

# Composition and Stability of the Vaginal Microbiota of Pregnant Women With Inflammatory Bowel Disease

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**Background:** Inflammatory bowel disease (IBD) is common in women of childbearing years, and active IBD during pregnancy is associated with increased rates of preterm delivery and low-birth-weight newborns. Changes in the vaginal microbiome have been associated with preterm delivery. We aimed to determine the taxonomic composition of the vaginal microbiota at 3 time points during pregnancy in a population of women with IBD.

**Methods:** Participants were recruited from the patient registry of the Preconception and Pregnancy IBD Clinic at Royal University Hospital in Saskatoon, Canada. Self-collected vaginal swabs were obtained from patients at each trimester. Microbiota profiles were created by cpn60 amplicon sequencing.

**Results:** We characterized the vaginal microbiota of 32 pregnant participants with IBD (33 pregnancies) during each trimester. A total of 32 of 33 pregnancies resulted in a live birth with 43.8% ( $n = 14$  of 32, 2 missing) by caesarean section; 2 of 32 were preterm. Microbiota compositions corresponded to previously described community state types, with most participants having microbiota dominated by *Lactobacillus crispatus*. In 25 of 29 participants in which samples were available for more than 1 time point, there was no change in the community state type over time. Prevalence of Mollicutes (*Mycoplasma* and/or *Ureaplasma*) was significantly higher in pregnant participants with IBD than in a previously profiled cohort of 172 pregnant women without IBD who delivered at term.

**Conclusions:** The vaginal microbiome of participants with IBD was stable throughout pregnancy. Prevalence of Mollicutes, which has been associated with preterm delivery, warrants further study in this patient group.

## Lay Summary

Composition of the vaginal microbiota was stable throughout pregnancy. Prevalence of Mollicutes was significantly higher in individuals with inflammatory bowel disease than in a previously profiled cohort of 172 pregnant women without inflammatory bowel disease who delivered at term.

**Key Words:** Inflammatory bowel diseases, Microbiota, Mollicutes, Pregnancy, Vagina

## INTRODUCTION

Inflammatory bowel disease (IBD) is a lifelong, chronic disease with no cure, and the incidence of IBD is increasing worldwide.<sup>1</sup> The majority of patients diagnosed with IBD are in their childbearing years, and the potential for complications continues to be a source of fear and anxiety for many patients and their physicians. Patient concerns are a contributor to the higher rate of voluntary childlessness in women with IBD compared with those without.<sup>2</sup> There have been several reports that the risk of preterm delivery and low-birth-weight babies is elevated in women with IBD,<sup>3-7</sup> and these women are also more likely to deliver by caesarean section than the general population.<sup>8,9</sup> The risk of negative pregnancy and neonatal outcomes, however, are greater for women with active disease in the preconception period than for those with quiescent disease.<sup>10</sup>

The importance of the intestinal microbiome in pathogenesis of IBD is well established,<sup>11</sup> and it has been observed that the microbiota of individuals with IBD is less diverse than in healthy individuals.<sup>12</sup> The lower diversity has in turn been associated with the initiation and maintenance of inflammation and a loss of tolerance to commensal bacteria.<sup>13-15</sup> Whether the abnormal immune response in IBD patients that results in inflammation in the gut affects microbiomes of other body sites is not known.

Pregnancy in women without IBD has been reported to affect the composition of the vaginal microbiota, resulting in greater stability, increased proportional abundance of *Lactobacillus* species, reduced prevalence of *Mycoplasma* and *Ureaplasma*, and reduced diversity compared with the vaginal microbiomes of nonpregnant women.<sup>16-20</sup> Whether the vaginal microbial communities of women with IBD undergo these same changes

is not known. In a recent study, pregnant women with inflammatory rheumatic and inflammatory bowel diseases were observed to have “abnormal vaginal microbiota” more frequently than healthy control subjects.<sup>21</sup> In this study, abnormal vaginal microbiota was defined as the occurrence of bacterial vaginosis, *Trichomonas vaginalis*, or *Candida* spp. as observed by microscopy of vaginal smear specimens, and women with IBD represented about one-third of the case group. Increased amounts of *Gardnerella vaginalis* (an organism strongly associated with bacterial vaginosis) in the urine of nonpregnant women with IBD has also been described.<sup>22</sup>

Given the established link of IBD to alternations of the intestinal microbiome, and the associations of inflammation and vaginal microbiome dysbiosis with negative pregnancy outcomes,<sup>23</sup> more detailed investigations of the vaginal microbiome in pregnant women with IBD are warranted. The objective of the current study was to determine the taxonomic composition of the vaginal microbiota at 3 time points during pregnancy in a population with IBD.

## Methods

### Ethical Considerations

This study was approved by the University of Saskatchewan Biomedical Research Ethics Board (Protocol 14-211).

### Study Population and Sampling

Participants were recruited from the patient registry of the Preconception and Pregnancy IBD Clinic at Royal University Hospital in Saskatoon, Canada, between February 2015 and August 2016. Pregnant participants  $\geq 18$  years of age with confirmed IBD were eligible for inclusion. The diagnosis of IBD was based on standard clinical, radiologic, endoscopic, and histologic criteria. Baseline maternal data were collected including information on demographics; IBD history; gynecological and obstetrical history (including history of sexually transmitted infections, number, dates, and outcomes of previous pregnancies); and medical, surgical, and social history. Self-collected vaginal swabs were obtained from patients who consented to the study at their first antenatal visit. Samples were collected at 3 time points in pregnancy: 12 to 16 weeks', 22 to 24 weeks', and 32 to 34 weeks' gestation, concurrent with routine clinic visits. Vaginal swabs were stored at  $-80^{\circ}\text{C}$  immediately after collection until batch processing and analysis.

### Vaginal Microbiome Analysis

Total DNA was extracted from vaginal samples using a magnetic bead-based kit (MagMAX Total Nucleic Acid Isolation Kit; Life Technologies, Burlington, ON, Canada). Reagent-only extraction negative control samples were included with each of 2 batches of extractions. Polymerase chain reaction (PCR) amplification of the cpn60 barcode sequence and sequencing library preparation was performed using an established protocol (described in detail elsewhere).<sup>24</sup> No template control samples were included with each of 2 batches of PCR reactions. Purified amplicons from each sample were modified by dual indexing to allow pooling of amplicon libraries into a single 500-cycle sequencing run on an Illumina MiSeq (Illumina, San Diego, CA, USA). Negative extraction control samples ( $n = 2$ ) and no template control samples ( $n = 2$ ) were carried through the entire sequencing process.

Four hundred cycles were performed for read 1 and 100 cycles for read 2; only read 1 sequences were used in downstream analysis.

### Bioinformatics

De-multiplexed Read 1 fastq files were processed with Cutadapt<sup>25</sup> to remove amplification primer sequences, and then quality filtered with Trimmomatic (minimum length 150, minimum quality 30).<sup>26</sup> Quality filtered reads were loaded into QIIME2 (<https://qiime2.org>) for sequence variant calling and read frequency calculation with DADA2 (truncation length 150). For taxonomic identification, variant sequences were aligned to the cpnDB\_nr reference database (downloaded from [www.cpnDB.ca](http://www.cpnDB.ca)) using watered-BLAST.<sup>27</sup> Only sequences with identities of  $>55\%$  to a cpnDB sequence were retained for downstream analysis.<sup>28</sup> Sequences with the same best match in the database were grouped into nearest-neighbor “species” by summing their total read counts within samples. Read count data were used to calculate Bray-Curtis dissimilarity values in QIIME2 with a sampling depth of 1000 reads per sample.

For community state type (CST) analysis and clustering, read counts were converted to proportions, and a Jensen-Shannon distance matrix was calculated in R (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria) using the *vegdist* function in the *vegan* package. This distance matrix was used for hierarchical clustering using the *hclust* function in R with Ward linkage. CSTs were labeled according to dominant species as described previously.<sup>29</sup>

### Mollicutes Detection

Because some Mollicutes lack the cpn60 gene, targeted PCR assays were used to detect these species in study samples. A family-specific semi-nested PCR that targets the 16S ribosomal RNA gene was used to detect Mollicutes (*Mycoplasma* and/or *Ureaplasma*).<sup>30</sup> A PCR targeting the gene encoding the multiple-banded antigen was used to detect *Ureaplasma* spp.<sup>31</sup> Results of these PCR assays were interpreted as positive or negative by electrophoresis and visualization of the reaction products on 1% (w/v) agarose gels stained with ethidium bromide. Results of Mollicutes and *Ureaplasma* detection were compared with previously reported results from 3 cohorts of Canadian women: pregnant women who delivered at term ( $n = 170$ ),<sup>16</sup> pregnant women who delivered preterm ( $n = 46$ ),<sup>32</sup> and nonpregnant reproductive-aged women ( $n = 310$ ).<sup>33</sup>

### Statistical Analysis

Mollicutes and *Ureaplasma* PCR results were compared between this study and previously reported pregnant and nonpregnant cohorts with a chi-square test to detect significant differences among the groups, followed by pairwise comparisons using Fisher's exact test. Statistical tests were performed in GraphPad Prism version 9.1.2 (GraphPad Software, San Diego, CA, USA).

## Results

### Description of the Study Population and Pregnancy Outcomes

Thirty-two participants provided samples for the study, with 1 participant providing samples from 2 pregnancies that

occurred during the study period. Demographic characteristics, IBD diagnosis, and birth outcomes are shown in Table 1. Most (n = 20 of 32, 62.5%) of the participants had a diagnosis of Crohn's, and the remainder had an ulcerative colitis diagnosis (n = 12 of 32, 37.5%). Thirty-two live births from 33 pregnancies resulted, with only 2 births occurring prior to 37 weeks gestational age.

### Vaginal Microbiome Composition and Stability

A total of 80 vaginal swabs were processed for cpn60 amplicon sequencing, including samples from 3, 2, or 1 time points for 16, 15, and 2 pregnancies, respectively. Two extraction-negative control samples and 2 no-template control samples were also included in the sequencing run. Following adapter removal and quality trimming, an average of 5935 reads per sample were available for analysis. Two samples (121-2T and 008-2T) were removed from the analysis due to low read counts (21 and 44 reads, respectively). For the remaining 78 samples, an average of 6161 reads per sample (range 768-16 118, median 5706). Most (3 of 4) negative control samples yielded no data, and 1 extraction negative control sample yielded 87 reads. When aligned to cpnDB\_nr, 243 unique sequence variants corresponded to 113 distinct nearest-neighbor species, and 32 of these neighbors comprised at least 1% of at least 1 sample. Sequence data have been deposited in the National Center for Biotechnology Information Sequence

**Table 1.** Description of study population (n = 32 women, n = 33 pregnancies)

Characteristic	
Age, y	30 ± 4 (20-38)
Body mass index, kg/m <sup>2</sup>	26.9 ± 5.6 (19.1-39.9)
Race	
White	28 (87.5)
Other	2 (6.3)
Missing	2 (6.3)
Parity	
0	15 (45.5)
1	8 (24.2)
2	5 (15.2)
3	1 (3.0)
Missing	3 (9.1)
IBD diagnosis	
Crohn's disease	20 (62.5)
Ulcerative colitis	12 (37.5)
Birth mode <sup>a</sup>	
Vaginal	16 (50.0)
C-section	14 (43.8)
Missing	2 (6.3)
Gestational age at delivery <sup>a</sup>	
≥37 wk (term)	28 (87.5)
<37 wk (preterm)	2 (6.3)
Missing	2 (6.3)

Values are mean ± SD (range) or n (%).

Abbreviation: IBD, inflammatory bowel disease.

<sup>a</sup>Birth mode and gestational age at delivery were reported for 32 live births.

Read Archive in association with BioProject Accession PRJNA759867.

Clustering of vaginal microbiome profiles resulted in the identification of 5 CSTs previously described for the human vaginal microbiome<sup>29</sup>: CST I dominated by *Lactobacillus crispatus*, CST II dominated by *L. gasseri*, CST III dominated by *L. iners*, CST IV containing a heterogeneous mixture of species, and CST V dominated or codominated by *L. jensenii* (Figure 1). Clustering of samples from individual women was apparent regardless of CST and was confirmed when the vaginal microbiome CSTs for each woman were compared across time points (Figure 2A). For the 29 pregnancies where multiple samples were available, a change in CST was observed in only 4 cases (patient IDs 105, 106 [second pregnancy], 124, and 131).

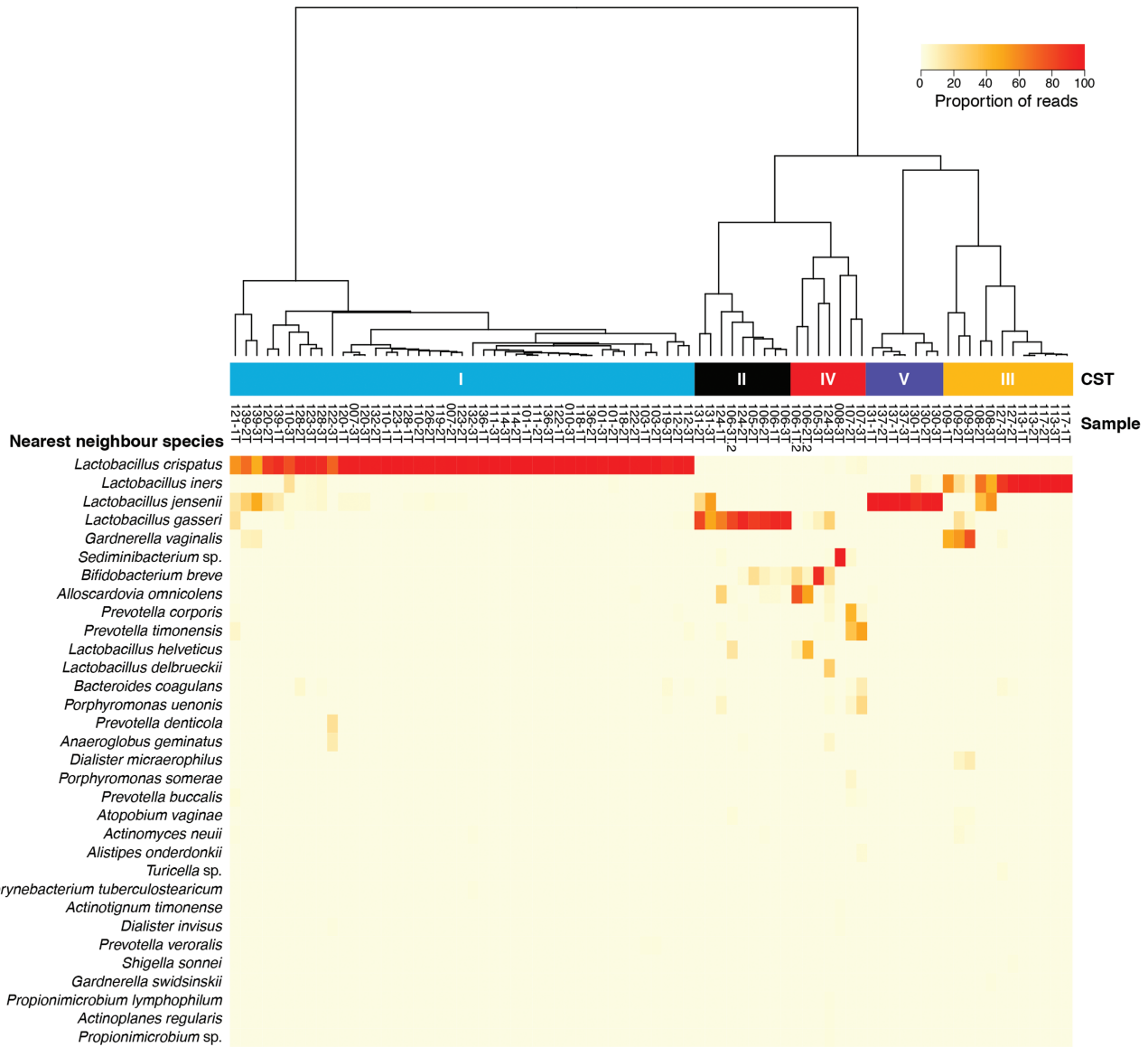
When microbiome profiles were compared among samples from individual participants ("within"), they were found to be more similar to each other (median Bray-Curtis dissimilarity 0.062) than to samples from different participants ("between"; median Bray-Curtis dissimilarity 0.999) (Figure 2B), further illustrating the relative stability of the microbiota composition throughout pregnancy. Participants with only single samples available, and 2 samples with <1000 reads (105-3T and 124-3T) were excluded from this analysis.

### Mollicutes Detection

Mollicutes (*Mycoplasma* and/or *Ureaplasma*) PCR detection was performed on vaginal swabs from 31 participants, and 25 (80.6%) of 31 were found to be positive for Mollicutes at least once in their pregnancy. Mollicutes status did not change for 10 of 14 participants for whom results were available for all 3 time points. Only 12 (38.7%) of 31 participants were positive for *Ureaplasma* spp. at any point during pregnancy. Mollicutes and *Ureaplasma* prevalence data generated using exactly the same method were available for comparison from previous studies of Canadian women delivering at term or preterm<sup>32</sup> and nonpregnant Canadian women.<sup>16</sup> For this comparison, only samples from the second time point in the current study were included (n = 29). Significant differences were detected among groups (chi-square test,  $P < .0001$ ). Subsequent pairwise comparisons showed that the proportion of pregnant participants with IBD that tested positive for Mollicutes was significantly higher than pregnant women who went on to deliver at term (Fisher's exact test,  $P = .0005$ ) and was not different from either women who delivered preterm or nonpregnant women (Fisher's exact test,  $P > .05$ ) (Table 2). *Ureaplasma* prevalence in participants with IBD was not significantly different than the other groups.

### Discussion

The vaginal microbiome plays an important role in reproductive health. In contrast to the intestinal microbiota, vaginal microbial communities are relatively sparse and tend to be dominated by one or a few bacterial species, usually one of several *Lactobacillus* species.<sup>29</sup> Reduced numbers of lactobacilli and an overgrowth of mixed aerobic and anaerobic bacteria are characteristic of bacterial vaginosis, a dysbiosis that can be associated with troubling symptoms, increased



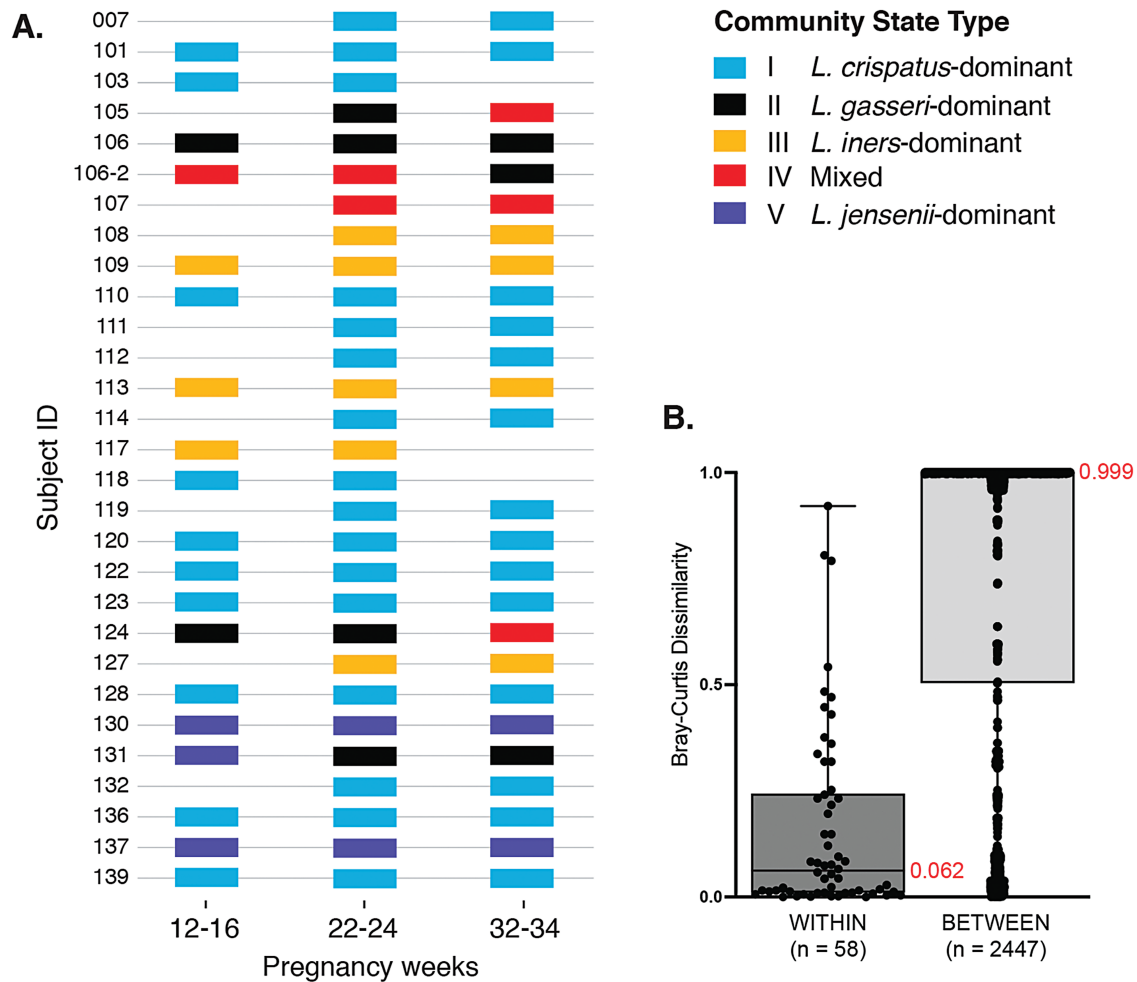
**Figure 1.** Clustering of vaginal microbiome profiles based on taxonomic composition. Bacterial species (nearest neighbors) are in rows, samples are in columns with patient ID and time point indicated above the heatmap (1T = 12-16 weeks’ gestation, 2T = 22-24 weeks’ gestation, 3T = 32-34 weeks’ gestation). Proportional abundance of each species in each sample is indicated by color in the heatmap (yellow to red, according to the legend). Only species accounting for at least 1% of at least 1 sample were included. The dendrogram above the heatmap indicates clustering by Jensen-Shannon distance, and community state types (CSTs) I-V are indicated immediately below the dendrogram.

transmission of sexually transmitted infections, and negative reproductive health outcomes including preterm birth.<sup>23</sup> Most of what is known about the vaginal microbiome in pregnancy, however, has come from studies of women without chronic inflammatory diseases. Given the complexities of management of IBD in pregnancy and established pregnancy risks associated with active inflammatory disease, foundational knowledge of the characteristics of the vaginal microbiome in IBD is needed.

In our current study, 14 (43.8%) of 32 participants had caesarean deliveries, which is higher than the overall caesarean delivery rate in Saskatchewan of 23.6% and the Canadian average of 29.1%<sup>34</sup> but is not unexpected for a group of individuals with IBD. For example, in a study of women who delivered between 2006 and 2014 at a hospital in Toronto, Canada, women with Crohn’s disease or ulcerative colitis had

caesarean delivery rates of 52% and 48%, respectively.<sup>35</sup> The authors of this retrospective study found that the main predictors of caesarean delivery rates in the IBD population were history of perianal disease, and prior caesarean delivery.<sup>35</sup> Preterm birth occurs more frequently in women with IBD, especially in patients with active disease either in the preconception period or throughout their pregnancy.<sup>3-7</sup> Only 2 participants in our study delivered before 37 weeks’ gestation, but disease activity status was not an eligibility criterion for the study, and it was not designed to address preterm birth as an outcome.

Vaginal microbiomes observed in our study were characteristic of well-established CSTs that have been reported in reproductive-aged women worldwide regardless of pregnancy status and determined using a variety of techniques.<sup>29,36-41</sup> Most (n = 43 of 78, 55.1%) samples were



**Figure 2.** A, Vaginal microbiome community state types of individual participants are indicated by colored blocks according to the legend. Patterns are shown for participants with at least 2 samples available. B, Samples from different participants were more different from each other (between) than from samples from the same participant (within). Median Bray-Curtis dissimilarity values for each set of comparisons are shown in red. Participants with single samples only and samples with <1000 reads ( $n = 2$ ) were excluded from this analysis.

**Table 2.** Mollicutes and *Ureaplasma* detection and comparison with previous studies

	PCR-Positive Women <sup>a</sup>			
	IBD (n = 29) <sup>b</sup>	Term (n = 170)	Preterm (n = 46)	Nonpregnant (n = 310)
Mollicutes	22 (75.9) <sup>A</sup>	68 (40.0) <sup>B</sup>	28 (60.8) <sup>A</sup>	217 (70.0) <sup>A</sup>
<i>Ureaplasma</i> spp.	11 (37.9) <sup>AB</sup>	40 (23.4) <sup>B</sup>	14 (30.4) <sup>B</sup>	149 (48.1) <sup>B</sup>

Values are n (%).

Abbreviations: IBD, inflammatory bowel disease; PCR, polymerase chain reaction.

<sup>a</sup>Significant differences are indicated by superscript uppercase letters (Fisher's exact,  $P < 0.05$ ).

<sup>b</sup>Subject 106: samples from 2 pregnancies gave same results.

classified as CST I, dominated by *L. crispatus*, and the remaining samples were approximately evenly distributed among CST II, III, and V (dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively), and CST IV (mixed species, low *Lactobacillus*) (Figure 1). Characteristic differences in intestinal microbiota composition such as elevated levels of adherent *Escherichia* and *Fusobacterium*, as well as reduced diversity, are well established to occur with IBD.<sup>42,43</sup> Our ability to compare our findings with previous reports of vaginal microbiome composition in pregnant women

without IBD is limited because differences in the sequencing method (pyrosequencing vs sequencing by synthesis) and bioinformatic methods (de novo assembly vs variant calling) used in previous studies preclude a direct quantitative comparison. Qualitatively, however, no conspicuous differences were noted between the microbiome profiles we observed in the current study and previous descriptions of pregnant and nonpregnant reproductive-aged women using a cpn60 barcode sequencing approach<sup>16,32,33,37</sup> or 16S ribosomal RNA amplicon sequencing studies.<sup>29,44</sup>

The longitudinal design of the present study did allow an examination of vaginal microbiome stability through pregnancy. Changes in CSTs were observed in only 4 of 29 participants for whom multiple samples were analyzed (Figure 2A). Furthermore, regardless of CST, any sample was likely to resemble another sample from the same participant more closely than a sample from another participant (Figure 2B). This stability is consistent with previous longitudinal studies that describe *Lactobacillus* abundance, reduced diversity, and stability over time as characteristics of the vaginal microbiome in pregnancy.<sup>18-20,45</sup> The explanation for these characteristics is not clear, but it has been suggested that the hormonal environment of pregnancy and the associated increase in thickness of the epithelium and increased glycogen deposition create an environment favorable for *Lactobacillus* spp. that maintain a low pH and prevent the growth of other bacteria.<sup>16</sup>

Because some species of *Mycoplasma* and *Ureaplasma* lack cpn60 genes, we used targeted PCR assays to detect Mollicutes in the vaginal samples. Using the same method on the same types of samples processed with the same DNA extraction method, Freitas et al<sup>16,32</sup> reported a lower prevalence of Mollicutes in pregnant women who delivered at term compared with either nonpregnant women or pregnant women who delivered preterm. In the present study, Mollicutes prevalence of pregnant participants with IBD was significantly higher than pregnant women without IBD who delivered at term, and similar to the prevalence in the nonpregnant and preterm birth groups (Table 2). We speculated that the higher prevalence of Mollicutes in the IBD group could reflect a higher species richness and diversity in the study population relative to pregnant women without IBD, but larger sample numbers and inclusion of a non-IBD control group would be required to investigate this relationship. *Mycoplasma* spp. have been linked to preterm birth,<sup>46</sup> and so their prevalence in women with IBD is certainly of interest given the higher preterm birth rates observed in this population and the lack of knowledge of the interaction of the microbiome and pregnancy outcomes in the context of IBD.

## Conclusions

Taken together, our results provide insight into the composition and stability of the vaginal microbiome of pregnant women with IBD. No obvious differences in composition from what has been described in other pregnant cohorts were observed, and in most cases, participants maintained a consistent microbiota throughout their pregnancy. Our results also suggest a difference in Mollicutes prevalence in the IBD group relative to pregnant women without IBD. Future studies should include a simultaneous sampling of nonpregnant age-matched women with IBD, and investigate the influence of preconception disease status on any pregnancy-associated changes in the vaginal microbiome. The collaboration of specialized preconception and pregnancy IBD clinics would be a major advantage in making such studies possible.

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## Conflicts of Interest

The authors have no conflicts of interest.

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