one case series published reporting neovaginal candidiasis in five individuals (de Haseth et al., 2018), warranting further characterization of neovaginal candida species and the development of clinical guidelines for prevention and treatment. The cause of neovaginal malodour also warrants further investigation. Malodour in the EDV has been associated with the production of biogenic amines by BV-associated bacteria (McMillan et al., 2015; Puebla-Barragan et al., 2021). Larger studies employing omics approaches, including metagenomics, metatranscriptomics and metabolomics to characterize the

TABLE 3 | Summary of the characteristics of the estrogen dominated vagina (EDV), post-menopause vagina, testosterone dominated vagina (TDV), sub-preputial penile skin, penile skin-lined neovagina, and sigmoid-lined neovagina.

	Epithelium	Dominant Hormone	Dominant Taxa in Microbiota
EDV	Stratified, squamous Thick (~300μm) Glycogen-rich	Estrogen	Optimal: *L. crispatus, L. gasseri, L. jensenii*; commonly dominated by a single bacterial species Molecular BV: *G. vaginalis, Ca.* L. vaginae*, A. vaginae, L. iners, Sneathia, Megasphaera, Prevotella, Anaerococcus*
Post-menopause vagina	Stratified, squamous Thinner (~150μm) Reduced glycogen	Low estrogen	*L. iners, Streptococcus, G. vaginalis, L. gasseri, Bifidobacterium, Anaerococcus, Corynebacterium, Atopobium, Enterococcus*
TDV	Stratified, squamous Thinner (~180μm) Reduced glycogen	Testosterone	*Anaerococcus, Corynebacterium, Finegoldia, Peptoniphilus, Streptococcus, Prevotella, Dialister, Gardnerella, Campylobacter, Fusobacterium, Parvimonas, Porphyromonas* Based on n=28
Penile skin	Stratified, squamous Thinner (70-100μm) No glycogen Dense keratin bundles and extracellular lipid deposition	Testosterone	*Prevotella, Peptoniphilus, Porphyromonas, Finegoldia, Anaerococcus, Dialister, Corynebacterium, Murdochiella, Ezakiella, Campylobacter, Negativococcus, Peptostreptococcus, Mobiluncus*
Penile skin-lined neovagina	Reduced cornified layer No glycogen	Estrogen	*Prevotella, Peptostreptococcus, Peptoniphilus, Corynebacterium, Porphyromonas*, *Campylobacter* Based on n=4
Sigmoid colon-lined neovagina	Unknown *Sigmoid colon is a simple, columnar epithelium with local mucus production*	Estrogen	*Bacteroidaceae, Enterobacteriaceae, Escherichia, Fusobacteriaceae, Actinomycetaceae* Based on n=1

neovaginal microenvironment in symptomatic individuals are urgently needed to inform treatment options.

Equally important is defining what constitutes an optimal neovaginal microbiota post penile inversion or sigmoid vaginoplasty. This information is essential to guiding treatment options, as what the treatment leaves untouched may be just as important as what it removes. A portion of metronidazole's efficacy in treating BV of the EDV is that it selectively spares *Lactobacillus*, and thus helps to promote an optimal microbiome that is resistant to re-colonization with inflammatory/pathogenic anaerobes (Petrina et al., 2017). While little is known of the microstructure of the penile-skin lined neovaginal epithelium, if it indeed lacks glycogen and retains cornification, it would be unlikely to promote *Lactobacillus* dominance. A minority of uncircumcised cM [~12% in Uganda (Prodger et al., 2021)] sustain a microbiota under the foreskin fold of the penis that is dominated by *Corynebacterium* with low abundance of Gram-negative anaerobes. This microbiota is associated with low inflammation and reduced risk of STI acquisition (Prodger et al., 2021); future larger studies will reveal if a similar microbiota is optimal in the penile skin-lined neovagina. Additional information on the microstructure of the neovaginal epithelium post penile inversion or sigmoid vaginoplasty would help to inform what type of bacterial commensals might promote an optimal, low-inflammation, protective neovaginal microenvironment.

There is paucity of data on the kind of practices that promote an optimal neovaginal microenvironment, both in the immediate post-operative period and for long-term hygiene and care. Due to a lack of evidence-based guidelines, neovaginal care recommendations vary substantially between centers (Grimstad et al., 2021). Frequent dilation is necessary post-operatively to prevent stenosis of the neovaginal canal (Goddard et al., 2007; Horbach et al., 2015; Buncamper et al., 2016; Loree et al., 2020); most centers recommend at least two dilations a day for the first six months decreasing to once weekly after a year (Buncamper et al., 2016). Ample water-based lubrication is recommended to increase the ease of the dilations and to protect the integrity of the surgical dilators. Frequently the use of a vaginal douche after dilation is recommended with varied solutions including water, soap, vinegar, or povidone iodine solutions (Goddard et al., 2007; Deutsch, 2016; Pan et al., 2019). Douching and the use of soaps or lubricants can promote molecular BV in the EDV of cF, but their effects on the neovaginal microbiota are unknown. Regular use of hygienic products and even boric acid [to lower the pH, promote colonization with *Lactobacillus*, and treat vaginal yeast infection (Donders et al., 2010; Iavazzo et al., 2011)] are also frequently reported, however, such efforts would be in vain, and potentially disruptive, if the optimal neovaginal microbiome is found to be dominated by *Corynebacterium* (penile skin-lined) or *Bacteroidaceae* (sigmoid-lined).

CONCLUSIONS

As access to gender-affirming hormone therapy and surgery increases, a growing number of TGD persons will need access to effective and evidence-informed gynecological care. There is

an urgent and growing need to identify the causative agents of the unique gynecological concerns of TGD populations and to define clinical guidelines to promote gynecological health. In tM individuals on testosterone therapy, vaginal pain, bleeding, atrophy, and non-*Lactobacillus*-dominated vaginal microbiota are common. Further research is warranted to establish the role of testosterone augmentation beyond that of estrogen deprivation. Anecdotal evidence suggests topical estrogen therapy may promote a *Lactobacillus*-dominated microbiome, justifying further studies to investigate if this approach can alleviate symptoms. In tF individuals with a neovagina, gynecological symptoms such as abnormal discharge, itching and malodor are common, but the etiology of these symptoms remains unknown, and treatments designed for cF may be ineffective. The limited data we have of the neovaginal microbiome (n=5) suggests that it is very unlike that of reproductive-aged or post-menopausal cF and may have more commonalities with the microbiota of the uncircumcised penis of cM or the vagina of tM on testosterone therapy. Importantly, the limited available data suggests the tissue used to create the vaginal canal may have a substantial impact on the subsequent microbiota and should be considered and reported in future research. What defines optimal vs. non-optimal microbiota in different types of neovaginas, and what bacteria are pathogenic, is yet to be defined and this information is critically needed to improve clinical management and treatment options.

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JP, JR, GB, YK, EP, HW, JH, and BM contributed to literature review, manuscript writing, and editing. All authors contributed to the article and approved the submitted version.

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Associations Between Vaginal Bacteria and Bacterial Vaginosis Signs and Symptoms: A Comparative Study of Kenyan and American Women

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Background: Bacterial colonization and associations with bacterial vaginosis (BV) signs and symptoms (Amsel criteria) may vary between populations. We assessed relationships between vaginal bacteria and Amsel criteria among two populations.

Methods: Kenyan participants from the placebo arm of the Preventing Vaginal Infections (PVI) trial and participants from a Seattle-based cross-sectional BV study were included. Amsel criteria were recorded at study visits, and the vaginal microbiota was characterized using 16S rRNA gene sequencing. Logistic regression models, accounting for repeat visits as appropriate, were fit to evaluate associations between bacterial relative abundance and each Amsel criterion.

Results: Among 84 PVI participants (496 observations) and 220 Seattle participants, the prevalence of amine odor was 25% and 40%, clue cells 16% and 37%, vaginal discharge 10% and 52%, elevated vaginal pH 69% and 67%, and BV 13% and 44%, respectively. BV-associated bacterium 1 (BVAB1) was positively associated with all Amsel criteria in both populations. *Eggerthella* type 1, *Fannyhessea* (*Atopobium*) *vaginae*, *Gardnerella* spp., *Sneathia amnii*, and *Sneathia sanguinegens* were positively associated with all Amsel criteria in the Seattle study, and all but discharge in the PVI trial.

Conclusions: Core vaginal bacteria are consistently associated with BV signs and symptoms across two distinct populations of women.

Keywords: bacterial vaginosis, vaginal microbiota, Amsel criteria, Kenya, United States, BVAB1

INTRODUCTION

Bacterial vaginosis (BV) is the most common cause of vaginal discharge worldwide, affecting approximately 26% of reproductive-age women (Peebles et al., 2019). BV is a polymicrobial condition characterized by a diverse microbiota of anaerobic and facultative bacteria, including *Gardnerella*, *Prevotella*, *Fannyhessea* (*Atopobium*), and *Sneathia* species. BV etiology remains unclear, in part because vaginal microbiota composition varies between individuals diagnosed with BV and over time within individuals (Srinivasan et al., 2010; Gajer et al., 2012; Srinivasan et al., 2012; Ravel et al., 2013; Bautista et al., 2016; Jung et al., 2017; Muzny et al., 2018; Muzny et al., 2019). Clinical BV diagnosis is based on the presence of at least three of four signs/symptoms termed Amsel criteria: amine odor on addition of potassium hydroxide to vaginal fluid; >20% clue cells on vaginal wet prep; thin, gray, homogeneous vaginal discharge; and elevated vaginal pH >4.5 (Amsel et al., 1983). In research settings, BV is often identified using a Gram stain-based classification system developed by Nugent and Hillier (Nugent et al., 1991). BV symptomatology may vary between women and between global regions, with some studies in sub-Saharan Africa reporting as few as 10% of women with Nugent-BV are symptomatic compared to one third of women with Nugent-BV in North America (Yen et al., 2003; Thoma et al., 2011; Peebles et al., 2019).

It is unclear what drives heterogeneity in BV presentation. A cross-sectional study of women in Seattle, USA identified independent associations between vaginal bacteria and individual Amsel criteria (Srinivasan et al., 2012). These findings suggest BV symptomatology may be driven by the presence or abundance of specific bacteria; however, this has not been assessed in non-US populations. Growing evidence demonstrates that vaginal microbiota composition and associations with clinical outcomes may vary between global populations (Jin et al., 2007; Spear et al., 2008; Lee et al., 2013; Brotman et al., 2014; Benning et al., 2014; Jespers et al., 2014; Balkus et al., 2016; Callahan et al., 2017; McClelland et al., 2018; Bayigga et al., 2019). To investigate the generalizability of findings from the Seattle study, we conducted a comparative analysis of the associations between vaginal bacteria and individual Amsel criteria in a population of Kenyan women enrolled in the Preventing Vaginal Infections (PVI) trial and the Seattle population described above. We performed parallel *de novo* statistical analyses in each study population to facilitate direct comparison of results between the populations.

MATERIALS AND METHODS

The PVI trial was a randomized, double-blinded, placebo-controlled trial of periodic presumptive treatment (PPT) for reducing BV and vulvovaginal candidiasis (VVC) (McClelland et al., 2015). The trial and the current study were approved by the Institutional Review Boards of Kenyatta National Hospital, the University of Washington, and the University of Alabama at Birmingham (ClinicalTrails.gov NCT01230814, October 6, 2014). All participants provided written informed consent prior to screening and a second written informed consent if they were eligible and agreed to enroll. The Seattle BV study was cross-sectional and was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center (Srinivasan et al., 2012). All participants provided written informed consent prior to enrolling.

PVI Trial Participants and Study Procedures

PVI trial participants were recruited from four research clinics: one in Mombasa, Kenya, two in Nairobi, Kenya, and one in Birmingham, Alabama (McClelland et al., 2015). The Mombasa clinic and one Nairobi clinic recruited cisgender women who reported transactional sex. The other two clinics recruited cisgender women from the general population. Eligible participants were 18-45 years old, HIV-1 seronegative, and sexually active. Women had to have a vaginal infection at screening: BV (Nugent score ≥7) (Nugent et al., 1991), VVC, and/or *Trichomonas vaginalis* (TV) infection. Women with ≥4 episodes of treatment for symptomatic vaginal infections in the prior year were excluded due to expected need for frequent open-label treatment. At screening, women with symptomatic BV or VVC received open-label treatment, and all women with TV infection received open-label treatment. Women who met enrollment criteria at screening were invited to enroll within 7-28 days. Returning women with no vaginal symptoms were eligible to enroll. During follow-up, participants with symptomatic vulvovaginitis, vaginal discharge, or vaginal itching received open-label treatment. Pelvic speculum examinations were performed and cervicovaginal swabs collected by clinicians at enrollment and follow-up months 2, 4, 6, 8, 10, and 12. At these visits, vaginal saline wet mounts were examined for clue cells, vaginal secretions tested for amine odor, vaginal pH measured using a test strip (EMD Millipore), and vaginal discharge characteristics recorded by clinicians.

Vaginal fluid was collected using a push-off polyester/polyethylene terephthalate swab (FitzCo) for PCR targeting the 16S rRNA gene (V3-V4 hypervariable regions) and Illumina MiSeq sequencing. Approximately 33,000 sequence reads were generated per sample, giving robust detection of minority species. Results were analyzed using a bioinformatics pipeline that overcomes the tendency of sequencing to overestimate species richness (Srinivasan et al., 2012). Sequence data were submitted to the NCBI Short Read Archive (PRJNA638104, **Supplementary Table 1**).

Seattle Study Participants and Study Procedures

Seattle study participants were recruited from the Public Health Seattle & King County STD Clinic (Srinivasan et al., 2012). Nonpregnant women age 18-50 were eligible, regardless of BV status. Pelvic speculum examinations were performed and cervicovaginal swabs collected by clinicians. Vaginal swabs were used for Gram staining, measuring vaginal pH, saline

microscopy, and testing for amine odor. Clinicians recorded vaginal discharge characteristics.

Vaginal fluid was collected using polyurethane foam swabs (Epicentre Biotechnologies) for PCR targeting the 16S rRNA gene (V3-V4 hypervariable regions) and 454 FLX pyrosequencing. Approximately 1,600 sequence reads were generated per sample. Results were analyzed using the same bioinformatics pipeline as above (Srinivasan et al., 2012). Sequence data were submitted to the NCBI Short Read Archive (SRA051298).

Statistical Analysis

The primary aim of this work was to characterize relationships between vaginal bacteria and Amsel criteria that are qualitatively consistent between the PVI and Seattle populations. Parallel *de novo* statistical analyses were conducted for both populations to enable direct comparison of results between the populations. The current PVI analysis was limited to participants enrolled in Kenya and randomized to placebo because the PPT intervention impacted vaginal bacterial colonization (Balkus et al., 2016). PVI trial observations immediately following open-label treatment were excluded from this analysis. Descriptive statistics were used to summarize participant demographic and behavioral characteristics; Amsel criteria prevalence; and bacterial detection and relative abundance. For each study population, 16S rRNA gene sequencing data were restricted to bacterial taxa whose mean relative abundance was in the top 25% of mean relative abundances for that study and whose prevalence of detection was ≥5% for that study. These filters limited the current analyses to taxa that were commonly detected and that are present at high enough abundances that they may reasonably have a biological impact on the vaginal environment. Amsel criteria (amine odor, clue cells, vaginal discharge, and elevated vaginal pH) were analyzed as individual outcomes and modeled as binary variables (Amsel et al., 1983). Hypothesis tests were considered significant at $p<0.05$, and a 5% Benjamini-Hochberg false discovery rate was applied to all hypothesis tests. All analyses were conducted separately in each study population, were complete case analyses, and were performed in R (version 3.6.1).

For each study population, taxa of interest for modeling were identified using Analysis of Composition of Microbiomes (ANCOM) (Mandal et al., 2015). ANCOM detects taxa that are differentially abundant between sample types (samples with and without a given Amsel criterion present). ANCOM was specifically designed to be used with compositional 16S rRNA gene sequencing data, as compared to elasticnet regression, which was used in the original Seattle analysis and does not account for compositionality (Fernandes et al., 2014; Mandal et al., 2015). ANCOM was performed separately for each outcome in both study populations.

Bacterial taxa identified as differentially abundant between samples with and without an Amsel criterion present in ANCOM analysis were included in modeling analysis. For each study, four sets of logistic regression models were fit. Each set of models had a single Amsel criterion as the outcome. Within each set, individual models were fit to evaluate associations between the Amsel criterion and each taxon identified as differentially abundant between samples with and without that Amsel criterion. Bacterial relative abundances were modeled using four-level ordinal variables with levels: no detection (reference), tertile 1, tertile 2, and tertile 3. Tertiles of bacterial relative abundance were calculated for each taxon separately in each study population using all samples in which the taxon was detected. Using ordinal exposure variables generates a single odds ratio (OR) for the association between a given taxon and a given Amsel criterion. This OR represents the relative change in odds of the Amsel criterion comparing: samples in relative abundance tertile 1 to samples with no detection; samples in tertile 2 to tertile 1; and samples in tertile 3 to tertile 2. Generalized estimating equations (independent correlation structure) were used for PVI models to account for correlated data due to repeated measures within participants and different numbers of measurements between participants. No exposure variables were lagged in PVI models to allow for cross-sectional interpretation, enabling direct comparison of PVI and Seattle results. All models for both study populations were unadjusted due to the *a priori* hypothesis that no available Seattle study covariates would act as confounders, and to enable direct comparison of PVI and Seattle results.

RESULTS

Of 234 PVI participants, 221 (94%) returned for at least one follow-up visit and consented to additional testing of stored specimens. One hundred ten (50%) of these participants were randomized to placebo, of whom 84 (76%) were enrolled in Kenya and included in this analysis. Median age was 29 years (interquartile range (IQR): 23.8–33 (**Table 1**). Of 242 Seattle participants, 220 (91%) participants' 16S rRNA gene sequencing data met previously-described quality measures and were included in this analysis (Srinivasan et al., 2012). Median age was 27 years (IQR: 22-33). Prevalence of BV assessed by Amsel criteria was 13% during PVI follow-up and 44% at the Seattle study visit (Amsel criteria prevalence in **Table 1**). Additional demographic and clinical data are presented in **Table 1**.

Prevalence of detection and relative abundance of bacterial taxa identified by ANCOM (for any outcome in either population) are summarized in **Figure 1**. *Lactobacillus vaginalis*, *Sneathia* spp. (closely related to *Sneathia amnii* and *Sneathia sanguinegens* but not sufficiently different in the V3-V4 region of the 16S rRNA gene sequences for species-level placement), *Streptococcus mitis*, and *Veillonella montpellierensis* were detected more frequently among PVI samples. *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, BV associated bacterium 1 (BVAB1), *Megasphaera lornae*, and *Prevotella bivia* were detected more frequently among Seattle samples. Among samples in which a given taxon was detected, median relative abundances of *L. crispatus*, *Lactobacillus iners*, BVAB1, Candidate Division TM7, and *Gardnerella* spp. were higher for PVI samples. Median relative abundance of *S. amnii* was higher for Seattle samples.

TABLE 1 | Baseline characteristics and Amsel criteria prevalence in the PVI trial and Seattle study populations.

Characteristic	PVI trial enrollment (N=84)		Seattle study (N=220)	
	N	%	N	%
Race				
American Indian or Alaskan Native	0	0	7	3
Asian	0	0	15	7
Black	84	100	75	34
Mixed race	0	0	12	6
Native Hawaiian or Pacific Islander	0	0	5	2
White	0	0	97	44
Age[a]	29	24 - 33	27	22 - 33
Nugent score				
BV Negative (0-3)	36	43	90	41
Intermediate (4-6)	18	21	13	6
BV Positive (7-10)	30	36	117	53
Clinical factors[b]				
Chlamydia trachomatis infection	6	7	–	–
Trichomonas vaginalis infection	0	0	15	7
Herpes simplex virus type-2 seropositive	55	66	–	–
Vulvovaginal candidiasis	17	20	17	8
Cervicitis	2	2	–	–
	PVI trial follow-up (N=496)		**Seattle study (N=220)[c]**	
	N	%	N	%
Amsel criteria & BV				
Amine odor	124	25	87	40
Clue cells	79	16	81	37
Vaginal discharge	51	10	103	52
Elevated vaginal pH	344	69	145	67
BV assessed by Amsel criteria[d]	66	13	97	44

[a]*Age is reported as median, interquartile range.*

[b]*Testing for Chlamydia trachomatis, Herpes simplex virus type-2, and cervicitis was not performed in the Seattle study. No PVI trial participants tested positive for Neisseria gonorrhoeae at enrollment, and testing for N. gonorrhoeae was not performed in the Seattle study.*

[c]*Outcomes were not assessed for all participants of the Seattle study. Amine odor, clue cells, and BV were only assessed for 219 participants, vaginal discharge for 200 participants, and vaginal pH for 218 participants. Percentages for these variables used the number of participants for which the outcome was assessed as the denominator.*

[d]*BV was defined based on the presence of at least three of four Amsel criteria. PVI, Preventing Vaginal Infections; BV, bacterial vaginosis.*

Associations between *Lactobacillus* species and Amsel criteria were largely population-dependent (**Figures 2**–**5**). Higher relative abundance of *L. crispatus* was associated with the absence of all Amsel criteria among Seattle participants, whereas it was only associated with the absence of elevated vaginal pH among PVI participants. Higher relative abundances of *L. gasseri* and *L. jensenii* were associated with the absence of all Amsel criteria among Seattle participants and were not associated with any criteria among PVI participants. Higher *L. iners* relative abundance was associated with the absence of amine odor and clue cells in both populations, the absence of vaginal discharge among Seattle participants, and the absence of elevated vaginal pH among PVI participants.

Several associations between BV-associated taxa and Amsel criteria were consistent between the populations (**Figures 2**–**6**). Higher BVAB1 relative abundance was associated with the presence of all Amsel criteria in both populations. Higher *Eggerthella* type 1, *Fannyhessea* (*Atopobium*) *vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens* relative abundances were associated with the presence of all criteria among Seattle participants, and all criteria except discharge among PVI participants. Higher *P. bivia* relative abundance was associated with elevated vaginal pH in both populations. The magnitude of these shared associations was consistently larger among Seattle participants than PVI participants (**Figures 2**–**5**).

For several additional BV-associated taxa, only some associations were shared between the PVI and Seattle populations (**Figures 2**–**6**). Higher *Prevotella timonensis* and BVAB2 relative abundances were associated with the presence of clue cells and elevated vaginal pH in both populations. Both taxa were associated with the presence of discharge among Seattle participants, and *P. timonensis* was associated with the presence of amine odor among Seattle participants. Higher *Parvimonas micra* relative abundance was associated with elevated vaginal pH in both populations. It was also associated with the presence of clue cells among PVI participants and with the presence of discharge among Seattle participants. Higher *Prevotella amnii* relative abundance was associated with elevated vaginal pH in both populations. It was also associated with the presence of amine odor and clue cells among PVI participants. Higher *Prevotella* genogroup 3 and *M. lornae* relative abundances were associated with the presence of clue cells in both populations. Both taxa were associated with the presence of amine odor and discharge among Seattle participants. *M. lornae* was also associated with elevated vaginal pH among Seattle participants.

DISCUSSION

In this comparative study, a core group of six vaginal bacteria, BVAB1, *Eggerthella* type 1, *F. vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens*, were associated with the presence of three to four Amsel criteria in research populations enrolled in Mombasa and Nairobi, Kenya and Seattle, USA. This core group of vaginal bacteria may play a key role in the manifestation of BV signs and symptoms across diverse populations. Although the etiology of BV remains unclear, these results support recent hypotheses that emphasize the formation of a polymicrobial biofilm, possibly initiated by *Gardnerella* and with early inclusion of *F. vaginae* (Swidsinski et al., 2008; Verstraelen and Swidsinski, 2013; Hardy et al., 2015; Jung et al., 2017; Muzny et al., 2018; Muzny et al., 2019). The shared associations between these six bacteria and Amsel criteria may reflect bacterial cooccurrence in the BV biofilm that persists across populations. They may also indicate bacterial interactions within the BV biofilm, which have been documented for *Gardnerella* and *F. vaginae* (Castro et al., 2020).

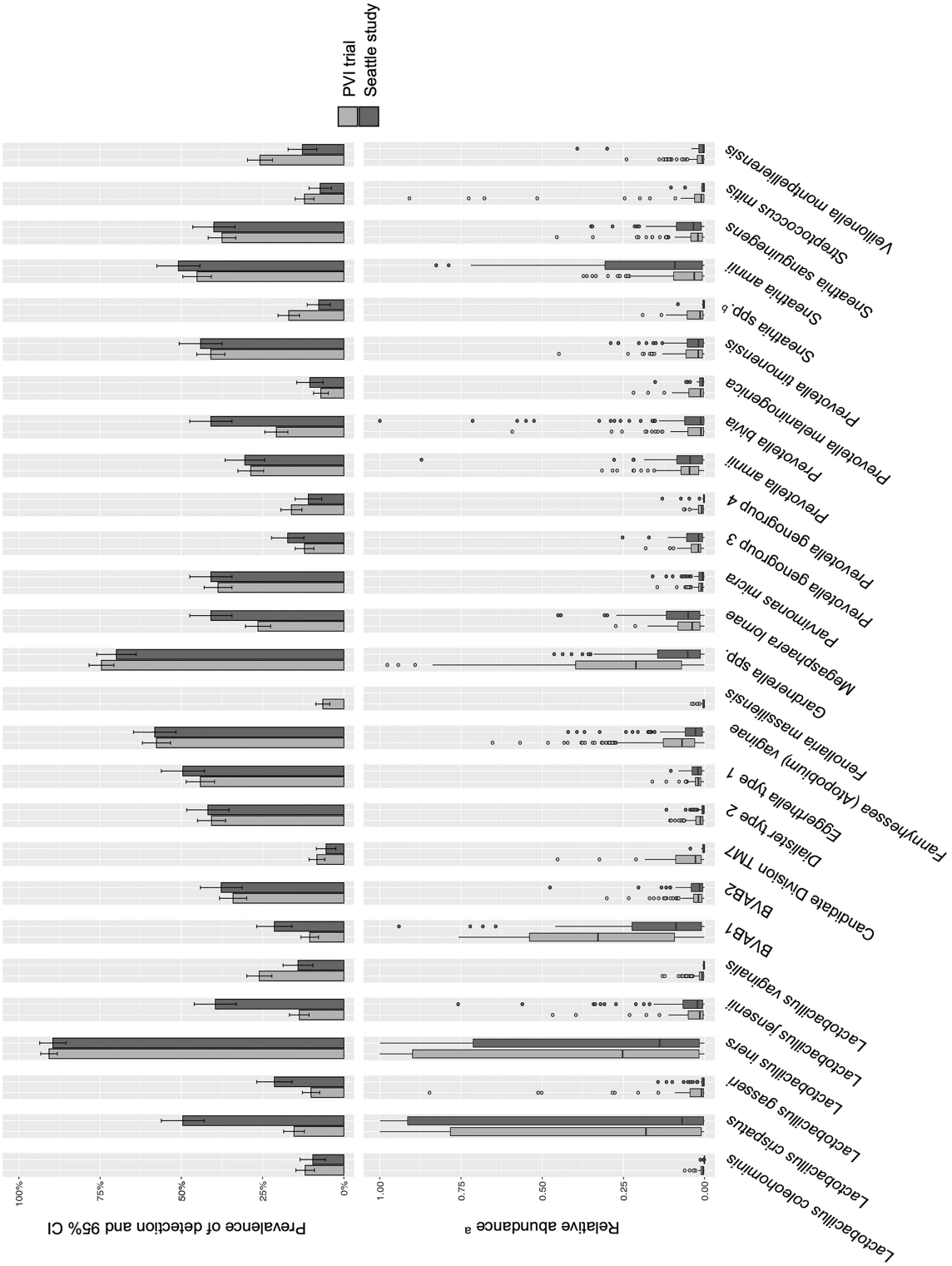

FIGURE 1 | Detection and relative abundance of bacteria included in modeling analysis. All bacteria identified by ANCOM as significantly differentially abundant between samples with and without an Amsel criterion in either study population were included as exposures in modeling analysis and are presented in this figure. [a]Relative abundances are presented for samples in which a given taxon was detected. [b]*Sneathia* spp. includes those not classified at the species level as *S. amnii* or *S. sanguinegens*, but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level. PVI, Preventing Vaginal Infections; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium; ANCOM, analysis of composition of microbiomes.

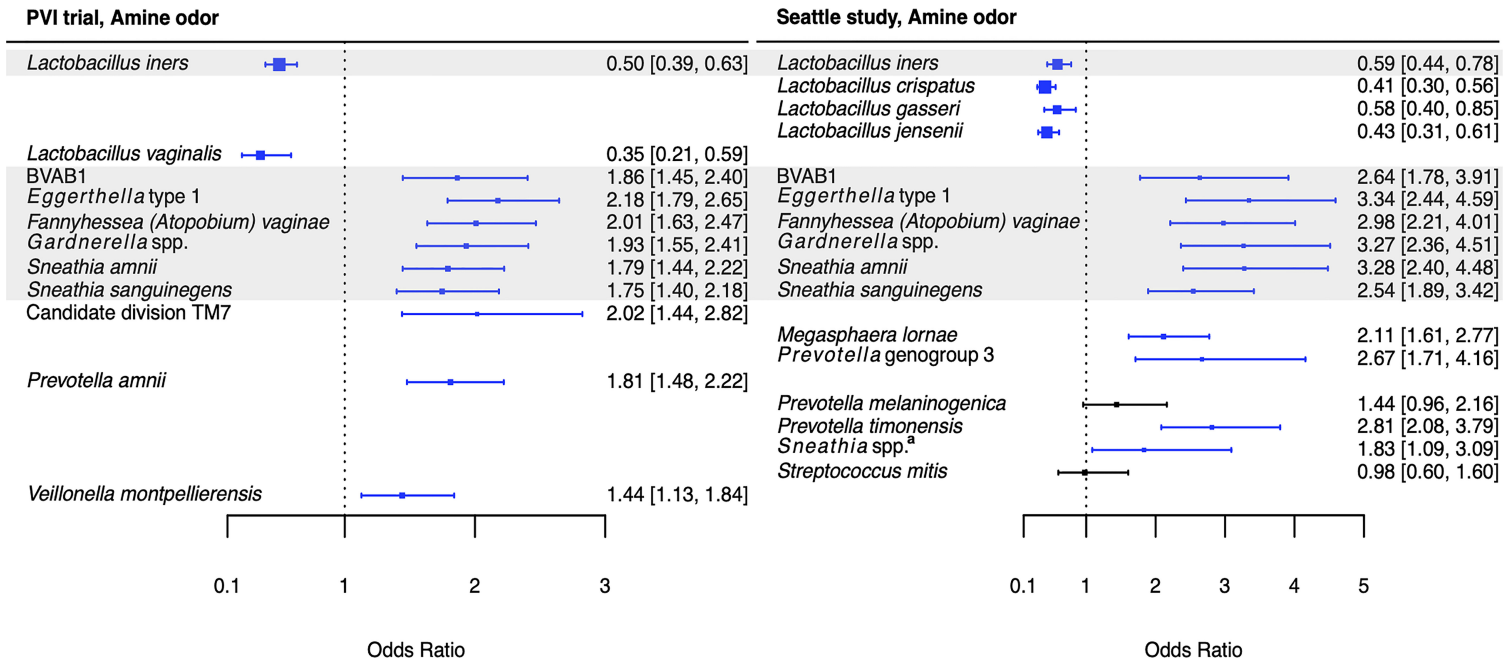

FIGURE 2 | Logistic regression modeling results for the association between vaginal bacteria and amine odor. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models. [a]*Sneathia* spp. includes those not classified at the species level as *S. amnii* or *S. sanguinegens*, but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level. PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

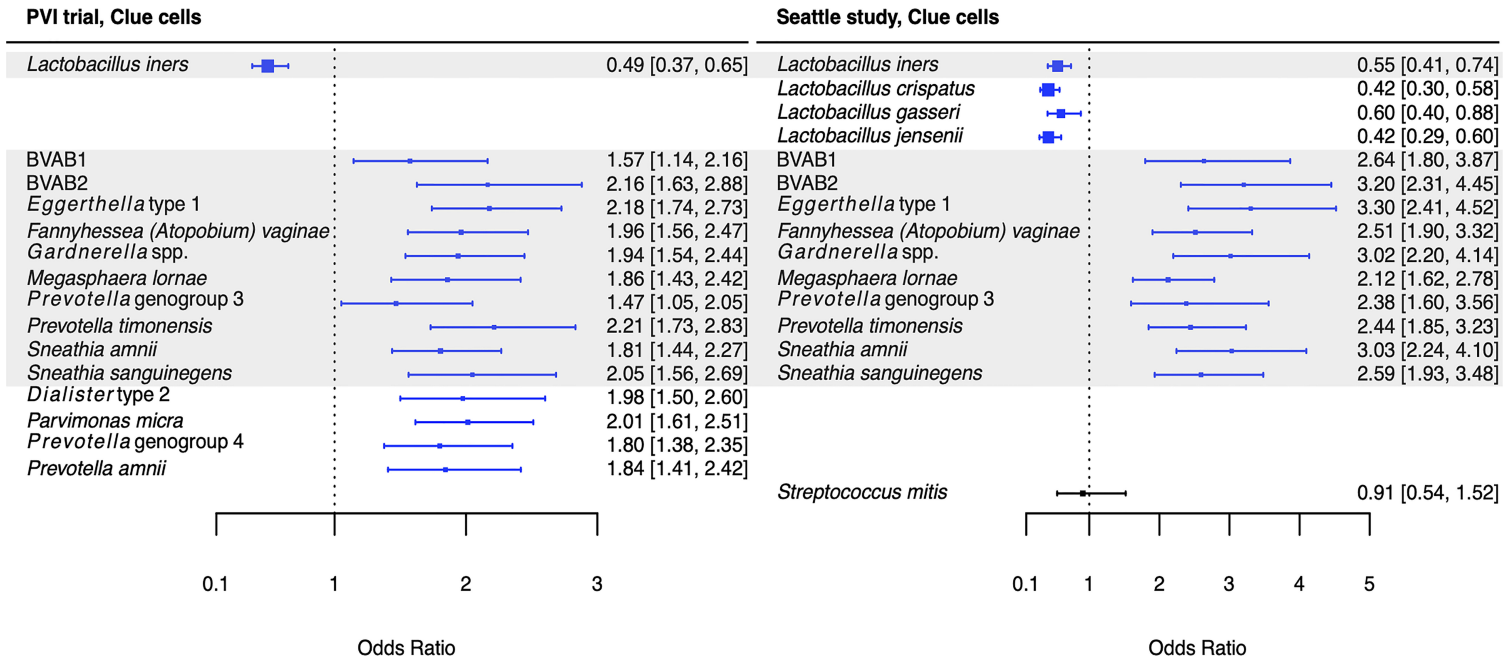

FIGURE 3 | Logistic regression modeling results for the association between vaginal bacteria and clue cells. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models. PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

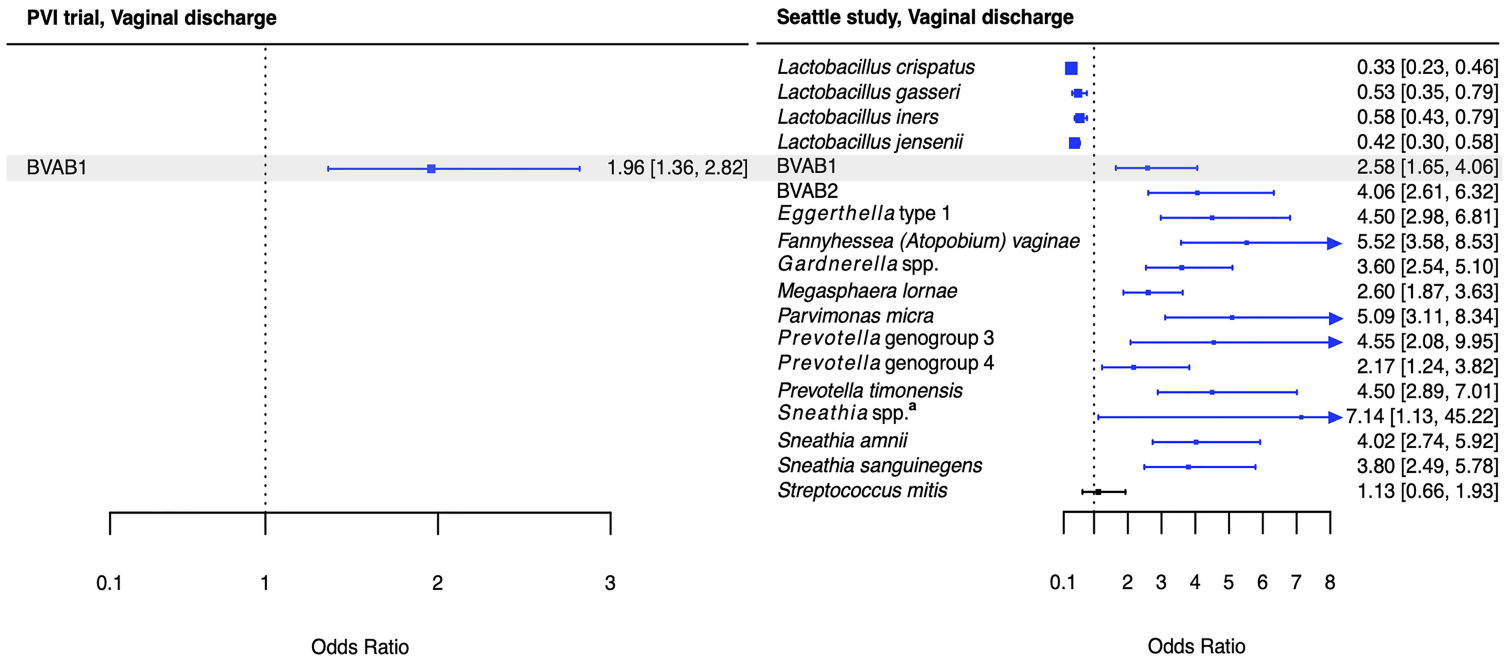

FIGURE 4 | Logistic regression modeling results for the association between vaginal bacteria and vaginal discharge. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models. [a]*Sneathia* spp. includes those not classified at the species level as *S. amnii* or *S. sanguinegens*, but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level. PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

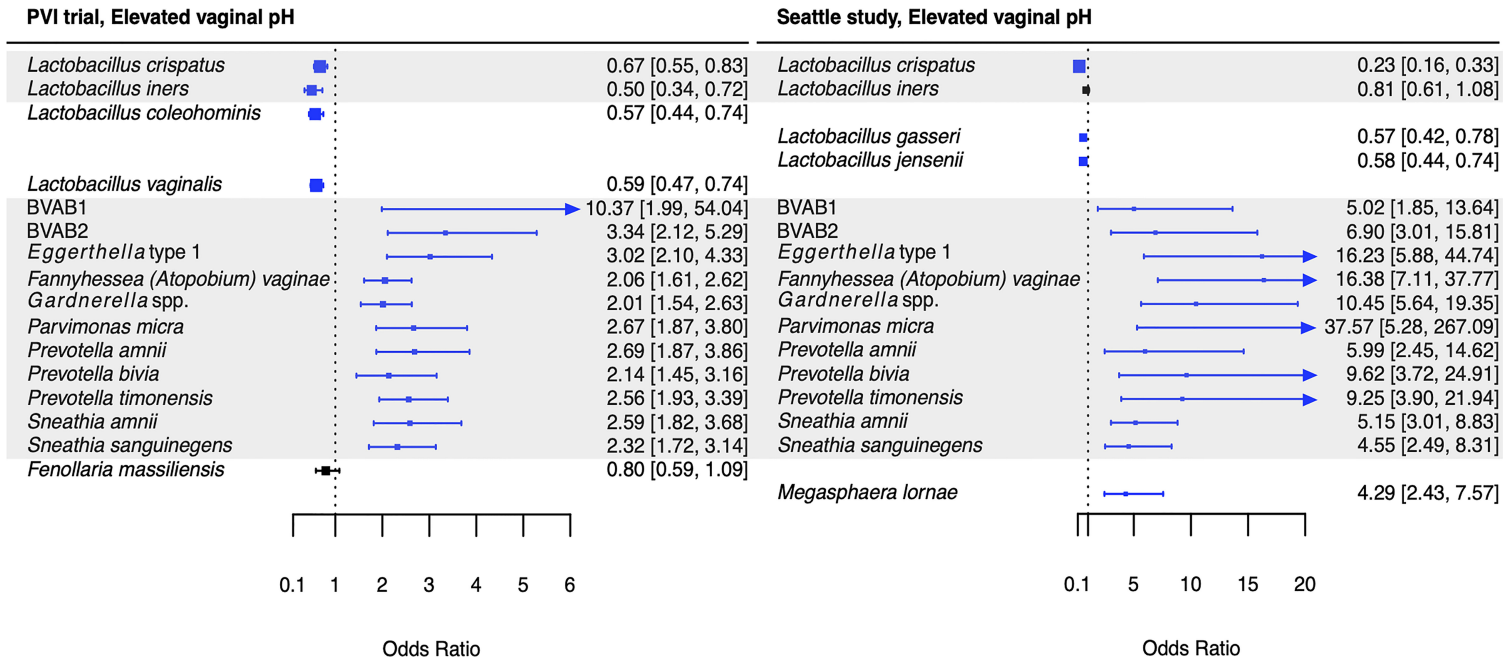

FIGURE 5 | Logistic regression modeling results for the association between vaginal bacteria and elevated vaginal pH. Gray shading indicates taxa that were modeled in both study populations. Blue box and whiskers indicate associations that were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate), and black box and whiskers indicate associations that were not significant following multiple comparisons adjustment. All models were univariable logistic regression models with the Amsel criterion as the outcome and bacterial relative abundance as the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI models. PVI, Preventing Vaginal Infections; OR, odds ratio; CI, confidence interval; BVAB, bacterial vaginosis associated bacterium.

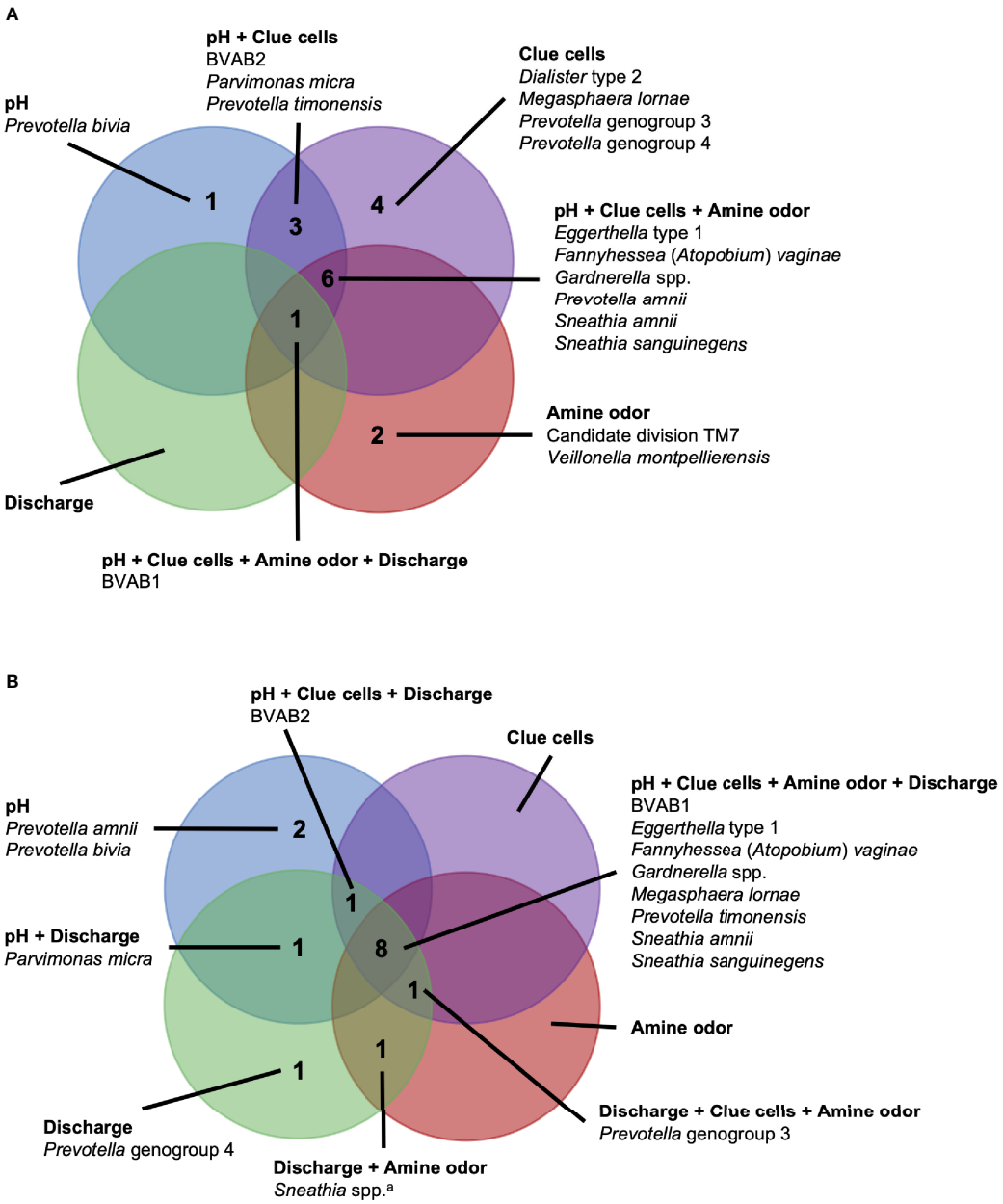

FIGURE 6 | Vaginal bacteria significantly positively associated with Amsel criteria in the PVI trial population and Seattle study population. Vaginal bacteria significantly positively associated with at least one Amsel criterion in **(A)** the PVI trial population and **(B)** the Seattle study population. Results are from logistic regression models fit separately for each study population. In each model, a single Amsel criterion was the outcome, and a single bacterial taxon was the exposure. Bacterial relative abundances were modeled as tertiles with the reference level being no detection (four-level ordinal variables with levels: no detection, tertile 1, tertile 2, tertile 3). Generalized estimating equations with an independent correlation structure were used for PVI trial models, and no variables were lagged in PVI trial models. All models were univariable. All associations represented in the Venn diagrams were significant following Benjamini-Hochberg multiple comparisons adjustment (5% false discovery rate). [a]*Sneathia* spp. includes those not classified at the species level as *S. amnii* or *S. sanguinegens*, but closely related to these two species. Sequences are not sufficiently different at the V3-V4 region of the 16S rRNA gene to classify at the species level, hence classified at the genus level. PVI, Preventing Vaginal Infections; BVAB, bacterial vaginosis associated bacterium.

Consistent with the current Seattle analysis, *Eggerthella* type 1 and *S. amnii* were associated with the presence of all Amsel criteria in the original Seattle analysis published by Srinivasan and colleagues, and *F. vaginae* and *Gardnerella* spp. were associated with three criteria (Srinivasan et al., 2012). However, BVAB1 was only associated with amine odor in the original analysis, and *S. sanguinegens* was not associated with any criteria (complete comparison of original Seattle, current Seattle, and

current PVI analyses in **Supplementary Table 2**) (Srinivasan et al., 2012). These and other differences in Seattle analysis results are likely due to differences between elasticnet regression and univariable logistic regression, used in the original and current analyses, respectively (Srinivasan et al., 2012). Elasticnet regression is a predictive modeling approach in which features retained in the final model are those that are most strongly predictive of the outcome, conditional on other features retained in the model (Zou and Hastie, 2005). The lack of association between Amsel criteria and BVAB1 and *S. sanguinegens* in the original analysis reflects that these taxa's relative abundances did not substantially improve the elasticnet model's accuracy in predicting Amsel criteria. In the current univariable logistic regression analysis, associations between higher BVAB1 and *S. sanguinegens* relative abundances and the presence of Amsel criteria indicate that these taxa's relative abundances were significantly higher among samples with Amsel criteria present. The original and current Seattle results are not necessarily inconsistent, and both analytic approaches are hypothesis-generating and require mechanistic studies to understand the role of vaginal bacteria in generating BV signs and symptoms.

Studies investigating vaginal bacterial functions during BV suggest two biologically plausible mechanisms by which this core group of six bacteria may contribute to BV symptomatology. The first potential mechanism is through biogenic amine production. Genomic analyses demonstrated that *Eggerthella* isolates contain genes encoding biogenic amine-producing proteins, and a metabolomics study found that *Eggerthella* type 1, *F. vaginae*, *Gardnerella* spp., *S. amnii*, and *S. sanguinegens* were highly correlated with several biogenic amines, which were in turn associated with BV and individual Amsel criteria (Nelson et al., 2015; Srinivasan et al., 2015). Biogenic amines may contribute to transudation of fluid into the vagina and squamous cell exfoliation, resulting in vaginal discharge and clue cell formation (Sobel, 2000; Yeoman et al., 2013; Srinivasan et al., 2015). They are also involved in amine odor, and production of biogenic amines may consume protons, increasing vaginal pH (Nelson et al., 2015). The second potential mechanism involves production of enzymes that degrade cervicovaginal mucus and epithelium, including sialidases and phospholipases. Studies evaluating the role of extracellular enzymes in vaginal epithelial cell sloughing showed that *Gardnerella* spp. produced sialidases and phospholipases, and high abundances of *F. vaginae* and *Sneathia* were associated with increased sialidase activity during BV (Yeoman et al., 2010; Marconi et al., 2013; Jung et al., 2017). These enzymes may contribute to squamous cell exfoliation and subsequent clue cell formation, and they likely enhance vaginal discharge (Yeoman et al., 2010).

While these potential mechanisms do not appear to involve BVAB1, it is notable that BVAB1 was the only taxon associated with the presence of vaginal discharge in both the PVI and Seattle populations. In addition to BVAB1, 12 other bacteria were associated with vaginal discharge among Seattle participants. The presence of vaginal discharge characteristic of BV was recorded by clinicians in both studies, so these differences are not due to differences in vaginal discharge self-report between the populations. Instead, they can be attributed, at least partially, to the fact that ANCOM only identified BVAB1 as differentially abundant between PVI samples with and without vaginal discharge, making BVAB1 the only bacterial exposure modeled for discharge in the PVI population. Vaginal discharge was only present at 10% of PVI visits, compared to 52% of Seattle visits, and ANCOM may be underpowered to detect taxa that are differentially abundant between PVI samples with and without discharge present. These differences may also indicate that vaginal discharge arises through differing microbial pathways in different populations. Metabolomics evidence again indicates that biogenic amines may contribute to vaginal discharge in the Seattle population. In addition to the core group bacteria, BVAB2, *M. lornae*, *P. micra*, *P. timonensis*, and *Sneathia* spp. were associated with the presence of discharge among Seattle participants. A metabolomics study reported that these taxa correlated strongly with the polyamine cadaverine, which was associated with vaginal discharge (Srinivasan et al., 2015).

Unlike for vaginal discharge, several taxa in addition to the core group bacteria were associated with the presence of clue cells in both the PVI and Seattle populations: BVAB2, *M. lornae*, *P. timonensis*, and *Prevotella* genogroup 3. Prior analysis of the Seattle data also found that BVAB2 and *M. lornae* were associated with clue cells (Srinivasan et al., 2012). Results from the current analyses and prior Seattle analysis are supported by observations from studies evaluating vaginal bacterial functions in BV. Fluorescence *in situ* hybridization targeting BVAB2 showed that BVAB2 can attach to vaginal epithelial cells resulting in similar appearance to clue cells (Fredricks et al., 2005). Other studies investigating vaginal epithelial cell sloughing showed *Prevotella* can produce sialidases and glycosidases, suggesting *Prevotella* may contribute to squamous cell exfoliation and the formation of clue cells (Briselden et al., 1992; Olmsted et al., 2003; Yeoman et al., 2010; Marconi et al., 2013). Likewise, high abundance of *Megasphaera* spp. during BV was associated with high sialidase activity (Marconi et al., 2013). Metabolomics analyses demonstrated that BVAB2, *M. lornae*, and *P. timonensis* were highly correlated with the lipid deoxycarnitine, which was in turn associated with the presence of clue cells (Srinivasan et al., 2015). *Prevotella* are also associated with the polyamine putrescine and contain genes encoding biogenic amine-producing proteins, and polyamines may be involved clue cell formation as discussed above (Sobel, 2000; Yeoman et al., 2013; Nelson et al., 2015). These reports suggest biologically plausible mechanisms that support the epidemiologic associations of BVAB2, *M. lornae*, *P. timonensis*, and *Prevotella* genogroup 3 with the presence of clue cells.

Our results also suggest *Prevotella* may contribute to elevated vaginal pH. In both populations, *P. amnii*, *P. bivia*, *P. timonensis*, BVAB2, and *P. micra* were associated with elevated vaginal pH, in addition to the core group bacteria. Srinivasan and colleagues' prior analysis of the Seattle data also reported that *P. bivia* was associated with elevated vaginal pH (Srinivasan et al., 2012).

Metabolomic and genomic evidence again suggests biogenic amines may underlie these associations. One metabolomics study observed that cadaverine and tyramine, both amines, and N-acetylputrescine, a degradation product of the amine putrescine, were associated with elevated vaginal pH (Srinivasan et al., 2015). These amines were highly correlated with BVAB2 and *P. timonensis*, and cadaverine and N-acetylputrescine with *P. micra* (Srinivasan et al., 2015). A second metabolomics study reported that *Prevotella* are associated with putrescine, and genomic data indicate that various *Prevotella* species contain genes encoding biogenic amine-producing proteins (Yeoman et al., 2013; Nelson et al., 2015). These prior reports and the current analyses' results are consistent with Nelson and colleagues' proposed model that biogenic amine production by vaginal bacteria consumes protons, increasing vaginal pH (Nelson et al., 2015).

Several features of the Seattle study, PVI trial, and current analyses are important to consider in interpreting these findings. Although statistical analyses were conducted to enable direct comparison of PVI and Seattle results, technical variation between the studies may influence differences in results. Mean sequencing depth of Seattle samples was 1,620 reads per sample, compared to 33,467 reads per sample for PVI samples as different sequencing platforms (pyrosequencing versus Illumina Mi-Seq, respectively) were used based on availability (Srinivasan et al., 2012). Minority taxa were less likely to be detected in Seattle samples due to lower sequencing depth, and relative abundances may be overestimated in Seattle data (Weiss et al., 2017). Additional study design differences and sources of technical variation between the parent studies may limit our ability to detect associations that are consistent between the two populations, which is why we focus on associations that are consistent between the populations. Moreover, that we were able to detect several shared associations between the populations despite this variation strengthens the evidence for these relationships. Finally, 16S rRNA gene sequencing facilitates taxonomic identification at the genus or species levels for most taxa. For example, 16S rRNA gene sequencing does not differentiate between the recently identified *Gardnerella* species (Vaneechoutte et al., 2019), which may have different relationships with BV signs and symptoms. We were unable to examine heterogeneity between *Gardnerella* species because both original studies used 16S rRNA gene sequencing and therefore classified *Gardnerella* at the genus level.

This comparison study indicates that a core group of vaginal bacteria may contribute to BV symptomatology across populations, and that additional vaginal bacteria may play a role in the manifestation of specific BV signs and symptoms. While these relationships should be investigated in additional populations, these findings help contextualize heterogeneity in BV symptomatology between women and across global regions. These findings are also consistent with growing evidence regarding the role of biogenic amines and extracellular enzymes in BV etiology and symptom manifestation. Taken together, this work and prior reports suggest that bacteria highlighted here may consistently contribute to BV symptomatology in various populations, despite differences in overall vaginal microbiota composition.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The Seattle study datasets analyzed for this study can be found in the NCBI Short Read Archive (SRA051298, https://www.ncbi.nlm.nih.gov/sra/?term=SRA051298) and the supplementary materials of the original Seattle study publication (Srinivasan et al. PLoS ONE. 2012. DOI: 10.1371/journal.pone.0037818, https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0037818, https://doi.org/10.1371/journal.pone.0037818.s006, https://doi.org/10.1371/journal.pone.0037818.s007). The PVI trial datasets analyzed for this study can be found in the NCBI Short Read Archive (PRJNA638104, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA638104/) and this publication's **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards of Kenyatta National Hospital, the University of Washington, and the University of Alabama at Birmingham (PVI trial, ClinicalTrails.gov NCT01230814, October 6, 2014); and the Institutional Review Board of the Fred Hutchinson Cancer Research Center (Seattle BV study). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TF, DF, and SS contributed to Seattle study design, implementation, and data collection. JB and RM contributed to PVI trial design. JB, OA, JK, VM, and RM contributed to PVI trial implementation and data collection. NH, TF, DF, and SS contributed to PVI trial and Seattle study microbiota characterization. NH, TF, DF, and SS contributed to the original Seattle study statistical analysis. KC, JB, and SS contributed to the current PVI trial and Seattle study statistical analyses. KC wrote the first manuscript draft and led manuscript writing. JB and SS contributed substantially to early manuscript revisions. KC, JB, OA, JK, NH, TF, VM, DF, RM, and SS contributed to, reviewed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.801770/full#supplementary-material

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A Deep Look at the Vaginal Environment During Pregnancy and Puerperium

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A deep comprehension of the vaginal ecosystem may hold promise for unraveling the pathophysiology of pregnancy and may provide novel biomarkers to identify subjects at risk of maternal-fetal complications. In this prospective study, we assessed the characteristics of the vaginal environment in a cohort of pregnant women throughout their different gestational ages and puerperium. Both the vaginal bacterial composition and the vaginal metabolic profiles were analyzed. A total of 63 Caucasian women with a successful pregnancy and 9 subjects who had a first trimester miscarriage were enrolled. For the study, obstetric examinations were scheduled along the three trimester phases (9-13, 20-24, 32-34 gestation weeks) and puerperium (40-55 days after delivery). Two vaginal swabs were collected at each time point, to assess the vaginal microbiome profiling (by Nugent score and 16S rRNA gene sequencing) and the vaginal metabolic composition (^{1}H-NMR spectroscopy). During pregnancy, the vaginal microbiome underwent marked changes, with a significant decrease in overall diversity, and increased stability. Over time, we found a significant increase of *Lactobacillus* and a decrease of several genera related to bacterial vaginosis (BV), such as *Prevotella, Atopobium* and *Sneathia*. It is worth noting that the levels of *Bifidobacterium* spp. tended to decrease at the end of pregnancy. At the puerperium, a significantly lower content of *Lactobacillus* and higher levels of *Gardnerella, Prevotella, Atopobium*, and *Streptococcus* were observed. Women receiving an intrapartum antibiotic prophylaxis for Group B *Streptococcus* (GBS) were characterized by a vaginal abundance of *Prevotella* compared to untreated women. Analysis of bacterial relative abundances highlighted an increased abundance of *Fusobacterium* in women suffering a first trimester abortion, at all taxonomic levels. *Lactobacillus* abundance was strongly correlated with higher levels of lactate, sarcosine, and many amino acids (i.e., isoleucine, leucine, phenylalanine, valine, threonine, tryptophan). Conversely, BV-associated genera, such as *Gardnerella*, *Atopobium*, and *Sneathia*, were related to amines (e.g., putrescine, methylamine), formate, acetate, alcohols, and short-chain fatty-acids (i.e., butyrate, propionate).

Keywords: vaginal microbiome, vaginal metabolome, pregnancy, puerperium, miscarriage, women's health

INTRODUCTION

In healthy reproductive-aged women, the vaginal microbiome is generally dominated by members of the *Lactobacillus* genus (van der Wijgert et al., 2014; Smith and Ravel, 2017). Lactobacilli promote the maintenance of the vaginal health, preventing the colonization and growth of adverse microorganisms through various mechanisms, such as vaginal pH lowering, bioactive compounds production, competition for adhesion, and modulation of immune response (Parolin et al., 2015; Petrova et al., 2015; Foschi et al., 2017). On the other hand, the reduction of lactobacilli combined with the increase of different species of anaerobic bacteria (e.g., *Gardnerella*, *Atopobium*, *Prevotella*, *Mobiluncus*) results in the switch from a normal vaginal ecosystem to a polymicrobial dysbiosis, namely bacterial vaginosis (BV) (Parolin et al., 2018; Ceccarani et al., 2019). This condition is accompanied by marked alterations in the composition of vaginal metabolites, being higher concentrations of various biogenic amines and short chain fatty acids (SCFAs) common fingerprints of BV condition (Yeoman et al., 2013).

On a regular basis, the composition of the vaginal microbiome can vary throughout a woman's life in response to various factors, such as hormonal status, diet, sexual habits, pharmaceutical treatments, and urogenital infections (Kroon et al., 2018; Noyes et al., 2018; Dall'Asta et al., 2021). Specifically, during pregnancy, the vaginal microbiome undergoes marked changes, with a significant decrease in overall bacterial diversity, an increased stability, and an enrichment of *Lactobacillus* spp. (Aagaard et al., 2012; DiGiulio et al., 2015; Gupta et al., 2020; Marangoni et al., 2021). Contrariwise, in the postpartum period (i.e., puerperium), the vaginal microbiome becomes less *Lactobacillus* spp. dominated, with increased biodiversity (MacIntyre et al., 2015).

It is well known that the composition of the vaginal bacterial communities and related metabolites play a crucial role in maternal-fetal health (Fox and Eichelberger, 2015; Nelson et al., 2016; Laghi et al., 2021). As for healthy vaginal environments, healthy pregnancies are usually characterized by a lactobacilli-dominated ecosystem, whereas reduced lactobacilli abundances, increased bacterial diversity, and higher levels of specific vaginal metabolites (e.g., acetone, formate, isopropanol, methanol) are associated with preterm birth and other complications (Prince et al., 2014; Ansari et al., 2020; Di Simone et al., 2020). For example, reduced prevalence of *Lactobacillus* spp. and higher levels of selected vaginal metabolites (inosine, fumarate, xanthine, benzoate, ascorbate) (Al-Memar et al., 2020; Xu et al., 2020; Marangoni et al., 2021) seem to be predictors of a higher risk of spontaneous miscarriage.

In this context, only few studies have investigated the association between the structure of the vaginal ecosystem and the first trimester miscarriage (Zhang et al., 2019; Al-Memar et al., 2020; Fan et al., 2020; Xu et al., 2020), while many aspects about the dynamic interactions between the inhabitants of the vaginal ecosystem, their metabolites, and the host remain to be fully elucidated despite the recent advances in the study of the human microbiome during pregnancy and puerperium (Vinturache et al., 2016; Gupta et al., 2020).

Therefore, the aim of this study was to deepen the characteristics of the vaginal environment in a cohort of Caucasian women with a normal pregnancy throughout their different gestational ages (i.e., first, second, third trimester) and puerperium. A group of women suffering a spontaneous first trimester miscarriage was also included for a wider characterization. For each subject and each time point, both the vaginal bacterial composition (16S rRNA sequencing) and the vaginal metabolic profiles (Proton nuclear magnetic resonance spectroscopy-^{1}H-NMR) were analyzed.

MATERIALS AND METHODS

Study Cohort and Samples Collection

Subjects were enrolled among all the Caucasian pregnant women presenting to the Family Advisory Health Centers of Ravenna (Italy) for prenatal care starting from April 2019.

Exclusion criteria were the following: (i) age < 18 years; (ii) HIV positivity; (iii) body mass index (BMI) > 33; (iv) medically assisted procreation; (v) use of any antibiotics in the month preceding the sampling; (vi) use of vaginal douches or topical agents in the two weeks before sampling; (vii) presence of uncontrolled chronic diseases (e.g., diabetes, autoimmune disorders, malignancies); (viii) drug addiction or heavy smokers (> 15 cigarettes/day). Moreover, women with urogenital infections due to sexually transmitted pathogens (i.e., *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium*), aerobic vaginitis (AV) or symptomatic vulvo-vaginal candidiasis (VVC) were further excluded after the laboratory testing.

At gestational age of 9-13 weeks (first trimester), 20-24 weeks (second trimester), 32-34 weeks (third trimester), and puerperium (40-55 days after delivery) women underwent an obstetric examination. For all patients, demographic data and information about urogenital symptoms were recorded.

Women colonized with Group B *Streptococcus* (GBS) at the third trimester of pregnancy received intrapartum antibiotic prophylaxis (IAP) (i.e., penicillin G or ampicillin), following international guidelines (Kolkman et al., 2020).

Two vaginal swabs were collected at each time point. The first one (E-swab, Copan, Brescia, Italy) was used for microbiological diagnostic tests and Nugent score assessment. The second was collected with a sterile cotton bud, re-suspended in 1 mL of sterile saline, and stored at -80°C until use. Frozen vaginal swabs were thawed, vortexed for 1 min and removed from the liquid. After centrifugation (10000 × *g* for 15 min), the cell-free supernatants were used for metabolomic analysis, whereas bacterial pellets were employed for vaginal microbiome profiling.

A written informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee of Romagna (CEROM) (n° 2032 of 21st February 2018). This study was carried out in accordance with the Declaration of Helsinki, following the recommendations of the Ethics Committee.

Microbiological Investigations

A commercial nucleic acid amplification technique (NAAT) was used for *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* and *M. genitalium* detection (Seeplex STI Master Panel 1; Seegene, Seoul, KR). VVC was excluded by the microscopic presence of fungal buds and a significant growth of *Candida* colonies by culture (Yano et al., 2019). AV were diagnosed by means of a microscopic examination (i.e., diminished/absent lactobacilli, presence of leukocytes, parabasal cells, small coliform bacilli, cocci, or chains), combined with the growth of aerobic microorganisms, mainly of intestinal origin, by culture (Donders et al., 2011).

A Gram stain scoring system (Nugent score) was used for a preliminary assessment of the vaginal flora composition (Nugent et al., 1991). Based on this score, women were grouped as follows: 'H' group (normal lactobacilli-dominated microbiota, score 0-3), 'I' group (intermediate microbiota/flora, score 4-6), 'BV' group (bacterial vaginosis, score 7-10) (Zozaya-Hinchliffe et al., 2010).

Vaginal Microbiome Profiling

Nucleic acids were extracted from vaginal swabs by means of the Versant molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) equipped with a sample preparation module designed for automated sample preparation (Marangoni et al., 2015).

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified, according to the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). Final indexed libraries were prepared by equimolar (4 nmol/L) pooling, denaturation, and dilution to 6 pmol/L before loading onto the MiSeq flow cell (Illumina). A 2×300 bp paired-end run was used.

Raw reads were analyzed according to a previously described procedure (Severgnini et al., 2021). Briefly, a single fragment from two overlapping pairs was generated using PandaSeq software (v2.5, Masella et al., 2012). Downstream analyses, such as filtering, zero-radius Operational Taxonomic Units (zOTUs) creation, taxonomy assignments, and diversity analyses were performed using the QIIME suite (release 1.9.0, Caporaso et al., 2010), unoise3 algorithm (Edgar, 2016), RDP classifier (Wang et al., 2007), and SILVA 16S rRNA database (release 132, https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_132_release.zip).

Characterization of *Lactobacillus* spp. was performed by BLAST-aligning all reads belonging to that genus to a custom reference database made up collecting all available reference sequences in NIH-NCBI database ftp://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt of 17 *Lactobacillus* species commonly found in the vaginal environment, with finishing status of "complete genome", "chromosome" or "scaffold". Potential matches were filtered in order to retrieve an unequivocal classification for each read, according to the procedures already described (Ceccarani et al., 2019, **Supplementary Material**). Since 2020, *Lactobacillus* taxonomy underwent major update, with the re-classification of the genus in 25 different genera (23 of which are novel) (Zheng J. et al., 2020). Old and new species names used in the present article are available as **Supplementary Table S1**.

Alpha-diversity evaluation was estimated according to several microbial diversity metrics (i.e., chao1, Shannon index, observed species, Good's coverage, and Faith's phylogenetic distance). Beta-diversity analysis was conducted using both weighted and unweighted Unifrac metrics (Lozupone et al., 2011), and through the Principal Coordinates Analysis (PCoA).

Metabolomic Analysis

Metabolomic analysis was performed by means of a ^{1}H-NMR spectroscopy starting from 700 µL of the cell-free supernatants of the vaginal swabs, added to 100 µL of a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM set to pH 7.0 (Foschi et al., 2018)

^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with Topspin software (Ver. 3.5) according to previously described procedures (Foschi et al., 2018). The signals originating from large molecules were suppressed by a CPMG filter of 400 spin-echo periods, generated by 180° pulses of 24 µs separated by 400 µs (Ventrella et al., 2016).

To each spectrum, line broadening (0.3 Hz) and phase adjustment was applied by Topspin software, while any further spectra processing, molecules quantification and data mining step was performed in R computational language (version 4.1.2., R Core Team, 2021) by means of in-house developed scripts.

The spectra were aligned towards the right peak of alanine doublet, set to 1.473 ppm. The spectra were then baseline-adjusted by means of peak detection according to the "rolling ball" principle (Kneen and Annegarn, 1996) implemented in the "baseline" R package (Liland et al., 2016). A linear correction was then applied to each spectrum, to make the points pertaining to the baseline randomly spread around zero.

The signals were assigned by comparing their multiplicity and chemical shift with Chenomx software data bank (ver 8.3, Chenomx Inc., Edmonton, Alberta, Canada). Quantification of the molecules was performed in the first sample acquired by employing the added TSP as an internal standard. To compensate for differences in sample amount, any other sample was then normalized to such sample by means of probabilistic quotient normalization (Dieterle et al., 2006). Integration of the signals was performed for each molecule by means of rectangular integration.

Data Analysis and Statistics

Statistical evaluation of α-diversity indices was performed by non-parametric Monte Carlo-based tests through the QIIME pipeline. Beta-diversity differences were assessed by a permutation test with pseudo F-ratios using the "adonis" function from R package "vegan" (version 2.0-10, Oksanen et al., 2013). Pairwise relative abundance analysis was performed using a non-parametric Mann–Whitney U test. For comparing relative abundances across multiple categories, we applied a Kruskal-Wallis test, followed by Dunn's *post-hoc* test for pairwise comparisons.

Metabolite concentrations were correlated to bacterial composition by calculating Spearman's correlation coefficient between metabolites and bacterial genera present ≥1% in at least 1 sample (n=51). In this analysis, we considered all data points at T1, T2, T3, and T4. We performed a Spearman's rank-based correlation between genus relative abundances and metabolite quantities, selecting only those with p-value< 0.05 (i.e.: correlation significantly different from 0). To better visualize patterns of positively correlated bacteria and metabolites, a heatmap was drawn, clustering correlation coefficients for metabolites and bacteria (using Pearson's correlation as clustering metric and average linkage).

For each statistical analysis, unless otherwise stated, p-values < 0.05 were considered as significant. Statistical analyses were performed using MATLAB software (Natick, MA, USA).

Data Availability

Raw sequencing data of 16S rRNA gene are available at NCBI Short-reads Archive (SRA) with BioProject accession number PRJNA766806 (https://www.ncbi.nlm.nih.gov/sra/PRJNA766806). Raw metabolomic data are available as a **Supplementary material (Data Sheet S1)**.

RESULTS

Study Population

A total of 63 Caucasian pregnant women with a mean age of 30.8 ± 5.1 years (min: 21, max: 44) and a mean BMI of 23.5 ± 3.5 (min: 16.0, max: 32.5) were enrolled and sampled during all gestational ages; for 30 of them, clinical and microbiological data were also available for the puerperium.

In addition, 9 women (mean age: 33.8 ± 6.4 years; mean BMI: 24.5 ± 4.7) who had a spontaneous miscarriage at the first trimester of pregnancy (gestational age: 11-13 weeks) during the study were included. From the first to the third trimester of pregnancy, we noticed a significant decrease of BV cases, together with an increase of samples characterized by a normal microbiota (p=0.001; **Table 1**). During the puerperium, only one third of the women (33.3%) showed a lactobacilli-dominated flora, being most of them characterized by an alteration of the vaginal bacterial composition (26.6% intermediate flora, 40.0% BV-condition).

Ten out of the 30 (33.3%) women with puerperium data available received an intrapartum antibiotic prophylaxis to prevent GBS neonatal infection.

Most cases of spontaneous abortion were associated with an altered vaginal microbiome (55.5% intermediate status (I); 22.2% BV condition).

Vaginal Microbiome Structure Characterization

Overall, microbiota composition assessed through 16S rRNA sequencing was in accordance with what expected for the vaginal environment, with the *Lactobacillus* genus having an average relative abundance of 77.9%, followed by *Gardnerella* (8.9% on average), *Bifidobacterium* (3.5%), *Atopobium* (2.1%), *Prevotella* (1.8%), and *Megasphaera* (1.3%). Other genera, such as *Sneathia, Ureaplasma, Aerococcus*, and *Dialister* had <1% abundance. The first 12 genera accounted for 97.7% of the overall relative abundance, confirming the relatively low biodiversity of the vaginal samples (**Supplementary Figure S1**).

Microbiota structure was evaluated according to the vaginal status derived from the Nugent score, for a total of 189 samples (63 women at 3 time points each), comparing healthy (H) with intermediate (I) and bacterial vaginosis (BV) status. As expected, BV condition was characterized by a profound alteration of the microbiota, with a dramatic reduction of *Lactobacillus* spp. (83.8% vs 29.8% for H and BV, respectively) and an increase of opportunistic bacteria, (i.e., *Gardnerella*, *Atopobium*, *Prevotella*, *Megasphaera*, *Sneathia*, *Aerococcus*). The I condition seemed to be composed by nearly the same bacterial members of the H samples, with little exceptions in low-abundant members of the community (i.e., *Dietzia, Actinomyces, Enterococcus, Cutibacterium,* and '*Eubacterium eligens group*'), all contributing with <0.2% of average abundance (**Figure 1A**). Among the lactobacilli species, a significant reduction of *L. crispatus* was highlighted in BV samples as compared to both H and I (avg. abundance: 4.1% BV vs 32.7% H, 42.9% I); on the other hand, *L. iners* (avg. abundance: 19.9% H, 17.4% I, 16.5% BV), as well as other *Lactobacillus* species, was not affected (**Figure 1B**). Differences in microbial composition were reflected in α-diversity analysis, which highlighted a significant increase in biodiversity in BV samples as compared to H and I ones (p=0.003 for all metrics) (**Figure 1C**). Moreover, we recorded a significant separation of microbial profiles (β-diversity) among BV, I, and H (p<0.039 for all pairwise group comparisons, unweighted

TABLE 1 | Characteristics of the vaginal environment (Nugent score), stratified by the different gestation periods and conditions.

	First trimester miscarriage (n=9)	Normal pregnancies (n=63)			Puerperium (n=30**)
		1st trimester	2nd trimester	3rd trimester	
Nugent score					
0-3 (H)	22.2% (2/9)	49.2% (31/63)	74.6% (47/63)	82.5% (52/63)	33.3% (10/30)
4-6 (I)	55.5% (5/9)	33.3% (21/63)	15.9% (10/63)	11.1% (7/63)	26.6% (8/30)
7-10 (BV)	22.2% (2/9)	17.5% (11/63)	9.5% (6/63)	6.4% (4/63)	40.0% (12/30)
			p=0.001*		

H, lactobacilli-dominated microbiome; I, intermediate flora; BV, bacterial vaginosis.

**statistical significance is referred to the differences in microbial composition (Nugent score) during the three trimesters of pregnancy, excluding women suffering a first trimester miscarriage.*

***clinical and microbiological data during the puerperium were available only for 30 women.*

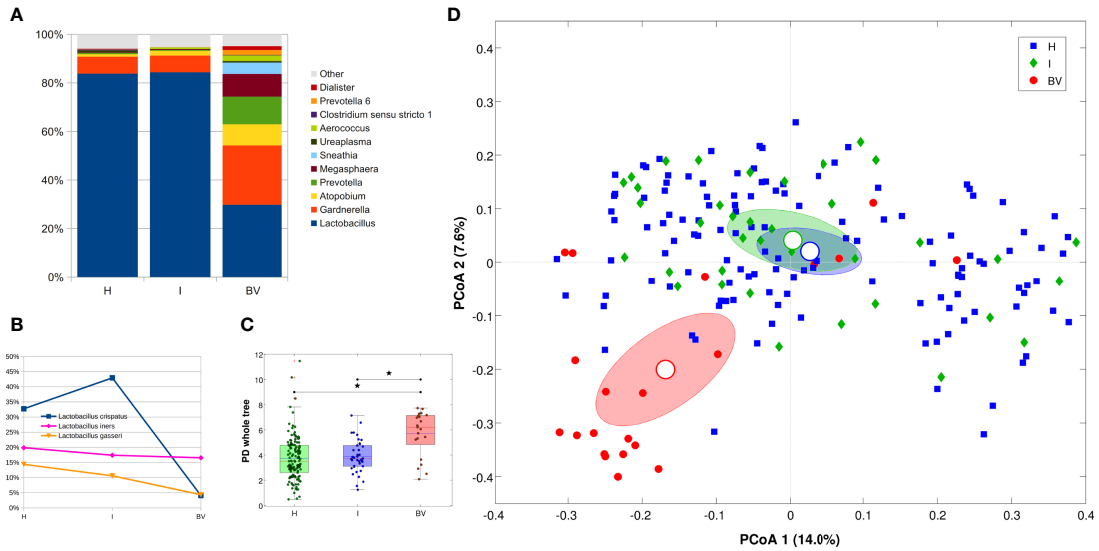

FIGURE 1 | Microbiota characterization according to the vaginal status (H, I or BV). **(A)** Barplot of average relative abundances at genus level. Genera with rel. ab. ≤1% were grouped in "Others" category; **(B)** Line plot of average *Lactobacillus* species abundance per vaginal status; only the 3 most abundant species are represented; **(C)** Boxplot of Faith's phylogenetic diversity of the samples (estimated at endpoint) for each vaginal status. Stars above the plots represent statistical significance ($p<0.05$); **(D)** Principal Coordinate Analysis (PCoA) based on unweighted Unifrac distance among samples. Each point represents a sample; ellipses are 95% SEM-based confidence intervals; point and ellipses are grouped according to vaginal status; the first and the second coordinate are represented.

Unifrac); at the same time, BV samples differed from the other two groups in major contributors of the microbiota (p=0.001 against both H and I conditions, weighted Unifrac) (**Figure 1D**). Similarly, distances between H and BV samples were higher than H vs I ones and I vs BV distances were higher than H vs I (both for weighted and unweighted Unifrac); at the same time, weighted Unifrac distances among BV samples resulted higher than that among H or I samples, confirming that BV status was characterized by a deeper alteration of the microbial composition with respect to other conditions.

Taxonomic Composition of the Vaginal Bacterial Communities During Pregnancy

We evaluated the vaginal microbiota dynamics along the three trimesters of pregnancy for a total of 63 women (189 total samples). The proportion of samples with the same vaginal status at each trimester was found to be statistically different for the H subjects (p<0.001, two-sided proportion test without continuity correction; increasing from 49.2% to 74.6% and 82.5% respectively at T1, T2, and T3) and the I subjects (p=0.005; decreasing from 33.3% at T1 to 15.9% at T2 and 11.1% at T3). On the other hand, no differences were highlighted for BV status (proportion of 17.5%, 9.5%, and 6.3% respectively at T1, T2, and T3).

There were no significant or noticeable differences in biodiversity over time, other than T1 vs T2 in chao1 (p=0.039) and T1 vs T3 for the Faith's phylogenetic diversity metric (p=0.015). With regards to microbial composition, T3 points were statistically separated from T1 and T2 sets ($p \leq 0.015$, unweighted Unifrac), which were indistinguishable from each other; no differences on the weighted Unifrac distance matrix were highlighted (**Supplementary Figures S2A, B**).

Analyzing bacterial genera co-abundance patterns, we were able to identify four co-abundance groups (CAGs) (**Supplementary Figure S3**): (i) *Ureaplasma* alone; (ii) *Lactobacillus* CAG (also including *Clostridium*); (iii) 'opportunistic' bacteria CAG (including *Bifidobacterium*, *Prevotella* and *Dialister*); (iv) BV-associated bacteria CAG (i.e.: *Gardnerella*, *Atopobium*, *Megasphaera*, *Sneathia* and *Aerococcus*). At all three time points, *Lactobacillus* CAG was inversely correlated to other CAGs, whereas opportunistic and BV-related CAGs were directly associated to one another, although with a different strength of correlation.

Many genera were statistically different over time, suggesting a deep reshaping of the microbiota between the first two trimesters: all groups were differential in both T1 vs T2 and T1 vs T3 comparisons, but not for T2 vs T3. In particular, we revealed increased *Lactobacillus* abundances and reduced levels of opportunistic (such as *Bifidobacterium* and *Prevotella*) and BV-related bacteria (*Atopobium* and *Sneathia*) (**Figures 2A–C**). Stratification by the vaginal status allowed a deeper evaluation of changes over time: in BV samples, we highlighted a shift between T1 and T2 among the phyla *Actinobacteria* (increased) and *Fusobacteria* (decreased), while at genus level *'Prevotella (group 6)'* was found decreased; for the I condition, no major members of the microbiota were statistically different among pregnancy trimesters (we observed only a total of 6 differential genera over time, all with average relative abundance <0.3%); finally, in the H samples, we observed a reduction of the phylum *Actinobacteria* and of its related genus *Bifidobacterium* (avg. rel. ab. of 7.5%, 2.1%, and 3.2% respectively at T1, T2, T3); the *Bifidobacterium*

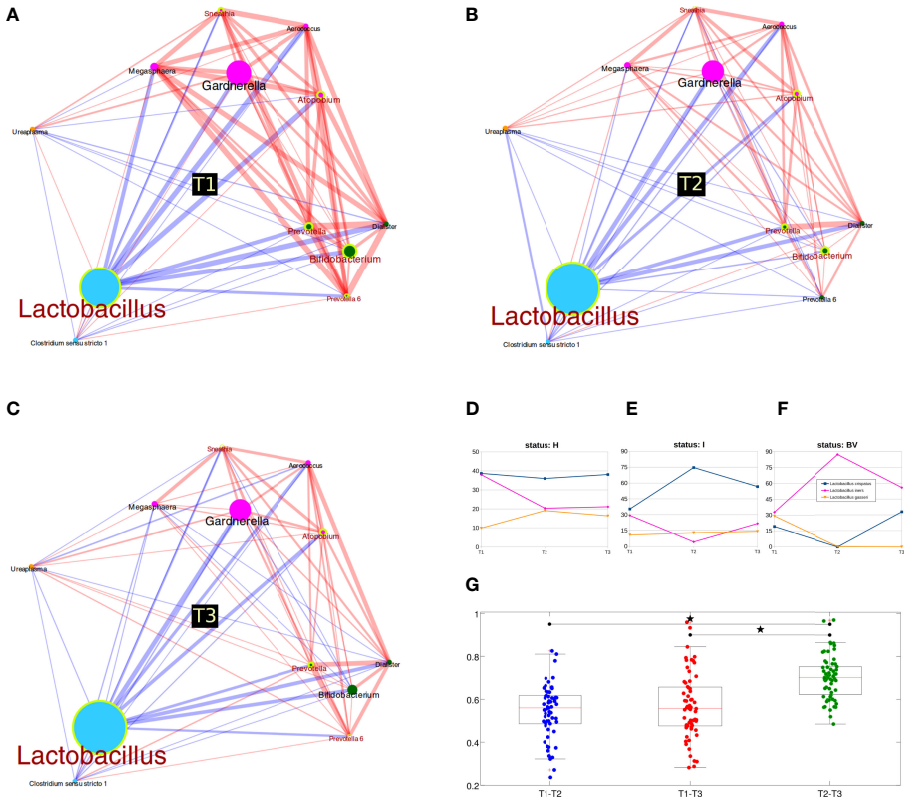

FIGURE 2 | Microbiota evolution during three trimesters of pregnancy. **(A–C)** Co-abundance networks of bacterial genera over time. Circle size is proportional to genus relative abundance for each time and colors are according to co-abundance groups (see also Suppl_Figure3_heatmap_CAGnetwork); edge size is proportional to the strength of correlation; red lines mean positive correlation, while blue lines indicate negative correlation. Genera resulting statistically different over time points are highlighted with a yellow circle and a red label; **(D–F)** *Lactobacillus* species abundance over time, stratified for vaginal status. Only the three most abundant species are represented; **(G)** Boxplot of unweighted Unifrac distances between samples over time. Distances were calculated for each pair of samples belonging to the same women, sampled at T1, T2 or T3; stars above the plots represent statistical significance ($p<0.05$).

reduction was also confirmed when limiting the analysis to the 22 women with a "healthy" microbiota (H group) at each pregnancy time-point (avg. rel. ab. 8.4%, 1.6%, and 1.0% respectively at T1, T2, T3). The *Streptococcus* genus was also decreased in the H group (entirely taken), but with a consistently lower abundance (avg. rel. ab. 0.5%, 0.1%, 0.2% respectively for T1, T2, and T3) (**Supplementary Figures S2C**).

As for evaluations within the *Lactobacillus* species, stratifying for the vaginal status, we observed several variations. In the H group, we highlighted the slight (non-significant) reduction of *L. iners* and the increase of *L. gasseri* in T1 vs T2, whereas abundances were nearly identical for T2 and T3; on the other hand, *L. crispatus* abundances were fundamentally unaltered. Considering the I group, a significant reduction of proportion of *L. iners* and a significant increase of *L. crispatus* was observed between T1 and T2. BV samples had the opposite trend, with a reduction of *L. crispatus* and an increase of *L. iners* between T1 and T2 (**Figures 2D–F**). No differences in *Lactobacillus* species were highlighted considering all samples together, regardless of their vaginal status.

Lastly, we analyzed the Unifrac distances among samples. The first interesting evidence was that the microbial profiles of all samples collected from one woman were more similar to each other than to those collected from the other women ($p<0.001$, intra- vs inter-distance for both weighted and unweighted Unifrac). When evaluating distances over time, we recorded that T2 vs T3 distance (unweighted Unifrac) was significantly higher than both T1 vs T2 and T1 vs T3 (comparisons not significantly different), indicating that between the second and third trimester of pregnancy the microbiota develops in a more independent way (**Figure 2G**). Considering the H samples alone, the evolution between T2 and T3 was confirmed; furthermore, our result suggests that T3 represents an evolution of the microbiota from T2 (as T1 had lower distance values to T2 than to T3), although average distances were very similar (T1-T2: 0.68, T1-T3: 0.71, T2-T3: 0.71).

Vaginal Microbiome at the Puerperium

In addition, we sought to evaluate the vaginal microbiome characteristics during the puerperium period in a group of 30 women, sampled a fourth time during the study (total subset time-points: T1, T2, T3 during pregnancy; T4 at puerperium, 40-55 days after delivery).

We did not observe a different biodiversity over time (p>0.05 for all α-diversity metrics tested); on the other hand, there seemed to be some separation in microbial composition within the β-diversity analysis, as T4 points were statistically different from T1, T2, and T3 (both unweighted and weighted Unifrac) (**Supplementary Figure S4A**). Over time, the analysis of microbial relative abundances at genus level suggested a composition variation at T4, with a lower content of *Lactobacillus* and a consistent presence of *Gardnerella*, *Prevotella*, *Atopobium*, and *Streptococcus*; those changes were significant (p<0.05) when compared to T1 (all except *Atopobium* and *Gardnerella*), to T2 (all except *Gardnerella*), and to T3 (all genera) (**Supplementary Figure S4B**). At species level, this was reflected in a significant decrease of all *Lactobacillus* species, *L. crispatus* and *L. jensenii* in particular (p<0.05), while *L. gasseri* was also found decreased but not significantly; contrariwise, *L. iners* was observed to be unchanged during the puerperium.

The stratification by vaginal status highlighted how these differences were mainly due to a change in bacterial members for the BV and I groups, whereas microbial profiles of H women resulted more stable, as evidenced by analyzing the correlation coefficients among average microbial profiles at genus level over time. Within the H group, microbial composition did not vary consistently, with an average Pearson correlation between T4 and all of the other three time-points of r=0.977, similar to the average r=0.998 between the paired comparisons of T1, T2, and T3; on the other hand, correlation coefficients for BV women were lower and slightly different over time (r=0.659 between T4 and the other three time points; r=0.826 among T1, T2, and T3); finally, the most substantial differences were observed for the I group, as the correlation coefficient dropped from r=0.994 to r=0.340 when comparing T4 to the other three time points (**Figures 3A, B**). BV samples at T4 were characterized by a significant reduction of the genera *Lactobacillus*, *Megasphaera*, and *Prevotella*, and by an increase of *Streptococcus* and *Finegoldia* with respect to T1; we observed a significant reduction of *Lactobacillus* and an increase of *Prevotella*, *Streptococcus*, and *Dialister* for the I condition in the comparison of T4 to all other gestational time-points; despite a slightly increase in the *Gardnerella* abundance, microbial profiles of the H group resulted very similar over all four time-points.

The *Lactobacillus* species analysis stratified by the vaginal status suggested that BV samples at puerperium had a switch compared to both the first and second trimester: *L. crispatus*

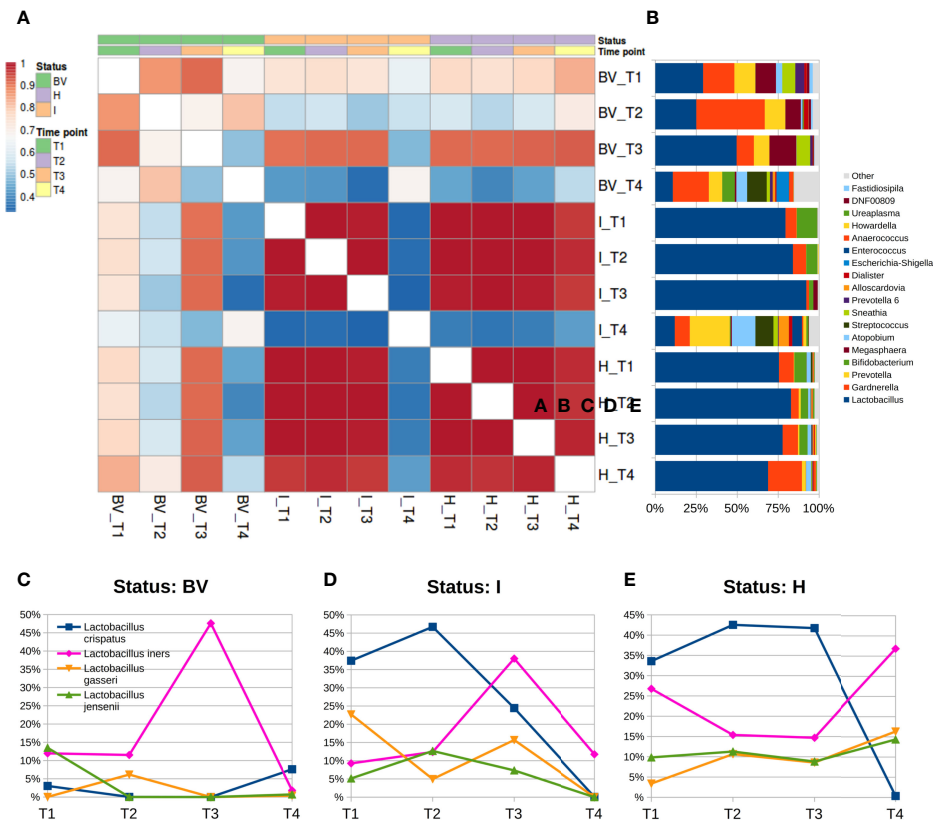

FIGURE 3 | Microbiota evolution during puerperium (T4). **(A)** Heatmap of Pearson's correlation coefficients calculated between average relative abundances at genus level over time and stratified for vaginal status; **(B)** barplots of average relative abundances at genus level over time and stratified for vaginal status; genera with rel. ab. ≤1% were grouped in "Others" category; **(C–E)** Line plots of average abundances of *Lactobacillus* species over time. Only the four most abundant species are represented.

showed a higher abundance (T4 7.6% vs T1 3.0% and T2 <0.1%; $p<0.05$ for T2 vs T4) while *L. iners* a lowered one (T4 1.8% vs T1 12.0% and T2 11.5%; $p<0.05$ for T1 vs T4). We could not evaluate the BV composition during the third trimesters (T3) since we had only 1 sample for this time-status combination. In the I samples, T4 microbiota displayed a dramatic decrease of *L. crispatus* (0.1% vs 36.2% of the gestational time-points average; $p<0.05$ forT2 vs T4). A similar decrease of *L. crispatus* was observed for H women as well (0.4% *vs* 39.3% on average of the gestational time points), together with a somewhat higher abundance of *L. iners* (36.8% *vs* 19.0% of the gestational time-points average). Due to the extreme variability among individuals, between T3 and T4 the sole *L. crispatus* reduction was statistically significant (**Supplementary Figures S3C–E**).

Among the women evaluated at T4, 10 out 30 (33.3%) received an intrapartum antibiotic prophylaxis for GBS. Microbial profiles of these women did not result significantly different from the untreated group (n=20) neither by alpha- ($p>0.05$ for all metrics tested) nor beta-diversity ($p=0.937$ and $p=0.112$ for unweighted and weighted Unifrac distances, respectively). Only one taxon, the *Prevotella* genus, was significantly altered, showing an increase in antibiotic-treated women, when compared to the untreated ones (rel. ab. of 20.0% with antibiotics vs 6.0% without antibiotics) (**Supplementary Figure S5**). At the same time, this difference was reflected in higher-level taxonomies as well (*Bacteroides*, *Bacteroidia*, *Bacteroidales*: 22.9% vs 8.0%; *Prevotellaceae*: 22.2% vs 7.3%, with vs without antibiotics). No differences were highlighted by the species-level characterization of the *Lactobacillus* genus.

Association Between Microbiome Composition and First Trimester Miscarriage

We compared the microbiome profiles at T1 of the 63 women with successful pregnancies to the ones of 9 women who suffered a first trimester miscarriage. No significant differences were found on both α- ($p>0.05$ for chao1, Shannon, Good's coverage, Observed species, Faith's phylogenetic diversity metrics) and β-diversity ($p=0.412$ and $p=0.110$ for unweighted and weighted Unifrac distances, respectively) analyses. Nevertheless, we observed an overgrowth of *Fusobacterium* (rel. ab. 1.1%, $p=0.02$) in the miscarriage group compared to successful pregnancies (0.1%). No significant differences were highlighted for the *Lactobacillus* species.

Vaginal Metabolites Composition and Metabolite-Microbiome Correlation

In the supernatants of the vaginal swabs, a total of 63 metabolites were detected and quantified by ^{1}H-NMR spectroscopy. Molecules mainly belonged to the groups of SCFAs, organic acids, amino acids, and biogenic amines (Data sheet S1).

We performed a correlation analysis aimed at relating microbial composition to metabolite concentrations, using Spearman's rank correlation to determine monotonically increasing or decreasing relationships. All samples collected over the four time-points were considered (n=219); miscarriage samples were analyzed separately.

We could define three main clusters of correlations: (i) *Lactobacillus* stood by itself, separated from all other bacteria, strongly positively correlated to lactate and sarcosine (r=0.62 and r=0.61, respectively). Moreover, positive correlations were evidenced for many amino acids (i.e., isoleucine, leucine, phenylalanine, aspartate, glutamate, valine, glycin, serine, threonine, tryptophan, with correlation values ranging from 0.26 to 0.65); (ii) BV-associated genera, such as *Gardnerella*, *Prevotella*, *Atopobium*, *Dialister*, *Aerococcus*, and *Sneathia*, were positively correlated to putrescine, methylamine, tyramine, formate, trimethylamine (TMA), alcohols (i.e., ethanol, isopropanol), and SCFAs (i.e., acetate, butyrate, propionate); (iii) other lower-abundance bacteria, such as *Bifidobacterium*, *Streptococcus*, and *Alloscardovia* correlated with nucleotides (i.e., adenine, glutamine, inosine, uracil), glucose, choline, benzoate, and fumarate (**Figure 4**).

Microbiome-metabolites correlation patterns were further refined by looking at the possible relationships with spontaneous miscarriages (n=9). Overall, only few correlations were significant (p-value of the linear model <0.05). Correlation patterns for *Lactobacillus* and BV-associated genera were in accordance with those described above for the samples of women with a successful pregnancy. Interestingly, *Fusobacterium* was found positively correlated to the nucleotides and their components (i.e., uracil, adenine, UDP, tyramine, r range 0.36-0.56), as well as to methionine (r=0.65), formate (r=0.54), choline, xanthine, and maltose (r=0.43-0.49), putrescine (r=0.38), and methylamine (r=0.31) (**Supplementary Figure S6**).

DISCUSSION

A deep comprehension of the vaginal ecosystem may hold promise for unraveling the pathophysiology of pregnancy and may provide novel markers to identify women at risk of complications, such as miscarriage and preterm births. Moreover, considering that microbial communities can be transferred from the mother's vaginal niche to the newborn gut, the study of the vaginal microbiome during pregnancy and puerperium can open new perspectives for infant's microbiome development and future health (Dominguez-Bello et al., 2010).

That is why in this study we characterized the vaginal environment in the situations of both a normal pregnancy, at the three gestational trimesters and the puerperium period, and a spontaneous first trimester miscarriage. In particular, we assessed the vaginal bacterial composition and the vaginal metabolic profiles.

At first, we confirmed that, irrespective of the period and type of pregnancy, BV cases were characterized by a dramatic reduction of *Lactobacillus* and an increase of anaerobic bacteria, such as *Gardnerella, Atopobium, Prevotella, Megasphaera, Sneathia*, *Aerococcus* (Deka et al., 2021).

In line with previous findings, the relative and absolute proportion of *L. crispatus*, a hallmark of vaginal eubiosis, inclined to decrease in the transition from H to BV conditions (Ceccarani et al., 2019). As for *L. iners*, the abundance of this species did not differ between H and BV groups in our cohort,

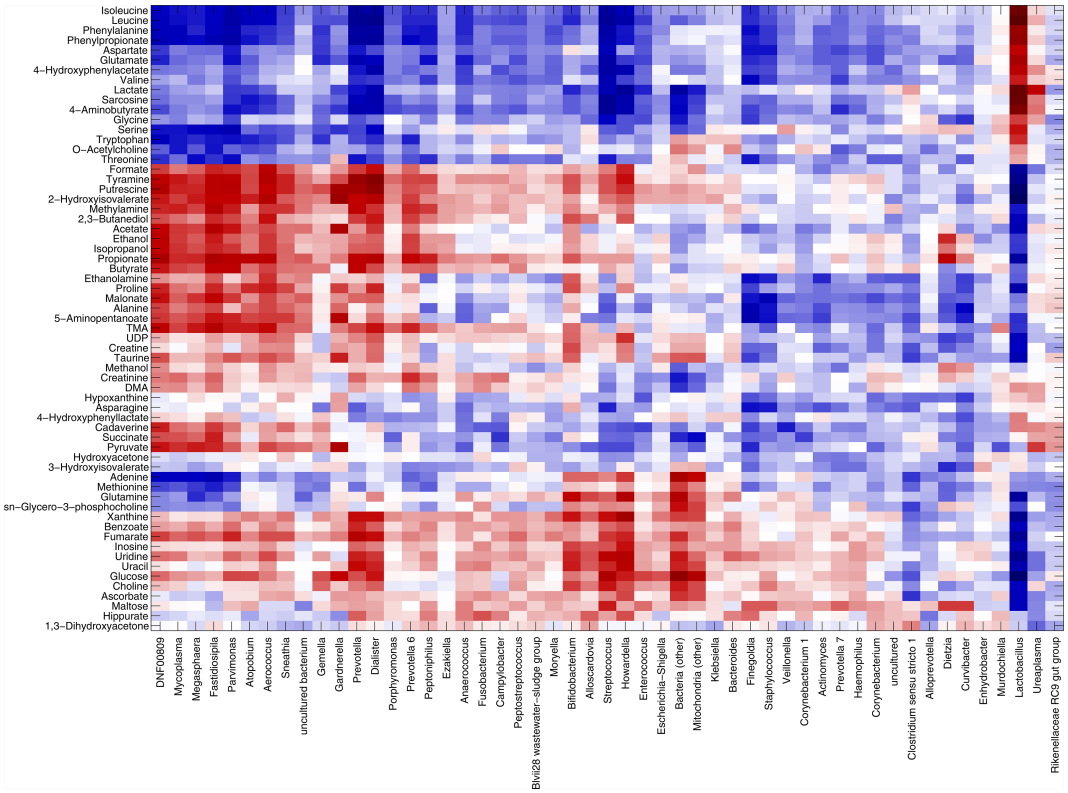

FIGURE 4 | Correlation between metabolome and microbiota. Heatmap showing the Spearman's correlation coefficient between metabolites concentration and the relative abundances of the main bacterial genera over all samples collected, excluding miscarriages (n=219). Only groups present at >1% of relative abundance in at least one sample were considered. Metabolite and microbial data were clustered using Pearson's correlation metric and average linkage.

even though it has been considered a transitional species typically associated with dysbiotic conditions (Yoshimura et al., 2020); on the other hand, *L. iners* has also been reported to be the dominating taxon in a large subset of women worldwide, being its presence associated with young age and unprotected sexual practices (France et al., 2020; Novak et al., 2021).

When considering the changes of the vaginal microbiome during the three trimesters of pregnancy, we observed that several bacterial genera were statistically different between the first and second trimester, suggesting a deep reshaping of the microbiome profiling towards a 'healthier condition': moving from the first to the third trimester, in line with the higher proportion of H cases, we found an increase of *Lactobacillus* genus and a decrease of BV-related genera (e.g., *Prevotella, Atopobium*, *Sneathia*), with no differences in *Lactobacillus* species.

Taken together, these data confirmed that the vaginal microbiome becomes more stable throughout the entire pregnancy, being less diverse and mainly dominated by lactobacilli (Li et al., 2020; Rasmussen et al., 2020; Marangoni et al., 2021; Pace et al., 2021).

It is worth noting that bifidobacteria, typical beneficial commensals inhabiting the human intestine, have tended to decrease their vaginal ecosystem abundances at the end of pregnancy. It has been shown that *Bifidobacterium* is the dominant genus of some vaginal microbiomes and that overall bifidobacteria have the potential to be as protective as lactobacilli, according to the current understanding of a healthy vaginal microbiome (Freitas and Hill, 2017). Nevertheless, Lee and colleagues recently observed that the relative abundance of *Bifidobacterium* spp. significantly increased during pregnancy in women with an intermediate and BV status compared to normal vaginal microbiota, and that some dysbiotic conditions were dominated by *Bifidobacterium breve* (Lee et al., 2020). In line with these observations, our data highlighted a co-abundant vaginal pattern, characterized by several BV-associated genera, such as *Prevotella* and *Dialister*, and *Bifidobacterium* spp. Since the role of this vaginal microbial group is yet to be understood, further studies are needed to investigate the clinical significance of the bifidobacteria reduction at the end of pregnancy, as well as to assess the potential impact on newborn's health (Bozzi et al., 2018).

Other interesting data emerged when looking at the vaginal environment after delivery. In agreement with previous reports (Nunn et al., 2021), at the puerperium we found a significantly lower content of *Lactobacillus*, and higher levels of *Gardnerella, Prevotella, Atopobium*, and *Streptococcus* compared to the third trimester of pregnancy. These variations are consequences of after-delivery vaginal alterations that profoundly altered the host

environment and, thus, led to changes in different bacterial species survival and proliferation capabilities (Nunn et al., 2021).

Moreover, we observed a significant increase in *Prevotella* abundance in women who received an intrapartum antibiotic prophylaxis (IAP) for GBS compared to untreated ones. This aspect deserves attention considering that members of *Prevotella* genera have been associated with negative 'outcomes' of the cervicovaginal environment, being responsible for strong inflammatory conditions, cytotoxicity, and alterations of the reproductive tract (Campisciano et al., 2020; Salliss et al., 2021). It is well known that IAP can negatively affect the gut microbiome of infants vaginally delivered, specifically in relation to microbial composition and occurrence of antibiotic resistance genes (Garcia, 2021). However, the effect of antibiotic prophylaxis on the vaginal microbiome after delivery is still little explored. Even if further studies are needed to clarify the reasons behind the increase in *Prevotella* levels in women receiving IAP, we can speculate that beta-lactam antibiotics could have selected this bacterial genus, as it is potentially able to produce β-lactamase enzymes (Toprak et al., 2020).

Moving to the analysis of bacterial relative abundances in women suffering a first trimester miscarriage, we highlighted a significant vaginal overgrowth of *Fusobacterium* in abortions compared to successful pregnancies, at all taxonomic levels. This microbial genus has been strongly associated with genital inflammation and dysbiosis, being *Fusobacterium* able to cooperate with other taxa to disrupt the normal vaginal bacterial composition, leading to microbial imbalance (Lennard et al., 2017; Agarwal et al., 2020). It has been shown that *Fusobacterium* has a mutualistic relationship with the BV-correlated bacteria: as they are major sialidase-producers, they enable *Fusobacterium* to consume sialic acids from the host-produced mucus. At the same time, *F. nucleatum* exposure to vaginal communities may encourage features of dysbiosis (e.g., increased sialidase activity and *G. vaginalis* abundance) in susceptible vaginal communities (Agarwal et al., 2020). In addition, *F. nucleatum* has been previously associated with preterm labor, since it was found in greater abundance in preterm placental membranes than at term (Doyle et al., 2014). To the best of our knowledge, this is the first time that *Fusobacterium* got linked to the risk of first trimester miscarriage, considering that previous investigations highlighted the potential role of other microorganisms, such as *Finegoldia, Coprococcus, Roseburia, Atopobium*, and *Prevotella* (Al-Memar et al., 2020; Xu et al., 2020; Jiao et al., 2021; Liu et al., 2021).

The vaginal bacterial community profiles found during pregnancy were accompanied by peculiar fingerprints in the composition of the vaginal metabolites. In agreement with recent observations, *Lactobacillus* abundance was strongly correlated with higher levels of lactate, sarcosine, and many amino acids, whereas BV-associated genera, such as *Gardnerella, Atopobium*, *Sneathia*, were correlated to amines (putrescine, methylamine, TMA), formate, alcohols (ethanol, isopropanol), and short-chain fatty-acids (SCFAs, as butyrate, acetate, propionate) (Ceccarani et al., 2019; Laghi et al., 2021). On the one hand, the lactate production by *Lactobacillus* species reduces the vaginal pH, contributing to the homeostasis against potentially endogenous or exogenous pathogens. These microorganisms are also known producers of branched-chain amino acids, thus the higher concentration of some of them, such as valine, leucine, and isoleucine, is another fingerprint of the prevalence of lactobacilli in 'healthy' women (Vitali et al., 2015). Conversely, during dysbiotic conditions, the proliferation of diverse bacterial genera, some of which typical of the gut microbiota, and the imbalance between lactobacilli and BV-related bacteria lead to higher levels of amines, organic acids, and SCFAs (Vitali et al., 2015). In this context, higher levels of *Fusobacterium*, associated with the higher risk of spontaneous abortion, were positively correlated to several vaginal molecules, including methionine, formate, putrescine, and methylamine. Considering the low number of data points (n=9), the exact role of the vaginal metabolome in first trimester miscarriages, as well as the causative relationship between microbiota and immune responses, remain to be further elucidated, to enable the best possible diagnosis and therapeutics of early pregnancy loss.

In conclusion, we deepened the existing literature knowledge about the composition of the vaginal ecosystem during pregnancy and puerperium, highlighting peculiar microbial/metabolic fingerprints.

Our data could help implement 'prognostic' criteria (e.g., evaluation of the risk of spontaneous miscarriage based on the microbiome/metabolome profiles), as well as strategies for the prevention of early pregnancy loss, based on the 'manipulation' of the vaginal bacterial inhabitants (e.g., use of probiotics and prebiotics). Moreover, the microbial changes induced by GBS prophylaxis (i.e., increase in *Prevotella* levels) deserve attention, leading to the idea of new approaches able to reduce the impact of antibiotics in maternal/neonatal health.

As a strength of our work, we excluded from the enrollment all the women harboring conditions able to perturb per se the vaginal microbiome composition (e.g., VVC, AV, presence of STIs) and we combined multiple 'omic' sciences (i.e., genomic and metabolomic) to decipher the vaginal environment in pregnancy and puerperium. On the other hand, we are fully aware of some limitations of the study, as the potential loss of low concentration molecules, due to the reduced sensitivity of ^{1}H-NMR compared to other metabolomic techniques (e.g., high resolution chromatographic separation techniques coupled to accurate tandem mass spectrometry).

To further understand the interactions between vaginal microbes and the host, future studies perspectives will include (i) the increase in number of women suffering a spontaneous first trimester miscarriage, to strengthen the conclusions regarding this group (ii) the evaluation of several inflammatory markers, (iii) the assessment of vaginal proteomic profile, and (iv) the evaluation of bacterial subspecies/clades.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for

Biotechnology Information (NCBI) BioProject database under accession number PRJNA766806.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Romagna (CEROM) (n° 2032 of 21st February 2018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM and CF conceived and designed the study. SZ and GP recruited the patients. LL, MP, SM, MS, CCo, CCe, and TC performed the experiments. CF, LL, MS, CCo, CCe, and TC analyzed the data. AM and VS contributed reagents/materials and analysis tools. CF, AM, and MS wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.838405/full#supplementary-material

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The diversity of vaginal microbiome in women infected with single HPV and multiple genotype HPV infections in China

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Introduction: The human papillomavirus (HPV) is the leading cause of cervical cancer globally. However, its microbial composition and association with the types of HPV infection remain elusive.

Methods: This study was designed to characterize the vaginal microbiota of 53 HPV-infected and 16 normal women (control group) by using high-throughput sequencing with the Illumina platform.

Results: In this study, the five leading phyla were *Firmicutes* (73.9%), *Actinobacteriota* (12.8%), *Proteobacteria* (6.2%), *Fusobacteria* (3.5%), and *Bacteroidota* (3.1%). We found that single HPV genotype–positive women had higher α-microbial diversity compared with HPV-negative and multiple HPV–positive women. In women with a single HPV genotype infection, the HPV-16 infection had significantly higher α-diversity than other genotype infections. In multiple HPV genotype–positive women, the highest α-diversity was found in women positive for HR–HR HPV genotype infection, compared with other infections. Furthermore, in single- and multiple-genotype infections, the abundance of s_unclassified_g_Lactobacillus decreased whereas the abundance of s_Gardnerella_vaginalis increased compared with control. Additionally, s_unclassified_f_Rhizobiaceae and s_sneathia_sanguinegens were only found in HPV-infected women.

Conclusion: This study showed that the type of HPV infection was associated with the composition of the vaginal microbiota. Further studies on HPV genotypes and vaginal microbiota are necessary to uncover more mysteries of their association and provide a promising therapeutic target as well as low-cost future therapeutic strategies.

KEYWORDS

vaginal microbiota, human papillomavirus (HPV), single infection, multiple infections, Cervical Cancer

Background

Cervical cancer is the fourth most common female cancer in the world. According to estimates, 570,000 cervical cancer cases and 311,000 deaths were reported in 2018 (Arbyn et al., 2020). Human papillomavirus (HPV) most commonly causes cervical squamous cell carcinoma (SCC) and its precursor lesions (cervical intraepithelial neoplasia; CIN) worldwide (zur Hausen, 2002; Muñoz et al., 2003; Bruni L et al., 2010; Forman et al., 2012; Crosbie et al., 2013). HPV, a small, double-stranded, non-enveloped DNA virus, infects genital tracts and oral mucosa. HPV genotypes have been divided into two groups depending on their carcinogenicity, i.e., high-risk and low-risk genotypes (Bodily and Laimins, 2011). The most frequent genotype is HPV-16 globally (Crow, 2012); however, the prevalence of other HPV genotypes varies from region to region (Crow, 2012; Baloch et al., 2016). According to estimates, most HPV infections are transient and cleared within a couple of years (Crow, 2012) (Myers et al., 2000; Richardson et al., 2003). Only 10%–20% of HPV infections persist latently, leading to disease progression and invasive cancer development (Shanmugasundaram and You, 2017). However, the underlying molecular mechanisms of HPV persistent infection are still not well understood.

The vaginal tract is populated with various types of microorganisms, which are collectively called the vaginal microbiome. It has been reported that vaginal microbiota protects women from different urogenital infectious diseases (Watts et al., 2005; Gillet et al., 2011; Guo et al., 2012; Lee et al., 2013; Vriend et al., 2015). The role of vaginal microbiota in the acquisition and persistence of HPV has been reported (Brotman et al., 2014; Mitra et al., 2016). However, the role of the diverse vaginal microbiome in single HPV and multiple HPV genotype infection has not been adequately investigated. Therefore, the current study was designed to investigate whether single-HPV and multiple HPV–genotype infections are associated with the diverse community of vaginal microbiota composition.

Methods

Ethical statement

This study was approved by the Ethics Committee of the Faculty of Life Science and Technology, Kunming University of Science and Technology, and the Center for Disease Control and Prevention (CDC) in Yunnan Province, China. Written consent was individually obtained from all participants.

Study design

A total of 69 women were recruited who visited the Out-Patient Department of the First People's Hospital of Yunnan Province from November 2016 to July 2017. For the sample collection, the vaginal swab was inserted ~2–3 cm into the vagina and swirled for ~30 s. The swab samples were immediately put into Fluidx tubes containing 0.8 ml of DNA/RNA Shield and transferred to the laboratory within 30 min. Samples were pelleted by centrifugation at ≥10,000× g (25°C) for 10 min and the sediment stored at −80°C until further analysis. Women infected with HIV, hepatitis B/C, or autoimmune disorders; who received antibiotics or pessaries within 14 days of sampling; or had a previous history of cervical therapy were excluded. A standardized questionnaire was used to collect information from each participant about their ethnicity, education, age, marital status, smoking, drinking, illness history, sexual activity, and profession.

DNA extraction

Bacterial and viral genomic DNA was extracted from the samples synchronously by using the TIANamp DNA Extraction Kit (Tiangen Biotech Co., Lai Chi Kok, Hong Kong) following the manufacturer's instructions. Quality control was carried out by gel electrophoresis, and nanograms per microliter of DNA and 260/280 OD were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., US). The purified DNA was stored at −20°C until further HPV genotyping and microbiota sequencing.

HPV genotype examination

DNA was amplified with broad-spectrum consensus primers (MY09/11) targeting the HPV L1 region using polymerase chain reaction (PCR) (Baloch et al., 2016). Isolated and amplified DNA from HeLa and CaSki cell lines was used as positive controls, and solutions without sample DNA were used as negative controls. GenoArray Test Kit (Hybribio, Chaozhou, China), an L1 consensus primer-based PCR assay kit, was used to amplify 23 HPV genotypes, namely, 13 HR-HPV genotypes (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68), three PHR-HPV genotypes (HPV-53, -66, and -81) and seven low-risk HPV (LR-HPV) genotypes (HPV-6, 11, 42, 43, 44, and 61), according to the manufacturer's recommendations.

16S rRNA gene sequencing and analysis

In this study, the V4 segment of the 16S rRNA gene was amplified, and the sequencing was performed using the Illumina MiSeq system, according to the manufacturer's recommendations. FASTQ file conversion of the raw data was performed following demultiplexing using the HiSeq reporter. High-quality reads (80% bases have Q score >20) were selected for analysis, and reads with

unknown bases (N) and poor-quality reads were discarded. Sequences were grouped into operational taxonomic units (OTUs) at a similarity threshold of 97%. The SILVA rRNA gene database was used to make taxonomic assignments for all OTUs and phylum, class, order, family, and genus and were identified at the species level.

Based on OTU information, rarefaction curves and α-diversity indices refer to community diversity (Shannon, Simpson), community richness (Chao1 and Ace), community evenness (Shannon-even, Simpson-even), and sequencing depth (Good's coverage) calculated by Mothur. A heatmap diagram showing the relative abundance of OTUs was generated using the Vegan Package in R 2.4. Phylogenetic beta diversities, including principal coordinate analysis (PCoA), were evaluated with the Bray–Curtis distance using QIIME 1.7.0. The linear discriminant analysis (LDA) and effect size (LEfSe) methods were used for potential biomarker detection. The threshold score of LDA was set at 3.0, and a significant p-value of 0.05 was employed.

Statistical analysis

A chi-square test was used to assess the differences between demographic and clinical characteristics of participants. Differences in microbiota between groups were measured using PERMANOVA (weighted UniFrac distance). Differences in the Chao index and Shannon index (α-diversity metrics) were tested according to the type of HPV infection using the Wilcoxon rank-sum test. Metagenomic potential biomarker discovery and associated statistical significance were assessed by analyzing the relative taxonomic abundances according to the linear discriminant analysis (LDA) effect size (LEfSe) methods. In LEfSe, the Kruskal–Wallis rank-sum test was used to distinguish features with significantly different taxon abundances in groups and LDA to calculate the size effect of each feature. A threshold of 3.0 on the logarithmic LDA score was used for discriminative microbial biomarkers. The association between microbial community structure and single-HPV-genotype or multiple-HPV-genotype infections was analyzed by multivariable logistic regression. The relationships among HPV infection type, ethnicity, married status, age, smoking, drinking, and sexual partner were compared by Fisher's exact test.

Results

Characteristics of participants

A total of 69 women were enrolled in this cohort study and were divided into three groups: healthy control (n = 16; 23.2%), single HPV-genotype infection (n = 31; 44.9%), and multiple HPV-genotype infection (n = 22; 31.9%). Among the total, single-infection, and multiple-HR-HPV genotypes, infected women were 26 and seven. HR-PHR-HPV-infected women were six, HR-LR-HPV infection was found in nine women, and LR-HPV/PHR-HPV infection was found in five women. Overall, 15, 12, 10, and eight participants were infected with HPV-16, 52, 33, and 58, respectively (Table 1). The demographic characteristics of participants are shown in Table 2. Among 69 participants, 53 were Han, 16 were from other ethnicities, 48 were married, and 21 were unmarried. Fifty-six women reported having a single sexual partner.

Composition of cervical microbiota

A 16S rRNA targeted metagenomics analysis was performed on individual vaginal swab samples collected from 69 women and allowed a comparison of the overall bacterial richness and phylogenetic composition of the vaginal microbiota. A heatmap analysis shows the vaginal microbiota composition, which is different among HPV-negative, single-genotype, and multiple-genotype infections in women (Figure 1). For conforming, α-diversity (the index of Sob, Chao, ACE, Shannon, Simpson, Coverage, Shannon, and Simpson even) and β-diversity (PCoA) were analyzed. α-Diversity analysis of the microbiota profile based on Shannon and Chao 1 diversity indicated substantial differences among different groups (Tables 3, 4). For β-diversity analysis, we performed a principal coordinate analysis (PCoA) to confirm the differences. We found a different clustering based on the HPV infection status (Figure 2A).

In this study, a total of 27 phyla, 62 classes, 156 orders, 262 families, 497 genera, 719 species, and 907 OTUs were classified. Among them, the five leading phyla were *Firmicutes* (73.9%), *Actinobacteriota* (12.8%), *Proteobacteria* (6.2%), *Fusobacteria* (3.5%), and *Bacteroidota* (3.1%) (Figure 2B). Furthermore, we found that HPV-negative and multiple-genotype-infected women had less microbiota diversity compared with single HPV genotype–infected women (Figures 3A, B).

At the genus level, we found that *Lactobacillus* and *Gardnerella* were the two leading bacterial genera in control, single HPV infection, and multiple HPV infection groups (Figure 4A). However, the abundance of s_unclassified_g_Lactobacillus decreased and that of g_Gardnerella_vaginalis increased in single- and multiple-genotype infections compared with control (Figure 4B). Further, g_unclassified_f_Rhizobiaceae was only found in HPV-positive women. Its abundance was higher in multiple HPV genotype–infected women compared with single HPV genotype–infected women (Figure 4A).

We further compared vaginal microbial diversity among different HPV genotypes. Our analysis showed that women infected with HPV-16, -52, and -58 had higher microbiota diversity than HPV-33 and other genotypes of infection (Figure 4C). Additionally, the proportion of s_Streptococcus_anginosis, s_lactobacillus_jemsenii,

TABLE 1 Different HPV distribution among single infection and multiple infections.

Genotypes	Single infection (n=31)	Multiple infections (n=22)	Total
HR-HPV	26	7	33
HR-PHR-HPV	3	3	6
HR-LR-HPV	0	9	9
LR-HPV or PHR-HPV	2	3	5
HPV-6	0	1	1
HPV-11	0	1	1
HPV-16	7	8	15
HPV-18	0	2	2
HPV-31	1		1
HPV-33	6	4	10
HPV-34	0	1	2
HPV-35	1	2	3
HPV-43	0	1	2
HPV-51	3	2	5
HPV-52	5	7	12
HPV-53	0	4	4
HPV-55	0	1	1
HPV-58	6	2	8
HPV-59	0	3	3
HPV-61	0	2	2
HPV-66	0	3	3
HPV-68	1	2	2
HPV-72	0	2	2
HPV-81	0	3	3
HPV-82	1		1
HPV-83	0	1	1
HPV-84	0	1	1

s_sneathia_amnii, etc., was higher in multiple HR–HR HPV genotype–infected women compared with LR–LR HPV infection (Figure 4D).

Identification of vaginal microbiota: Potential markers of HPV infection

In this study, we further extended our investigation to explore the possible bacterial biomarkers for HPV infection; therefore, we compared the relative abundance of different bacteria among women in the single HPV genotype, multiple HPV genotype, and control groups. Linear discriminant analysis (LDA) effect size (LEfSe) modeling was adopted, and 38 clades were detected (Figure 5A). We found that p_Proteobacteria, f_Burkholderiaceae, g_Peptostreptococcus, and *s_uncultureed_bacterium_g_Peptostreptococuss* were abundant in single infections, whereas o_*Rhizobiales* and *c_α-Proteobacteria* were more prevalent in multiple HPV genotype infections. Among multiple HR-HPV genotype infections, 10 clades were detected, and 15 clades were identified in multiple HR-HPV and LR-HPV genotype infections (Figure 5B). Among single HPV genotype infections, a total of 19 clades were confirmed (Figure 5C). These differentially abundant taxa could be considered as potential biomarkers.

Discussion

The effect of multiple HPV genotype infections on cervical cancer progression is still unclear (Pista et al., 2011). However, according to estimates, multiple HPV genotype infections have been commonly reported among young compared with old women as well as women with an impaired immune system or cytological anomalies (Ho et al., 1998; Herrero et al., 2000; Chaturvedi et al., 2005). The relationship between multiple HPV genotype infections and cervical cancer progression is inconsistent. Some reports have shown that multiple HPV genotypes increase the development of cervical cancer lesions (Fife et al., 2001; van der Graaf et al., 2002; Pista et al., 2011). However, some reports suggested no difference in cervical cancer lesion developments with multiple HPV genotype infection

TABLE 2 Demographic characteristics of participants.

Parameter	Control (n = 16)	Single infection (n = 31)	Multiple infections (n = 22)
Age mean ± SD	35.75 ± 7.94	39.55 ± 10.05	37.82 ± 9.21
Age range	23~49	22~59	23~55
Ethnicity			
Han	11 (68.8)	26 (83.9)	16 (72.7)
Others	5 (31.2)	5 (16.1)	6 (27.3)
Education level			
Primary or less	4 (25)	9 (29)	4 (18.2)
Middle	7 (43.7)	13 (41.9)	11 (50.0)
College or above	5 (31.2)	9 (29)	7 (31.8)
Marriage			
Married	16 (100)	21 (67.7)	11 (50)
Single	0	10 (32.2)	11 (50)
Smoking			
Yes	1 (6.2)	1 (3.2)	0 (0.0)
No	15 (93.8)	30 (96.8)	16 (100)
Drinking			
Yes	1 (6.2)	3 (9.7)	0 (0.00)
No	15 (93.8)	28 (90.3)	16 (100)
Sexual partner			
1	15 (93.8)	23 (74.2)	18 (81.8)
2 or more	1 (6.2)	8 (25.8)	4 (18.2)

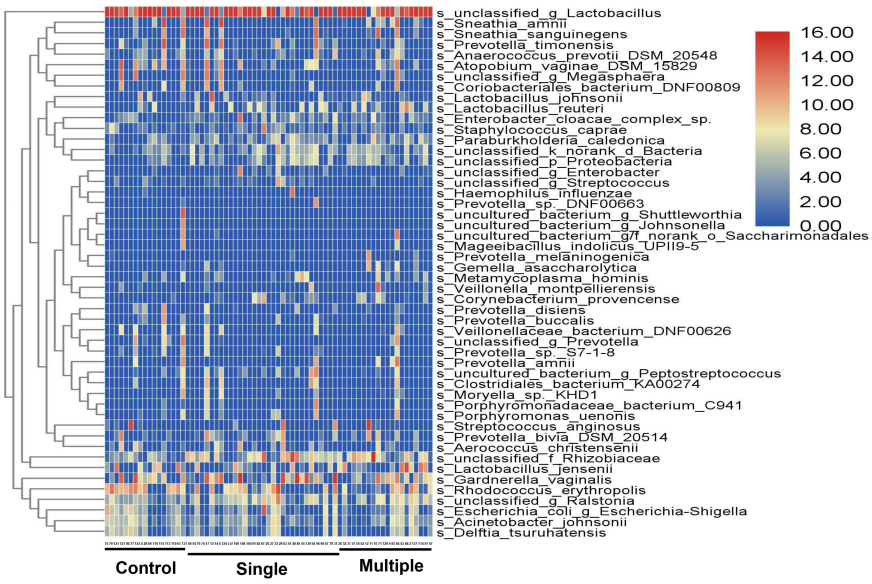

FIGURE 1
Heatmap shows diverse microbial composition in HPV-infected and control women. Color represents the relative abundance of bacterial specie; red indicates a high proportion, and blue indicates a low abundance.

compared with single HPV genotype infection (Herrero et al., 2000; Bosch et al., 2002; Cuschieri et al., 2004; Levi et al., 2004).

In this study, we evaluated the vaginal microbial profiles of single HPV genotype– and multiple HPV genotype–infected subjects for the first time. We applied high-throughput sequencing to evaluate whether single HPV genotype and multiple HPV genotype infections were associated with the diversity of the vaginal microbiota composition. We found that vaginal microbiota composition differed from single HPV genotype and multiple HPV genotype infections compared with controls. Overall, *Lactobacilli* was the most populated species among all participants, but its abundance was decreased in single HPV genotype and multiple HPV genotype infections compared with control, which is in line with previous studies (Lee et al., 2013; Mitra et al., 2015). The Simpson index of α-diversity showed an increasing trend among single HPV genotype and multiple HPV genotype infections and controls. In single HPV genotype–infected subjects, HPV-16-infected subjects showed the highest α-diversity compared with other HPV genotypes. In multiple HPV genotype–infected groups, the highest α-diversity was found in HR–HR HPV–infected subjects. In this study, we further analyzed β-diversity to evaluate species complexity among single HPV genotype–infected, multiple HPV genotype–infected, and control individuals. We observed that the proportion of *Lactobacilli* was decreasing in single HPV genotype– and multiple HPV genotype–infected subjects compared with controls.

With the application of deep metagenomic sequencing, 396 HPV genotypes have been identified (Bzhalava et al., 2014). Among them, 14 HPV genotypes are considered high-risk due to their oncogenic potential. In this study, HPV-16 was the most prevalent genotype, in line with previous research (Crow, 2012).

TABLE 3 The α-diversity of the vaginal microbial community compound at the genus level.

Estimators	Control	Single infection	Multiple infections	p-value
Sobs	33.63 ± 14.91	52.13 ± 35.18	47.91 ± 31.57	0.159
Shannon	0.54 ± 0.55	0.70 ± 0.71	0.48 ± 0.64	0.423
Simpson	0.77 ± 0.26	0.71 ± 0.29	0.82 ± 0.24	0.347
Ace	42.59 ± 26.60	74.09 ± 46.68	74.66 ± 58.07	0.069
Chao	39.74 ± 18.95	68.02 ± 41.42	62.54 ± 43.96	0.06
Coverage	0.9999 ± 0.00013	0.9996 ± 0.00024	0.9997 ± 0.0030	0.011
Shannon-even	0.16 ± 0.16	0.17 ± 0.16	0.12 ± 0.14	0.545
Simpson-even	0.06 ± 0.04	0.04 ± 0.02	0.05 ± 0.04	0.38

TABLE 4 The α-diversity of the vaginal microbial community compound at the species level.

Estimators	Control	Single infection	Multiple infections	p-value
Sobs	40.25 ± 20.03	64.16 ± 44.84	58.59 ± 41.26	0.146
Shannon	0.67 ± 0.58	0.80 ± 0.79	0.62 ± 0.64	0.621
Simpson	0.71 ± 0.26	0.69 ± 0.30	0.76 ± 0.25	0.65
Ace	52.39 ± 28.45	94.51 ± 59.97	95.70 ± 93.84	0.05
Chao	47.63 ± 24.08	84.97 ± 51.15	80.68 ± 57.48	0.042
Coverage	0.9998 ± 0.00016	0.9995 ± 0.00030	0.9996 ± 0.00041	0.010
Shannon-even	0.18 ± 0.16	0.19 ± 0.17	0.15 ± 0.15	0.732
Simpson-even	0.05 ± 0.04	0.04 ± 0.03	0.05 ± 0.04	0.414

Generally, microbial diversity is considered a sign of health (Turnbaugh et al., 2007). However, the female reproductive system was dependent on the lower diversity of the microbial community and was dominated by specific microorganisms (Mendling, 2016; Miller et al., 2016). *Lactobacilli* constitute more than 50% of the total commensal ecology and maintain a pH of 3.8 and 4.5 with other bacterial species, which is considered normal (Miller et al., 2016). Together with their antibacterial properties and immunological factors, *Lactobacilli* form the first line of defense against dysbiosis and infections. To date, more than 120 *Lactobacillus* species have been recognized and dozens of them are populated in the vagina (Mendling, 2016). In this study, s_unclassified_g_Lactobacillus was the most dominant species whose proportion was higher in control compared with single HPV genotype and multiple HPV genotype infections. Our results suggested that the proportion of *Lactobacillus* and other bacterial spp. was associated with HPV infection, and the types of HPV genotype infection such as s_unclassified_g_Lactobacillus abundance were substantially lower in HPV-52, -58, and -16 compared with HPV-33, other HR-HPV, and LR-HPV.

The underlying molecular mechanisms of how vaginal microbiota might influence the HPV persistent infection are still unknown. *Lactobacillus* spp. can produce several antimicrobial agents, including lactic acid (Boskey et al., 2001), which protect against sexually transmitted and other infections (Alakomi et al., 2000; O'Hanlon et al., 2011; Motevaseli et al., 2013). Our results show a relatively low abundance of *Lactobacilli* in women with single HPV genotype or multiple HPV genotype infection, which is consistent with a previously reported study in which they found overall high HPV infectivity and an elevated risk of multiple HPV genotype infections. Women have higher pH levels due to lack of *Lactobacillus* spp. (Clarke et al., 2012). Further, a high inflammatory response could improve viral clearance, but the immune-modulating bacteria may play a role in the progression of diseases (Khan et al., 2012; Quevrain et al., 2016). In our study, *Proteobacteria*, s_unclassified_f_Rhizobiaceae, and s_sneathia_sanguinegens were only found in HPV-infected women, which requires further investigation to confirm their role in pathogenesis. Further investigations are needed to establish the role of bacteria in immunomodulation in HPV infection and its persistence.

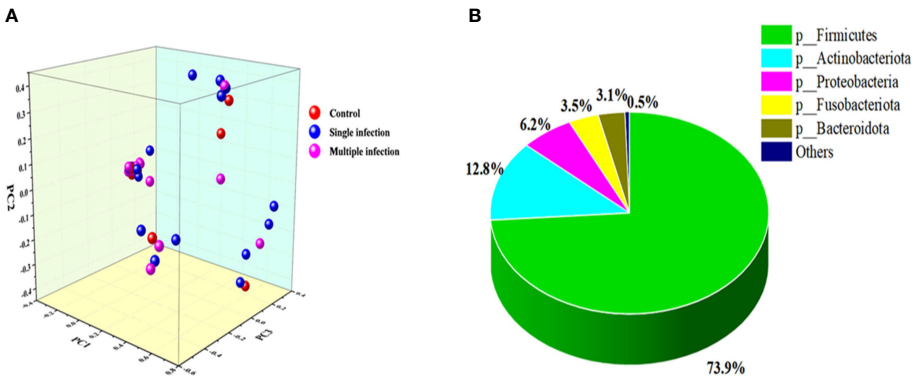

FIGURE 2
(A) PCoA profile of the cohort associated with HPV-infected status displayed with weighted UniFrac distance. Each dot presents a sample, and the color is according to grouping. **(B)** The dominant phyla in the vaginal microbial community.

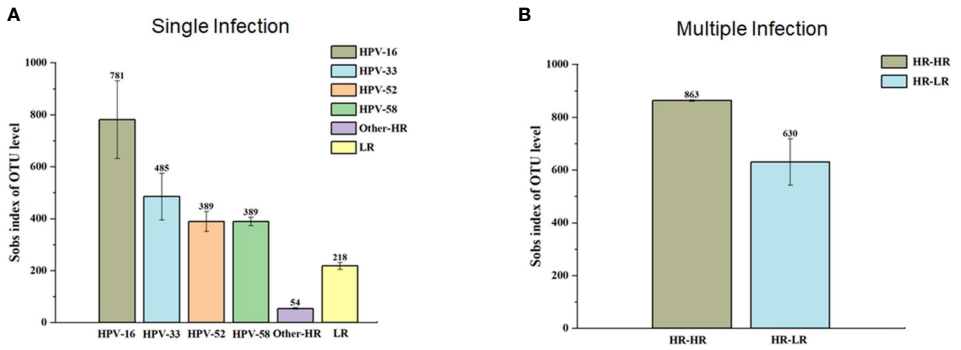

FIGURE 3
The α-diversity (Sobs index of OUT level) between single infection group **(A)** and multiple infection **(B)** groups. In single infection group, samples with HPV-16 infection presented the highest α-diversity in contrast to HPV-33, 52, 58 and others. In multiple infection groups, the highest α-diversity is in HR-HR HPV infection group compared to HR-HPVE and LR-HPV group.

Nowadays, HPV vaccines (Gardasil and Cervarix) have been approved in P.R. China and have become a hope for the possible elimination of HPV-related diseases (Brotherton, 2017; Huh et al., 2017). Despite these exciting scientific breakthroughs, many low-resource populations will not benefit from the vaccines due to technological, economic, and religious barriers. Consequently, it is important to identify alternative, evidence-based, and cost-effective intervention strategies that can be implemented to reduce HPV infection. Improving understanding of the vaginal microbiota offers opportunities to maximize a woman's first line of defense and contribute to developing practical and low-cost interventions to reduce her susceptibility to HPV infection. In a pilot study of 54 HPV-positive women diagnosed with low-grade squamous intraepithelial lesions, their daily consumption of a probiotic drink containing *Lactobacillus casei* appeared helpful in the clearance of HPV infection (Verhoeven et al., 2013). Therefore, we hypothesize that a personalized selection of probiotics would be most effective for women's health and protection. It will be

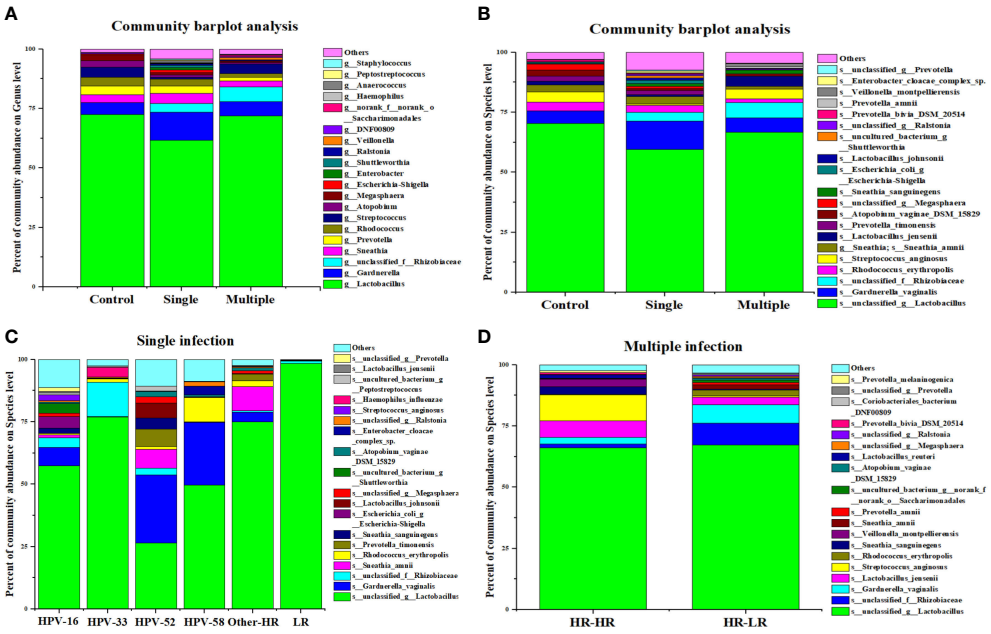

FIGURE 4
The composition of vaginal microbiota dependent on the HPV infection status. **(A)** Community barplot analysis on genus level among control, single infection and multiple infections. **(B)** Community barplot analysis on species level among control, single infection and multiple infections. **(C)** Community barplot analysis on species level related to HPV genotype in single infection group. **(D)** Community barplot analysis on species level related to genotype combination in multiple infection groups.

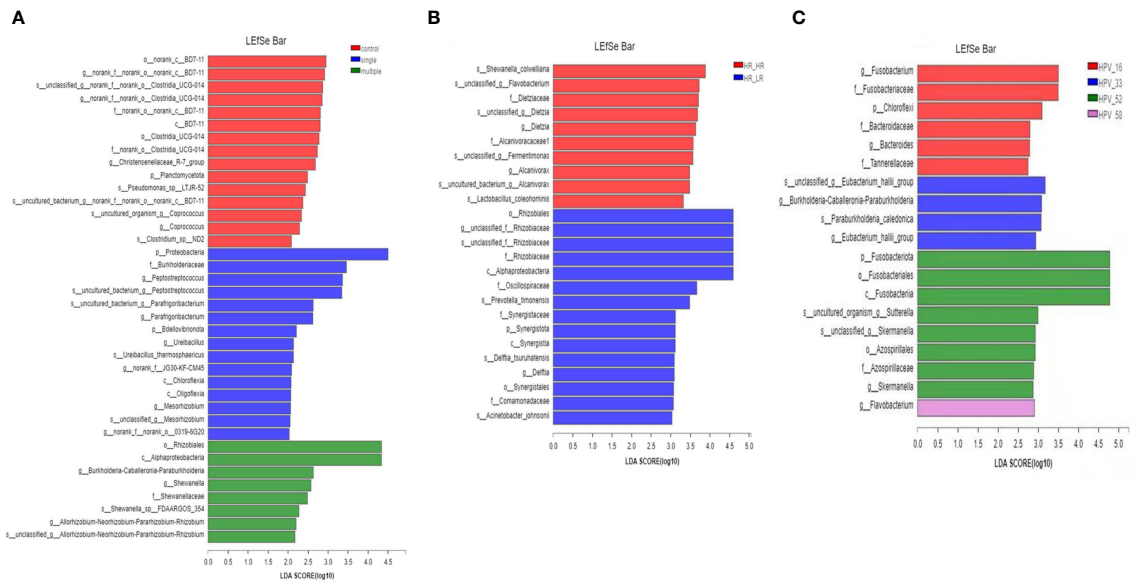

FIGURE 5
Taxonomic biomarkers. **(A)** The linear discriminant analysis (LDA) effect size (LEfSe) analysis among control, single-infection, and multiple-infection groups. **(B)** The linear discriminant analysis (LDA) effect size (LEfSe) analysis between HR-HPV, HR-HPV and LR-HPV, and HR-HPV genotype infection groups. **(C)** The linear discriminant analysis (LDA) effect size (LEfSe) analysis in single infection associated with the HPV genotype.

interesting and valuable to determine whether probiotic intervention can effectively reduce productive viral infection and lead HPV to a clinically "latent" situation in future studies.

The strength of this study includes the comparison of microbial composition and diversity of vaginal microbiota between single infections, multiple infections, high-risk infections, and low-risk infections, as well as different combinations of HPV genotypes such as HR–HR, HR–LR, and LR–LR. The weaknesses of our study include a small sample size, lack of HPV burden data, and other potential risk factors such as contraceptive methods, which would be critical for a better understanding of the association between HPV and vaginal microbiota.

Conclusions

Our results suggest that HPV infection types, i.e., single HPV, HR-HPV, and multiple HPV genotype infections, influence the composition of the vaginal microbiota, particularly non-*Lactobacillus*. Further studies on HPV genotype level infection and vaginal microbiota diversity are necessary to uncover more mysteries of their association and provide a promising therapeutic target as well as low-cost future therapeutic strategies.

Data availability statement

The datasets generated for this study were submitted to the NCBI under the accession number (PRJNA799456).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee at Kunming University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XX and ZB conceived and designed the research plan and supervised the study. ZB, YS, YL and SL finished the lab work, bioinformatics process, and statistical analysis. XW and YL performed sample collection. XX and ZB were responsible for manuscript writing and revising. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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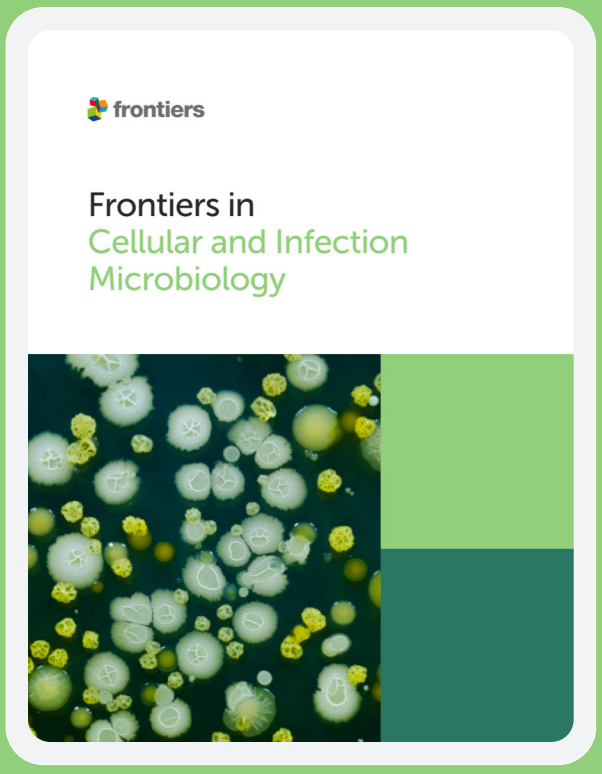

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