



Temporal Changes in Vaginal Microbiota and Genital Tract Cytokines Among South African Women Treated for Bacterial Vaginosis

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The standard treatment for bacterial vaginosis (BV) with oral metronidazole is often ineffective, and recurrence rates are high among African women. BV-associated anaerobes are closely associated with genital inflammation and HIV risk, which underscores the importance of understanding the interplay between vaginal microbiota and genital inflammation in response to treatment. In this cohort study, we therefore *investigated* the effects of metronidazole treatment on the vaginal microbiota and genital cytokines among symptomatic South African women with BV [defined as Nugent score (NS) ≥ 4] using 16S rRNA gene sequencing and multiplex bead arrays. Among 56 BV-positive women, we observed short-term BV clearance (NS < 4) in a proportion of women six weeks after metronidazole treatment, with more than half of these experiencing recurrence by 12 weeks post-treatment. BV treatment temporarily reduced the relative abundance of BV-associated anaerobes (particularly *Gardnerella vaginalis* and *Atopobium vaginae*) and increased lactobacilli species (mainly *L. iners*), resulting in significantly altered mucosal immune milieu over time. In a linear mixed model, the median concentrations of pro-inflammatory cytokines and

chemokines were significantly reduced in women who cleared BV compared to pre-treatment. BV persistence and recurrence were strongly associated with mucosal cytokine profiles that may increase the risk of HIV acquisition. Concentrations of these cytokines were differentially regulated by changes in the relative abundance of BVAB1 and *G. vaginalis*. We conclude that metronidazole for the treatment of BV induced short-term shifts in the vaginal microbiota and mucosal cytokines, while treatment failures promoted persistent elevation of pro-inflammatory cytokine concentrations in the genital tract. These data suggest the need to improve clinical management of BV to minimize BV related reproductive risk factors.

Keywords: vaginal microbiota, genital tract cytokines, bacterial vaginosis, metronidazole treatment, BV recurrence microbial and cytokine profiles by BV treatment

INTRODUCTION

Vaginal microbiota dominated by *Lactobacillus* species are considered optimal for the female genital tract (FGT), because they regulate host immunity and inhibit pathogen colonization by producing antimicrobial substances, like lactic acid and bacteriocins (1, 2). However, this paradigm of an optimal vaginal microbiota may not apply as clearly to women of African descent, because high proportions of non-*Lactobacillus*-dominant microbiota have frequently been found in asymptomatic women (3). Culture-independent 16S rRNA gene sequencing studies have shown that the majority of young, healthy, black South African women have vaginal microbiota with low *Lactobacillus* abundance and high diversity (4–6). These types of microbiota are reminiscent of those found associated with the condition known as bacterial vaginosis (BV), which is also characterized by a lack of *Lactobacillus* species, high pH and can present clinically with vaginal discharge, a fish-like odor, vaginal discomfort and urinary symptoms (7). BV is common in women of African descent and characterized by the colonization with a diverse spectrum of primarily anaerobic bacteria (7), elevated genital inflammation and thus increased HIV risk (4–6). Several studies have demonstrated that in the vagina, an increased bacterial diversity or a shift to higher quantities of specific bacterial species correlates with genital inflammation. Specifically, non-*Lactobacillus* species and BV-associated anaerobes enhanced pro-inflammatory cytokines and chemokines production (8, 9). *In vitro* studies have further shown that the relative abundance of *Prevotella bivia* was closely associated with increased levels of vaginal pro-inflammatory cytokines and chemokines (4, 10).

Despite not being considered an infection, per se, symptomatic BV is usually treated with either oral or topical metronidazole or clindamycin (11). However, antibiotic treatment of BV is often ineffective, and recurrence rates following treatment are high (12). After antibiotic treatment of BV, several studies have shown a modest reduction in bacterial counts of BV-associated microorganisms, which was inversely associated with increased relative abundances of *Lactobacillus* species (13–15). Verwijs et al. (2019) showed that only 16% of Rwandan women experienced a reduction of $\geq 50\%$ in mean

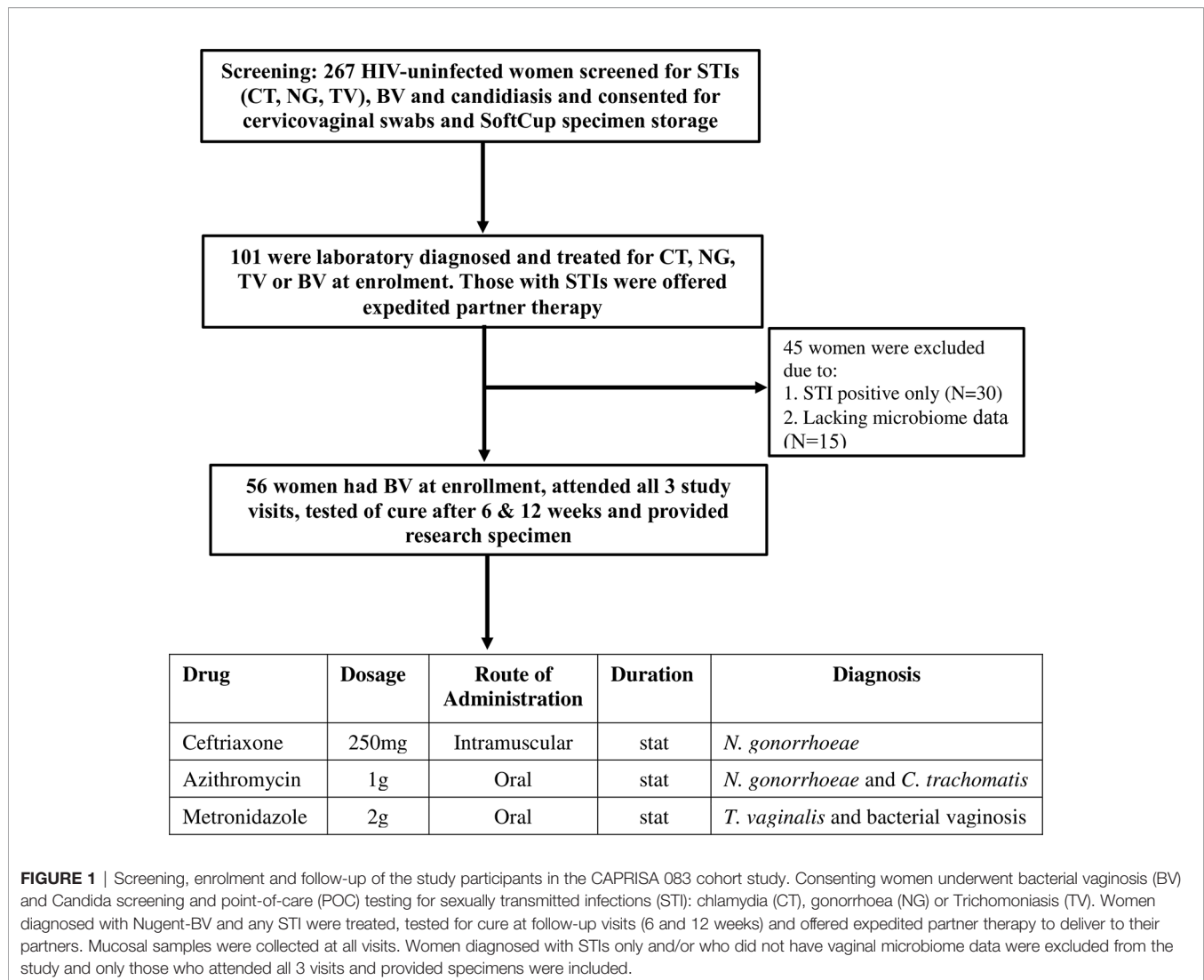
concentrations of BV-associated bacteria after metronidazole treatment. In Kenyan women, BV treatment was successful in reducing recurrence of BV by 10% (16), and a 36% increase in the proportion of women with a *Lactobacillus*-dominant vaginal microbiota (17), but this was short-lived after stopping oral metronidazole (18). The use of probiotic *L. crispatus* after treatment (Lactin-V) with vaginal metronidazole resulted in a significantly lower incidence of BV recurrence versus placebo at 12 weeks (19).

Given the association of both diverse vaginal communities and inflammation with poor reproductive health outcomes, a better understanding of the impact of current BV treatment on the vaginal microbial environment and genital inflammation is needed. In this cohort study among young South African women, we set out to investigate the longitudinal impact of standard BV treatment on the vaginal microbiota. We hypothesized that metronidazole treatment would reduce the abundances and diversity of vaginal bacteria, as well as genital inflammation, and that women with persistent or recurrent BV would have distinctly different mucosal cytokine and chemokine concentration compared to women who cleared the BV.

MATERIALS AND METHODS

Study Design, Participants, and Sample Collection

The CAPRISA 083 study screened 267 HIV-uninfected women, and enrolled 101 women with a laboratory-diagnosed STI and/or BV-intermediate or positive into a cohort (flow diagram in **Figure 1**) (20). Women who were pregnant or who had received antibiotic treatment within seven days of sampling were excluded from the study. STI screening was conducted at the clinic by GeneXpert (Cepheid, CA, USA) and by standard PCR multiplex assay (21) was performed. General physical examination, followed by a speculum examination to assess for vulval, vaginal and cervical abnormalities was also performed at each visit. Participants diagnosed with *Chlamydia trachomatis* (azithromycin 1g oral), *Neisseria gonorrhoeae* (ceftriaxone 250mg intramuscular and azithromycin 1g oral), *Trichomonas vaginalis* (metronidazole 2g oral), symptomatic BV with Nugent



score ≥ 4 (metronidazole 2g oral single dose) or candidiasis (clotrimazole 500mg pessary and clotrimazole 1% cream) were treated with regimens recommended in the South African STI treatment guidelines (22). At follow-up visit, women who remained BV positive after initial treatment were further offered metronidazole 400mg for 5 days. Enrolled and treated women were asked to return after 6 weeks and 12 weeks for follow-up examinations, further collections of specimens and retesting for STIs, Nugent-BV and candidiasis. Prostate-specific antigen (PSA) was measured in SoftCup supernatants by ELISA (R&D Systems, MN, USA) to account for the effects of recent sexual activity on measured parameters. The protocol for this study was approved by the Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE403/16).

BV Classification Using Nugent Score Across Visits

A vaginal swab was used for Gram staining for the diagnosis of candidiasis and BV using Nugent's criteria (a score of 0 - 3 was

considered BV negative, 4 - 6 intermediate BV, and 7 - 10 BV positive) (23). Nugent-BV in this analysis was defined as Nugent score ≥ 4 (13, 24). BV groups were then classified into women who "cleared", those who "persisted", and "recurrent" BV. The term "cleared" was defined as having a Nugent score ≥ 4 at baseline and 6 weeks follow-up visits, but < 4 at the 12 weeks follow-up visit. The term "persistence" was defined as Nugent Score ≥ 4 at baseline visit and remain so at the next consecutive visits. The term "recurrent" was defined as Nugent score < 4 at 6 weeks follow-up visit, but Nugent score increase to > 4 at the next visit.

Bacterial DNA Extraction

Bacterial DNA was extracted from the genital swabs for 16S rRNA gene sequencing using the PowerSoil DNA kit (MoBio, CA, USA), after mechanical and enzymatic disruption using 5 μ l of lysozyme at 10 mg/ml (Sigma-Aldrich, MO, USA); 13 μ l of mutanolysin at 11,700 U/ml (Sigma-Aldrich, MO, USA) and 3.2 μ l of lysostaphin a 1mg/ml (Sigma-Aldrich, St. Louis, MO)

and was incubated at 37°C for 60 min. Mechanical disruption was then performed using a Precellys 24 tissue homogenizer (Bertin instruments, Montigny-le-Bretonneux, France) at 3x30s at speed setting m/s 5.5 (equivalent to 5500rpm). DNA was then purified from the using the Agencourt AMPure XP (Beckman Coulter, CA, USA). DNA was quantified using Qubit 2.0 Fluorometer (Invitrogen, MA, USA).

Characterization of the Vaginal Microbiota

The V3-V4 hypervariable region was amplified using universal 319F/806R primers, and purified libraries consisting of ~120 pooled samples were sequenced on the Illumina MiSeq platform (paired-end sequence reads with v3 chemistry). FastQC was used to perform data quality control of raw sequences and to infer parameters for upstream processing. Divisive Amplicon Denoising Algorithm (DADA) 2 was used to infer amplicon sequence variants (ASVs) (25). The SILVA ribosomal RNA database (25) was used for taxonomic assignment. The ASVs for key genera such as *Lactobacillus*, *Prevotella*, *Sneathia*, *Mobiluncus*, were further refined with speciateIT (version 1.0, <http://ravel-lab.org/speciateIT>) to the species level. The resulting phylogenetic tree, ASV table, and taxonomic table combined with relevant metadata were consolidated into a phyloseq object using the phyloseq R package (26). Community state types were inferred using VALENCIA (VAGinal community state type Nearest Centroid classifier) (27), a nearest centroid classification method for vaginal microbial communities based on composition, implemented in python (version 3.6) and has the pandas module as a dependency (28). VALENCIA uses distance matrices to classify similarity of bacterial community structures between individual samples based on species proportions to each reference centroid were calculated using the Yue-Clayton θ (29).

Measurement of Cytokine Biomarkers of Genital Inflammation

Cervicovaginal SoftCup supernatants collected at matching time points with vaginal swabs were used to measure concentrations of 48 cytokines by Bio-Plex Pro-Human Cytokine Group I (27-Plex Panel) and Group II (21-Plex Panel) kits (Bio-Rad Laboratories, USA) kits as previously reported (30). The cytokine panel included chemokines, pro-inflammatory cytokines, adaptive, growth factors and anti-inflammatory: Interleukin (IL)-1 β , IL-1R α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-16, IL-18, IL-1A, IL-2RA, IL-3, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF-basic), cutaneous T-cell attracting chemokine (CTACK), Eotaxin, granulocyte colony-stimulating factor (G-CSF), GM-CSF, GRO- α , hepatocyte growth factor (HGF), Interferon (IFN)- γ , IFN- α 2, interferon- γ -inducible protein (IP)-10, leukemia inhibitory factor (LIF), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage colony-stimulating factor (M-CSF), monokine induced by gamma- interferon (MIG), Macrophage migration inhibitory factor (MIF), macrophage inhibitory protein (MIP)-1 α , MIP-1 β , nerve growth factor-beta (NGF- β), platelet derived growth factor (PDGF- β), regulated upon activation normal T cell expressed

and presumably secreted (RANTES), stem cell factor (SCF), stem cell growth factor-beta (SCGF- β), stromal cell-derived factors 1-alpha (SDF-1 α), tumor necrosis factor alpha (TNF)- α , TNF- β , TNF-related apoptosis-inducing ligand (TRAIL), and vascular endothelial growth factor (VEGF). Supernatants were filtered and centrifuged prior to testing. Standard curves were used to calculate cytokine concentrations in the samples using a 5-parameter logistic regression model. The inter-and intra-assay variability was performed by comparing cytokine concentrations in replicates plated on the same or across plates, with Spearman r values >0.8 considered acceptable per cytokine. Cytokine levels below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentrations measured for each cytokine.

Statistical Analysis

Descriptive data of continuous variables were summarized using medians and interquartile ranges, whilst categorical data were summarized with both frequency counts and percentages. A generalized estimating equation model using a binomial distribution and accounting for repeated measures was used to determine the effect of time on each binary clinical characteristic. Wilcoxon matched-pairs signed-ranks test was used to compare continuous data in two-time points. Linear mixed models were used to evaluate the associations between CSTs and inflammatory markers at women level with visits nested within a woman. The fixed term effects are CST, age, PSA, any STI and a constant term. The random effect are participant ID and time/visit. Time/visit was considered a categorical variable with three levels and an independent/simple covariance structure was assumed. In addition, linear mixed models were also used to estimate the effect of CSTs, metronidazole treatment, BV recurrence or persistence on the concentrations of each cytokine, adjusting for age, STI and recent sexual activity, as measured by PSA. Principal component analysis (PCA) was used to obtain summary measures for the multivariable cytokine set and performed using the FactoMineR package (<https://cran.r-project.org/web/packages/FactoMineR/index.html>) in R (www.r-project.org). Relative abundance of the most common taxa in cervicovaginal bacterial communities and log₁₀-transformed fold change in various cytokines at baseline and after treatment were used for PCA, as previously described (14). The cos2 value of the variable indicates its contribution in driving the input data into principal components. Pearson correlation was used to determine associations between factors and principal components. All statistical calculations were carried out using R and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Adjustment of multiple comparisons was performed using False Discovery Rate (FDR) (31). P-values less than 0.05 were considered statistically significant.

RESULTS

Characteristics of Study Participants

Of the 101 HIV-negative women enrolled in the CAPRISA 083 cohort, 56 women with median age of 24 years [interquartile

range (IQR) 21-27 years] had symptomatic intermediate BV or BV by Nugent score (NS) (Nugent-BV defined as NS ≥ 4) at baseline, who attended all three visits, and had genital specimen stored to conduct the longitudinal microbiota and cytokine analysis were used for this sub-analysis (**Figure 1**). At baseline, all 56 women received treatment with a single dose of 2g metronidazole. Twenty-eight of the 56 women also had sexually transmitted infections (STIs) and were treated with 1g of azithromycin (n=17), 2g of metronidazole (n=5), and 250mg (n=6) of ceftriaxone. Characteristics of study participants at all time points of sampling are reported in **Table 1**.

Changes in the Vaginal Community State Types Following Metronidazole Treatment

To describe the vaginal CSTs, we sequenced the V3-V4 region of the 16S rRNA gene of bacterial DNA in swabs collected from the women. We identified three CSTs based on the composition and relative abundance of bacterial species by VALENCIA (**Figure 2** and **Supplementary Figure 1**). These were CST I dominated by *L. crispatus*, CST III dominated by *L. iners*, and CST IV

characterized by a wide array of strict and facultative anaerobes without a consistent dominant species. These CSTs could be further divided into sub-CSTs: CST IV was sub-divided into CST IV-A representing samples with high relative abundance of BVAB1, CST IV-B characterized by *G. vaginalis* dominance and CST IV-C characterized by an even community with moderate abundance of *Prevotella bivia*.

Among the 56 women, only 1/56 (2%) were assigned to CST I and 15/56 (27%) to CST III (**Figure 2A**), while the most prevalent CSTs at baseline were CST IV-A (30%, 17/56) and IV-B (41%, 23/56). Six weeks after treatment, the number of women with *L. iners* (CST III) increased to 39% (22/56) from 27% (**Figure 2B**), CST IV-A decreased to 20% (11/56) from 30% and CST IV-B remained relatively stable (41% (23/56) at baseline versus 36% (20/56) at 6-weeks). At 12 weeks post-treatment, the proportion of women with CST I vaginal microbiota increased slightly to 5% (3/56) and CST III (46%, 26/56) was the most common CST. CST IV-A reduced further to 12.5% (7/56), while the proportion of CST IV-B again remained relatively stable (36%, 20/56) (**Figure 2C**).

TABLE 1 | Clinical characteristics of study population at baseline and follow-up (N=56).

Variable	Level	Baseline	Week 6 % (n/N)	Week 12	P-value
Age (years)	Median (IQR)	24 (21-27)	–	–	–
Condom use	Yes	71.4 (40)	–	–	–
	No	28.6 (16)	–	–	–
Frequency of condom use	Always	5.3 (3)	–	–	–
	Sometimes	66.1 (37)	–	–	–
	Never	28.6 (16)	–	–	–
Contraceptive use	Yes	37.5 (21)	–	–	–
	No	62.5 (35)	–	–	–
Type of contraception	Injection	52.4 (11/21)	–	–	–
	IUD	4.8 (1/21)	–	–	–
	Oral	14.3 (3/21)	–	–	–
	Subdermal implant	28.6 (6/21)	–	–	–
Genital examination	Abnormal	62.5 (35)	–	–	–
	Normal	37.5 (21)	–	–	–
Candidiasis	Yes	14.3 (8)	12.5 (7)	19.6 (11)	0.451
	No	85.7 (48)	87.5 (49)	80.4 (45)	
BV status (Nugent score)	No BV (0-3)	0 (0)	30.4 (17)	23.2 (13)	<0.0001
	Intermediate BV (4-6)	39.3 (22)	33.9 (19)	46.4 (26)	
	BV (7-10)	60.7 (34)	35.7 (20)	30.4 (17)	
<i>Chlamydia trachomatis</i>	Yes	30.4 (17)	5.4 (3)	3.4 (2)	0.001
	No	69.6 (39)	94.6 (53)	96.4 (54)	
<i>Neisseria gonorrhoea</i>	Yes	10.7 (6)	0.0 (0)	1.8 (1)	0.113
	No	89.3 (50)	100 (56)	98.2 (55)	
<i>Trichomonas vaginalis</i>	Yes	8.9 (5)	3.6 (2)	0.0 (0)	0.013
	No	91.1 (51)	96.4 (54)	100 (56)	
Any STI	Yes	50.0 (28)	8.9 (5.0)	5.4 (3)	<0.0001
	No	50.0 (28)	91.1 (51)	94.6 (53)	
Co-conditions	BV only	51.8 (29)	60.7 (34)	73.2 (41)	–
	STI only	0.0 (0)	0.0 (0)	1.8 (1)	
	BV and STI	48.2 (27)	8.9 (5)	3.6 (2)	
	No BV or STI	0 (0)	30.4 (17)	21.4 (12)	
PSA	Yes	19.6 (11)	21.4 (12)	25 (14)	0.785
	No	80.3 (45)	78.6 (44)	75 (42)	

BV, bacterial vaginosis; IQR, interquartile range; IUD, intrauterine device; PSA, prostate specific antigen; STI, sexually transmitted infection. Any STI includes all STIs tested excluding candidiasis.

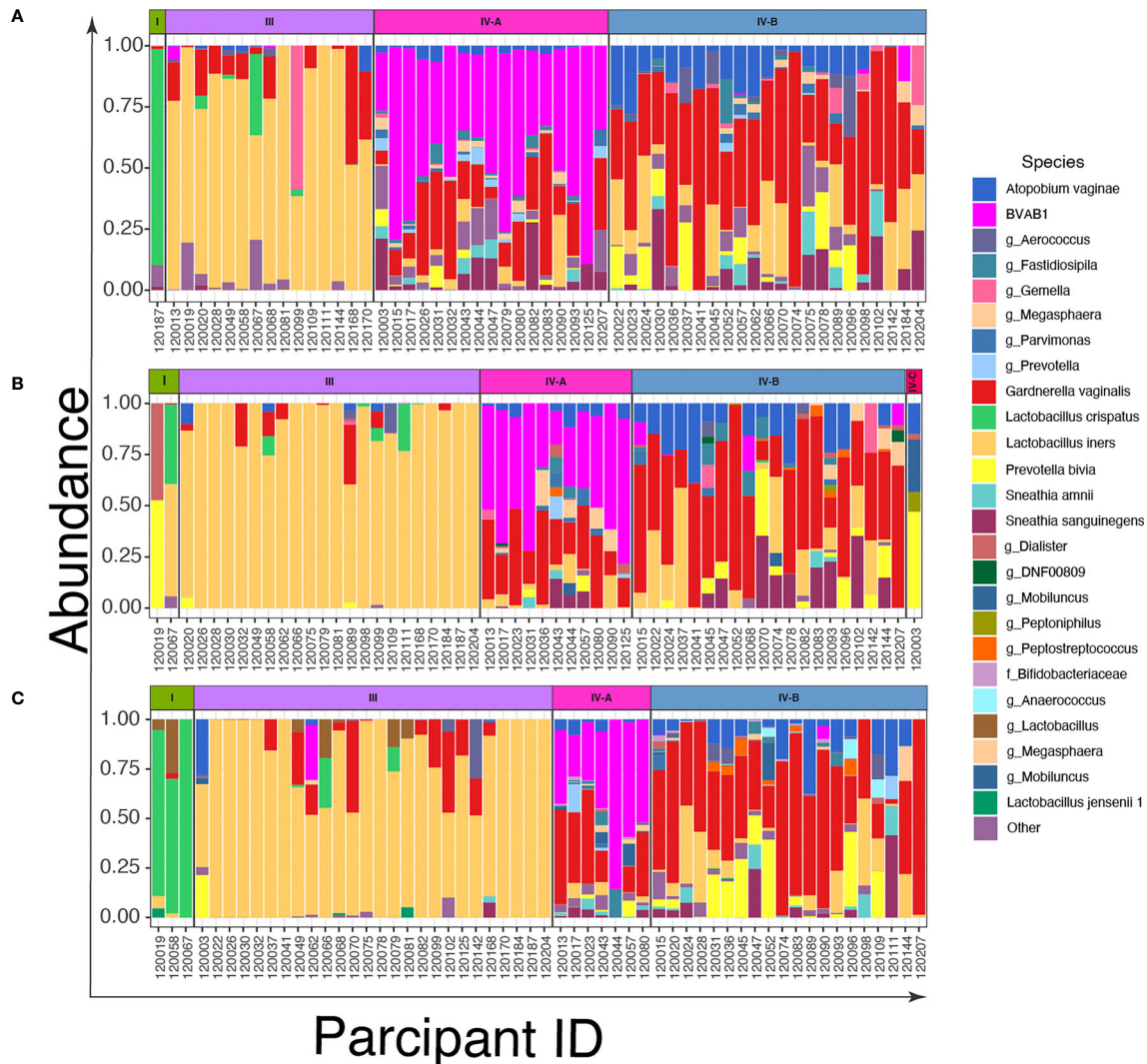


FIGURE 2 | Bar plots of the relative abundance of the twenty most common taxa in cervicovaginal bacterial communities in women (n=56) before and after treatment with metronidazole with and without sexually transmitted infection (STI) treatment **(A)** at baseline; **(B)** at 6 weeks post-treatment, and **(C)** at 12 weeks post-treatment.

Changes in the Vaginal Community State Types Following Antibiotic Treatment for STIs

We also determined whether the STI treatment had any effect on the vaginal microbiota by assessing CSTs of six women treated with azithromycin for present or suspected infection with *Chlamydia trachomatis*. All six women did not have BV by Nugent score at baseline and were excluded from the downstream analysis. **Supplementary Figure 2** depicts the fifteen most common bacterial species shown per patient. At baseline, 2/6 women had *L. iners* dominated vaginal microbiota, another 2/6 had communities dominated by both BVAB1 and *G. vaginalis* while the other 2 had a by a wide array of bacterial without a consistent dominant species. At 6 weeks after

treatment, only 1 woman remained dominated by *L. iners* with while 2 changed to an *L. crispatus* dominated microbiota. *L. iners* became a dominant species in three women 12 weeks after treatment. One woman did not have 16S data at 6 weeks and 3 did not have at 12 weeks after treatment.

Intra-Individual Transitions Across CSTs

We next assessed whether metronidazole treatment caused individual participant transitions between CSTs, particularly towards *Lactobacillus* dominated CSTs. We found that 25% (14/56) of women remained in their baseline CST [CST III (36%, 5/14), CST IV-A (29%, 4/14), CST IV-B (36%, 5/14)] despite treatment (**Figure 3** and **Supplementary Table 1A**). Twenty percent (11/56) of women transitioned from a profile of BV-associated CSTs [CST

IV-A (27%, 3/11) or CST IV-B (73%, 8/11)] at baseline into CST III at 6 weeks post-treatment and only 18% (2/11) reverted back CST IV-B and none for CST IV-A. Furthermore, 27% (15/56) transitioned across CSTs at 12 weeks post-treatment despite remaining in their baseline CSTs at the 6-week follow-up visit. These include 27% (4/15) women that transition from CST III to CST IV-B, 7% (1/15) from CST III to CST I, 7% (1/15) from CST IV-A to CST III, 13% (2/15) from CST IV-A to CST IV-B and 47% (7/15) from CST IV-B to CST III. The highest percentage of women that transition to CST III at 12 weeks had CST IV-B (9/20) at the baseline. None of these individual participant transitions between CSTs were significant using pairwise symmetry tests (**Supplementary Table 1B**). Furthermore, women who transitioned from one CST at baseline to another at 6 weeks post treatment had BV and/or STIs, with no distinct separations between those who transitioned compared to those who did not transitioned.

We then assessed whether the women who transitioned between CSTs from baseline to 6 weeks post-treatment either (i) remained in their new CSTs, (ii) reverted to their baseline CSTs, or (iii) transitioned to other CST at 12 weeks post-treatment (**Figure 3**). Only 36% (20/56) of women remained in their new CSTs following treatment, and did not transition to another CST [CST I (10%, 2/20), CST III (45%, 9/20), CST IV-A (15%, 3/20), CST IV-B (25%, 5/20)] at 12 weeks post-treatment. Seven percent (4/56) of women initially changed their CST at baseline to another CST at 6 weeks, and then transitioned back to baseline CST [CST III (25%, 1/4), CST IV-B (75%, 3/4)] at 12 weeks. Only 5% (3/56) of women who transitioned between CSTs from baseline to 6 weeks post-treatment further transitioned to other CST [CST III (67%, 2/3), CST IV-B (33%, 1/3)] at 12 weeks post-treatment. There were no significant differences amongst women who transitioned between baseline and follow up visits CSTs (**Supplementary Table 1**).

Relationship Between CSTs and Genital Tract Cytokines

To test the hypothesis that vaginal microbiota dominated with non-*Lactobacillus* species would be associated with increased cytokine concentrations compared to communities dominated by *Lactobacillus* species, we performed linear mixed effect regression analysis comparing genital cytokine concentrations with prevalent CSTs over time. Here, CST I and CST III were combined to represent the *Lactobacillus* group and to avoid analysing using CST I number alone. Women with vaginal CST IV-A communities had higher concentrations of hepatocyte growth factor interleukin (IL-5, $\beta = 0,651$, 95% confidence interval (CI) 0,079 – 1,223; $p = 0,026$), IL-7 ($\beta = 0,518$, 95% CI 0,108 – 0,928; $p = 0,013$), IL-18 ($\beta = 0,643$, 95% CI 0,181– 1,105; $p = 0,006$), and leukemia inhibitory factor (LIF, $\beta = 0,620$, 95% CI 0,227– 1,012; $p = 0,002$); compared to those with *Lactobacillus* dominance (CST I and CST III) and lower concentrations of growth-regulated oncogene-alpha (GRO- α , $\beta = -0,722$, 95% CI -1,262– (-0,182); $p = 0,009$), IL-12p40 ($\beta = -1,288$, 95%CI -1,989– (-0,588); $p = <0,0001$), interferon- γ -inducible protein (IP)-10 ($\beta = -1,378$, 95%CI -2,048 – (-0,709); $p = <0,0001$), monokine induced by gamma- interferon (MIG, $\beta = -0,631$, 95% CI -1,036 – (-0,227); $p = 0,002$), RANTES

($\beta = -0,682$, 95% CI -1,323 – (-0,041); $p = 0,037$) and stem cell factor (SCF, $\beta = -1,099$, 95% CI -1,906 – (-0,291); $p = 0,008$) (**Supplementary Figure 2** and **Supplementary Table 2**), adjusting for age, recent sexual activity and STIs. Women with vaginal CST IV-B communities had higher concentrations of IL-1 α ($\beta = 0,890$, 95% CI 0,011 – 1,786; $p = 0,044$), IL-18 ($\beta = 0,589$, 95% CI 0,220 – 0,957; $p = 0,002$), LIF ($\beta = 0,466$, 95% CI 0,149– 0,782; $p = 0,004$) and macrophage migration inhibitory factor (MIF, $\beta = 0,610$, 95% CI 0,261 – 0,960; $p = 0,001$) compared to women with *Lactobacillus* dominance (CST I and CST III) and reduced concentrations of GRO α ($\beta = -0,670$, 95% CI -1,100 – (-0,241); $p = 0,002$), IP-10 ($\beta = -1,369$, 95% CI -1,881– (-0,857); $p = <0,0001$) and MIG ($\beta = -0,381$, 95% CI -0,690 – (-0,072); $p = 0,016$), respectively (**Supplementary Figure 2**).

Impact of Metronidazole Treatment on Temporal Dynamics of Mucosal Cytokines Profiles

We next assessed whether women who cleared BV (NS <4) at week 6 post-treatment and did not recur by week 12 also had lower genital cytokines. Compared to pre-treatment samples, a significant reduction in concentrations of certain cytokines was noted in post-treatment SoftCup supernatants of women who cleared BV, including tumor necrosis factor (TNF)- α ($p = 0,027$), IL-1 β ($p = 0,002$); IL-8 ($p = 0,040$); LIF ($p = 0,043$). In contrast, cytokine granulocyte macrophage colony-stimulating factor (GM-CSF, $p = 0,033$) increased following metronidazole treatment (**Supplementary Figure 3**).

We hypothesized that episodes of BV persistence or recurrence may distinctly alter mucosal cytokine and chemokine concentrations compared to BV clearance. To test our hypothesis, we used a linear mixed model to compare the concentrations of cytokines in women who cleared BV (at 12 weeks post-treatment) with those who had persistent BV (at 12 weeks post-treatment) (**Figure 4** and **Supplementary Table 3**). After adjusting for age, recent sexual activity and STIs, we observed higher concentrations of several cytokines in women with persistent BV relative to the BV cleared group: including IL-1 α ($\beta = 0,466$, 95% CI 0,011 – 0,921; $p = 0,045$), IL-18 ($\beta = 0,621$, 95% CI 0,164 – 1,078; $p = 0,009$), MIF ($\beta = 0,481$, 95% CI 0,054 – 0,908; $p = 0,028$), IL-7 ($\beta = 0,363$, 95% CI 0,012 – 0,713; $p = 0,043$), and LIF ($\beta = 0,621$, 95% CI 0,236 – 1,005; $p = 0,038$). Interestingly, women who tended to experience persistent BV also had significantly lower concentrations of IP-10 ($\beta = -0,999$, 95% CI -1,757 – (-0,241); $p = 0,011$) and monocyte chemotactic protein (MCP)-1 ($\beta = -0,687$, 95% CI -1,331 – (-0,043); $p = 0,037$) relative to those who cleared BV. However, none of these associations remained significant after adjusting for multiple comparisons.

Next, we assessed whether BV recurrence at 12 weeks post-treatment, after initial BV clearance at 6 weeks, influenced changes in mucosal cytokines relative to BV clearance by week 12. Only IL-16 ($\beta = -1,004$, 95% CI -1,986 – (-0,022); $p = 0,045$) appeared to be significantly reduced in women who had BV recurrence relative to those who cleared BV, although not after adjustment for confounders.

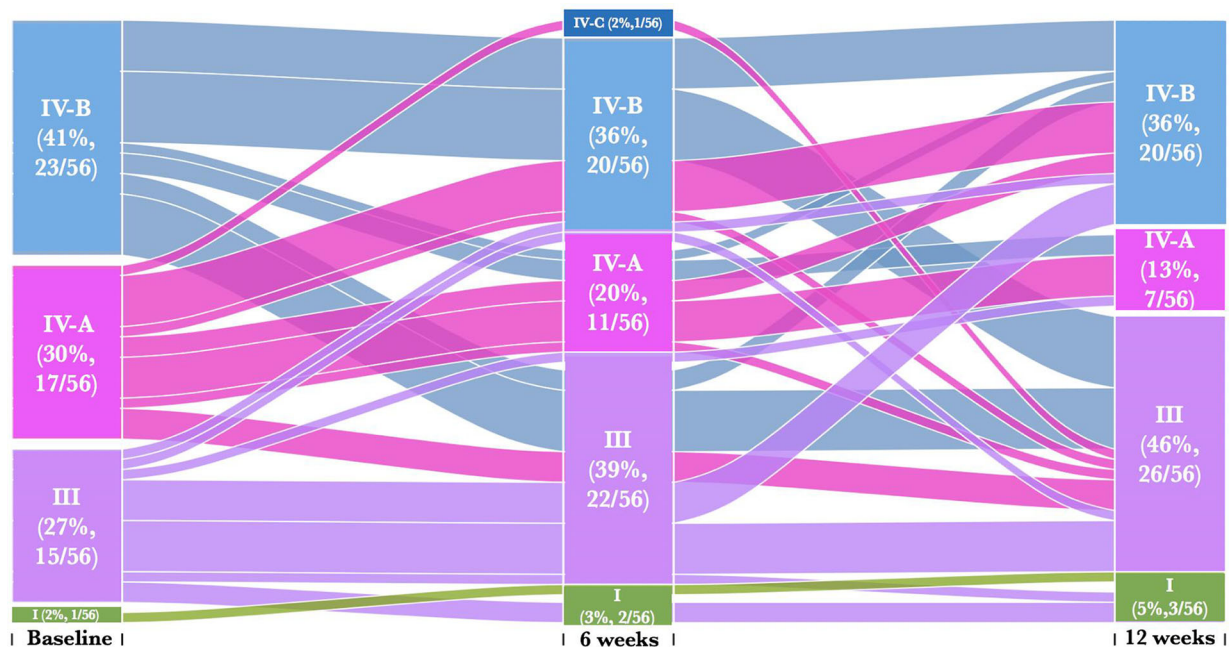


FIGURE 3 | Alluvial diagrams are showing participant transition (n=56) between CSTs pre- and post-treatment. CST I (green) lacked a consistent dominant species, but all communities included *L. crispatus*. CST III (purple) *L. iners* dominance, CST IV-A (pink) had diverse bacterial communities dominated by BVAB1, CST IV-B characterized by *G. vaginalis* dominance and CST IV-C characterized by an even community with moderate abundance of *Prevotella bivia*.

RELATIONSHIP BETWEEN VAGINAL MICROBIAL TAXA AND CYTOKINES

Focusing on the most abundant vaginal bacterial taxa across all three visits and the cytokines that were significantly associated with BV clearance (including TNF- α , IL-1 β , IL-8, LIF, GM-CSF), we determined whether the observed alterations in cytokine concentrations post-treatment were associated with relative abundance of any specific bacterial taxa (**Figure 5A**). At any time point, concentrations of TNF- α , IL-1 β , IL-8, and LIF appeared to be influenced by the relative abundance of BVAB1, commonly found in women with BV. In contrast, GM-CSF appeared to be associated with the abundance of *L. crispatus*. Next, we used cytokines (IL-1 α , IL-7, IL-18, IP-10, MCP-1, MIF and LIF) that were significantly associated with persistent BV and correlated with the relative abundance of the 20 most abundant vaginal bacterial taxa found in both CST IV-A and CST IV-B. At any time point, concentrations of IL-1 α , IL-18, MIF, and LIF clustered with the relative abundance of *G. vaginalis*, also commonly found in women with BV (**Figure 5B**). Furthermore, the relative abundance of *L. crispatus* was clustered with MCP-1 and IP-10 in women with persistent BV.

DISCUSSION

BV is common among women of African descent (32) and is often recalcitrant to treatment with metronidazole or clindamycin (33). However, little is known about the temporal relationships between

microbial shifts and genital inflammation. Here, we investigated the effects of metronidazole treatment on the vaginal microbiota and concentrations of mucosal soluble immune mediators in women pre- and post-treatment for BV, including those who had treatment failure or BV recurrence. We found that complete BV clearance rates were suboptimal and recurrence rates were high post-treatment. The post-treatment *16S rRNA gene sequencing* data showed that treatment mediated a gradual shift away from a BV-associated strict anaerobic species dominated profile towards an increased relative abundance of *Lactobacillus* species, particularly *L. iners*. Women classified as having persistent BV had high concentrations of mucosal cytokines and chemokines relative to BV clearance and this response was likely driven by BV-associated strict anaerobes.

In agreement with previous studies that used Nugent scoring to diagnose BV (34) clinical, we observed short-term BV clearance (NS <4) rates in our cohort, with more than half of those who cleared BV by 6 weeks experiencing recurrence (NS \geq 4) by 12 weeks post-treatment, suggesting that stability in the vaginal microbiota was not achieved after short-term gains. Furthermore, the majority of women experienced persistent BV despite treatment. Metronidazole treatment resulted in the decreased prevalence of diverse CSTs and a shift toward the increased proportion of women having *L. iners* dominant CST III. The increase in the relative abundance of *L. iners* is consistent with earlier studies showing a positive correlation between the abundance of lactobacilli species and metronidazole treatment (11, 13). While metronidazole treatment was also associated with a shift toward increased

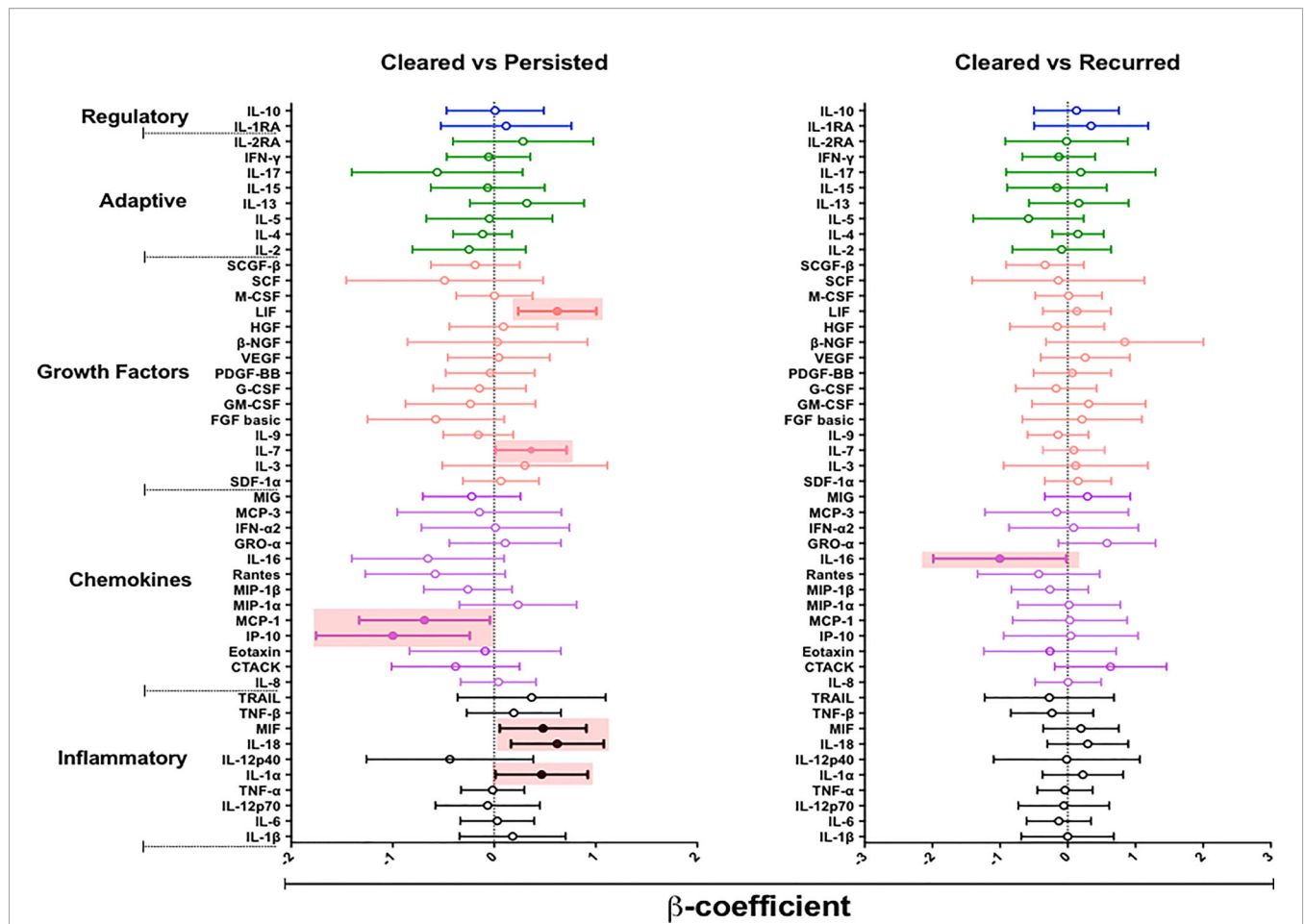
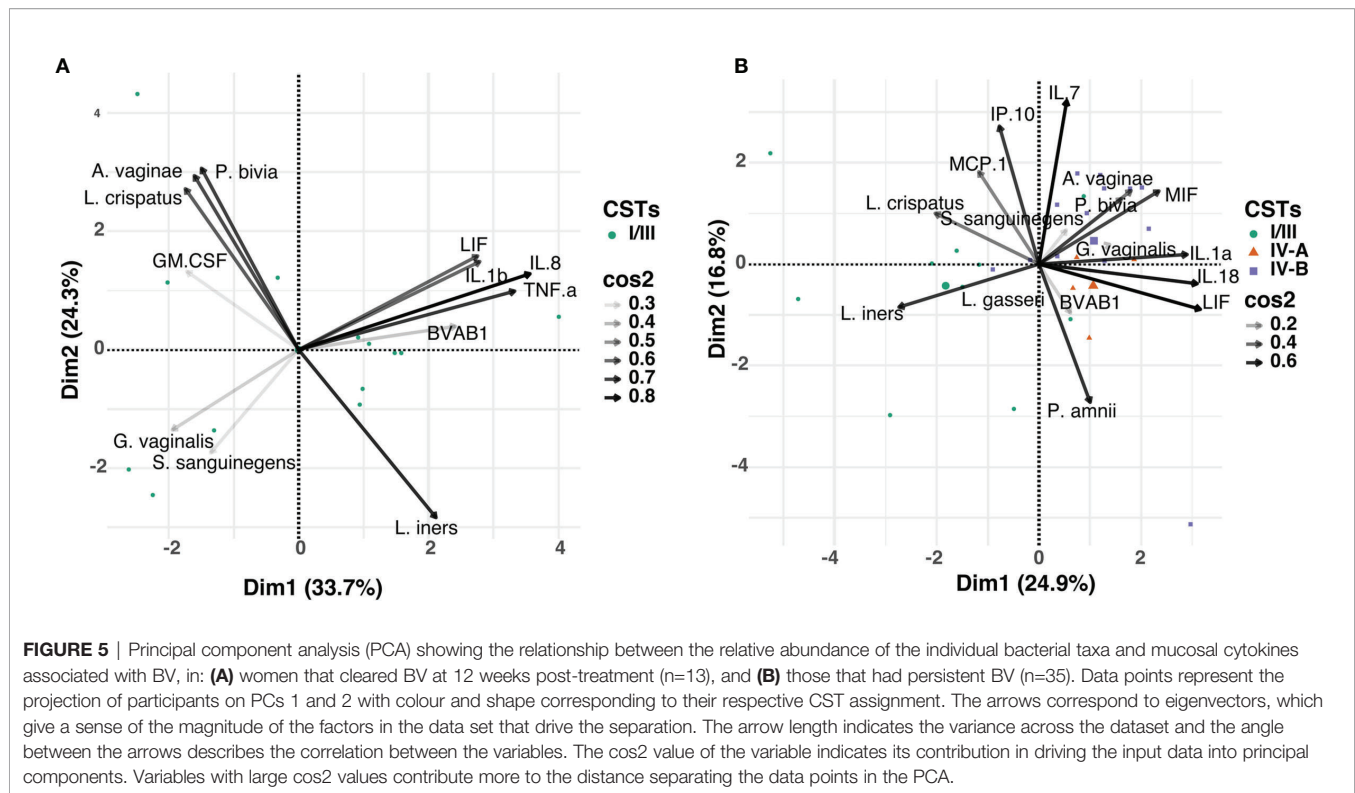


FIGURE 4 | β -coefficients from linear mixed models to determine the effect of persistent (n= 35) or recurrent BV (n= 9) on the cytokine milieu compared to women who cleared (n= 13) their BV. Individual associations are shown between BV status and pro-inflammatory cytokines (black), chemokines (purple), growth factors (orange), adaptive response cytokines (green), and regulatory cytokines (blue), with error bars depicting standard error. Shaded shapes represent significant associations, after adjusting for age, recent sexual activity, and sexually transmitted infections.

relative abundance of *L. iners*, the relative abundance of *L. crispatus* was extremely modest. The metronidazole induced *L. iners*-dominant state, an unstable community, with high probability of transitioning back to a high diversity state is particularly concerning and may explain high rates of BV occurrence and recurrence among women of sub-Saharan Africa descent. Our findings emphasize a need to extensively investigate *L. iners* role in both lactobacilli-dominated and diverse microbial communities. Given the specific health benefits afforded by a *Lactobacillus*-dominant vaginal microbiota against infections and the limited efficacy of antibiotics against BV-associated sequelae, a viable alternative such as probiotics to improve *Lactobacillus* representation as part of curative or preventive vaginal dysbiosis interventions are needed. In addition, there is a need to include rapid molecular assays that are able to quantify key beneficial bacteria and those with pathogenic potential, including biofilm producers in a point of care management.

In this cohort of African women with BV, BVAB1, and *G. vaginalis* were found to be the most abundant species in the non-

Lactobacillus-dominated CSTs, with BVAB1 being the most easily cleared by metronidazole treatment (35). Metronidazole treatment was associated with the reduced relative abundance of *G. vaginalis*, but the extent of the reduction was modest and appeared to re-establish at 12 weeks post-treatment. This was confirmed by the number of women who transitioned out of CST IVA or B into CST III, but reverted back to either of their original CSTs at 12 weeks post-treatment. Our findings are consistent with other studies that suggested that women with a high *G. vaginalis* (36) were the most likely to fail treatment. Studies have hypothesized that metronidazole may be ineffective against *G. vaginalis* due to their ability to form complex biofilms and/or harbor metronidazole resistance (37, 38). Another study suggests that high levels of CRISPR-associated Cas genes can undermine the efficacy of metronidazole against *G. vaginalis* (39). Although more longitudinal studies are needed to understand the effect of treatment on BV, these findings suggest that treatment may successfully eradicate BV in women colonized by BVAB1 communities.



Recent studies showed the effect of azithromycin on the vaginal microbiome. Although limited by sample size, our findings are in agreement with other studies that showed a shift towards increased relative abundance of lactobacilli (mainly *L. iners*) following 1g of oral azithromycin treatment. It has been shown that women with high relative abundance of *L. iners* were associated with increased susceptibility to *C. trachomatis* infection, suggesting that azithromycin treatment increase probabilities of transitioning to diverse disease-associated states or reinfection (40, 41). Considering that both metronidazole and azithromycin favor increased relative abundance of unstable *L. iners* communities, there is a need to use Gardnerella specific-biofilm dissolving treatment, boosted with Lactobacilli-based live biotherapeutic products to restore an optimal vaginal microbiota after antibiotic treatment.

We determined whether vaginal microbiota dominated with non-*Lactobacillus* species is associated with increased genital cytokine production using linear mixed effect regression analysis. In agreement with previous studies (42, 43), we demonstrated that high concentrations of proinflammatory cytokines, adaptive growth factors and hematopoietic cytokines (IL-1 α , IL-5, IL-7, IL-18, LIF and MIF) were associated with non-optimal vaginal microbiota while communities dominated by *Lactobacillus* species (mainly *L. iners* in this cohort) were associated with lower concentrations of inflammatory cytokines and chemokines (GRO- α , IL-12p40, IP-10, MIG, RANTES and SCF). In contrast, other studies reported that women with communities dominated by *L. iners* had increased concentrations of IP-10 (14, 43, 44). Pro-inflammatory

cytokines and chemokines such as IL-12p40, IP-10, RANTES and are each involved in recruitment of T cells, neutrophil and antigen presenting cells to infection sites (45–48). Our results suggest that vaginal microbiota dominated by non-*iners-Lactobacillus* species, metronidazole induced communities dominated by *L. iners* may have similar beneficial effect on inflammatory response and HIV susceptibility.

Unlike previous studies that only investigated the impact of BV clearance with metronidazole at one-month post-treatment (14, 39), we also extended our analysis to determine the long-term impact of treatment on the vaginal microbiota and the impact of BV clearance, persistence and recurrence on mucosal cytokine concentrations. In this study, BV clearance was associated with decreased concentrations of TNF- α , IL-1 β , IL-8, and LIF compared to matched pre-treatment visits. Our data suggest that treatment over time or BV clearance had a desirable effect by downregulating cytokines that induce an inflammatory response (LIF) and those that are linked to increased risk of acquiring HIV infection (TNF- α , IL-1 β ; IL-8) (4). In contrast, clearing BV was also associated with increased concentrations of the growth factor GM-CSF, which is known to induce proliferation and differentiation of hematopoietic cells such as neutrophils and macrophages in response to invading pathogen (49). Relative to cleared BV, persistent BV had reduced concentrations of cytokines that may influence the trafficking of lymphocytes (MCP-1 and IP-10). Our findings are consistent with previous studies that found *in vivo* IP-10 suppression concentrations in BV positive women (50–52). Studies suggest that IP-10 concentration are increased in viral infections, and

decreased by pathogenic bacterial infection (51, 53, 54) but the role and significance of IP-10 in BV remains unclear. Persistence was also associated with increased concentrations of cytokines (IL-1 α , IL-18, MIF, IL-7 and LIF) associated with HIV susceptibility. IL-1 α , IL-18, and LIF are known to induce an inflammatory response and acute immune response, including the proliferation of Th17 type CD4+ cells (55). These findings suggest that BV persistence may alter lymphocyte chemotaxis and potentially increase susceptibility to HIV infection.

Although BV is a multifactorial condition, with no one organism being linked to increased or decreased concentrations of mucosal cytokines, our study showed that TNF- α , IL-1 β , IL-18, MIF, and IL-8 appeared to be differentially regulated by changes in the relative abundance of BVAB1 and *G. vaginalis*. This is consistent with previous studies from our group and others that found a correlation between Nugent-defined BV status (including high microbial diversity by 16S rRNA sequencing) in women and changes in pro-inflammatory cytokine and chemokine concentrations (4, 5, 44, 56). In contrast, the relative abundance of *L. crispatus* clustered with the concentrations of GM-CSF, MCP-1, and IP-10 confirming an anti-inflammatory effect mediated by lactobacilli species dominance (57, 58). Although these results suggest a relationship between vaginal microbiota and altered cytokine concentrations post-treatment, mechanistic studies are required to confirm this relationship further.

The strength of this study includes longitudinal assessment of the impact that efforts to normalize the vaginal microbiome have on mucosal cytokine concentrations in a prospective cohort of women undergoing STI and BV management. However, this study had limitations, including the sample size that restricted the ability to investigate in detail the effects of BV clearance, persistence, or recurrence on vaginal microbiota and how changes triggered by treatment would affect genital inflammation. Another limitation is that almost half of participants had STI and also treated with azithromycin and ceftriaxone and this may have confounded the results. The lack of the control group also limited our ability to investigate microbial and cytokine changes between the treatment experienced and naïve groups. We also did not investigate the role cellular markers of genital inflammation on BV clearance and persistence. Likewise, we were unable to investigate the impact of other potential co-factors, including human papillomavirus, herpes simplex virus, hormonal contraceptives, diets, hormonal status, other vaginal disorders (e.g., aerobic vaginitis) on microbiota, although these may have different biological effects.

In conclusion, metronidazole treatment induced a shortlived reduction of BV-associated microbiota and an increase in vaginal *Lactobacillus* species, which resulted in altered mucosal cytokine concentrations. Our findings suggest that the efficacy of BV treatment may depend on the pre-treatment microbial species present in the female genital tract, suggesting value in screening for biofilm-producing bacteria before administering treatment. Only when novel and more effective BV treatment regimes that target and disrupt biofilm-producing bacteria are developed will we be able to reduce the burden of BV.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE403/16). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AMt, SN, and LL conceived and designed the analysis and AMt, SN, SEJ, KM, JR and FO performed the analyses. AR, AMi and NG were the principal investigators of the CAPRISA 083 study. AMt, SN, JS, FO, NG, CB, JG, HO, KM, GM, TO, AR, AMi, SA, JR, J-AP, QA, HJ, and LL contributed to the interpretation and discussion of the results and writing 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/fimmu.2021.730986/full#supplementary-material>

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