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Longitudinal profiling and correlations of vaginal and fecal microbiomes throughout the menstrual cycle: A pilot prospective study

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Abstract

Hormonal and endometrial fluctuations across the menstrual cycle may influence both vaginal and fecal microbiomes. Since no longitudinal study examining both matrices together in this context exists, our study aimed to evaluate microbiome changes in three phases of the menstrual cycle and to investigate correlations between bacteriomes and mycobiomes in vaginal and fecal samples.

Over the course of three consecutive menstrual cycles, vaginal and stool swabs were self-collected in the follicular, ovulatory, and luteal phases from eight parous women of reproductive age who had regular menstrual cycle. Microscopic fungi in all vaginal samples were cultured and identified by mass spectrometry. DNA from 72 vaginal and 72 fecal samples were analyzed using quantitative PCRs, 16S and ITS rRNA amplicon sequencing for bacteriome and mycobiome profiling. Metabolic pathways were analyzed with a focus on the members of the Lactobacillaceae family.

Bacteriome diversity in vaginal samples remained stable across studied months and individual phases, while changes in bacteriome alpha diversity (Shannon index) across phases were observed in fecal samples, with the highest diversity observed during the luteal phase. As expected, the genus *Lactobacillus* (mostly *L. crispatus*) predominated in the vaginal samples. Furthermore, different patterns of the predicted metabolic potential were observed in vaginal samples dominated by *Lactobacillus iners* or *Gardnerella vaginalis* with *Lactobacillus jensenii*, compared to the profile dominated by *L. crispatus*. Yeasts, such as *Candida albicans*, *Nakaseomyces glabratus*, and *Pichia kudriavzevii*, were found in some vaginal samples. The presence of vaginal yeasts correlated with relative abundances of *L. crispatus* (negatively) and *L. iners* (positively) in vaginal samples, and with genus *Streprococcus* in stool samples (positively). Relative abundances of *L. jensenii* in vaginal samples correlated with genera *Bifidobacterium* (negatively) and *Dialister* (positively) in stool samples.

The results indicate that when investigating female fecal microbiome, the phase of menstrual cycle should be considered. Some strong correlations between the relative abundance/presence of vaginal lactobacilli and yeasts and fecal bacteria were found, suggesting a possible link between the composition of microbial communities of these two anatomical sites.

Introduction

The vaginal microbiota plays a crucial role in maintaining female reproductive health. In healthy individuals, this ecosystem is typically dominated by bacteria of the family Lactobacillaceae, which creates a protective environment through the production of lactic acid, hydrogen peroxide, and antimicrobial peptides [1]. These metabolites lower vaginal pH and inhibit the growth of microorganisms with pathogenic potential, thereby reducing the risk of infections, inflammation, and fertility-related complications [2], [3].

Lactobacillus crispatus, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Lactobacillus iners* are among the most prevalent species of the family Lactobacillaceae present in the

vaginal environment [4]. While *L. crispatus* is widely considered a marker of vaginal health due to its ability to maintain a stable acidic environment, *L. iners* is frequently detected in transitional or dysbiotic states, and its role remains under investigation [4], [5], [6], [7], [8]. The specific composition of vaginal microbiome, particularly lactobacilli, has been associated with a lower incidence of bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), human papillomavirus (HPV) infections, and adverse pregnancy outcomes [9], [10].

Vaginal dysbiosis, defined as a disruption of the microbial balance, is typically characterized by a reduction in the abundance of Lactobacillaceae and overgrowth of anaerobic bacteria, which increases the susceptibility to infections and inflammatory conditions. Such imbalances have also been linked to gynecological disorders, including endometriosis, where the vaginal microbiome often exhibits reduced levels of protective lactobacilli and higher prevalence of opportunistic pathogens, such as *Gardnerella vaginalis*, species *Streptococcus*, and *Escherichia coli* [11], [12], [13], [14], [15]. Emerging evidence suggests that microbial dysregulation may contribute to disease progression, modulate immune responses, and negatively impact fertility [16].

Microscopic fungi, particularly yeasts, represent another important component of the vaginal ecosystem. *Candida albicans* is the most common cause of VVC, affecting up to 75% of women at least once in their lifetime [17], [18]. Its pathogenicity is driven by virulence factors such as hyphal formation, biofilm development, and phenotypic switching, which enable adaptation to changing microenvironments and immune evasion [19]. *C. albicans* has gained clinical relevance due to its persistence and antifungal resistance profiles, resulting in treatment strategy complications [20]. Despite growing interest in the vaginal microbiome, longitudinal studies examining its stability throughout the menstrual cycle remain limited, and even fewer have integrated fungal community analysis [21], [22], [23], [24].

Interestingly, recent evidence indicates a relationship between the gut microbiota and the menstrual cycle [25]. Several human and animal studies have suggested that cyclical hormonal fluctuations can modulate gut bacteriome composition, with associations reported between specific bacterial taxa and menstrual disorders, premenstrual symptoms, or changes in gut permeability during different phases of the menstrual cycle [26], [27], [28], [29]. Consequently, many healthy women experience cyclical changes in gastrointestinal function and symptomatology across the menstrual cycle, reflecting the broader physiological impact of sex-hormone–microbiome interactions (Bharadwaj et al., 2015; Mohib et al., 2018). Understanding these dynamics is essential for elucidating the interplay between hormonal fluctuations, microbial resilience, and potential dysbiosis.

We hypothesize that the vaginal bacteriome remains relatively stable throughout the menstrual cycle, whereas the gut bacteriome may exhibit subtle cyclical fluctuations. Furthermore, we propose that integrating fungal community analysis will reveal additional complexity and potential clinical relevance within these ecosystems. In this study, we aimed to (i) assess vaginal and fecal bacteriome diversity and composition across different phases of the menstrual cycle, (ii) explore predicted metabolic profiles of

the vaginal and fecal bacteriomes, (iii) describe the presence of microscopic fungi in vaginal and fecal samples, and (iv) investigate correlations among relative abundances/presences of vaginal bacteria and yeasts with fecal bacteria.

Material and Methods

In this pilot prospective observational study, eight women (W1-W8) of reproductive age (25–45 years) who already had children were enrolled and their vaginal and fecal bacteriome and mycobiome were analyzed. Over the course of three consecutive menstrual cycles (C1-C3), vaginal and stool swabs were collected by self-sampling at defined time points corresponding to the three phases of the menstrual cycle: follicular (days 4–11 of the menstrual cycle), ovulatory (days 11–17), and luteal (days 18–24). The ovulatory phase was determined using over-the-counter ovulation tests. No samples were taken during the menstrual phase.

Informed consent

was obtained from all participants in accordance with the principles outlined in the Helsinki Declaration.

The study was approved by the Ethics Committee of CELSPAC, Czech Republic (Ref. No. CELSPAC/EK/1/2018, dated 12th March 2018).

Inclusion and exclusion criteria, and collection of exposure and behavioral variables

Both sexually active and non–sexually active women were eligible for inclusion in the study, with a regular menstrual cycle lasting > 21 days (the median length was 26 days) being the crucial inclusion criterion. Exclusion criteria were current pregnancy, lactation, the use of hormonal contraceptives, and treatment with antimicrobials within three months prior to study initiation.

Throughout the study, participants were monitored using questionnaires for additional exposure and behavioral factors potentially influencing their microbiomes, including intercurrent illnesses, use of antimicrobials, probiotic supplementation, sexual intercourse, and the use of lubricants or condoms during intercourse.

Sample collection and microbiological examination of microscopic fungi

Sampling was conducted at each of the nine time points; participants self-collected vaginal and stool samples using sterile flocked swabs (FLOQSwabs, COPAN, Italy).

At each sampling point, an additional vaginal swab was placed in a tube with the Amies medium (TransystemTM, COPAN, Italy) and transported at 4°C for microbiological examination of microscopic

fungi as described previously [30]. The presence of microscopic fungi was monitored using conventional culture techniques; subsequently, the isolated strains were identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker, Switzerland).

Preprocessing and DNA isolation

Immediately after collection, swabs were transferred into sterile cryogenic tubes and stored at − 80°C until further processing. DNA isolation from all collected samples was performed using the DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol optimized for microbial DNA isolation. Prior to DNA isolation, samples were homogenized in two 50-second cycles using 1.4 mm ceramic beads (VWR International, USA) and subjected to enzymatic pretreatment with lyticase (20 mg/mL) to enhance cell lysis efficiency. All procedures were conducted in a sterile handling box to minimize contamination. A bacterial internal standard for bacteriome analysis (ZymoBIOMICS Spike-In Control I, High Microbial Load; Zymo Research), as well as negative controls (NC), were also included in the isolation process. All DNA samples were eluted in a final volume of 50 µL and stored at − 20°C until further analysis.

Quantification of the total content of bacterial and fungal DNA by qPCRs

The total contents of bacterial and fungal DNA were quantified by qPCRs using universal primers targeting the gene for 16S rRNA (UNI 515/806) [Kurina I, et *al.*,2020] and the gene for ITS rRNA region (FungiQuanti_F/FungiQuanti_R) [Liu CM, et *al.*, 2012], respectively. Reactions were carried out with SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad, USA) on a LightCycler® 480 Instrument II (Roche, Switzerland). For quantification of the gene for 16S rRNA, the thermal profile comprised an initial denaturation at 98°C for 3 min, followed by 40 cycles of 98°C for 10 s and 55°C for 15 s, using each primer at a final concentration of 0.5 µM [31]. For quantification of the gene for ITS rRNA, the protocol was identical except for the annealing step that was performed at 60°C for 15 s, and each primer was used at 0.4 µM. Fluorescence data were processed using LightCycler® 480 Software v1.5.1.62 (Roche, Switzerland). Technical duplicates were evaluated for concordance, and Cp differences of ≤ 0.5 were considered acceptable. Values corresponding to fewer than 50 gene copies were regarded as below the limit of detection.

16S and ITS rRNA amplicon sequencing and bioinformatic analysis

DNA isolates (N = 144) and NCs (N = 12) were spiked with bacterial internal standard, and amplification of the hypervariable V3–V4 region of the gene for 16S rRNA was performed using IL primers (Supplementary Table S1) with Q5® High-Fidelity 2× Master Mix (New England BioLabs, USA). The PCR master mix (excluding template DNA) was decontaminated by the addition of 40 µM 8-methoxypsoralen (Sigma-Aldrich, USA).

For the general description of vaginal and fecal mycobiomes, individual DNA isolates from all nine samples collected from each participant were pooled into a single sample and spiked with an internal fungal standard. Eight pooled vaginal, eight pooled fecal samples, and two NCs were spiked with fungal internal standard, and amplification of the ITS2 region was performed using ITC2_C primers (Supplementary Table S2) with the same polymerase and process as described above.

For both bacteriome and mycobiome analysis, thermal cycling was carried out as follows: initial denaturation at 98°C for 30 s; 30 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s; a final extension at 72°C for 2 min; and a hold at 4°C. Following amplification, PCR products were purified using SPRIselect beads (Beckman Coulter, USA), quantified on a Synergy HTX fluorimeter (BioTek, USA), and combined in equimolar proportions. Indexed libraries were generated via secondary PCR using Nextera XT indices (Illumina, USA). Sequencing was conducted on a MiSeq system with the MiSeq Reagent Kit v3, in accordance with the manufacturer's instructions (Illumina, USA).

Both datasets were processed separately using nf-core/ampliseq version 2.11.0 [32] of the nf-core collection of workflows [33], utilizing reproducible software environments from the Bioconda [34] and Biocontainers [35] projects.

Data quality was evaluated with FastQC and summarized with MultiQC [36]. Sequences were processed with DADA2 tool, an open-source software package that denoises and removes sequencing errors from Illumina amplicon sequence data [37]. Processing steps include elimination of PhiX contamination, trimming reads (before median quality drops below 25 and at least 85% of reads are retained; forward reads at 300 bp and reverse reads at 253 bp, reads shorter than this were discarded), discarding reads with more than 3 expected errors, correcting errors (denoising), merging read pairs, and removing PCR chimeras.

Taxonomic classification was performed in DADA2 using the naïve Bayesian classifier [38] with the SILVA 138.1 prokaryotic SSU database [39] for 16S rRNA amplicon data and the UNITE database for ITS data [40], [41]. Additionally, the BLAST algorithm [42] was used to identify the species, and all taxa with the maximum identity and minimum e-value were selected for each ASV.

ASV sequences, abundance table, and DADA2 taxonomic assignments were loaded into QIIME2 [43]. Within QIIME2, the final microbial community data were processed into bacteriome and mycobiome profiles separately.

PICRUSt2 [44] was used to predict hypothetical abundances of KEGG orthologs in each vaginal and fecal sample of each woman and summarized into higher functional processes. Normalization of gene copy numbers that were used for determining bacterial functional profiles was performed using the MUSICC algorithm [45]. Additionally, the Predicted Relative Metabolomic Turnover (PRMT) [46] approach was applied to the normalized data to generate community-wide metabolic potential scores (PRMT scores) for each metabolite annotated in the KEGG database. To investigate the temporal dynamics of bacterial metabolic potential, we performed single-sample enrichment analysis on metabolites grouped into KEGG

pathways across collected samples. Only pathways covered by at least 25% of their constituent metabolites in our dataset were included in the final presentation.

Statistical analysis

Only samples and NCs with a sequencing depth of at least 2000 reads (including the specific internal standard) were retained for downstream analysis. While reads mapped to internal standards were used to assess sequencing performance and subsequently removed from the dataset, NCs were retained throughout the analysis to monitor for potential contamination and are displayed in relevant figures for comparison.

Alpha diversity indices, including the Shannon index and the number of distinct amplicon sequence variants (ASVs), were computed using the vegan package (v.2.6–6.1). Differences between groups were tested using the Wilcoxon test, a paired non-parametric test, for testing the differences between vaginal and fecal samples. Friedman's ANOVA test was used to test the differences between phases of the menstrual cycle. Evaluation of the stability of individual variables in each phase within a given matrix was performed to find out if measurements across the months could be aggregated. If no significant difference was detected, the median of the three measurements was used for each phase of the menstrual cycle.

Principal component analysis (PCA) was performed on CLR-transformed abundance data using the PCAtools (v.2.20.0) package to visualize differences in microbial community composition. To statistically confirm the observed differences between sample types, a PERMANOVA analysis was conducted using Euclidean distances, as appropriate for CLR-transformed data.

In the correlation analysis, Spearman's correlation coefficient was used to assess associations between microbial taxa. Spearman correlation was applied to the relative abundances of the 25 most abundant bacterial genera detected in fecal samples, as well as five selected bacterial species and one bacterial genus present in vaginal samples. In addition, information on the presence or absence of yeasts in vaginal samples at each sampling time point (binary variable obtained by conventional microbiological methods) was incorporated into the analysis. To evaluate associations between yeasts occurrence and bacterial relative abundances, the rank-biserial correlation coefficient was calculated, and statistical significance was assessed using the Wilcoxon rank-sum test. Correlations with an absolute magnitude ≥ 0.6 were considered strong. The resulting correlation network was visualized using the igraph package (v.2.2.2).

P-values ≤ 0.05 were considered statistically significant. All resulting *p*-values were adjusted for multiple hypotheses testing using the Benjamini–Hochberg procedure. All analyses were conducted using R version 4.5.1 (2025-06-13).

Results

In the context of 16S and ITS rRNA amplicon sequencing, all vaginal (N = 72) and fecal samples (N = 72) passed the quality control process. Importantly, although the genera *Flavobacterium*, *Pseudoxanthomonas*, and *Chryseobacterium*, as well as members of the class *Parcubacteria*, were detected in one of the twelve NCs, none of these genera appeared in any vaginal or fecal sample included in the study.

Longitudinal stability of bacteriome profiles within individual phases of the menstrual cycle

To assess the consistency and potential month-to-month variation of the vaginal and fecal bacteriome within specific phases of the menstrual cycle, bacteriome diversity represented by the Shannon index (and, for the vaginal samples, the abundance of the family Lactobacillaceae) across three consecutive cycles was analyzed. In the vaginal bacteriome, no statistically significant differences were observed between months within any of the phases, indicating an overall temporal stability of the dominance of the genus *Lactobacillus*. Complementing these analyses, Table S3 summarizes the results of statistical testing for the top 25 most abundant taxa in both vaginal and fecal samples, comparing their relative abundances within each phase across the three consecutive menstrual cycles.

This allowed us to aggregate data, where the median values of studied parameters were calculated from three measurements for each woman and menstrual cycle phase. This strategy allowed robust comparisons across phases while minimizing the influence of short-term fluctuations.

Bacteriome alpha diversity across menstrual cycle phases

Log-scaled total content of bacterial DNA and Shannon diversity indices from 16S rRNA amplicon sequencing across follicular, ovulatory, and luteal phases in both vaginal and fecal samples were compared to assess the changes throughout the menstrual cycle (Fig. 1).

No statistically significant differences in the total content of bacterial DNA were observed between phases of the menstrual cycle in either vaginal or fecal samples (Fig. 1A,B). No significant differences in Shannon diversity were detected between phases in vaginal swabs (Fig. 1C), while phase-dependent change in fecal bacteriome diversity was observed (Fig. 1D). Shannon diversity indices showed a gradual increase from the follicular through the ovulatory phase, reaching the highest values in the luteal phase; this trend resulted in a statistically significant difference between the follicular and luteal phases (p = 0.028).

Bacteriome composition across menstrual cycle phases

In vaginal samples, PCA revealed substantial overlap between follicular, ovulatory, and luteal phases (Fig. 2A), with no clear clustering by phase of the menstrual cycle. This lack of separation was confirmed by PERMANOVA analysis, which showed no significant effect of the phase on vaginal bacteriome composition (R^2 = 0.016, F = 0.554, p = 0.826).

Similarly, PCA of fecal samples showed overlapping distributions of samples from all phases of the menstrual cycle (Fig. 2B, PC1 = 13.2%, PC2 = 10.9% of explained variance), indicating high inter-individual variability but no phase-specific structuring of the fecal bacteriome. Consistently, PERMANOVA analysis revealed no significant association between the phase and fecal bacteriome composition ($R^2 = 0.02$, $F = 0.694$, $p = 0.914$).

Samples from the follicular, ovulatory, and luteal phases were distributed throughout the vaginal and fecal clusters without forming any phase-specific subgroups. Within each matrix type, points tended to cluster according to individual women rather than the phase of the menstrual cycle (Figure S1).

To further explore the bacteriome composition across sample types and phases of the menstrual cycle, the 25 most abundant bacterial genera across the entire dataset were selected (Figure S1). This selection predominantly included genera typical of the gut bacteriome, such as *Faecalibacterium*, *Segatella* (formerly *Prevotella*), *Blautia*, *Bacteroides*, and *Bifidobacterium*, which were highly abundant in fecal samples. *Lactobacillus* and *Gardnerella*, which were present only in vaginal samples, were also among the top genera. Genera *Streptococcus*, *Clostridium, Dialister, Bifidobacterium*, and some others (such as *Escherichia*) were found in both vaginal and fecal samples.

Taxonomic structure and metabolic functional landscape of the vaginal bacteriome

Upon closer examination of vaginal swab samples at the species level using BLAST, we observed that in most samples, *L. crispatus* was the dominant species within the family Lactobacillaceae (Fig. 3). One woman (W5), however, exhibited *L. iners* as the predominant species, and in another one (W1), in whose samples a high presence of *G. vaginalis* was observed, the predominant lactobacillus was identified as *L. jensenii*.

The heatmap in Fig. 3 also presents the enrichment scores based on the predicted metabolic potential of selected bacterial metabolic pathways in vaginal samples for individual women and phases of three menstrual cycles. Notably, pathways associated with environmental adaptation and pathogenicity (such as the *two-component system* and *metabolic pathways, carbon metabolism*, and *glycerolipid metabolism*) were markedly enriched in samples with reduced relative abundance of *L. crispatus* and increased relative abundances of *G. vaginalis*.

Interestingly, one woman (W5) displayed a distinct metabolic profile despite the fact that at the genus level (on which the PRMT calculations are based), her bacteriome composition was comparable with women in whom *L. crispatus* dominated in vaginal swabs (Fig. 3). However, BLAST-based species identification in this woman revealed the dominance of *L. iners*, a species frequently associated with transitional or dysbiotic states (Fig. 3). This may suggest that the predicted metabolic profile in this woman was influenced by the genera in the “others” category, which might have been different from those found in women with predominance of *L. crispatus* in their vaginal bacteriome. A similar pattern was observed in another woman (W4), whose samples from time points dominated by *L. iners* exhibited

a different metabolic potential than those from periods with *L. crispatus* dominance. The similarities between periods dominated by *L. iners* in women W4 and W5 are striking, showing clear differences from the metabolic profiles of *L. crispatus*-dominated samples across all women (Fig. 3). Notably, another participant (W1), characterized by a high abundance of *G. vaginalis* and dominance of *L. jensenii*, also showed a distinct metabolic potential in vaginal samples.

In addition, the selected exposure and behavioral factors, such as recent sexual activity, use of lubricants or condoms, antimicrobial treatment, probiotic intake, and reported illness, were recorded. A reduction in the relative abundance of *G. vaginalis* in W1 following the supplementation with oral probiotics and an increase in the relative abundance of the same bacteria following infections in W7 were observed (see Fig. 3). Still, the low number of women in this pilot study does not allow us to perform any formal statistical analysis.

Bacteriome composition and predicted metabolic potential of fecal samples

Figure 4 displays a combined heatmap of the 25 most abundant bacterial genera in fecal samples alongside the predicted enrichment of key bacterial metabolic pathways across women and phases of three menstrual cycles.

Analysis of fecal samples revealed a bacterial community dominated by genera commonly associated with a healthy gut microbiome, including *Faecalibacterium, Segatella*, *Blautia*, *Bacteroides*, *Dialister*, *Bifidobacterium*, and members of the families Lachnospiraceae and Ruminococcaceae (Fig. 4). Supplementary Table S4 summarizes the results of statistical testing for the top 25 most abundant taxa on the aggregated data (the approach mentioned above).

The predicted metabolic potential of the fecal bacteriota was also consistent across time points and reflected a distinct compositional pattern of each participant (Fig. 4). Pathways belonging to *taurine and hypotaurine metabolism*, *nitrogen metabolism*, and *butanoate metabolism* were among the most enriched pathways across the participants. On the other hand, *tryptophan metabolism* and *benzoate degradation* showed consistently lower enrichment scores.

Mycobiome profiles of vaginal and fecal samples

In total, presence of yeasts was detected by conventional microbiological culture with subsequent identification by mass spectrometry in fourteen vaginal samples collected from five women (Fig. 3). *C. albicans* was found in four cases and *Nakaseomyces glabratus* (formerly *Candida glabrata*) in one woman. The microbiological examination revealed no more than one microscopic fungal species per woman across the entire study period (nine time points). This allowed us to pool vaginal samples from individual women for mycobiome analyses, and the resulting profiles from each woman was compared with the corresponding microbiological findings. Pooling was also applied to fecal samples.

No statistically significant difference between vaginal and fecal samples was observed in the total content of fungal DNA ($p = 0.19$). However, Shannon diversity indices differed significantly ($p = 0.008$). In agreement with results of the conventional microbiological examination, *C. albicans* was identified by ITS rRNA amplicon sequencing in vaginal samples from four women (W2, W4, W6, W7) and *Nakaseomyces glabratus* in samples from one woman (W5). Moreover, *Pichia kudriavzevii* (formerly *Candida krusei*) not revealed by conventional methods was detected by ITS rRNA amplicon sequencing (74 reads) in one woman (W3). Fecal samples showed a broader fungal spectrum, including environmental and food-associated genera such as *Saccharomyces uvarum*, *Penicillium* spp., and *Maudiozyma barnettii*.

Correlations among relative abundances/presence of vaginal bacteria and yeasts with fecal bacteria

Presence of vaginal yeasts positively correlated with relative abundances of genus *Streprococcus* in fecal samples ($r = 0.76$). In vaginal samples, the presence of yeasts positively correlated with the relative abundance of *L. iners* ($r = 0.61$) and negatively correlated with the relative abundance of *L. crispatus* ($r = -0.69$). While *the* relative abundance of *L. jensenii* in vaginal samples correlated positively with the relative abundances of the genus *Bifidobacterium* ($r = -0.63$), a negative correlation with this lactobacillus was observed with genus *Dialister* ($r = 0.83$). Additional strong correlations ($r \geq 0.6$) among fecal bacterial genera are also presented in Fig. 6.

Discussion

Our longitudinal analysis of vaginal and fecal microbiomes across three consecutive menstrual cycles provides a comprehensive view of microbial dynamics in relation to matrix type and phase of the menstrual cycle. Importantly, this study extends current knowledge by incorporating fungal community profiling through ITS amplicon sequencing, offering a more holistic perspective on microbial ecosystems in healthy women.

Vaginal and fecal bacteriome diversity and composition across phases of the menstrual cycle

The findings confirm fundamental differences between the vaginal and gut microbiomes in terms of total bacterial load, bacteriome diversity, and taxonomic composition [21], [24]. Fecal samples exhibited significantly higher microbial richness and diversity, reflecting the complexity of the gut environment [47], whereas vaginal samples were dominated by *Lactobacillus*, consistent with the typical profile of a healthy vaginal microbiome [4], [48], [49], [50], [51], [52]. As expected, the two sample matrices separated well in PCA, while differences among phases of the menstrual cycle were minor. Significant changes in alpha diversity between follicular and luteal phases were only detected in fecal samples. This pattern supports previous findings that anatomical site and individual factors are dominant drivers of microbiome profile, whereas hormonal fluctuations exert only minor influence [10].

While the vaginal microbiome remained relatively stable across phases of the menstrual cycle, both in terms of alpha diversity represented by Shannon index and Lactobacillaceae dominance, fecal samples displayed modest but statistically significant variation in alpha diversity across phases, with the highest diversity observed during the luteal phase [53]. This pattern suggests potential hormonal modulation of gut microbial richness, possibly mediated by estrogen and progesterone-driven changes in gut motility and immune function [26], [27], [28], [29]. These findings align with previous reports indicating that the gut microbiome is predominantly shaped by long-term host-specific factors [54]. Although the effect size was small, these findings warrant further investigation into the interplay between hormonal cycles and gut microbiome dynamics. On the other hand, the results suggest that, where the vaginal microbiome is concerned, the phase of the menstrual cycle is not a major driver of compositional changes in healthy women. These results align with previous studies reporting resilience of the vaginal microbiome across phases of the menstrual cycle studied in this paper [52], [55].

Our results indicate that the taxonomic composition and functional potential of fecal microbiome of healthy women is stable across the menstrual cycle. This sugests that inter-individual variability is the dominant driver of fecal bacteriome composition, overshadowing any potential influence of menstrual cycle–related hormonal shifts. This is consistent with existing literature demonstrating that gut microbial communities are generally stable in healthy adults and strongly modulated by genetics, diet, immune, and lifestyle. The stability observed in our dataset thus supports the view that short-term endocrine oscillations alone are unlikely to drive robust shifts in gut bacterial communities under healthy conditions.

The taxonomic composition of fecal samples in our cohort, which was dominated by *Faecalibacterium*, *Blautia*, *Bacteroides*, *Bifidobacterium*, and members of the Lachnospiraceae and Ruminococcaceae families, corresponds to a typical gut microbiome. This composition remained largely consistent across all menstrual cycle phases. Predicted metabolic pathway profiles showed strong individual-specific signatures with minimal temporal variation.

Predicted metabolic profiles of the vaginal and fecal bacteriomes

Our analysis of enrichment scores based on the predicted metabolic potential on 16S rRNA sequencing data of selected microbial metabolic pathways provides important insights into the functional dimension of vaginal dysbiosis. The enrichment of adaptive systems, such as the *two-component system* in samples with increased abundance of *L. iners* (at the expense of *L. crispatus*), suggests that potential pathogens may exploit these mechanisms to persist and thrive in the vaginal environment, possibly utilizing these systems to adapt to changing conditions within the vaginal environment to enhance their survival and virulence [56]. While *L. crispatus* is widely considered a marker of vaginal health due to its ability to maintain a stable environment and inhibition of *E. coli* and *Candida* spp. [57], [58], *L. iners* is frequently associated with transitional or dysbiotic states, and its role remains under investigation [4],

[59], [60]. These findings suggest that metabolic potential might serve as an early indicator of community instability even when taxonomic composition appears stable [5], [49], [61], [62], [63].

In the woman W1, who exhibited elevated levels of *G. vaginalis*, species-level analysis revealed *L. jensenii* as the dominant *Lactobacillus* species rather than *L. crispatus*. While *L. jensenii* is generally considered a health-associated species, its protective capacity may differ from that of *L. crispatus*. Previous studies suggest that *L. jensenii* produces lower amounts of lactic acid and hydrogen peroxide compared to *L. crispatus*, potentially resulting in a less acidic vaginal environment and reduced antimicrobial activity [57], [64], [65]. This could facilitate the persistence of opportunistic pathogens such as *G. vaginalis*, contributing to a state of suboptimal vaginal health.

Microscopic fungi in vaginal and fecal samples

Our study extends current knowledge by incorporating fungal community analysis through ITS sequencing. Vaginal swabs were characterized by *C. albicans*, *N. glabratus*, and *P. kudriavzevii*, while fecal samples exhibited a broader fungal spectrum, including environmental and food-associated taxa such as *S. uvarum* and *Penicillium* spp. *C. albicans* remains the leading cause of vulvovaginal candidiasis, with pathogenicity linked to hyphal formation, biofilm development, and phenotypic switching [17], [18], [66].

The results of the analysis of the vaginal microbiome in participant W5 are particularly interesting. Although the taxonomic analysis at the genus level suggested a typical *Lactobacillus*-dominated profile, species-level identification revealed *L. iners* as the predominant species, accompanied by markedly higher fungal load of *N. glabratus* on the mycobial analysis and a distinct metabolic signature predicted from 16S rRNA sequencing data.

N. glabratus is an opportunistic yeast that has gained clinical importance due to its ability to persist in the vaginal environment and its intrinsic tolerance to azole antifungals. Unlike *C. albicans*, *N. glabratus* does not form true hyphae, which limits its invasive potential but enhances its capacity for biofilm formation and survival under stress conditions [67], [68]. Its growth is favored in environments with reduced acidity, which can occur during dysbiosis or following shifts in the *Lactobacillus* species composition (note that *L. crispatus* is known for its ability to reduce pH). The dominance of *L. iners* in this sample highlights the limitations of the genus-level analysis for clinical interpretation. The predicted enrichment of core metabolic pathways and pyruvate metabolism supports its association with transitional or dysbiotic states. If oxidative stress is present (e.g., inflammation or immune response), *L. iners* may redirect pyruvate toward acetate instead of lactate, creating a less acidic environment that can facilitate pathogen colonization. *N. glabratus* is a facultative anaerobic yeast and grows better in conditions with higher pH, that is, less acidic. The higher fungal load in both quantification and sequencing depth compared to other participants indicates a persistent infection with *N. glabratus* throughout the study period. Interestingly, the same metabolic patterns were found in another woman (W4) who showed the predominance of both *L. crispatus* and *L. iners* at different periods. The PRMT-based metabolic profiles (determined at the genus-level) in the periods dominated by these two bacteria

were still distinct, with those dominated by *L. iners* being markedly similar to those detected in woman W5.

Correlations among relative abundances/presence of vaginal bacteria and yeasts with fecal bacteria

The correlations observed between vaginal yeasts and both vaginal and fecal bacterial communities suggest a tightly interconnected relationship that may be facilitated by the potential transfer of bacteria, yeasts, and their metabolites across the rectal tract. This also suggests a bidirectional relationship between the mycobiome and bacteriome, spanning both the vaginal and gut environments.

The presence of vaginal yeasts was positively associated with the relative abundance of *Streptococcus* in fecal samples, indicating the possible existence of broader dysbiotic patterns along the gut–urogenital tract. Within the vaginal environment, yeast presence correlated positively with *L. iners* and negatively with *L. crispatus*. This pattern is consistent with the characteristics of these species, where *L. iners* often dominate in transitional or less stable vaginal states, whereas *L. crispatus* is typically linked to a stable, protective, and anti-yeast vaginal microbiome. The associations involving *L. jensenii* positively correlated with *Bifidobacterium* and negatively with *Dialister* may indicate broader interactions between anti-inflammatory and pro-inflammatory bacterial taxa.

Overall, these findings highlight the potential systemic nature of bacteria–yeast interactions and emphasize the importance of studying vaginal and gut microbial communities as interconnected ecosystems rather than isolated compartments.

Limitations and strengths

One of the limitations of our study was that the pooling of samples for mycobiome analysis made it impossible to track dynamics during the menstrual cycle; however, the results of microbiological examination showed that the composition of vaginal samples was very similar throughout the observation period.

Our pilot study was also limited by its small sample size. However, the longitudinal design, collection of two types of matrices, and analysis of both the bacteriome and mycobiome have enabled us to expand our knowledge of the female microbiome during the menstrual cycle. The sample size was limiting especially for the analysis of the microbiome in relation to exposure and behavioral data. Our analysis of exposure and behavioral variables revealed that most factors, including recent sexual activity, use of lubricants or condoms, and probiotic intake, had minimal impact on vaginal microbial diversity and Lactobacillaceae dominance. In our study, reduced relative abundance of family Lactobacillaceae was associated with an increased presence of potential pathogens linked to BV, such as *Gardnerella vaginalis* and the genus *Streptococcus* [11], [12].

Conclusions

Our findings confirm that anatomical site and interindividual variation are the primary determinants of microbiome composition in reproductively healthy women, with phases of menstrual cycle playing only a minor role. The stability of the vaginal microbiome across phases of menstrual cycle reflects its resilience and supports its potential protective role against dysbiosis and infection. The observed metabolic differences between *L. crispatus, L. iners, L. jensenii*, and *G. vaginalis*-dominated communities highlight the importance of functional profiling for early detection of instability. Finally, the inclusion of mycobiome analysis reveals an additional layer of complexity in host-microbe interactions, emphasizing the need to consider fungal communities in studies of women's health.

Conversely, the observed cyclical variation in fecal bacteriome diversity may influence metabolic and immune processes, suggesting a need for further research into systemic effects of hormonal cycles. Importantly, our correlation analyses highlight a deeper level of interconnection between body sites by revealing that the presence of vaginal yeasts is linked to shifts in both vaginal and fecal bacterial communities. These patterns, together with the possibility of microbial and metabolite transfer across the rectal tract, underscore a dynamic interplay between the bacteria and microscopic fungi that extends beyond local microbial environments. Although this is only a pilot study the results of which need to be verified on a larger sample, the results indicate that when investigating female fecal microbiome, the phase of menstrual cycle should be considered.

Declarations

Supplementary materials

Figure S1. Heatmap of the 25 most abundant bacterial genera across all samples. The heatmap displays the log-transformed relative abundances of the top 25 bacterial genera across all vaginal and stool samples collected over three menstrual cycles. Squares highlighted by framing indicate taxa reaching a relative abundance greater than 20% in the corresponding sample. Grey squares indicate taxa that were not detected in the corresponding sample. W1, woman 1; C1, menstrual cycle 1.

Table S1. Illumina primer sequences used in this study.

Table S2. ITC2_C primers used in this study.

Table S3. Results of statistical testing for the top 25 most abundant taxa in both vaginal and fecal samples, comparing their relative abundances within each phase across the three consecutive menstrual cycles.

Table S4. Results of statistical testing for the top 25 most abundant taxa on the median value of the three measurements for each phase of the menstrual cycle.

Data availability statement

Sequencing data were uploaded to the European Nucleotide Archive under accession number PRJEB108971.

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Author contribution

BZ: Bioinformatic Analysis, Statistical Analysis, Visualization, Writing – Original Draft.

JH: Writing – Revision.

OT: Investigation.

PB: Methodology.

PH: Methodology.

LM: Writing – Revision.

MJ: Writing – Revision.

FR: Methodology, Investigation, Writing – Revision.

PBL: Conceptualization, Methodology, Supervision, Writing – Original Draft, Funding.

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Use of AI statement

The authors declare that they have used generative artificial intelligence, specifically Microsoft Copilot only to improve R scripts for handling data formatting and generation of figures.

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Figures

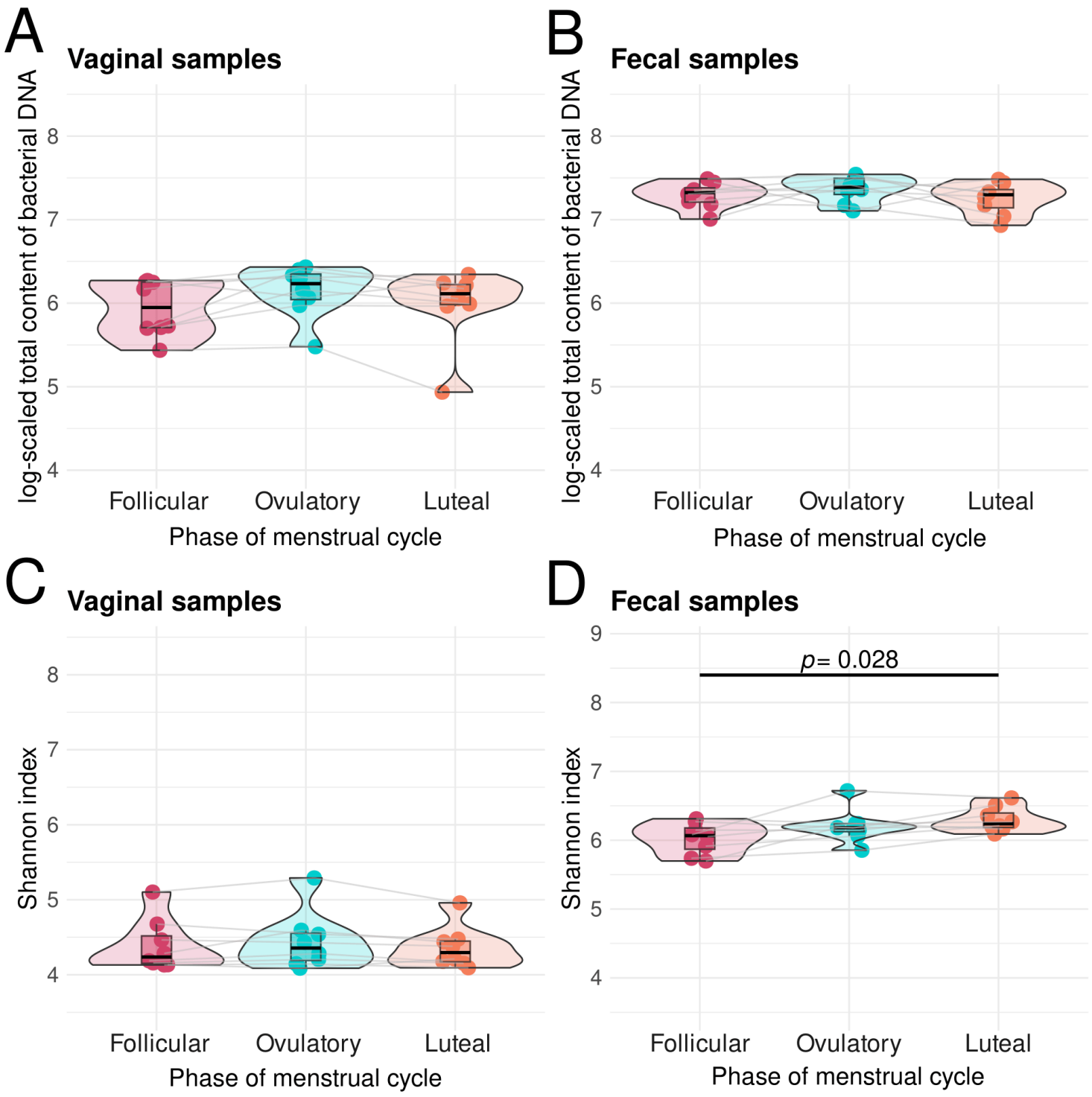

Figure 1

The total content of bacterial DNA and bacteriome alpha diversity in vaginal (A and B, respectively) and fecal samples (C and D, respectively) from eight women across three phases of the menstrual cycle. Data shown as medians derived from three independent measurements. Paired samples are connected by a grey line.

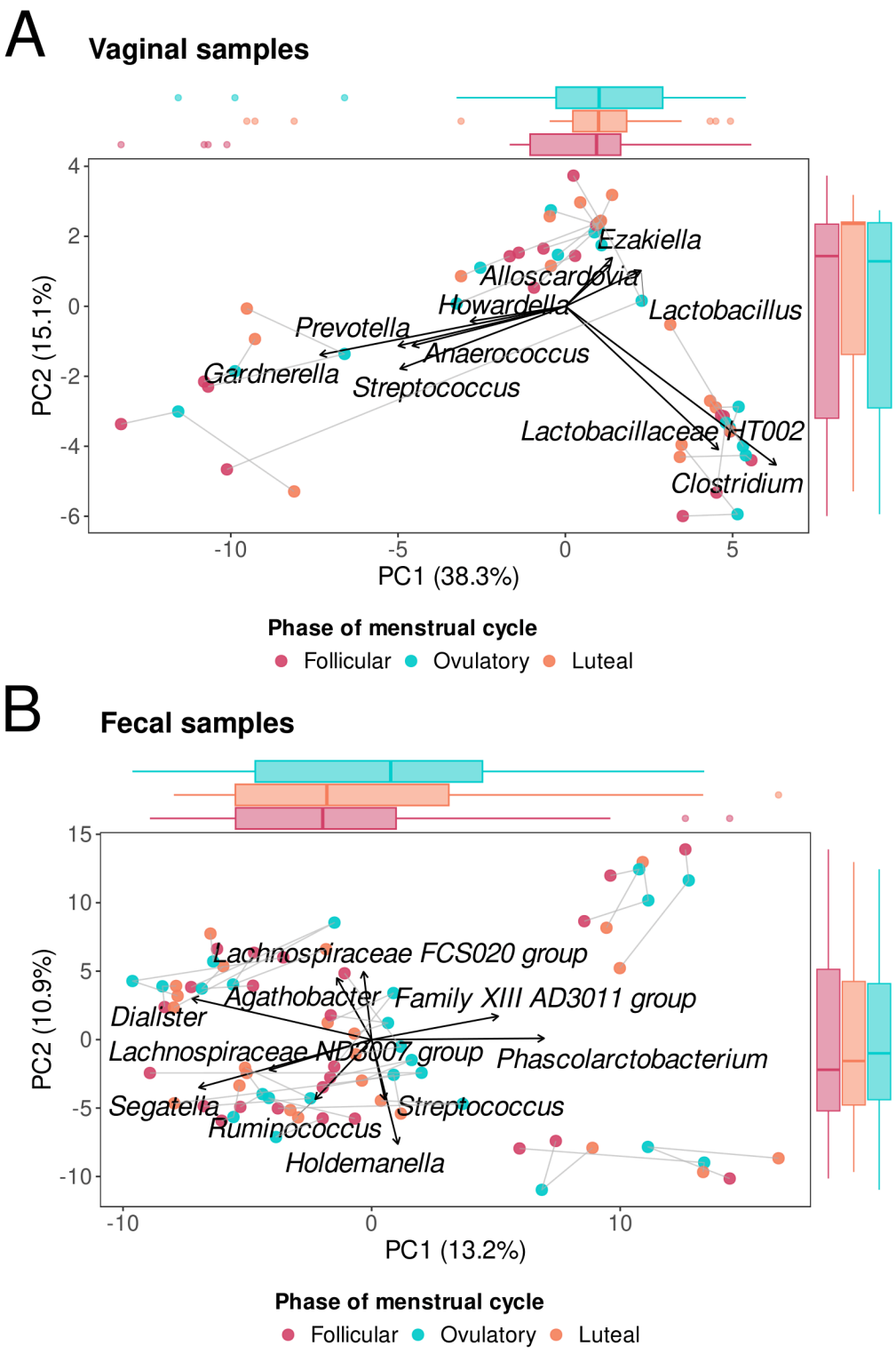

Figure 2

Principal component analysis (PCA) of bacteriome composition in vaginal (A) and fecal samples (B) from eight women across three phases of three menstrual cycles. Paired samples are connected by a grey line.

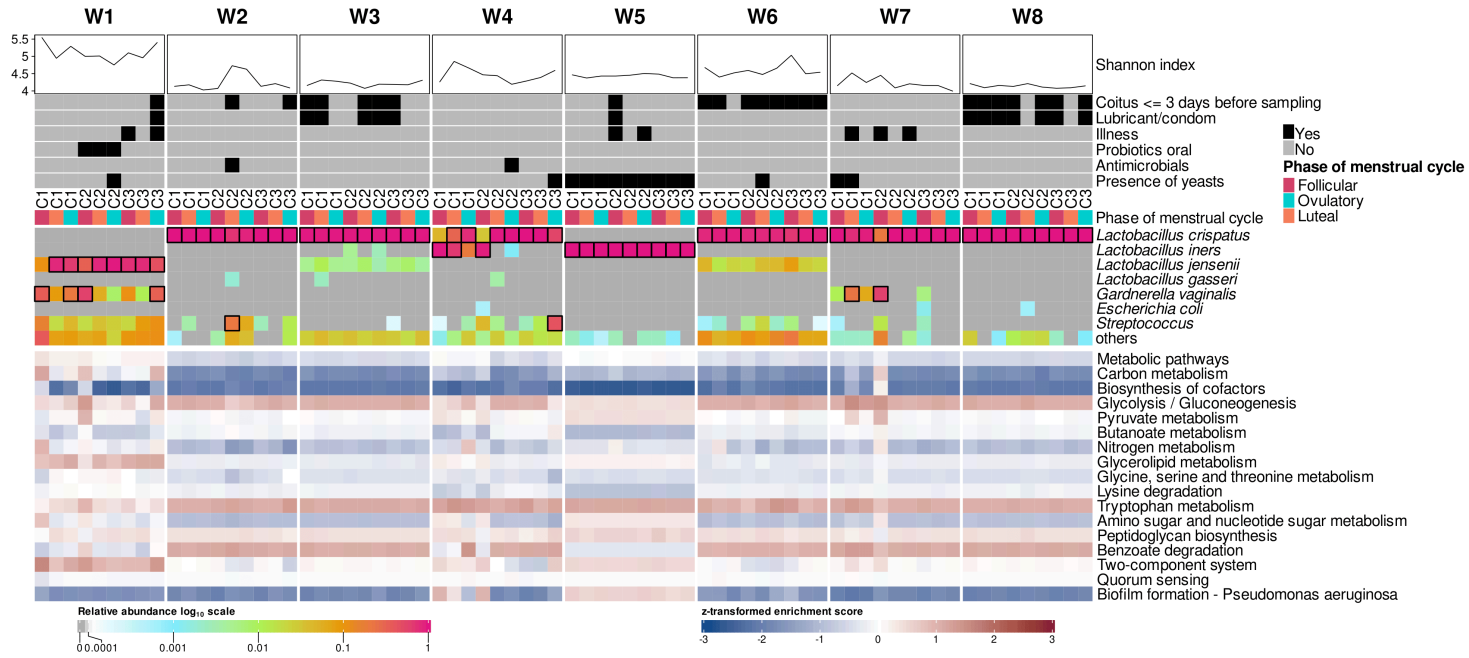

Figure 3

Shannon indices, relative abundances of selected bacterial species, and predicted enrichment of bacterial metabolic pathways in the vaginal samples of eight women across three phases of three menstrual cycles. Squares highlighted by framing indicate taxa reaching a relative abundance greater than 20 % in the corresponding sample. Grey squares indicate taxa that were not detected in the corresponding sample. The heatmap of metabolic pathways displays z-score-transformed enrichment scores based on the predicted metabolic potential from 16S rRNA amplicon sequencing data for selected metabolic pathways. The presence of vaginal yeasts, as well as exposure and behavioral factors potentially influencing the vaginal microbiome, are also shown. W1, woman 1; C1, menstrual cycle 1.

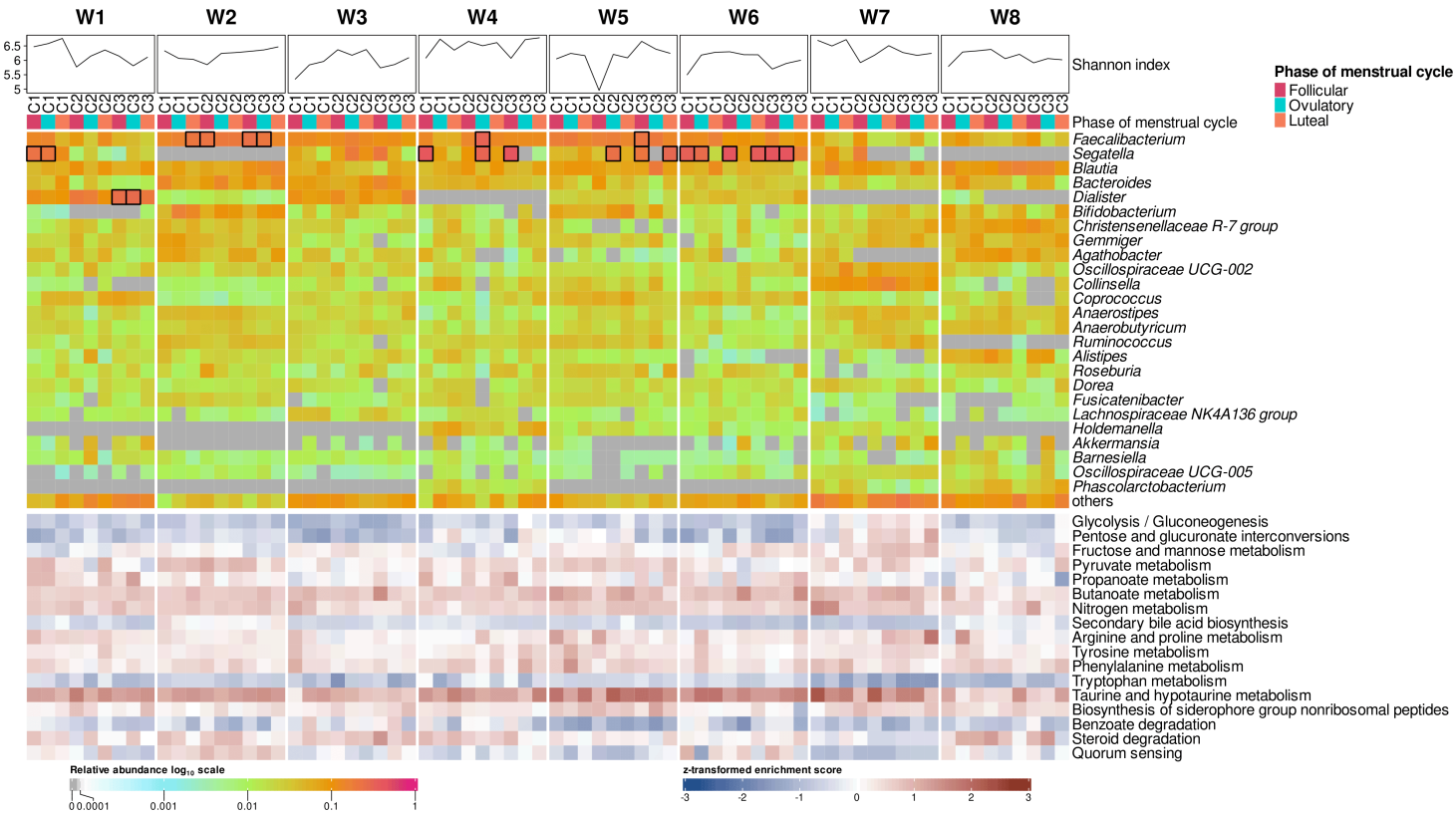

Figure 4

Shannon indices, relative abundances of the 25 most abundant genera, and predicted enrichment of bacterial metabolic pathways in fecal samples from eight women across three phases of three menstrual cycles. Squares highlighted by framing indicate taxa reaching a relative abundance greater than 20% in the corresponding sample. Grey squares indicate taxa that were not detected in the corresponding sample. The heatmap of metabolic pathways displays z-score-transformed enrichment scores based on the predicted metabolic potential from 16S rRNA amplicon sequencing data for selected metabolic pathways. W1, woman 1; C1, menstrual cycle 1.

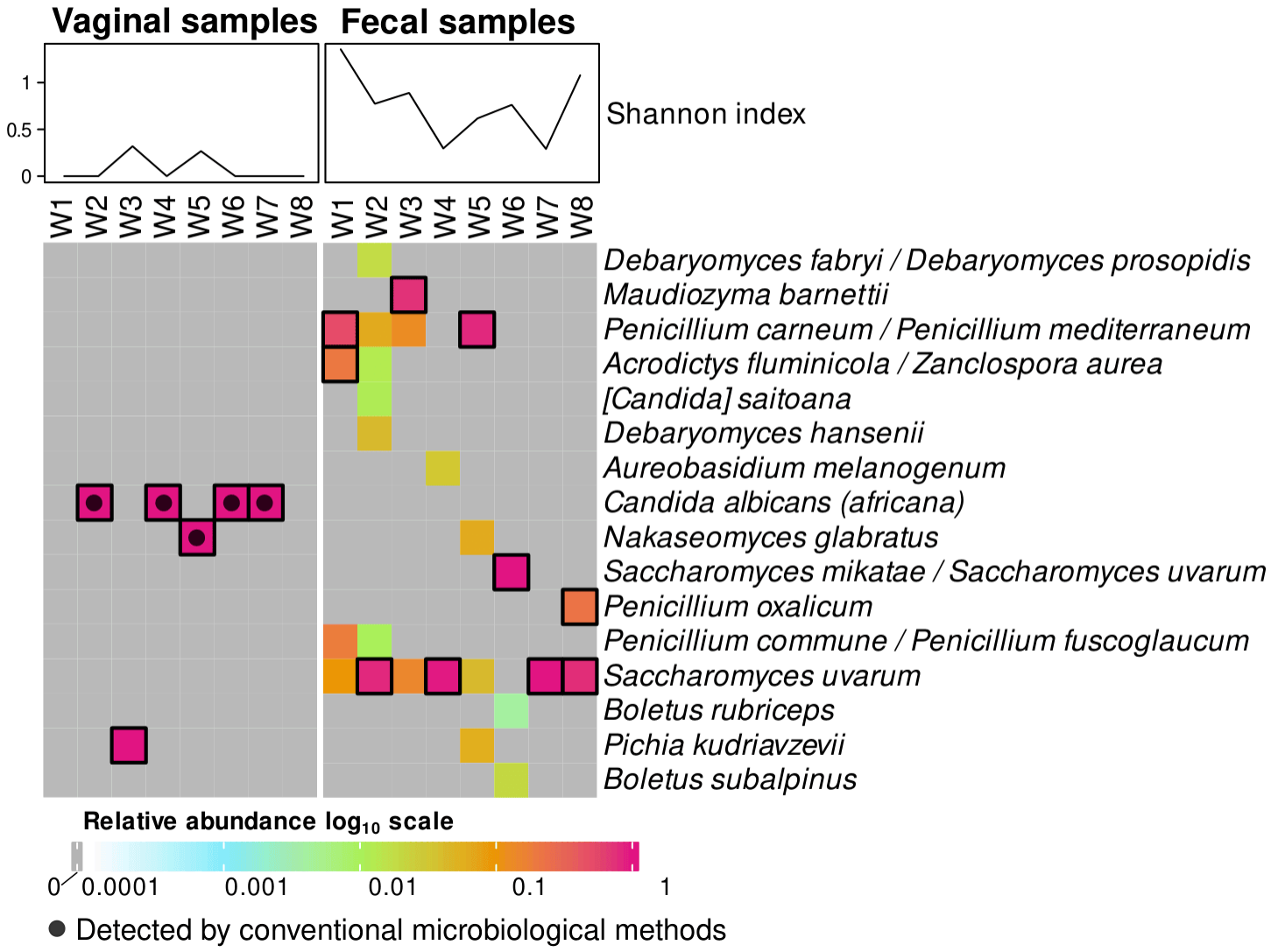

Figure 5

Mycobiome diversity and composition of vaginal and fecal samples from eight women. The heatmap displays the relative abundance of fungal taxa detected across pooled samples from eight participants, representing nine time points in three menstrual cycles per individual. Relative abundances are shown on a $\log_{10}$ scale. Taxa in vaginal samples confirmed by conventional microbiological methods are marked accordingly. Squares highlighted by framing indicate taxa reaching a relative abundance greater than 20% in the corresponding sample. Grey squares indicate taxa that were not detected in the corresponding sample.

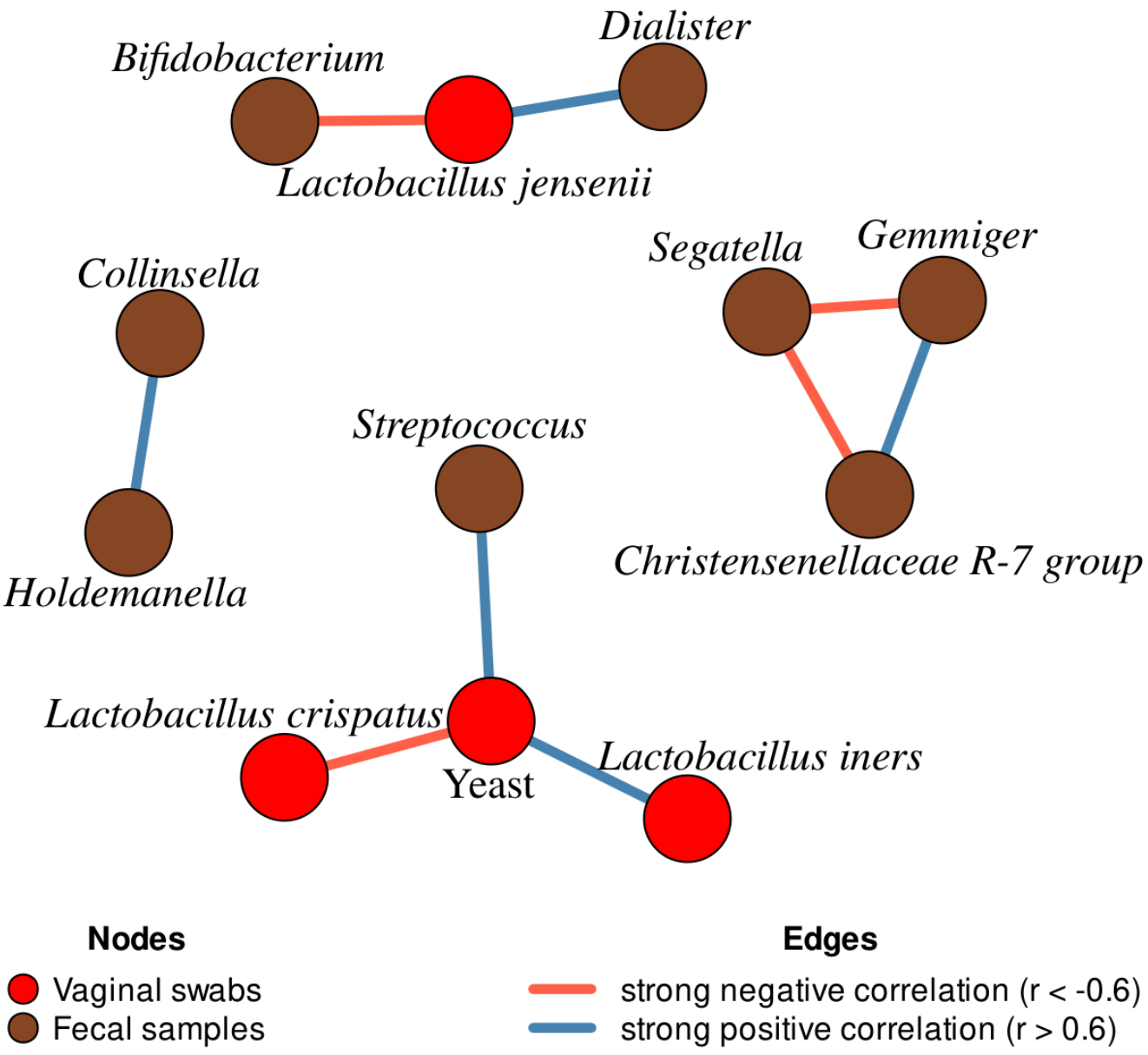

Figure 6

Spearman correlation among relative abundances/presence of vaginal bacteria and yeasts with fecal bacteria. Note that the distances between individual bacteria correspond to the calculated correlations, even where no connecting lines are shown (worse correlation corresponds to longer distance; only the strongest correlations with absolute value of >0.6 are shown)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.xlsx

- TableS3.xlsx
- TableS4.xlsx
- FigureS1.pdf