Title: Sub-communities of the vaginal ecosystem in pregnant and non-pregnant women

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SH, LS, PJ, DR conceived and designed the analyses.

DR, JR, DK, SB, EC, MF collected and annotated the data.

LS, PJ performed the analysis.

LS, SH, EC, DR wrote the manuscript draft.

All authors contributed to the final version of the manuscript.

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Abstract:

Diverse and non-*Lactobacillus*-dominated vaginal microbial communities are associated with adverse health outcomes such as preterm birth and acquisition of sexually transmitted infections. Despite the importance of recognizing and understanding the key risk-associated features of these communities, their heterogeneous structure and properties remain ill-defined. Clustering approaches have been commonly used to characterize vaginal communities, but they lack sensitivity and robustness in resolving community substructures and revealing transitions between potential sub-communities. We used a more highly resolved approach based on mixed membership topic models with multi-domain longitudinal data from cohorts of pregnant and non-pregnant subjects to identify several non-*Lactobacillus*-dominated sub-communities common to women regardless of reproductive status. These sub-communities correlated with clusters of metabolites. In non-pregnant subjects, we identified non-*Lactobacillus*-dominated sub-communities that were more common during menses but did not predict an increased likelihood of other non-*Lactobacillus*-dominated communities during the rest of the menstrual cycle. The menstrual cycle was a strong driver of transitions between sub-communities and was correlated with changes in levels of cytokines and metabolites, such as elevated TNF-α concentrations at the time of ovulation, and elevated kynurenine concentrations during menses, respectively. In pregnant women, some metabolite clusters were predictive of temporal instability in vaginal microbiota structure. Overall, our analyses based on mixed membership models revealed new substructures of the vaginal ecosystems which may have potentially important clinical or biological associations.

Main Text

Introduction

Several critical aspects of women's health are linked to the structure of the vaginal microbiota (1–3). When associated with positive health outcomes, the vaginal microbiota is dominated by *Lactobacillus* species (3). A paucity of *Lactobacillus* and a diverse array of strict and facultative anaerobes, however, are associated with negative health outcomes such as preterm birth (4, 5) and susceptibility to sexually transmitted infections (6–9), including HIV (10–12). Some *Lactobacillus* species, such as *L. crispatus* or *L. gasseri*, are more robust to invasion or take-over by non-*Lactobacillus* species and create greater vaginal ecosystem stability during and outside of pregnancy (13–15). Other *Lactobacillus* species, such as *L. iners*, are more frequently associated with non-optimal communities (13–15). In some individuals, the vaginal microbiota structure may change several times over the course of a few months or even a few days (4, 13, 14, 16). In non-pregnant menstruating women, menses are associated with drastic changes in microbiota structure (13). A cross-sectional study showed that recent sexual intercourse was associated with the presence of specific bacteria such as *Corynebacterium* (17). These associations suggest that changes in women's physiology, the interactions with the penile microbiome, and/or other factors may perturb the vaginal microbiota (3). However, the underlying mechanisms driving changes, and predictors of change in microbiota structure are still not identified, especially at times of the menstrual cycle other than menses. It is unclear why the vaginal microbiota of some individuals easily transitions back to an optimal state from a non-optimal one, while other microbiotas do not (4, 13, 14, 18).

In this study, we pursued three objectives. First, we sought to deepen our understanding of the fine structure of non-optimal vaginal microbiotas. To address this objective, we used mixed membership statistical methods to explore whether distinct sub-communities could be identified in a robust manner. Second, we evaluated potential associations between the reproductive status of the host (gestational age and phase of the menstrual cycle) and variation in features of the vaginal ecosystem, defined here as the set of bacteria, microbial and host metabolites, and host cytokines present in the vagina. Third, we sought to describe the temporal dynamics of the vaginal microbiota and identify metabolites or cytokines predictive of changes in vaginal microbiota structure.

Our first objective was motivated by the fact that non-optimal vaginal microbiotas are highly heterogeneous among and across individuals (4, 13, 14). Clustering approaches are commonly used to define community structure and have led to the adoption of the concept of community state types (CST) (19, 20). However, while this categorization serves as an important dimensionality reduction tool for these complex datasets, it tends to oversimplify community structure in non-optimal microbiota (often labeled Community State Type IV), and importantly, hides transition states between clusters. For example, the vaginal microbiota of two individuals could switch from one CST to another and be described by the same sequence of CSTs, but one individual might have experienced a progressive shift in her vaginal microbial structure (*e.g.,* the proportion of *Gardnerella* might have been slowly decreasing over time) while the other might have experienced an abrupt change (*e.g., Gardnerella* completely disappearing over a few days). These two situations may be driven by different mechanisms and have different health implications. Moreover, while clustering approaches can identify sets of species that frequently co-occur, they are not well suited to identify subsets of species that may have similar functions but that are not frequently found together. Consequently, to address our first objective, we turned to mixed membership models, and in particular topic models, to describe vaginal microbiota structure.

Topic models, developed to infer population structures (21) and formally described as Latent Dirichlet Allocation (LDA) (22), have been adopted for analyzing microbiota to identify bacterial sub-communities (23). Microbial samples are modeled as a mixture of topics (sub-communities), and each topic is characterized by a particular distribution of bacterial species or strains. For example, if a sample were described as 70% topic 1 and 30% topic 2 this would mean that the species subsumed in topic 1 accounted for 70% of the sample, while the species in topic 2 accounted for the remaining 30%. Some species can be found in several topics (*e.g.,* a species can co-exist within two distinct sub-communities). Topics may be composed of a few species or strains (sparse topics) or include a larger number. Here, we have applied topic analysis to vaginal samples from two longitudinal cohorts of women to identify specific sub-communities and report their temporal dynamics and the transitions between them. The first cohort was composed of pregnant subjects who provided weekly samples throughout pregnancy. The second cohort was composed of non-pregnant subjects of reproductive age who provided daily samples for ten weeks.

Our second and third objectives, which were to identify ecosystem features associated with reproductive status (*i.e.,* in pregnant and non-pregnant individuals) and predictive of changes in microbiota structure, required multi-domain data (data quantifying distinct biological components) acquired longitudinally from both menstruating and pregnant women. While several studies have collected longitudinal vaginal microbiota data (4, 5, 14, 18, 24, 25), relatively few also quantified metabolite concentration (13, 26) or cytokine levels (27–29), and none collected samples in both pregnant and non-pregnant subjects or accounted for potential menstrual cycle effects on the vaginal environment. In this study, metabolites and cytokines were quantified longitudinally from five samples per subject in each of forty subjects from each cohort (40 pregnant and 40 non-pregnant participants). The integration of 16S rRNA gene sequence, metabolite, and cytokine data allowed us to characterize the relationships among these different kinds of data and to describe the vaginal ecosystem throughout the menstrual cycle and pregnancy. Finally, we investigated the associations between metabolite and cytokine concentrations and stability of the vaginal microbiota composition.

Results

Topic analysis reveals shared and unique bacterial sub-communities across pregnant and non-pregnant women.

Topic models were fit to the count data of 16S rRNA amplicon sequence variants (ASVs) in 2,179 vaginal samples collected weekly from 135 pregnant individuals enrolled at two sites and 2,281 vaginal samples collected daily from 40 non-pregnant individuals enrolled at three sites (Methods, Table S1 for demographic data, Fig S1-3 for CST time series). To facilitate linkage with the published literature, “G1/G2/G3” labels were added to the three most abundant *Gardnerella* ASVs as these were previously found to be differentially associated with preterm birth risk (4) and may correspond to specific strains of Gardnerella (30) (SI, Fig S4).

Topic analysis requires choosing K, the number of topics, for the modeling of the provided count data. This number can be estimated using cross-validation or, as recently proposed (30), by performing topic alignment across models with different resolutions (*i.e.,* with different K). This latter approach offers the advantage of providing diagnostic scores to characterize individual topics and evaluate deviations from LDA assumptions. In pregnant subjects, the alignment suggested that 10 topics provided the best compromise between dimension reduction and accurate modeling of the ASV counts, while in non-pregnant subjects, 11 topics was best suited (Methods, SI).

Our analysis showed that several bacterial sub-communities (*i.e.,* topics) were shared across pregnant and non-pregnant women (Fig 1a). Besides the topics dominated by Lactobacillus spp., several topics without *Lactobacillus* showed strong agreement between cohorts and were robustly identified (Method, SI). For example, pairs of ASVs could be identified in three topics from both pregnant and non-pregnant women. Specifically, in topics E and F, ASV 15 labeled as *Gardnerella piotii* ("G3") was frequently observed together with ASV 5 - *Gardnerella leopoldii /swidzinski* ("G1") - but much less so with ASV 7, *Gardnerella vaginalis* ("G2") which co-occurred with ASV10 (*Atopobium vaginae*) in topic G. These two ASVs ("G2" and *A. vaginae*) constituted a robust topic in both pregnant and non-pregnant women and showed an ability to dominate (*i.e.,* explain almost 100% of the sample composition), especially in pregnant individuals (Fig S5-6). Topic E highlighted the observation that ASV 8 (*Megasphaera lornae*) was more frequently observed together with ASV 9 (*Ca*. Lachnocurva vaginae, "BVAB1") than with other non-*Lactobacillus* species (Fig S7-8).

Topic alignments (Fig 1b-c) constructed from the pregnant and non-pregnant women's samples provided insights into the coherence of the identified topics and relationships between them. Overall, we observed several similarities when comparing the alignment structures in both groups. Of particular interest, two topics at K = 4 were identified as "parents" of two distinct topic sets similar across groups. The first set of topics contained topic E, F, G, and – in non-pregnant participants - topic K, in which *Gardnerella*, *Megasphaera*, *Ca*. Lachnocurva and specific *Prevotellas* ASVs had high prevalence. The second set contained topic H, and, in non-pregnant participants, topics I and J. In this second set, *Finegoldia*, *Streptococcus*, *Enterococcus*, and *Staphylococcus* ASVs were highly prevalent. Topic alignments repeated on technical replicates of selected samples from both cohorts yielded similar results (SI, Fig S9). Examples of time series displays showing the topic composition of two participants are shown in Fig 2. These examples also show how metabolites and cytokines concentrations varied over time.

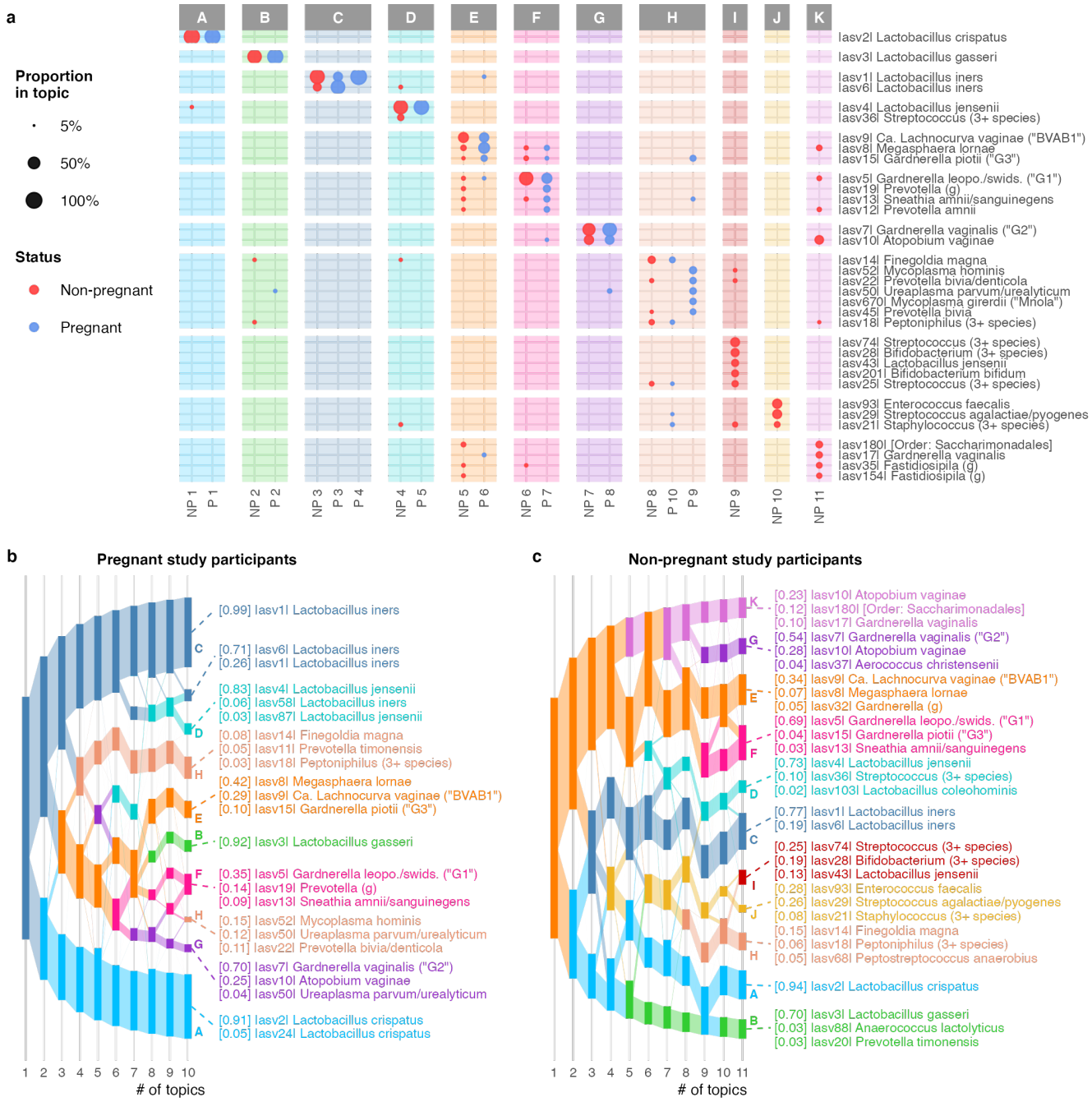

Figure 1: Topic analysis on samples from pregnant and non-pregnant women reveals shared and unique sub-communities.

(a) Composition of topics identified with samples of pregnant (P) and non-pregnant (NP) subjects considered separately. Dots size indicates the proportion of a specific ASV in each topic. Dot color indicates whether the topic was identified in samples of pregnant (blue) or non-pregnant (red) participants. To improve the readability of the figure, ASVs were displayed if they accounted for at least 5% of the topic composition. Topics were grouped (A to K) by similarity based on the Jensen-Shannon divergence between topic composition. Topic background color matches topic colors in panels (b), and (c). ASVs (rows) were ordered within topics by prevalence. **(b & c)** Alignment of topics (rectangles) identified separately with samples of pregnant subjects **(b)** and non-pregnant subjects **(c),** as the number of topics (x-axis) was varied from 1 to 10 or 11, the optimal number of topics for samples of pregnant or non-pregnant subjects respectively (SI). The height of the rectangles is scaled according to the total proportion of the corresponding topic in all samples: taller rectangles represent topics more frequently found among all samples than those represented by shorter rectangles. The numbers in brackets in front of each ASV indicate the proportion of that ASV in the topic.

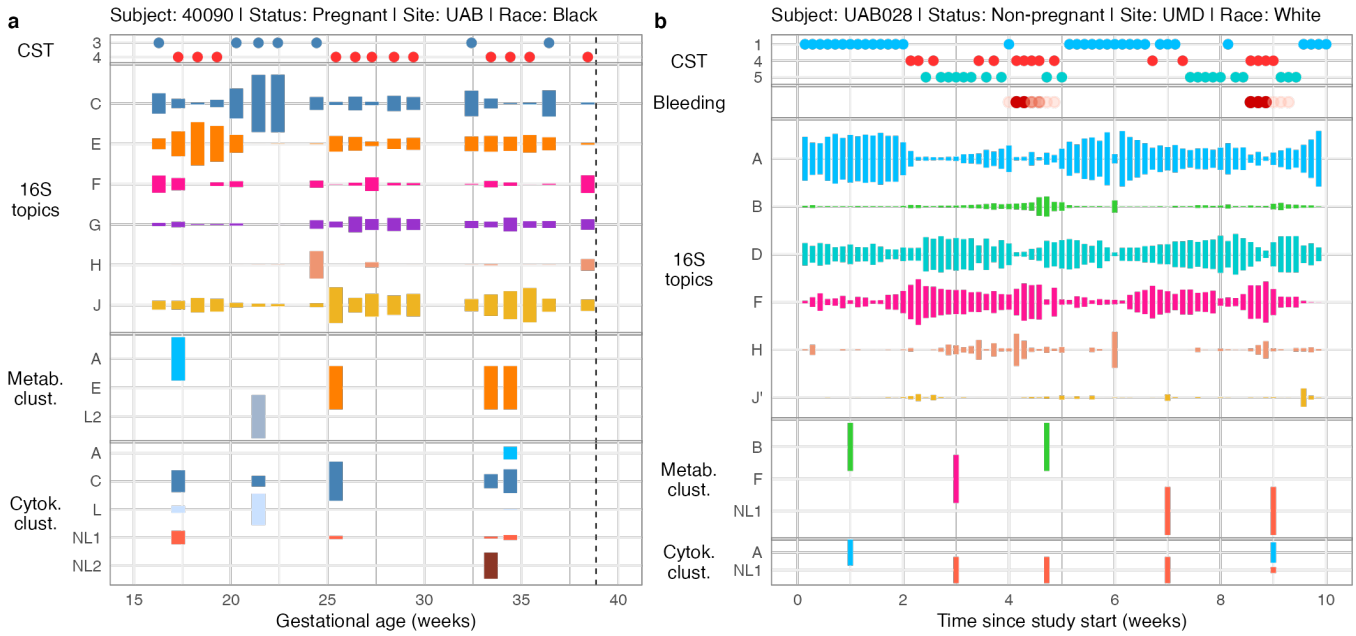

Figure 2: Examples of time-series displays of data from two subjects

Time-series displays of data from one pregnant participant (subject 40090, panel a) and one non-pregnant participant (subject UAB028, panel b). From the top, for each sample (x-axis): CSTs as defined in refs (14, 19), reported bleeding (just for the non-pregnant participant), topics based on 16S rRNA data, metabolite clusters, and cytokine clusters. For the 16S rRNA topics, the height of the bar is proportional to the topic proportion in each sample. For each participant, topics were included if they accounted for at least 10% of sample composition in at least one sample from that participant. For the metabolite or cytokine clusters, the height of bars is proportional to the inverse log10 probability of the sample belonging to that cluster (a higher bar means that the sample was closer to the cluster mean). Similarly, clusters were filtered such that their relative transformed probability was higher than 10%.

Metabolite concentrations are strongly correlated with microbial sub-communities.

Metabolite and cytokine concentrations were available at a lower temporal resolution than ASVs (Methods, Fig 2). Their concentrations were measured in 5 samples per participant in 40 pregnant and 40 non-pregnant women. Using a mixture model (Methods) to identify clusters in metabolite and cytokine concentrations, we found that metabolite clusters, and less so, cytokine clusters, were closely related to microbial structure (Fig 3). The UMAPs (Uniform Manifold Approximation and Projection) of cytokine and metabolite concentrations further showed that the menstrual cycle, and in particular menstruation, had a strong effect on both metabolite and cytokine concentrations (Fig 3).

Vaginal concentrations of cytokines were, in general, more strongly associated with the menstrual cycle than with microbial community structure: cytokine clusters were weakly aligned with 16S rRNA topics (Fig 3d) and only a few cytokines were associated with the proportion of non-*Lactobacillus* species in a sample when adjusting for menstrual bleeding (Fig 3a, Fig S10). IP10, MIG, and IL8 were positively associated with *L. iners* and *L. jensenii* (but not with *L. crispatus* or *L. gasseri*) and negatively associated with several topics dominated by non-*Lactobacillus* species (Fig S11). One topic (H'), not strongly dominated by any specific ASV, but composed of one *Finegoldia* ASV and over ten *Corynebacterium* and seven *Prevotella* ASVs, was associated with several cytokines, including IL1α, IL1β, IL8, MIG, and IP10 (Fig S11). Pregnancy status was also not associated with differences in cytokine concentration, except with MIP3α, IL1α, and IL1β, which were mildly elevated in samples collected before 5 months of gestation (Fig S12).

Metabolite concentrations were strongly associated with microbiota structure. Both in pregnant and non-pregnant women, most metabolites (58% in pregnant, 48% in non-pregnant, 34% in both) were associated with the proportion of *Lactobacillus* species in a sample (Fig 3b-c, SI) when adjusting for gestational age (pregnant) or bleeding (non-pregnant). Further, there was a strong correlation between metabolite clusters and 16S rRNA topics (Fig 3e-f), indicating that metabolite concentrations are strongly associated with specific sub-communities of the vaginal microbiota. In pregnant women, metabolites were associated with gestational age, as hinted by the mild gradients on the UMAP (earlier gestational age in the center on the left panel of Fig 3b). Nine metabolites were significantly associated with gestational age (SI, Fig S13). In non-pregnant women, menses was associated with metabolite concentrations, as most samples collected during menstruation were located in the same area of the UMAP (Fig 3c).

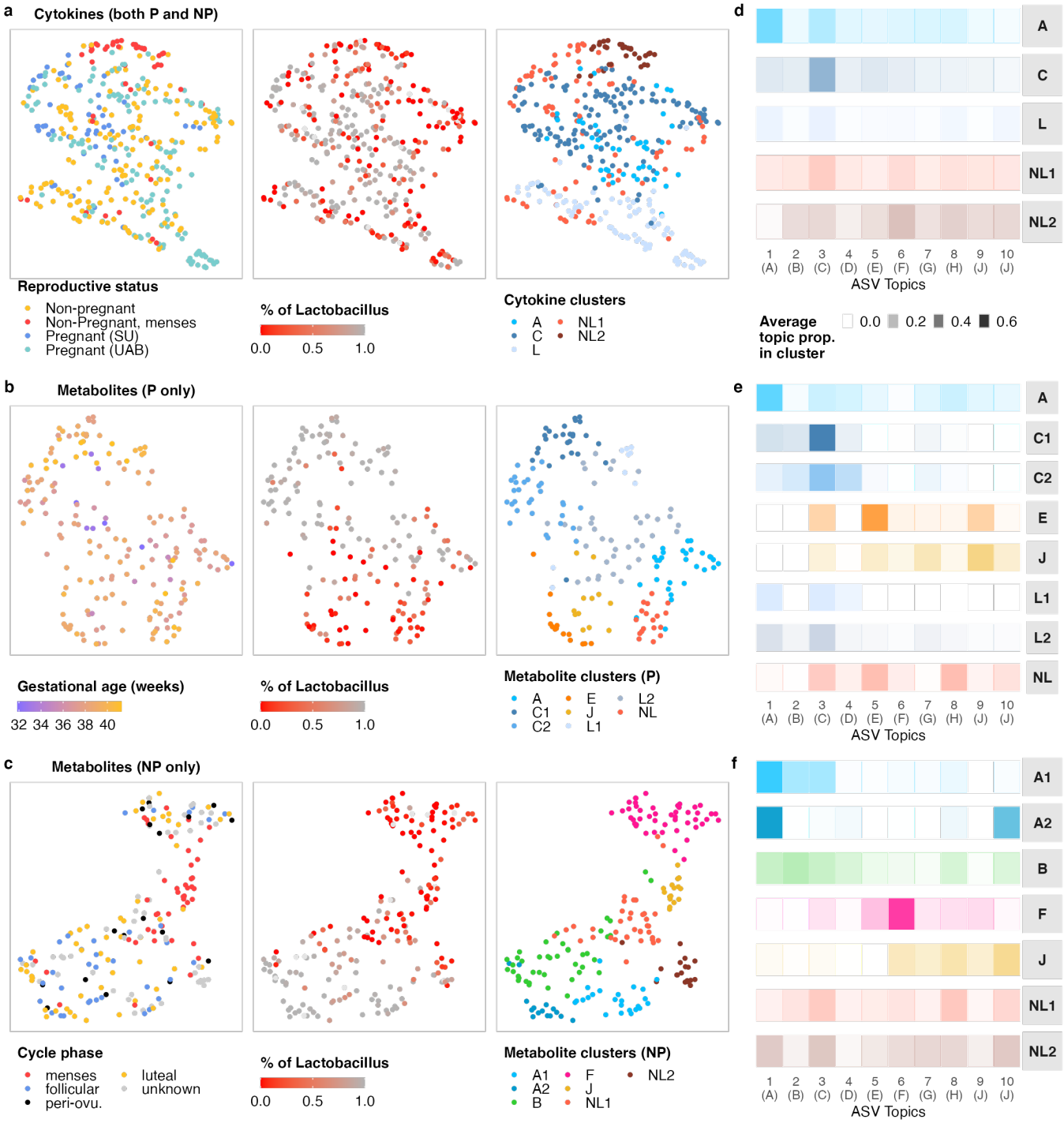

Figure 3: Integration of metabolite and cytokine clusters with microbial sub-communities

(a-c) UMAP (Uniform Manifold Approximation and Projection) of cytokine (a) and metabolite (b-c) concentrations (each dot is a sample). Because samples from pregnant and non-pregnant women were processed slightly differently for metabolite measurements, batch effects prevented a combined analysis of samples from the two groups. For each modality, the same UMAP was reproduced three times with different colors. Samples were colored by reproductive status, gestational age, or cycle phase in the left panels; by the proportion of *Lactobacillus* species in each sample in the center panels; and by their respective clusters on the right. **(d-f)** Color intensity indicates the proportion of each 16S rRNA sub-community (topic from analysis on all P and NP samples, displayed horizontally as columns) in each cluster (displayed vertically as rows). Color hues match cluster designations in corresponding panels on the left.

The menstrual cycle shapes the vaginal ecosystem.

Among the 40 non-pregnant subjects, 33 had reported vaginal bleeding patterns that allowed for the identification of at least one menstrual cycle within the ten study weeks (Methods). For 24 subjects, two menstrual cycles could be identified reliably. Given that the luteal phase (after ovulation) is known to vary less in duration than the follicular phase (before ovulation) (31, 32), cycles were standardized starting from 18 days before to 7 days after the first day of menses (Fig 4a, Methods). Ovulation was assumed to occur around 2 weeks before the first day of menses based on the average luteal phase duration (Methods).

Consistent with past results (19), the vaginal pH of *Lactobacillus*-dominated samples was lower (4.4, 90% 4.0-5.3) than that of non-*Lactobacillus*-dominated samples (5, 90% 4.0-5.8). The pH remained stable throughout the cycle (*Lactobacillus*-dominated: 4.3, 90% 4.0-5.3; non-*Lactobacillus* dominated: 4.9, 90% 4.0-5.5), except during menses when it increased by about 0.5 units in *Lactobacillus*-dominated samples (4.7, 90% 4.0-5.8) and non-*Lactobacillus*-dominated samples (5.4, 90% 4.4-7.0) (Fig 4b).

Half of the measured cytokines showed concentration changes associated with the menstrual cycle (Fig 14-15, Table S3). Most cytokines had elevated levels during menses (Fig S14). However, a few cytokines (e.g., INF-γ) increased around the predicted time of ovulation or, like MIP3α, exhibited a gradual increase throughout the luteal phase (Fig 4c). The abundance of about 45 metabolites (out of 355) was associated with the menstrual cycle (Table S4, Fig S16). While the largest changes were often found around the time of menses (Fig S16), several metabolites also exhibited changes during specific phases of the menstrual cycle, such as following ovulation (e.g., isoleucine), or in the mid- or late-luteal phase (e.g., aspartate) (Fig 4d, Fig S16).

To assess possible associations between vaginal microbiota structure and menstrual cycle, we used a longitudinal approach and compared vaginal microbiota structure between two consecutive cycles of a given participant. Vaginal structure, characterized by ASV rank (or relative abundance), showed a high correlation between consecutive cycles (Fig 4e-f, Fig S17). This longitudinal (within-subject) approach was used because the absolute abundance of ASVs cannot be established from our 16S rRNA gene amplicon sequence data, so we could not test for their association with time in the menstrual cycle as we did for metabolites and cytokines. We still used a cross-sectional approach to evaluate the impact of the menstrual cycle on the probability of an ASV being detected on a given cycle day. We found a strong association for 91 ASVs (Fig S18), among which 55 (60%) had a higher probability of detection during menses compared to the rest of the cycle (Fig S19). A total of 17 (20%) ASVs, including four *Prevotella* ASVs and one *Atopobium* ASV, were associated with the late luteal phase (Fig 4g).

Given that specific non-*Lactobacillus* subcommunities, with a high prevalence of *Finegoldia* or *Pseudomonas* (FigS20, Table S5), were often found during the menses, including in participants with *Lactobacillus*-dominated

microbiota throughout the rest of their cycle, we tested whether these menses-associated subcommunities were predictive of other non-*Lactobacillus* species, such as *Gardnerella* spp., at other phases of the menstrual cycle. We found no association (Fig S21, Table S6-7). Participants whose pre-menses samples were dominated by *Lactobacillus* species were as likely to see an increase in non-*Lactobacillus* species during menses (SI). Similarly, the presence of these menses-specific species (or topics H and J, Fig S21, Table S7) was not associated with a higher risk of non-*Lactobacillus* species following menses. Finally, we found that some menses associated ASVs are also found around the time of ovulation in some individuals (Fig S21). Because many participants used a combination of pads and tampons during their menses (Table S8), we did not find significant associations between topics and menstrual protection use (Fig S22). Our analysis also confirmed that behaviors suspected of disrupting the vaginal microbiota, such as sexual intercourse or the use of vaginal gels or powder, were associated with changes in the vaginal microbiota. Indeed, we observed more changes in microbiota structure among participants who more frequently reported these behaviors (Fig S23). However, this study did not have the statistical power to identify which behaviors were most disruptive.

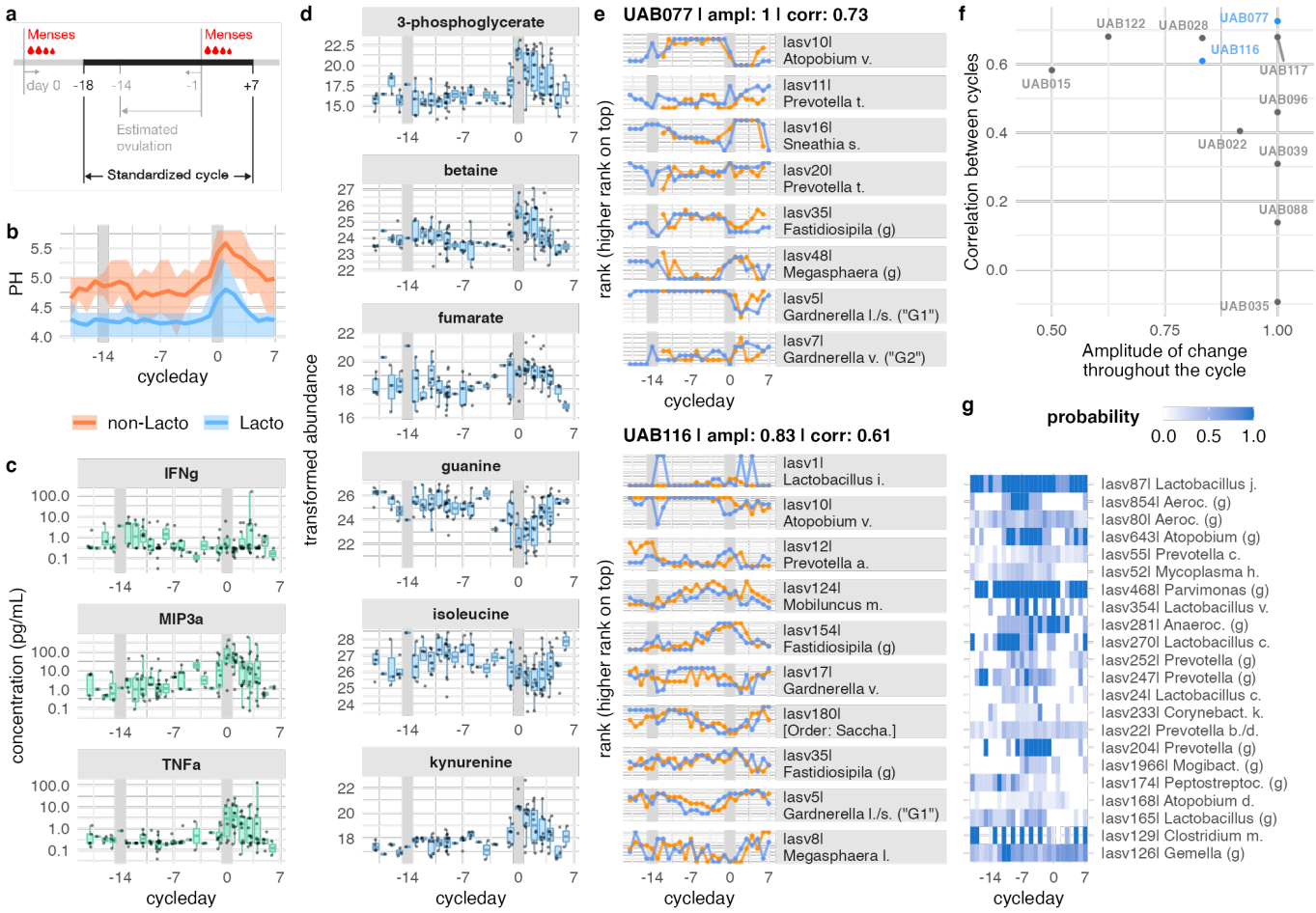

Figure 4: The menstrual cycle shapes the vaginal ecosystem.

(a) Schematic illustrating the features of standardized cycles. **(b)** Vaginal pH throughout the menstrual cycle for *Lactobacillus* or non-*Lactobacillus*-dominated vaginal microbiotas. **(c)** Vaginal cytokine concentrations (pg/mL) throughout the menstrual cycle for three cytokines correlated with the menstrual cycle. Profiles of all measured cytokines are shown in Fig S14-15 **(d)** Vaginal metabolite relative concentrations throughout the menstrual cycle for 6 metabolites showing a strong association with time in the menstrual cycle. Profiles of all metabolites associated with time in the menstrual cycle are shown in Fig S16 **(e)** Vaginal microbiota structure of two subjects with data available for at least two full menstrual cycles. The first menstrual cycle is displayed in orange, the second in blue. These two subjects were selected to show the diversity of temporal profiles. The time series display shows

ASV rank (y-axis, a higher rank value indicates that this ASV was more abundant than other ASVs) on each cycle day (x-axis). For each study participant, ASVs were included if they were part of a pool accounting for 98% of the total composition in a sample and if they were present in at least ten samples from that participant. **(f)** The scatter plot, in which each dot is a participant, shows the relationships between correlation in vaginal microbiota structure between two cycles of the same individual (y-axis), and the relative amplitude of change throughout the cycle (x-axis). **(g)** Heatmap of the probabilities that ASVs are present on a given cycle day. ASVs whose probability were significantly higher in the luteal phase were selected for this panel. A similar heatmap with ASVs associated with other phases of the menstrual cycle is shown in Fig S18.

Metabolite clusters are associated with changes in vaginal microbiota structure.

Intrigued by the fact that samples with different metabolite structures (A, C1, C2, L1, L2, fig 3e) shared a similar topic (and ASV) composition, we investigated whether metabolite profiles were indicative of microbial community stability. We conducted this analysis using the pregnant participants' samples, excluding the samples from the non-pregnant participants to avoid confounding with menstrual cycle effects. We compared microbial community stability in each metabolite cluster identified in pregnant women's samples (Fig 5). Specifically, for each sample, we computed the percentage of the microbial composition that was different between that sample and the sample at the next or previous time-point. We observed that samples categorized in the A and L2 metabolite clusters were more likely to be associated with subsequent changes in topic composition than those categorized in the C1, C2, and L1 metabolite clusters (Fig 5). In particular, we observed that samples fully dominated by *L. crispatus* were more likely to still be dominated by *L. crispatus* in the previous or next sample if their metabolic state was best represented by C1, C2, or L1 metabolite profiles.

In addition, we fitted sparse regressions on the pregnant subjects' samples to predict the relative abundance (proportion) of *Lactobacillus* species or the proportion of specific ASV topics at the next time-point (SI). We found that metabolite concentrations were better predictors than cytokine concentrations (Fig S24). Metabolites positively associated with increases in *L. crispatus* were often negatively associated with increases in *L. iners* and those positively associated with increases in topics of group E and F (*i.e.,* containing "G1" and "G3" *Gardnerella* ASVs) were negatively associated with increases in either *L. crispatus* or *L. iners* (Fig S25).

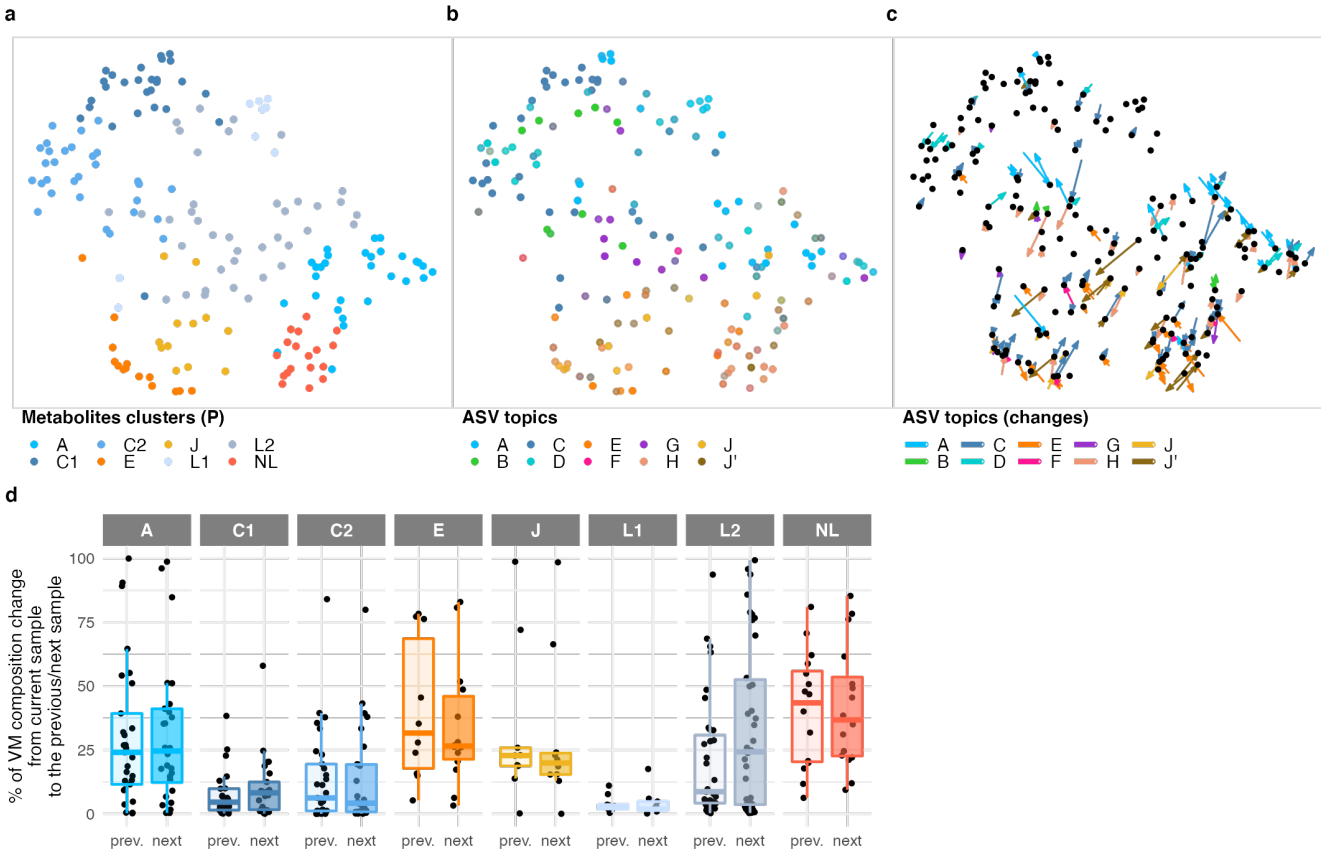

Figure 5: Metabolite clusters are associated with instability in the vaginal microbiota.

(a-c) UMAP of the metabolite concentrations in samples (dots) from pregnant women (same as in Fig 3b). **(a)** Same as in Fig 3b right-hand display. **(b)** UMAP of metabolite concentrations where samples are colored by 16S rRNA topic proportion. When samples are composed of several topics, dot colors are a mix of each individual topic colors. **(c)** Arrows show changes in topic proportion from the previous or the next time-point. Incoming arrows show the difference in topic proportion from the previous time-point to the current time-point. Outgoing arrows show the difference in topic proportion from the next time-point to the current time-point. The angle of the arrow is related to the topic. The length of the arrow is proportional to the difference in topic proportion. For example, a dot with two large light sky-blue arrows indicates that both the previous and following time-points were dominated by topic A, but not the current one. **(d)** Distribution of changes in vaginal microbiome (VM) composition from one sample to the next (right boxes in each panel) or from the previous sample to the current sample (left boxes in each panel) for each metabolite cluster (horizontal panels).

Discussion

In this study, we identified bacterial sub-communities within non-*Lactobacillus*-dominated samples from both pregnant and non-pregnant women. Using topic analysis, we found several non-*Lactobacillus* sub-communities (*i.e.,* topics) to be shared by the two groups, refining the structure of samples traditionally categorized in the community state type (CST) IV (19). Within these subcommunities, we identified several pairs of co-existing bacterial ASVs to be typical of some sub-communities. For example, *Gardnerella* ASVs (G1 and G3) co-occurred more frequently than other Gardnerella ASVs (e.g. G2 and G3), and the most common *Atopobium* ASV defined a dominant, robust sub-community with G2 in 42% of the subjects for which *Atopobium* was present. It remains to be seen if these bacteria are symbiotic and if specific conditions, such as hormonal milieu, are favorable to their joint emergence.

The main difference between the approach used here (topic analysis) and clustering approaches traditionally used to identify sub-groups in the vaginal microbiota lies in the mixed membership nature of topic models, thereby allowing samples to be associated with multiple topics in different proportions. This property presents the advantage of revealing longitudinal transitions between sub-communities and the rate at which they occur, which is not possible with clustering approaches. In addition, topic models allow for synonyms, which may reflect potential functional equivalences in a microbial community context. Indeed, if two species are found interchangeably (but not simultaneously) with a specific group of other species, these two species will be found in the same topic. In contrast, clustering approaches will tend to create two clusters, one containing each of these two species, artificially increasing the number of sub-communities. In fact, the comparison of the subcommunities identified here with the CST IV sub-types more recently introduced (20) suggests that topics I and J encapsulate the 5 CST IV-C subtypes.

Our study also quantified vaginal metabolite and cytokine concentrations in pregnant and non-pregnant study participants. When integrating data across domains (microbiota composition, metabolite concentrations, and cytokine concentrations), we found that clusters fitted on metabolite concentrations correlated well with microbiota sub-communities. There was a weaker association between cytokine concentrations and microbiota structure. In non-pregnant participants, samples collected during menses showed a distinct profile across domains.

Almost all non-pregnant subjects with data available for two menstrual cycles showed a high between-cycle correlation in their vaginal microbiota variation. However, some species reached their maximal relative abundance at different menstrual cycle phases in different individuals. These differences could simply be due to our inability to detect absolute levels (ASV relative levels or rank prevent a reliable identification of the peak phase of each ASV). Besides this technical limitation, these inter-individual differences could be due to (i) inter-individual differences in menstrual timing (for example, one subject might have a 10-day luteal phase while another one might have a 14-day luteal phase); (ii) inter-individual differences in hormone levels (or the rates of change in these levels); or (iii) the set of species present in each subject and how each of these species might respond differently to the menstrual cycle while competing for resources. Future clinical studies should include the measurement of hormones and methods to estimate the timing of ovulation. Similarly, additional data would be necessary to understand if the substantial changes in vaginal microbiota composition during menses are driven by abrupt hormonal changes, the presence of blood, the transfer of uterine microbes carried in menstrual blood, or the use of menstrual protections such as pads or tampons.

Over 50% of measured metabolites showed an association with the proportion of *Lactobacillus and* about 45 metabolites, such as kynurenine or isoleucine, showed an association with a specific phase of the menstrual cycle.

Kynurenine is a tryptophan catabolite via a pathway involving IDO1-mediated degradation and is known to play a role in blood vessel dilatation during inflammatory events (33). The elevated levels of kynurenine during menses found in our study are thus consistent with these roles and with past studies showing varying levels in kynurenine serum and urinary levels through the cycle (34, 35). Isoleucine is a branched-chain amino acid, and as such, plays important metabolic functions (36). In our vaginal samples, isoleucine levels were found to be highest in the luteal phase and lowest during menses. Interestingly, serum levels of isoleucine show opposite trends (37).

Cytokines mostly showed an association with the menstrual cycle (half of them have a significant association) but not with pregnancy stages. Previous studies in non-pregnant subjects found similar variations in cytokine levels throughout the menstrual cycle (27, 28). Regarding the associations between cytokine concentrations and microbiota structure, we found that most cytokines were not associated with the proportion of *Lactobacillus* or with the microbiota characterized by its topic composition. Only a few cytokines showed associations with the proportion of specific topics. IP-10 is positively associated with topic C (dominated by *L. iners*), which is consistent with previous findings (27), and negatively associated with topics E, F, and H (dominated by non-*Lactobacillus* ASVs). IL-1β also showed a strong negative association with the abundance of *Lactobacillus crispatus* species in our dataset and was also previously found elevated during and after BV events (27, 38, 39). Our results showed limited overlap with those reported in a study of a large cohort of young South African women (12), but these differences could be explained by the fact that about half of the participants in that study were on an injectable progestin contraceptive and samples were not collected during menses, preventing the adjustments for the time in the menstrual cycle.

When investigating temporal changes in the vaginal microbiota of pregnant subjects, we found two metabolite clusters associated with increased instability in microbial composition. We also used supervised learning to identify specific metabolites or cytokines associated with the composition of the vaginal microbiome at the next time point and found that metabolites were better predictors of changes than cytokines. These results suggest that the metabolic state is associated with both the microbial composition and its stability. This highlights the important role of metabolomics for vaginal health as specific metabolites were also found to be predictive of spontaneous preterm births (26). The causal relationships between metabolite profiles and microbiota instability are still however unclear as the differences in metabolite concentrations could be explained by differential metabolite production by the host and/or be the result of differences in microbial transcriptional activity. It has indeed been recently suggested that differential transcriptional activities were associated with changes in metagenomic composition (40).

Conclusions

Topic analysis revealed bacterial sub-communities (topics) shared across pregnant and non-pregnant women. Compared to clustering approaches traditionally used to describe microbial composition, topics provide an expanded characterization of the heterogeneity of the previously described community state type IV (CST IV) and a high-resolution view of transitions between communities. We found that metabolite concentrations highly correlated with the make-up of microbial sub-communities and that the menstrual cycle had a strong impact on the vaginal ecosystem, defined here as the composition of the vaginal bacteria, metabolites, and cytokines. In-vitro studies will provide further functional insights into the identified sub-communities, their ecological network, and their effect on the vaginal epithelium. Finally, specific metabolic states and a few cytokines were found to be associated with changes in microbial composition. In vitro confirmation of these associations could improve the development of products supporting the growth of *Lactobacillus* species and the restoration of an optimal vaginal microbiota.

Material and Methods

Cohorts and sample collection

Daily samples from non-pregnant women. For this study, the samples from two cohorts with identical recruitment criteria and sample collection methods were used. The samples from 10 participants from a larger multi-site cohort recruited at Emory (EM) and the University of Maryland, Baltimore (AYAC) were used along with samples from 30 participants recruited at the University of Alabama Birmingham (UAB). Each participant self-collected daily vaginal swabs for 10 weeks, resulting in a maximum of 10 x 7 = 70 samples per individual. For further detail about recruitment criteria and sample collection, see (16).

Weekly samples from pregnant women. We used the samples from both cohorts presented in (4). 39 pregnant subjects were recruited at Stanford University (SU) and 96 pregnant subjects were recruited at the University of Alabama Birmingham (UAB). Participants recruited at UAB received intra-muscular injections of progesterone throughout pregnancy. Participants were enrolled from the 4th month of their pregnancy (earliest enrollment at week 8, latest at week 22) and vaginal swabs were collected weekly (approximately) until delivery.

Selection of the "VMRC samples". All samples described above were used for 16sRNA sequencing. Metabolites and cytokine concentrations were quantified on a subset of these samples. Specifically, 5 samples from 40 pregnant and 40 non-pregnant women were selected and labeled "VMRC samples". The 40 pregnant women were selected out of the initial pool of 135 participants as a representative sample of the larger pool based on their Jensen-Shannon stability index, the CST composition, and gestational age at delivery. 5 samples were then selected so that they were equally distributed in time throughout pregnancy. The 5 samples from the 40 non-pregnant women were similarly selected so that samples were distanced by approximately 2 weeks.

Ethics

All participants provided written informed consent. Ethical approval was obtained from the Institutional Review Boards of Stanford University, the University of Alabama, Birmingham, and the University of Maryland. All research was conducted in compliance with relevant guidelines and regulations.

Vaginal microbiome sequencing

Daily samples from the 10 non-pregnant participants recruited at EM and AYAC. the V1V4 regions of the 16S rRNA gene were amplified then sequenced using 454 sequencer, as described in (16).

Daily samples from the 30 non-pregnant participants recruited at UAB (1534 samples). The V3V4 regions of the 16S rRNA gene were amplified then sequenced with Illumina HiSeq/MiSeq

Weekly samples from pregnant participants of both cohorts (SU and UAB) (2179 samples): Raw sequence data from pregnant participants samples were generated and processed as described in (4). In brief, genomic DNA was extracted from vaginal samples using a PowerSoil DNA isolation kit (MO BIO Laboratories). Barcoded primers 515F/806R (41) were used to amplify the V4 variable region of the 16S rRNA gene from each sample. Pooled amplicons were sequenced on Illumina HiSeq/MiSeq instruments at the Roy J. Carver Biotechnology Center, University of Illinois, Urbana-Champaign.

VMRC samples (393 samples) from the 40 pregnant and 40 non-pregnant participants were re-sequenced (technical replica) using the same protocol as for the pregnant participants' samples.

Demultiplexed raw sequence data from the Illumina HiSeq/MiSeq (samples of pregnant subjects, of the 30 UAB non-pregnant participants, and VMRC samples) were resolved to amplicon sequence variants (ASVs) as described in the DADA2 Workflow for Big Data (https://benjjneb.github.io/dada2/bigdata.html) (42).

Taxonomic assignment. Automated taxonomic calls were made using DADA2's implementation of the RDP naive Bayesian classifier (43) and a Silva reference database (version 132) (44). The assignment of sequences of the most abundant ASVs were refined and standardized by BLASTing against NCBI RefSeq type strains. This is the case for *Lactobacillus, Candidatus* Lachnocurva vaginae (previously referred to as BVAB1), *Gardnerella*, and *Megasphaera lornae* species-level assignments,

following recently published work on these species (45, 46). *Gardnerella* ASVs were tagged as G1, G2, or G3 sensu (4) based on exact matching.

ASV harmonization across assay. While most analyses were performed the data from each assay separately, we were interested to also fit the topic model on the combined assays. However, given that two different sequencing technologies were used for the samples from the pregnant and the non-pregnant subjects, the sequenced region of the 16S rRNA gene differed between assays but shorter regions were all included within the longer regions. ASV sequences were thus matched across assays. We used exact matches (*i.e.,* a shorter sequence had to be fully and exactly included into a longer sequence). If several longer sequences exactly matched a given shorter sequence, the counts of the corresponding longer ASVs were aggregated. The taxonomic assignment of the shorter sequence was used for the matched ASVs.

Phylotype level data for all samples of non-pregnant participants. To harmonize the data from all non-pregnant participants, the V3V4 (HiSeq/MiSeq) and V1V3 (454 sequencer) 16S count data were aggregated at the species level (or at a higher taxonomic level when taxonomic data was not available at the species level) resulting in a table of dimension 225 species x 2281 samples (approximately 40 subjects x 70 samples). This table was used to correlate metabolite and cytokines concentrations with the relative abundance of Lactobacillus species. Topic analysis was also performed on this data and presented in the SI.

Metabolite concentration quantification

Untargeted metabolomics was performed on the 400 VMRC samples by ultra-high-performance liquid chromatography/tandem mass spectrometry (Metabolon, Inc.). Metabolite identification was performed at Metabolon based on an internally validated compound library and results were expressed in relative concentrations, following the same protocol as in (47). Samples were sent in two groups to Metabolon. All samples from the non-pregnant women and 80 samples from the pregnant women were sent in a first batch. The 120 remaining samples from the pregnant women were sent in a second batch. We observed a mild batch effect between the samples from the pregnant and non-pregnant women, likely due to slightly different collection and storage procedures, and between the samples of the first and second batch in pregnant women's samples. While it was not possible to remove the batch effect between the samples from pregnant and non-pregnant women because of potential confounding from the biological state, we could re-align samples from the two batches in pregnant subjects after the variance stabilizing transformation (see below) because there were no other variables found to be different in these two batches (well-mixed populations).

Data transformation. We transformed the raw metabolite relative concentrations using a variance stabilizing method (48). Raw data included the concentrations of 855 (P) and 853 (NP) metabolites. However, the abundance of 465 (P) and 517 (NP) metabolites was missing in more than 50% of the samples. We removed these metabolites from the analysis. The remaining 390 (P) and 336 (NP) metabolites were still missing in at least one sample for most of them. Missing metabolites might be missing because their abundance is lower than the detection limits or because the overall quality of a sample was lower (the proportion of missing metabolites varies widely from one sample to another and from one batch to another). 21 (P) and 1 (NP) samples with more than 60% missing metabolites were excluded for the rest of the analysis. Metabolite distributions were re-aligned between the two batches of pregnant women's samples by subtracting the median differences between the two batches.

Imputation. For some analyses, such as dimension reduction of the metabolite space (e.g., UMAP) or future VM composition prediction, missing metabolite abundances were imputed using a mixture between a fraction (0.95) of the metabolite-specific lowest observed value and the imputed value using a k-nearest neighbor (KNN) imputation method. The mixture was based on the quality of the sample. Missing values in samples of high quality (with few missing metabolites) had a higher weight on the imputation to a fraction of the lowest value for that metabolite. Missing values in samples of low quality had a higher weight on the KNN imputation. For the KNN imputation, the Euclidean distance between samples was computed based on the values of co-present metabolites in sample pairs. Then, for each sample, the value of missing metabolites is imputed as the weighted mean of the values of the k=5 closest samples for which this metabolite was quantified. The weight (weighted mean) is inversely proportional to the distance between the considered sample and its k nearest neighbors.

Cytokine concentration quantification

20 cytokines were quantified in the 400 VMRC samples using a Luminex-based assay with a custom kit of 20 analytes (IFNγ, IL-1a, IL-1b, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, IL-21, IL-23, IP-10, ITAC, MIG, MIP-1a, MIP-1b, MIP-3a, and TNFα) following the same protocol as in (12). The assay was run on a Luminex FLEXMAP 3D instrument. For measurements that were below the limit of quantification for a given cytokine, values were imputed at half the lower limit of quantification (LLOQ / 2). For measurements that were above the limit of quantification for a given cytokine, values were imputed as equal to the upper limit of quantification (ULOQ). Values reported here represent medians of two technical replicates. The medians were calculated after imputation in one or both replicates (if necessary) as described above. Missing cytokine values represent technical failures of the assay for that analyte.

Data transformation and imputation. Raw cytokine abundances were log-transformed. Raw data included the abundance of 20 cytokines. Most of the cytokines could be quantified and only 14/8000 data points were missing. These missing data points were imputed using the same KNN imputation method as for the metabolite KNN imputation.

Data integration into a multi-assay experiment (MAE) object

All analyses were done in R (49). Specific packages used for the analyses are referred to in the next sections. The raw datasets were loaded and minimally processed before being formatted into SummarizeExperiment objects of the SummarizedExperiment bioconductor package (50), then combined into a single S4 object using the MultiAssayExperiment bioconductor package (51).

Identifying bacterial sub-communities using topic analysis

Microbial communities were estimated based on LDA (latent Dirichlet allocation) (22, 23). LDA models were fitted to the data for K (the number of topics) = 1 to 20 using the R package "topicmodels" (52). Models were fitted on the count data of each 16S rRNA assay separately and on the combined samples of the ASV-resolution assays.

Identifying robust topics across K (the number of topics). Topics were aligned across K using the topic alignment method described in (30). To identify robust topics across K, we used the alignment summary scores for topic coherence as defined in the same reference.

Matching topics across cohorts. Topics identified in different cohorts were matched using hierarchical clustering on the combined topics, with the Jensen-Shannon divergence used as the distance metric between topics.

Clustering of metabolite and cytokine concentrations

Samples were clustered using a parameterized finite Gaussian mixture model as implemented in the mclust R package (53). Specifically, Gaussian mixture models were fitted on the imputed transformed (see above) concentrations and the model with the highest BIC was selected, determining the shape, orientation, and number of clusters. For each sample, the most likely cluster was reported as well as the probability of belonging to each cluster.

Identification of phases of the menstrual cycle

Menstrual cycles were identified from bleeding flows reported by participants on a scale from 0 (none) to 3 (heavy). Specifically, a hidden semi-Markov model was specified to account for empirically observed distribution of cycle length and bleeding patterns across the menstrual cycle (54). Data of participants who reported too few days with bleeding (*i.e.*, less than 3/70 study days) or too many (*i.e.,* more than 30/70 study days) were excluded from the menstrual cycle analyses. Once cycles were identified, cycle days were numbered forward and backward from the first day of the period. In order to align the two major menstrual events, i.e. ovulation and menses, across participant and given that the luteal phase has been well documented to vary less than the luteal phase (32), cycles were standardized starting from day -18 (*i.e.* 18 days before the start of the next cycle) and ending on day +7 (i.e. 7 days after the first day of the menses). This definition ensures that the standardized cycles would include the days leading to ovulation, estimated to happen around days -12 to -14 (32) and allows for the best possible alignment of the two major menstrual events (ovulation and menses) in the absence of hormonal and/or ovulation markers.

Testing for differential abundance throughout the menstrual cycle

To identify metabolites, cytokines or ASVs with differential abundance (metabolites or cytokines) or differential probabilities of being present at specific phases of the menstrual cycle, a linear model (for abundances) or logistic regression (presence/absence probabilities) was fitted to circular splines parametrized with 5 degrees of freedom (R package "pbs"). Analysis of deviance was used to report p-values of the F-statistics and corrected for multiple testing using the Benjamini-Hochberg method.

Correlation in VM composition between two consecutive cycles

To evaluate how the menstrual cycle affects the vaginal microbiome, we first identified the most abundant species for each individual with at least two complete consecutive cycles. Then, the Spearman (rank) or Pearson (relative abundance) correlation between the ASV counts of two consecutive standardized cycles were computed.

Predicting changes in the composition of the vaginal microbiota in pregnant women

To identify metabolites or cytokines predictive of changes in the vaginal microbiota of pregnant women, sparse logistic regression (lasso) was used to predict the *Lactobacillus* proportion at the next time point. To increase the robustness of the model, sparse regressions were fitted ten times on sub-samples of the available data (70% of samples were randomly considered for each fit). The coefficients of the 15 first non-zero features (metabolites or cytokines) were identified for each fit. In parallel, a random forest model was fitted similarly so that the most important features identified by the random forest models could be compared to those identified using sparse regression. The glmnet and randomForest R packages were used for these predictions.

Availability of data and materials

The sequence data are available in SRA under BioProject accession numbers PRJNA208535 (samples beginning with UAB) and PRJNA575586 (samples beginning with AYAC and EM) and, for the samples of the pregnant subject, on the NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (accession no. SRP115697). The SI contains the R code that enables the reproduction of the analyses.

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