OXFORD

human reproduction

Human Reproduction, 2025, **00(0)**, 1–11
https://doi.org/10.1093/humrep/deae270
Original Article

Reproductive endocrinology

The reproductive tract microbiome in women with polycystic ovary syndrome and across different menstrual cycle phases

S. Lee [1,2,†], O. Aasmets[3,†], R.K. Arffman [1], J. Laru [1], H.R. Rossi[1], A. Salumets[2,3,4,5], T.T. Piltonen [1], and E. Org [3,*]

[1]Department of Obstetrics and Gynecology, Research Unit of Clinical Medicine, Medical Research Center, Oulu University Hospital, University of Oulu, Oulu, Finland
[2]Department of Obstetrics and Gynaecology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia
[3]Institute of Genomics, Estonian Genome Centre, University of Tartu, Tartu, Estonia
[4]Division of Obstetrics and Gynaecology, Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institute and Karolinska University Hospital, Stockholm, Sweden
[5]Competence Centre on Health Technologies, Tartu, Estonia

*Correspondence address. Institute of Genomics, Estonian Genome Centre, University of Tartu, Tartu 51010, Estonia. E-mail: elin.org@ut.ee https://orcid.org/0000-0001-8688-9717

[†]Joint first authors.

ABSTRACT

STUDY QUESTION: Do polycystic ovary syndrome (PCOS), menstrual cycle phases, and ovulatory status affect reproductive tract (RT) microbiome profiles?

SUMMARY ANSWER: We identified microbial features associated with menstrual cycle phases in the upper and lower RT microbiome, but only two specific differences in the upper RT according to PCOS status.

WHAT IS KNOWN ALREADY: The vaginal and uterine microbiome profiles vary throughout the menstrual cycle. Studies have reported alterations in the vaginal microbiome among women diagnosed with PCOS.

STUDY DESIGN, SIZE, DURATION: This prospective case-control study included a cohort of 37 healthy control women and 52 women diagnosed with PCOS. Microbiome samples were collected from the vagina as vaginal swabs (VS) and from the uterus as endometrial flushing (EF) aspirate samples, and compared according to PCOS diagnosis, the menstrual cycle phases, and ovulatory status, at Oulu University Hospital (Oulu, Finland) from January 2017 to March 2020.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 83 VS samples and 80 EF samples were collected. Age and body mass index (BMI) were matched between women with and without PCOS. Clinical characteristics were assessed using blood samples collected between cycle days 2 and 8, and microbial DNA was sequenced on the Ion Torrent platform. Microbial alpha diversity (i.e. the observed number of unique genera and Shannon diversity index) was analysed across sample types, PCOS diagnosis and menstrual cycle phases. Linear mixed-effects models were utilised to identify microbial features in relation to PCOS and the menstrual cycle phases. Associations between the beta diversity of the RT microbiome and PCOS- and cycle-related clinical features were calculated using PERMANOVA.

MAIN RESULTS AND THE ROLE OF CHANCE: Microbial alpha diversity showed no difference with PCOS (VS: $P_{observed\ feature} = 0.836$, $P_{shannon} = 0.998$; EF: $P_{observed\ feature} = 0.366$, $P_{shannon} = 0.185$), but varied with menstrual cycle phases (VS: $P_{observed\ feature} = 0.001$, $P_{shannon} = 0.882$; EF: $P_{observed\ feature} = 0.026$, $P_{shannon} = 0.048$). No difference was observed in beta diversity based on either PCOS or the menstrual cycle phases (VS: $P_{PCOS} = 0.280$, $P_{cycle} = 0.115$; EF: $P_{PCOS} = 0.234$, $P_{cycle} = 0.088$). In the endometrial flushing samples, we identified two novel microbial features, characterised by the ratio of differential abundance of two genera, associated with PCOS (FDR ≤ 0.1) and 13 novel features associated with the menstrual cycle phases (FDR ≤ 0.1).

LIMITATIONS, REASONS FOR CAUTION: Although this was the first study to simultaneously analyse, the lower and upper RT microbiome in women with and without PCOS, the limited sample size of anovulatory cases may hinder the detection of differences related to PCOS and ovulatory status.

WIDER IMPLICATIONS OF THE FINDINGS: The main finding suggests that PCOS and the menstrual cycle phases are associated with specific microbial features in the upper RT, indicating that the analysis of the upper RT microbiome can potentially identify biomarkers for both PCOS and menstrual cycle phases.

STUDY FUNDING/COMPETING INTEREST(S): This research was funded by the Research Council of Finland (grants no. 315921, 321763, 336449), the Sigrid Jusélius Foundation, Novo Nordisk Foundation (grant no. NNF21OC0070372), and the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant (MATER, grant no. 813707). This research was also funded by the Estonian Research Council (grants no. PRG1076, PRG1414), the Horizon Europe grant (NESTOR, grant no.

Received: May 15, 2024. **Revised:** October 20, 2024. **Editorial decision:** November 18, 2024.

101120075) of the European Commission, and EMBO Installation Grant (grant no. 3573). The funders did not participate in any processes of the study. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

TRIAL REGISTRATION NUMBER: N/A.

Keywords: polycystic ovary syndrome / PCOS / microbiome / female reproductive tract microbiome / vaginal microbiome / endometrial microbiome / uterine microbiome / menstrual cycle

Introduction

With numerous studies leveraging advanced sequencing technologies to examine the communities of microorganisms, termed the microbiome, the analysis of the female reproductive tract (RT) microbiome has also become possible (Baker *et al.*, 2018; Molina *et al.*, 2020). The vagina is predominantly inhabited by *Lactobacillus* (Chen *et al.*, 2017), which helps to maintain an acidic environment with a pH ranging between 3.5 and 4.5, effectively preventing pathogen infections (Skarin and Sylwan, 1986; Hawes *et al.*, 1996). The vaginal microbial community shifts in response to hormonal changes during the menstrual cycle, with an increasing abundance of *Lactobacillus* from menses during the proliferative phase (PP) and into the secretory phases (SP), under high oestrogen levels, accompanied by a decrease in alpha diversity (Gajer *et al.*, 2012; Chaban *et al.*, 2014; Chen *et al.*, 2017; Krog *et al.*, 2022). Importantly, alterations in the vaginal microbial community have been associated not only with gynaecological diseases, such as bacterial vaginosis (BV) (Skarin and Sylwan, 1986), chronic endometritis (Liu *et al.*, 2019), and endometriosis (Hernandes *et al.*, 2020), but also with reproductive complications, such as *in vitro* fertilisation (IVF) failure (Hyman *et al.*, 2014; Benner *et al.*, 2018). However, thus far, the majority of research on the RT microbiome has focused on the vaginal microbiome, leaving a significant gap in understanding the upper RT microbiome, including its composition in fertility and infertility-associated diagnoses (Molina *et al.*, 2020).

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder affecting one out of eight women (Teede *et al.*, 2023). PCOS is not only characterised by systemic low-grade inflammation and insulin resistance but also by the increased risk of adverse reproductive outcomes, such as anovulation, pre-eclampsia, and preterm birth (Palomba *et al.*, 2015; Teede *et al.*, 2023). The aetiology of PCOS is complex and multigenic (Bruni *et al.*, 2022; Stener-Victorin *et al.*, 2024), while some data also suggest a role for altered gut microbiome (Lüll *et al.*, 2021; Sola-Leyva *et al.*, 2023; Lee *et al.*, 2024). Recently, the involvement of the RT microbiome in PCOS has gained attention (Sola-Leyva *et al.*, 2023). Previous studies have demonstrated an increased alpha diversity and a decreased relative abundance of *Lactobacillus* in the vaginal microbial communities of women with PCOS (Hong *et al.*, 2020; Tu *et al.*, 2020). Nevertheless, research on the RT microbiome lags behind gut microbiome studies. Moreover, changes in the upper RT microbiome in relation to PCOS, particularly in the endometrium, remain unexplored.

In this study, we aimed to characterise and compare the vaginal and uterine microbiome using less-invasively acquired samples, namely vaginal swab (VS) and endometrial flushing (EF) samples, in contrast with tissue biopsies. Secondly, we analysed the associations between microbial compositions and PCOS, considering both ovulatory and anovulatory status, as well as their associations with menstrual cycle phases, irrespective of PCOS diagnosis. To our knowledge, this is the first study to simultaneously explore the lower and upper RT microbiome in women with and without PCOS.

Materials and methods

Study population

A total of 83 VS samples from 28 healthy controls and 33 PCOS women and 80 EF samples from 30 healthy controls and 37 PCOS women were collected at Oulu University Hospital (Oulu, Finland) from January 2017 to March 2020 (Fig. 1). The study was approved by The Regional Ethics Committee of the Northern Ostrobothnia Hospital District, Finland (65/2017), and all study participants provided signed informed consent. We followed the principles of the Declaration of Helsinki in designing and conducting the study. Women who had used hormonal medication during the 3 months prior to sample collection and smokers were excluded. Some women donated multiple samples; however, only one sample was collected in each cycle. Control women were in good health, had regular menstrual cycles, and did not have a diagnosis of PCOS (Table 1). PCOS was diagnosed based on the Rotterdam consensus, which requires the presence of a minimum of two of the following three clinical features: oligo-anovulation, hyperandrogenism, and polycystic ovarian morphology (Teede *et al.*, 2023). Within the PCOS group, women were divided according to the presence or absence of ovulation defined by urinary LH testing, clinical evaluation in ultrasonography, and histological analysis of the endometrial biopsy. In those with anovulatory PCOS (no positive LH surge after longer tracking), ultrasonography revealed ovaries with arrested antral follicles that fail to progress to the preovulatory stage, while histological analysis confirmed an inactive endometrium characterized by quiescent glands and a lack of mitotic activity (Franks and Hardy, 2020).

Clinical measurements

Clinical parameters, including sex hormones and metabolic indices, were measured on the day of the clinical visit. Blood samples collected between cycle days 2 and 8 were analysed. Serum levels of LH, follicle-stimulating hormone (FSH), anti-Müllerian hormone (AMH), and sex hormone-binding globulin (SHBG) were measured by Elecsys assays (Roche) using a cobas e411 analyser, and serum testosterone and progesterone were measured by an Agilent 1290 Rapid Resolution LC System (Agilent, San Jose, CA, USA) (Häkkinen *et al.*, 2019). Free androgen index (FAI) was calculated by dividing testosterone (nmol/L) by SHBG (nmol/L) and multiplying by 100. Plasma glucose was analysed by an enzymatic dehydrogenase method (Advia 1800, Siemens Healthcare Diagnostic Inc., Tarrytown, NY, USA), and serum insulin was measured by a chemiluminometric immunoassay (Advia Centaur XP, Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Homeostatic model assessment insulin resistance (HOMA-IR) was calculated using the following formula: (fasting serum insulin [μU/ml] × fasting plasma glucose [mmol/l/22.5). Demographic and anthropometric traits of the study subjects are summarised in Table 1.

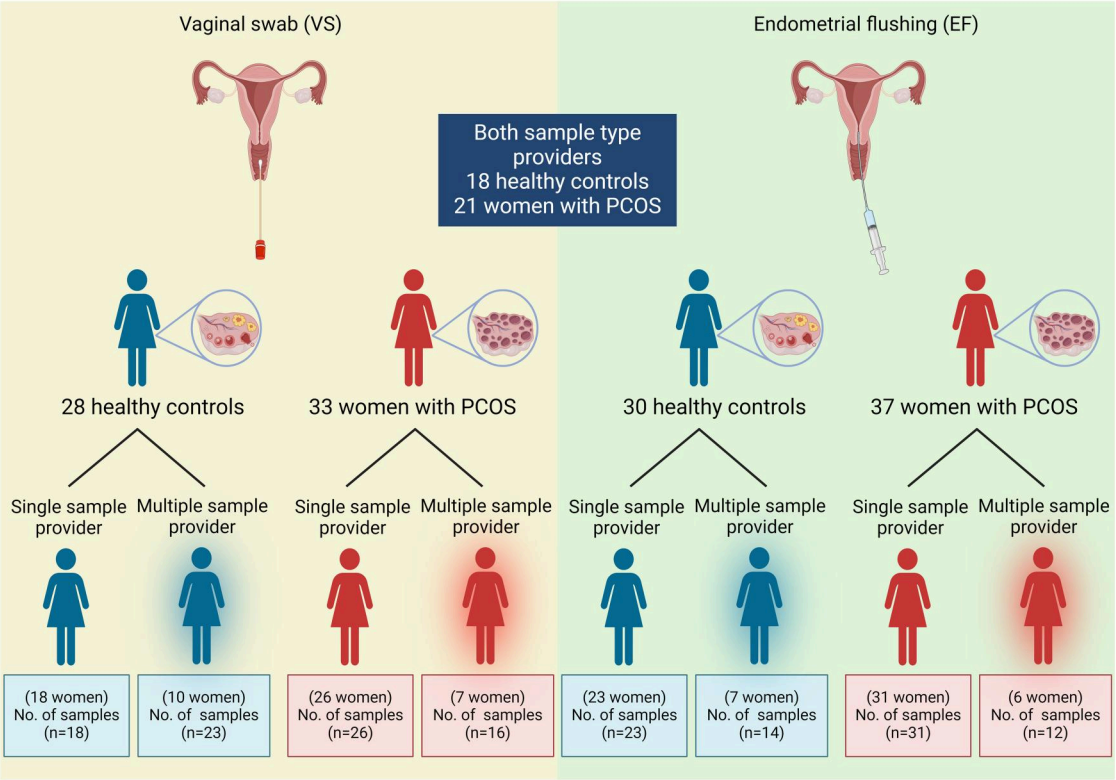

Figure 1. Study cohort overview. A total of 83 VS samples were obtained from 28 controls and 33 women with PCOS, and a total of 80 EF samples were collected from 30 healthy controls and 37 women diagnosed with PCOS. A subject who provided two or more samples, collected from different menstrual cycle phases, was represented as a 'multiple sample provider'. EF, endometrial flushing; VS, vaginal swab.

Table 1. Clinical characteristics of women with and without PCOS.

	Controls	PCOS ovulatory	PCOS anovulatory	P-value
Age, years	31.00 [28.00; 37.50] (37)	35.00 [30.00; 38.00] (43)	32.00 [24.00; 38.50] (9)	0.238
BMI, kg/m^2	26.40 [23.15; 31.65] (37)	27.60 [22.80; 32.00] (43)	28.60 [23.75; 37.70] (9)	0.432
Fasting glucose, mmol/l	5.20 [4.80; 5.50] (37)	5.30 [4.98; 5.60] (42)	5.60 [5.25; 6.35] (9)	**0.038**
Fasting insulin, mU/l	7.10 [5.00; 9.85] (37)	8.10 [5.50; 10.80] (43)	9.90 [7.15; 19.15] (9)	0.121
HOMA-IR	1.63 [1.08; 2.55] (37)	1.87 [1.16; 2.63] (42)	2.81 [1.98; 4.99] (9)	**0.024**
AMH, ng/ml	2.51 [1.54; 4.06] (37)	4.73 [2.53; 5.82] (42)	9.08 [4.35; 10.78] (9)	**<0.001**
Testosterone, nmol/l	0.89 [0.80; 1.33] (37)	1.19 [0.95; 1.40] (43)	2.22 [1.76; 2.71] (9)	**<0.001**
SHBG, nmol/l	62.00 [41.10; 89.57] (37)	49.43 [40.36; 70.27] (42)	34.62 [19.97; 56.97] (9)	**0.038**
FAI	1.85 [1.14; 2.33] (37)	2.43 [1.48; 3.10] (42)	6.42 [3.36; 10.37] (9)	**<0.001**
LH, IU/l	6.93 [5.94; 10.22] (31)	7.87 [6.47; 9.33] (36)	16.46 [11.57; 25.23] (9)	**0.005**
FSH, IU/l	7.14 [6.36; 8.34] (31)	6.49 [5.56; 7.63] (36)	6.59 [5.59; 8.86] (9)	0.571
Progesterone, nmol/l	0.54 [0.34; 0.77] (31)	0.48 [0.30; 0.75] (37)	0.36 [0.22; 0.67] (9)	0.129

Clinical parameters were assessed using blood samples collected between cycle days 2 and 8 (median with [Q1; Q3]). The number of subjects is indicated in parentheses. A P-value was determined by the Kruskal–Wallis test, and bold values represent $P < 0.05$.
AMH, anti-Müllerian hormone; BMI, body mass index; FAI, free androgen index; FSH, follicle-stimulating hormone; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; LH, luteinizing hormone; SHBG, Sex hormone-binding globulin.

Microbiome sample collection and DNA extraction

Ovulatory samples were collected during either the PP (cycle days 6–8) or the SP following the LH surge, at LH +2–4 (2–4 days post-LH-peak, early SP (ESP)), +7–8 (7–8 days post-LH-peak, mid-SP (MSP)), or +11–12 days (11–12 days post-LH-peak, late SP (LSP)). The LH surge was identified using a Clearblue digital urine test in the morning, and the presence of the corpus luteum was confirmed through transvaginal ultrasonography using Voluson E8 (GE Healthcare Technologies, USA). The anovulatory samples were obtained on any day convenient for the women.

Following the examination of the corpus luteum via transvaginal ultrasonography, a swab was gently inserted into the vagina, brushed on the vaginal wall, and then removed. The swab was immediately placed into a sterile tube and stored in a −80°C deep freezer before microbial DNA extraction. Subsequently, 1 ml of sterile 0.9% saline solution was administered into the uterine cavity using a syringe connected to a Pipelle® catheter. Following a ten-second interval, the flushing was aspirated and promptly transferred into a sterile tube. The EF samples were then placed on ice and stored at −80°C deep freezer for subsequent microbial DNA extraction. There were 18 control women and 21 women with PCOS provided who both types of samples (i.e. VS and EF samples) (Fig. 1). Finally, a total of 83 VS samples (controls n = 41 and PCOS n = 42) and 80 EF samples (controls n = 37 and PCOS n = 43) were acquired, which were categorised according to the menstrual cycle phases as follows (PP: controls VS n = 11, EF n = 8, PCOS VS n = 18, EF n = 8; ESP: controls VS n = 4, EF n = 11,

PCOS VS n = 8, EF n = 8; MSP: controls VS n = 12, EF n = 10, PCOS VS n = 4, EF n = 11; LSP: controls VS n = 14, EF n = 8, PCOS VS n = 7, EF n = 10; anovulatory: PCOS VS n = 5, EF n = 6).

Microbial DNA was extracted from the frozen VS and EF samples using the DNeasy PowerSoil PRO kit (Qiagen) following the manufacturer's instructions, and the quantity and quality of DNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The hypervariable regions V4-V5 of the 16S small ribosomal unit gene were amplified with a forward primer 519F (5′-CAGCMGCCGCGGTAATWC-3′) with a 30 bp long Ion Torrent adapter sequence, a 9-bp long barcode sequence, and a single nucleotide linker A, and a reverse primer 926R (5′-CCGTCAATTCCTTTRAGTTT-3′), which included an Ion Torrent adapter sequence. Polymerase chain reactions (PCRs) were conducted in duplicates. A total reaction volume of 20 µl included a 1×Phusion HF buffer, 0.5 µM of forward and reverse primers, 200 µM of dNTPs, 0.5 U of Phusion enzyme (Thermo Scientific, Finland), 20 ng of genomic community DNA as the template, and molecular-grade water. After an initial 3 min denaturation at 98°C, the following cycling conditions were used: 26 cycles at 98°C, 10 s; at 64°C, 10 s; and at 72°C, 20 s. PCR products were pooled and purified using the AMPure XP PCR clean-up kit (Agencourt Bioscience, CA, USA), and the purified DNA was quantified with a Bioanalyzer DNA chip (Agilent Technologies, CA, USA). The DNA samples were sequenced on a 316 Chip v2 with Ion Torrent 400 bp chemistry (Life Technologies, USA) at the Biocenter Oulu Sequencing Center (Oulu, Finland) with an Ion Torrent PGM sequencer.

Microbiome data analyses

The sequences were processed and analysed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) (Bolyen *et al.*, 2019). A sequence quality threshold was set as 20 and reads shorter than 200 bp were removed. In the demultiplexing step, primer, barcode sequences and chimeric reads were filtered out from the dataset, and the reads were denoised using QIIME2-implemented DADA2 (Callahan *et al.*, 2016). The reads were trimmed at base 18 and truncated at base 30 based on the quality plots. After the demultiplexing step, a total of 2 086 749 reads (range: 1644–8262) with an average of 12 802 reads per sample was identified: 1 049 311 reads from the VS samples (range: 368–48 048) with an average of 12 988 reads per sample and 1 008 696 reads from the EF samples (range: 1644–54 166) with an average of 12 608 reads per sample. The sequences were clustered into observed features, and taxonomy assignment was conducted using a pre-trained naïve Bayes taxonomy classifier against the SILVA database (version 138.1) with a similarity threshold of 99%. To eliminate contaminant reads from the negative control samples used during RT-PCR and sequencing, an R package decontam (version 1.8.0) was performed, resulting in the removal of 65 amplicon sequencing variants (ASVs). In total, 2636 ASVs were identified. For downstream analysis, the ASVs were aggregated into the genus level, which resulted in a total of 578 genera.

Filtering microbiome data

We removed seven EF samples with exceptionally low numbers of reads (<1000), resulting in 83 samples for VS and 73 samples for EF. In total, 114 genera were identified from the VS samples and 545 genera, from the EF samples. These genus-level microbiome profiles were used to calculate the alpha diversity metrics and inter-individual microbiome differences for beta-diversity analysis for each sample type separately. For differential abundance analysis, we focused on genera detected in at least 20% of the samples for the corresponding sample type to limit the number of tests carried out. This resulted in 13 genera for the VS samples and 33 genera for the EF samples.

Statistical analysis

The baseline characteristics of the study subjects were analysed using IBM SPSS Statistics version 27 (IBM Corporation, Armonk, NY, USA). For continuous variables, statistical differences were analysed using the Kruskal–Wallis test.

The observed number of unique genera and the Shannon diversity index were used to assess the alpha diversity of the microbial community using the vegan package (version 2.5.6). Linear mixed models were used to assess the association between alpha diversity, PCOS, and menstrual cycle phases. Likelihood ratio tests were used to assess the statistical significance of PCOS and menstrual cycle phases, with the baseline model having the covariate of interest removed from the model description.

The Euclidean distance on the unfiltered centred log-ratio (CLR) transformed genus-level microbiome profile was used to calculate the between-sample distances for the beta diversity analysis. Permutational analysis of variance (PERMANOVA) on the Euclidean distances was used to test the associations between the phenotypes (i.e. PCOS diagnosis, menstrual cycle phases) and microbiome composition using 10 000 permutations for the *P*-value calculations (Anderson, 2001). PERMANOVA was carried out using the adonis function in the vegan package (v.2.5-6.). To apply the CLR transformation, zero counts were imputed with a pseudocount of 0.5.

For differential abundance analysis, linear mixed-effects models on the pairwise log-ratios (PWLRs) of the microbial genera were applied considering PCOS status and menstrual cycle phases as fixed effects (78 log-ratios for VS and 595 log-ratios for EF). Similar to beta diversity analysis, zero counts were imputed with a pseudocount of 0.5 before the PWLR transformation. The analysis was conducted for VS and EF separately. Likelihood ratio tests were used to assess the significance of PCOS and menstrual cycle phases, with the baseline model having the covariate of interest removed from the model description. The Benjamini–Hochberg procedure was used to account for multiple tests.

Results

Clinical characteristics of study subjects

We refer to VS as the lower RT microbiome sample and EF as the upper RT microbiome sample. PCOS cases and the controls were matched for age (controls, median 31; PCOS ovulatory, median 35; PCOS anovulatory, median 32; $P = 0.238$) and BMI (controls, median 26.40; PCOS ovulatory, median 27.60; PCOS anovulatory, median 28.60; $P = 0.432$) (Table 1). The women with PCOS in our study displayed significant dysregulation in both endocrine and metabolic features, which was particularly severe in those who experienced anovulation. For example, the PCOS cases showed higher HOMA-IR values compared to the controls (controls, median 1.63; PCOS ovulatory, median 1.87; PCOS anovulatory, median 2.81; $P = 0.024$). Additionally, the PCOS cases exhibited significantly increased testosterone (controls, median 0.89; PCOS ovulatory, median 1.19; PCOS anovulatory, median 2.22; $P < 0.001$) and FAI levels (controls, median 1.85; PCOS ovulatory, median 2.43; PCOS anovulatory, median 6.42; $P < 0.001$), along with decreased SHBG levels (controls, median 62.00; PCOS ovulatory, median 49.43; PCOS anovulatory, median 34.62; $P = 0.038$) in comparison to the controls (Table 1).

Landscape and compositional differences within the RT microbiome

A total of 114 genera (mean ± sd per sample 9 ± 5.8) were detected for the microbiome obtained from the VS samples, while 545 genera (mean ± sd per sample 33 ± 23.7) were detected for the microbiome obtained from the EF samples. Similar to previous studies, *Lactobacillus* was the most abundant genera in both the VS and EF microbiomes (Chen *et al.*, 2017; Tao *et al.*, 2017; Kyono *et al.*, 2018), with the average abundance being slightly higher in the VS microbiome (VS: 85.0 ± 30.0; EF: 74.4 ± 35.0%) (Fig. 2a, Supplementary Table S1). The most prevalent genera were largely shared by the vaginal and uterine microbial communities, with *Atopobium*, *Streptococcus*, *Prevotella*, and *Shuttleworthia* being the most abundant (Fig. 2a, Supplementary Table S1).

Our first aim was to compare the lower and upper RT microbiome profiles. At the compositional level, there was a remarkable difference between the VS and EF microbiome, as illustrated by the genus-level taxonomic profiles and principal component analysis biplot (PERMANOVA R^2 = 4.16%, $P < 0.0001$) (Fig. 2b). The EF profiles exhibited significantly higher observed richness (mean ± sd VS = 8.9 ± 5.85; EF = 32.9 ± 23.70; $P < 2.2*10^{-16}$) and Shannon index (mean ± sd VS = 0.30 ± 0.47; EF = 0.81 ± 0.84; $P = 2.6 * 10^{-8}$) when compared to the VS profiles (Fig. 2c). Concerning the secondary analysis, a subset of samples from individuals who provided both VS and EF sample types was investigated to assess the variation within and between individuals as well as the composition of the VS and EF microbiome. We found that the VS and EF microbiome profiles from the same individual were more similar than those from two distinct subjects ($P = 0.021$), as indicated by Aitchison distance (Aitchison *et al.*, 2000) (Supplementary Fig. S1). This shows that the taxonomic profiles obtained with different sample types (i.e. VS and EF)

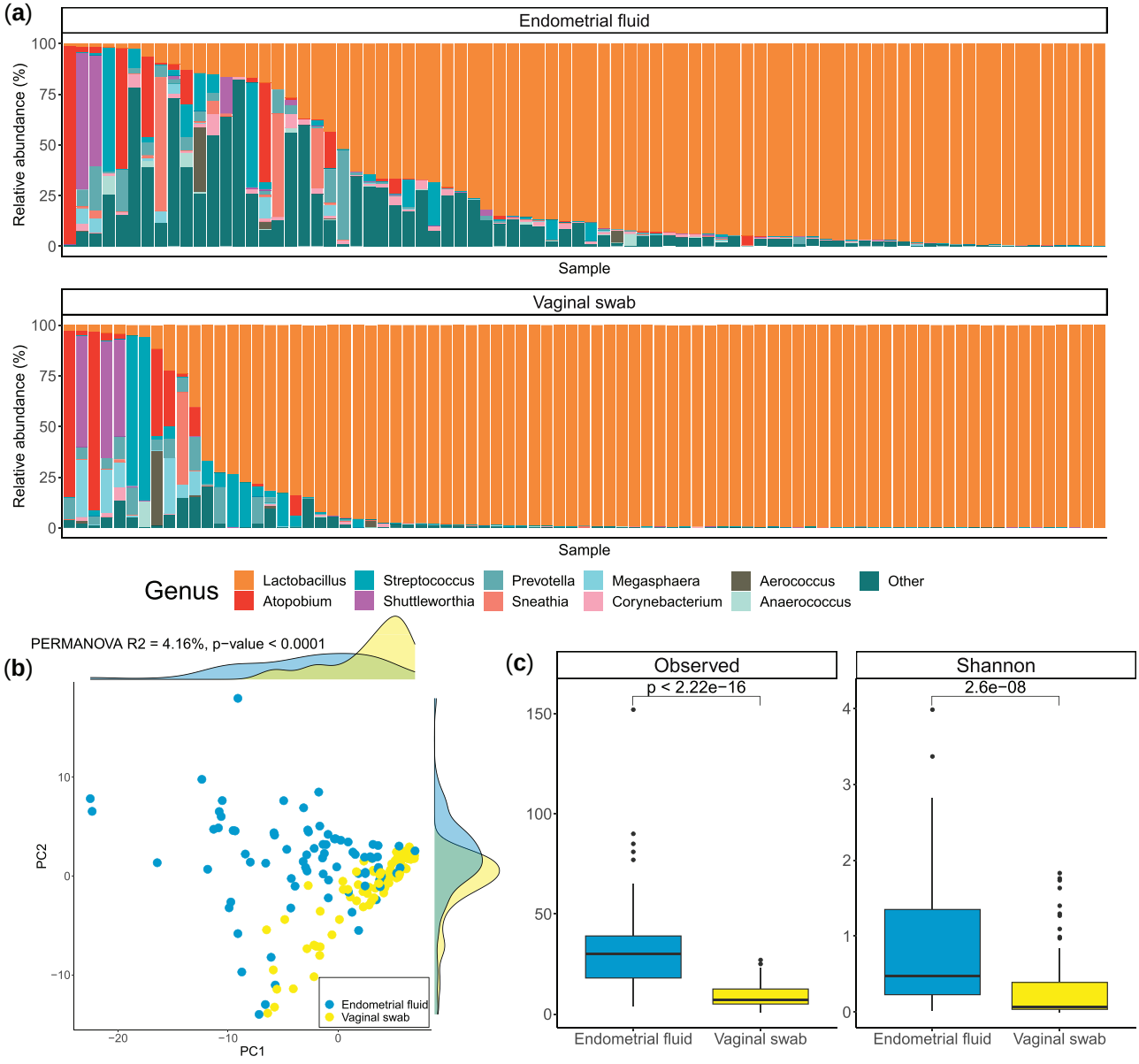

Figure 2. Microbial landscape and comparison of the lower and upper RT microbiome profiles. (**a**) genus-level taxonomic profiles for VS (upper panel) and EF (lower panel) samples. The top ten most abundant genera according to the average relative abundance are shown, with remaining genera grouped as 'other'. (**b**) principal component analysis biplot for the genus-level CLR-transformed microbiome profile, coloured by the lower and upper RT samples. (**c**) differences in microbiome alpha diversity between the lower and upper RT samples. CLR, centred log-ratio; EF, endometrial flushing; PC, principal component; PERMANOVA, Permutational analysis of variance; RT, Reproductive tract; VS, vaginal swab.

identify individual-specific signals. Additionally, we observed that the microbiome profiles obtained from the same individual VS samples (n = 13) during different menstrual cycle phases were more similar when compared to VS samples from random individuals ($P = 0.0001$). This indicates that the individual-specific signals are also identifiable across menstrual cycle phases in VS microbiome. Nevertheless, this pattern was not observed for the EF microbiome ($P = 0.32$), likely due to the low number of samples (n = 7).

Microbiome alterations associated with PCOS and menstrual cycle phases

Next, we analysed the associations between the RT microbiome and PCOS diagnosis, considering the ovulatory status of women with PCOS, as well as between the RT microbiome and the menstrual cycle phases. Given the significant differences in composition between the VS and EF microbiome profiles, we performed separate analyses for each sample type. We observed no statistically significant differences in alpha diversity regarding PCOS status in either the VS or EF profiles (linear mixed-effects models for controls, PCOS ovulatory, and PCOS anovulatory samples, VS: $P_{observed} = 0.836$, $P_{shannon} = 0.988$; EF: $P_{observed} = 0.366$, $P_{shannon} = 0.185$) (Fig. 3a, Supplementary Fig. S2). Although PCOS microbial communities exhibited increased Shannon diversity compared to the control community in the EF microbiome, these differences did not reach statistical significance. Similarly, no PCOS-related differences in microbiome beta diversity were observed (PERMANOVA, VS: $R^2 = 1.4\%$, $P = 0.280$, EF: $R^2 = 1.6\%$, $P = 0.234$) (Fig. 3b). On the other hand, the menstrual cycle phases were significantly associated with microbiome alpha diversity (linear mixed-effects models for the five different cycle phases, VS: $P_{observed} = 0.001$, $P_{shannon} = 0.882$; EF: $P_{observed} = 0.026$, $P_{shannon} = 0.048$), when all PCOS cases and controls were analysed together (Fig. 3c, Supplementary Fig. S2). In both VS and EF microbiome profiles, microbial diversity clearly decreased from the PP into the SP. The anovulatory samples did not exhibit any statistically significant differences in alpha diversity compared to all ovulatory samples. Similar to PCOS, no statistically significant association between the menstrual cycle phases and microbiome beta diversity was observed (PERMANOVA, VS: $R^2 = 6.5\%$, $P = 0.115$; EF: $R^2 = 6.6\%$, $P = 0.088$) (Fig. 3d). We additionally examined the association between RT microbiome composition and PCOS- and menstrual cycle phases-related clinical parameters, including endocrine and metabolic factors. We found that the microbial community was strongly associated with SHBG (PERMANOVA, VS: $R^2 = 3.73\%$, $P = 0.009$; EF: $R^2 = 2.58\%$, $P = 0.023$) and fasting

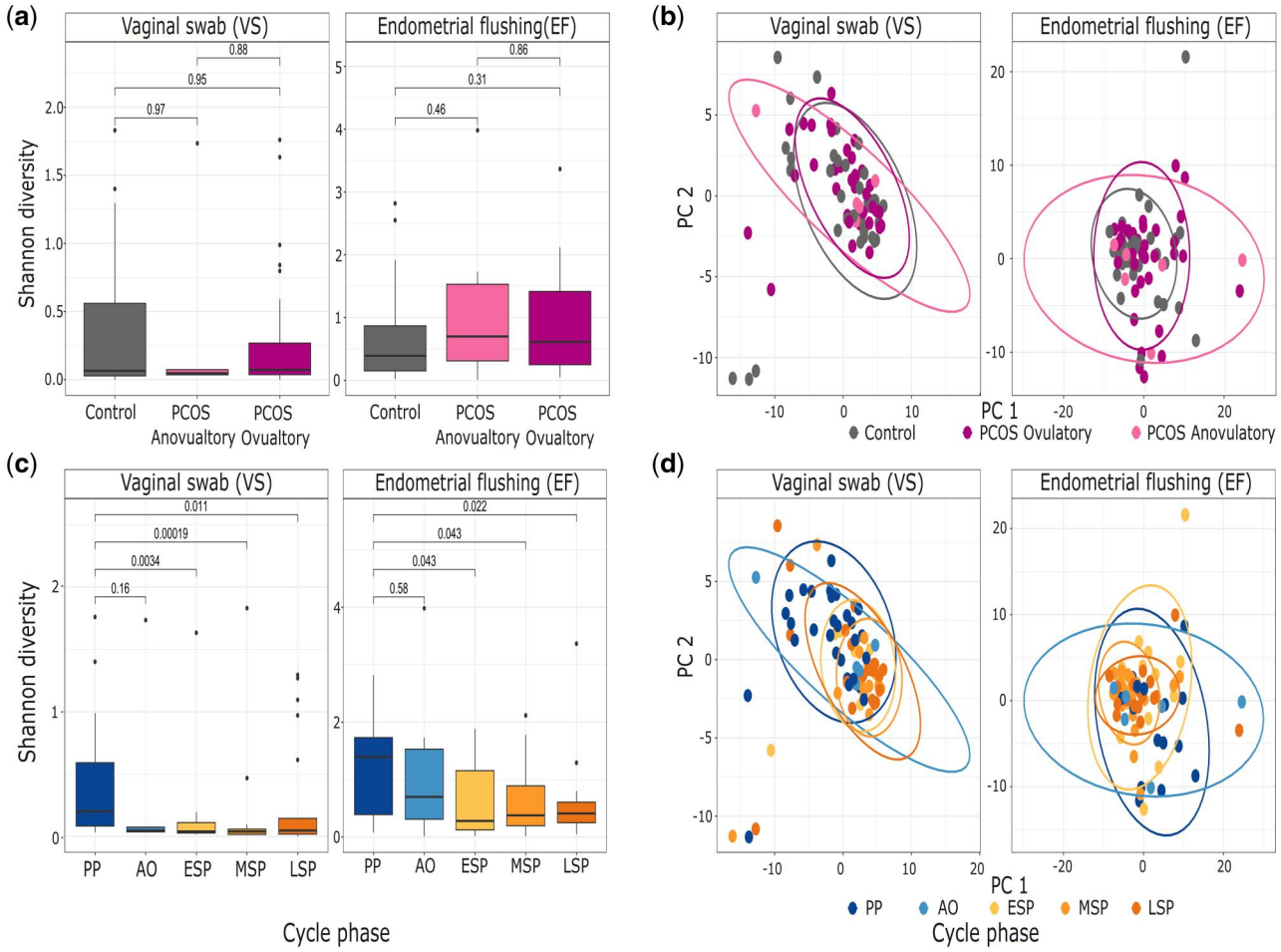

Figure 3. Microbiome changes related to PCOS and menstrual cycle phases. Differences in alpha diversity (Shannon index) by (**a**) PCOS status and (**c**) menstrual cycle phases. *P*-values on the plot correspond to post-hoc pairwise *t*-tests. Principal component analysis biplot for the genus-level CLR-transformed microbiome beta diversity by the (**b**) PCOS status and (**d**) menstrual cycle phases. AO, anovulatory; CLR, centred log-ratio; EF, endometrial flushing; ESP, early secretory phase; LSP, late secretory phase; MSP, mid-secretory phase; PC, principal component; PP, proliferative phase; VS, vaginal swab.

Table 2. Reproductive tract microbiome variance explained by clinical parameters.

	VS (R^2)	P-value	EF (R^2)	P-value
PCOS-related parameters				
Fasting glucose	0.73%	0.779	2.02%	0.070
Fasting insulin	3.26%	**0.028**	1.67%	0.219
HOMA-IR	2.24%	0.093	1.62%	0.234
AMH	0.61%	0.877	1.34%	0.532
Testosterone	0.84%	0.719	1.24%	0.797
SHBG	3.73%	**0.009**	2.58%	**0.023**
FAI	2.24%	0.097	1.65%	0.317
Cycle-related parameters				
LH	0.93%	0.649	1.07%	0.610
FSH	1.14%	0.504	1.22%	0.434
Progesterone	2.59%	0.050	1.98%	0.115

Variance explained (R^2) according to the PERMANOVA model with Euclidean distance on the CLR-transformed microbiome profiles. Bold values represent $P < 0.05$.
AMH, anti-Müllerian hormone; CLR, centred log-ratio; EF, endometrial flushing; FAI, free androgen index; FSH, follicle-stimulating hormone; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; LH, luteinizing hormone; PERMANOVA, Permutational analysis of variance; SHBG, sex hormone-binding globulin; VS, vaginal swab.

insulin levels (PERMANOVA, $R^2 = 3.26\%$, $P = 0.028$) in relation to PCOS (Table 2). However, no significant associations were found with the menstrual cycle phases.

To study the microbiome alterations related to PCOS and the menstrual cycle phases in greater detail, we focused on the PWLR between two bacterial genera. Microbiome sequencing data contain only relative information about bacterial abundances, a property known as compositionality, which can invalidate the analytical results if not properly accounted for (Gloor *et al.*, 2017). The aim of analysing log-ratios, which has become a widely used approach for analysing microbiome data, is to alleviate issues with data compositionality (Aitchison, 1982; Gloor *et al.*, 2017; Quinn *et al.*, 2021). Using linear mixed-effects models, we identified two log-ratios of bacterial taxa associated with PCOS related to the abundance of *Rhodoferax* in the EF microbiome. Specifically, the ratios of Acetobacteraceae uncultured/*Rhodoferax* and *Bryobacter*/*Rhodoferax* were significantly lower in PCOS anovulatory communities compared to control and PCOS ovulatory communities (FDR ≤ 0.1) (Fig. 4a, Supplementary Table S2). Additionally, we observed 13 log-ratios of bacterial taxa associated with the menstrual cycle phases in the EF microbiome (FDR ≤ 0.1) (Fig. 4b, Supplementary Table S2). Focusing on PWLR further allowed us to directly compare the effects of PCOS and the menstrual cycle phases on the studied log ratios obtained from both the VS and EF microbiomes (Fig. 4c and d). Following the small number of associations between the RT microbiome and PCOS, the effect sizes for PCOS obtained from VS and EF were not correlated ($r = -0.037$, $P = 0.769$) (Fig. 4c). Therefore, our findings suggest that PCOS-associated signals found in EF microbiome are not shared with the lower (VS) RT microbiome. On the other hand, the effect sizes for the menstrual cycle phases showed a strong correlation ($r = 0.503$, $P < 2.2 * 10^{-16}$), indicating that the lower (VS) and upper (EF) RT microbiome convey similar signals and could partially serve as an alternative for each other (Fig. 4d).

Discussion

Previous studies have identified alterations in the vaginal and endometrial microbiome profile during the menstrual cycle phases (Chaban *et al.*, 2014; Chen *et al.*, 2017; Sola-Leyva *et al.*, 2021; Krog *et al.*, 2022) and associations between the vaginal microbiome and PCOS (Sola-Leyva *et al.*, 2023). Nevertheless, our understanding of the vaginal and uterine microbiome profiles in individuals with PCOS, as well as those with inherent anovulatory conditions, remains relatively limited. In this study, we addressed this gap by comprehensively investigating both the lower and upper RT microbiome: (i) among women with and without PCOS, also considering the ovulatory status of the participating women, and (ii) throughout the menstrual cycle.

Our study is the first to investigate the lower and upper RT microbiome in women with PCOS in parallel. We observed considerable community diversity of the lower and upper RT microbiome between PCOS cases and controls. Although the vaginal microbiome of women with PCOS has been reported to have increased alpha diversity and compositional changes, including a decreased abundance of *Lactobacillus* (Hong *et al.*, 2020) and an increased abundance of *Mycoplasma* and *Prevotella* (Hong *et al.*, 2020; Tu *et al.*, 2020), our study did not confirm these findings. This may be explained by the fact that previous studies did not account for age, BMI, and ovulatory status, when comparing women with and without PCOS (Hong *et al.*, 2020; Tu *et al.*, 2020). Given that these confounding factors are associated with PCOS characteristics such as hyperandrogenism and insulin resistance (Hsu, 2013; Moran *et al.*, 2015), as well as with the microbiome (Allen *et al.*, 2022; Medina-Bastidas *et al.*, 2022), our careful matching of cases and controls for these factors likely explains comparable microbiome profiles between women with and without PCOS. Additionally, despite the more pronounced hormonal imbalances in anovulatory cases, we found comparable microbiome profiles between ovulatory and anovulatory PCOS women. Given that previous studies have demonstrated sex hormone-associated microbial changes in the RT microbiome (Xu *et al.*, 2020; Lu *et al.*, 2021; Wu *et al.*, 2021), the lack of significant differences may be attributed to the limited number of anovulatory samples analysed.

We also identified potential features of PCOS that may influence RT microbiome profiles. Our results revealed strong correlations between the RT microbiome profiles and fasting insulin and SHBG levels, consistent with the clinical characteristics of PCOS, including insulin resistance and reduced SHBG levels (Stener-Victorin *et al.*, 2024). SHBG plays a crucial role in regulating serum sex hormone levels by binding to oestrogen and testosterone (Dunn *et al.*, 1981; Yan *et al.*, 2024). Additionally, SHBG can be indirectly modulated by chronic inflammation, which may affect liver function (Osmancevic *et al.*, 2023), and by the presence of metabolic syndrome (Allan and McLachlan, 2010). Our findings provide new insights into the impact of endocrine-metabolic dysregulation in PCOS on the RT microbiome.

The ratios of Acetobacteraceae to *Rhodoferax* and *Bryobacter* to *Rhodoferax* in the upper RT microbiome were significantly lower in PCOS cases compared to those in controls. This suggests a lower abundance of Acetobacteraceae or *Bryobacter* or a higher abundance of *Rhodoferax*. The family Acetobacteraceae, a group of acetic acid-producing bacteria, not only demonstrates antimicrobial properties but also plays a crucial role in glucose metabolism. This family of microbes impedes the growth of pathogens by employing organic acids, which reduce intracellular pH and disrupt cellular processes (Chen *et al.*, 2016). Moreover, the family Acetobacteraceae contributes to lowering blood glucose levels through a decrease in disaccharidase activity (Ogawa *et al.*, 2000), leading to increased glucose uptake and its conversion to glycogen (Hlebowicz *et al.*, 2007), and the adenosine monophosphate-activated protein kinase pathway (Sakakibara *et al.*, 2006). Meanwhile, an elevated abundance of *Rhodoferax* has been noted

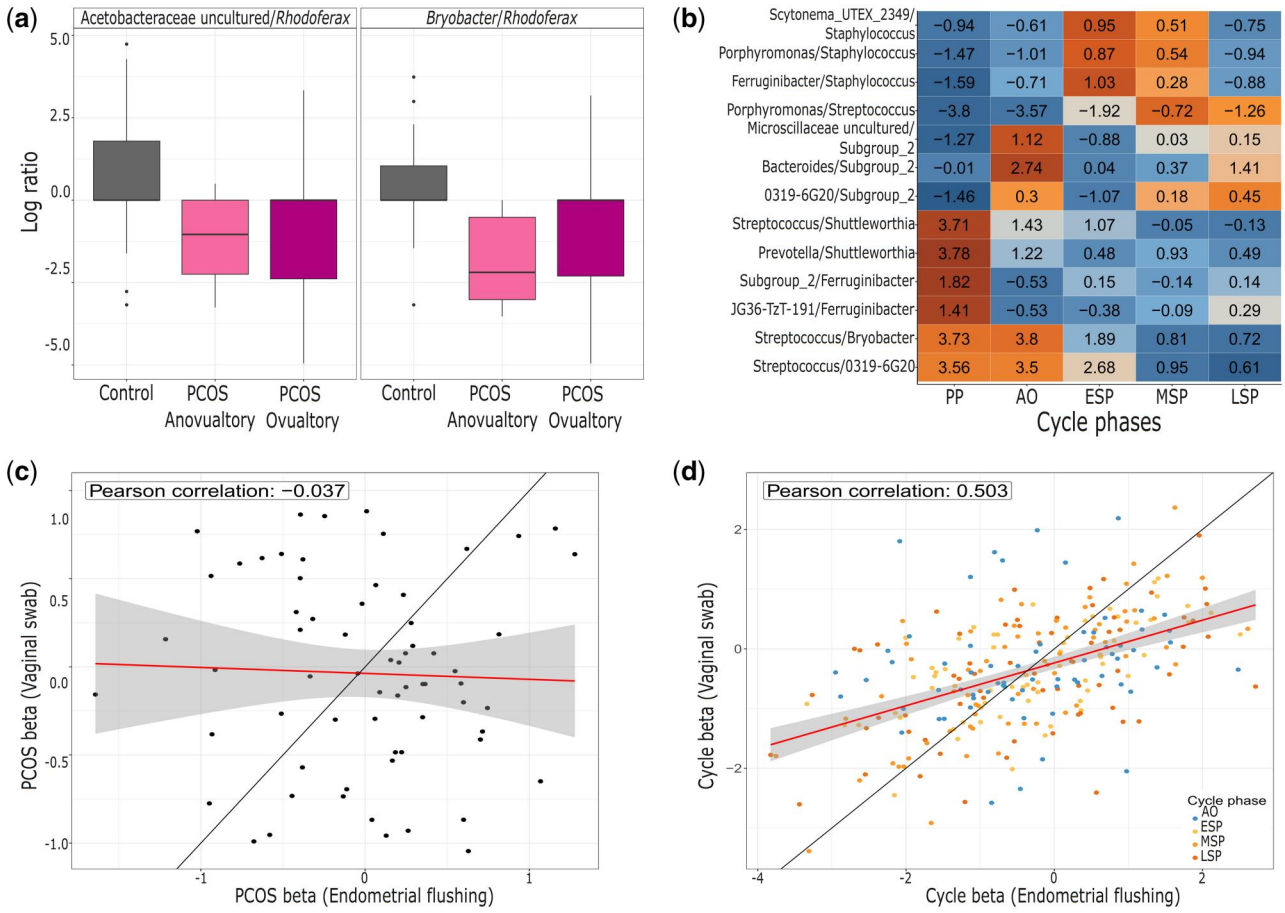

Figure 4. Changes in the abundances of microbial genera based on PCOS status and the menstrual cycle phases. (**a**) Log ratios of genera associated with PCOS in the EF samples (FDR ≤ 0.1). (**b**) CLR abundances by menstrual cycle phases for log ratios of genera associated with the menstrual cycle phases in the EF samples (FDR ≤ 0.1). Colours represent z-scores after scaling the data row-wise. Similarity of fixed regression coefficients (beta) for (**c**) PCOS and (**d**) menstrual cycle phases for PWLR obtained from the lower (VS; vertical axis) and upper (EF; horizontal axis) RT microbiome profiles using the linear mixed-effects model. AO, anovulatory; CLR, centred log-ratio; EF, endometrial flushing; ESP, early secretory phase; FDR, false discovery rate; LSP, late secretory phase; MSP, mid-secretory phase; PP, proliferative phase; PWLR, pairwise log-ratio; VS, vaginal swab.

within the endometrial microbial community of patients experiencing recurrent implantation failure (Chen *et al.*, 2022). This may potentially indicate a less supportive upper RT microbiome environment against pathogens, metabolic regulation and fertility in women with PCOS compared to the microbiome in non-PCOS controls.

The decrease in alpha diversity observed from the proliferative phase to the secretory phase is known to be associated with sex hormone changes (Chen *et al.*, 2017; Krog *et al.*, 2022). The PP samples were collected at cycle days 6–8 before oestradiol peak, while the SP samples were obtained after the LH surge. Oestrogen causes glycogen accumulation in the vaginal epithelium, while progesterone triggers the release of glycogen into the vaginal lumen by lysing the epithelium (Shen *et al.*, 2022). Consequently, in the SP, increased nutrient availability leads to an increase in the abundance of *Lactobacillus*, along with decreased species richness and evenness within the community (Chen *et al.*, 2017; Song *et al.*, 2020; Krog *et al.*, 2022). This may imply a microbial community that is more stable and less diverse during the SP compared to the PP. On the other hand, the community diversity of anovulatory samples was comparable to that of ovulatory samples, despite severe hormonal imbalances present in the anovulatory cases. Further research with increased sample sizes is necessary to examine the reproductive tract microbiome in intrinsic anovulatory cases and its associations with hormonal and metabolic imbalances.

Several log ratios exhibited significant changes throughout the menstrual cycle, including the ratio between *Streptococcus* and *Staphylococcus*. Both *Staphylococcus* and *Streptococcus* are opportunistic pathogens related to endometritis and adverse reproductive outcomes (Cicinelli *et al.*, 2008; Kitaya *et al.*, 2018; Medina-Bastidas *et al.*, 2022). Given the heightened endometrial immune status in the LSP, which creates a more protective environment against pathogens compared to earlier SP (Yang *et al.*, 2019), one would expect decreases in their abundance. Therefore, further investigation into this association is warranted, and employing techniques such as absolute quantification would facilitate a better understanding of the role of these bacteria.

Our results indicate that EF samples can identify specific PCOS and menstrual cycle phase-related signals more distinctly when compared to the VS samples. This can be attributed to the unique niche of the endometrium. With its low biomass of microbes and isolated location from the external environment (O'Callaghan *et al.*, 2020), the substantial changes in endometrial tissue environment may cause slight, but significant alterations in the local microbial community. Additionally, the

endometrium maintains a neutral pH (7.5–7.8), fostering bacterial proliferation, contrasting with the acidic nature of the lower RT (Lykke *et al.*, 2021). Consequently, these unique anatomical and biochemical characteristics of the endometrium may lead to a more pronounced response, leading to PCOS-related changes in the microbiome, becoming visible only in the endometrial samples and not in the VS samples. Therefore, we encourage researchers to study the upper RT microbiome in relation to PCOS and other infertility-associated conditions, as it may provide unique and potentially novel biomarkers for reproductive complications.

Successive sample collection following the exact determination of the menstrual cycle phases, and even encompassing anovulatory cases, enhanced the reliability of our findings. This study pioneered the employment of anovulatory samples arising from intrinsic hormone imbalances, an area that remains relatively unexplored compared to studies on anovulation induced by hormonal contraception (Song *et al.*, 2020; Krog *et al.*, 2022). Furthermore, by considering the menstrual cycle phases, which have significant effects on the RT microbiome (Molina *et al.*, 2021; Lüll and Org, 2023; Sola-Leyva *et al.*, 2023), as well as matching the age and BMI of cases and controls, both of which impact the development of PCOS (Hsu, 2013; Moran *et al.*, 2015), we mitigated the potential influence of confounding factors on the results. However, the small sample size of anovulatory cases may have hindered the detection of microbial differences in this particular condition. Moreover, the limited number of VS samples in certain menstrual cycle phase may affect the attainability of statistical power. Furthermore, we cannot rule out the possibility of contamination of the EF samples from the lower RT, despite using a catheter and conducting careful sample collection by experienced gynaecologists. As summarised in Lüll and Org (2023), sampling methods are critical in endometrial microbiome research, as they can introduce significant biases in microbial composition. Given the significant roles of oestrogen in reproductive health and its influence on RT microbial communities, the fact that serum oestrogen levels fell below the detection limit of liquid chromatography-mass spectrometry may restrict the investigation of the interactions between oestrogen and the RT microbiome. Additionally, employing metagenomic approaches like whole shotgun sequencing could enhance the resolution for identifying bacteria at the genus and strain levels, as well as their functional capabilities. This could potentially reveal underlying mechanisms linking to PCOS or menstrual cycle phases that were beyond the scope of this study. Despite these limitations, this study revealed specific alterations in the RT microbiome among women with PCOS and its ovulatory sub-phenotypes, and across the menstrual cycle phase, as well as in changes in microbial diversity according to the menstrual cycle phase, in well-characterised study participants and samples by exploring both the lower and upper RT microbiome concurrently.

Conclusion

This study simultaneously examined the lower and upper RT microbiome in relation to PCOS and the menstrual cycle phases. We found specific novel microbial features, represented by taxonomic ratios, associated with PCOS, as well as with the menstrual cycle phases in the upper RT microbiome. Additionally, the PCOS RT microbiome showed significant associations with fasting insulin and SHBG levels, and there were significant changes in microbial diversity across the menstrual cycle. This descriptive study offers initial insight into the upper RT microbiome in women with PCOS, establishing a foundation for future research into the role of the RT microbiome in the aetiology and potential treatment of PCOS.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The 16S RNA sequencing data were submitted in the Sequence Read Archive (SRA) (Reference No. PRJNA1079033) (https://www.ncbi.nlm.nih.gov/bioproject/1079033).

Acknowledgements

We extend our gratitude to all the subjects for their participation. Special thanks go to research nurse Elina Huikari for her assistance in sample collection and to Marko Suokas for preprocessing the sequencing data.

Authors' roles

This study was designed by S.L., R.K.A., and T.T.P. and supervised by T.T.P., R.K.A., A.S., O.A., and E.O. T.T.P., S.L., and R.K.A. chose the study population and conducted microbiome sequencing. S. L. and O.A. performed the data analysis and statistical analysis. All authors (including J.L. and H-R.R.) revised and approved the final version.

Funding

This research was funded by the Research Council of Finland (315921, 321763, 336449); the Sigrid Jusélius Foundation, Novo Nordisk Foundation (NNF21OC0070372); and the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant (MATER, 813707). This research was also funded by the Estonian Research Council (PRG1076, PRG1414); the Horizon Europe grant (NESTOR, 101120075) of the European Commission; and EMBO Installation Grant (3573). The funders did not participate in any processes of the study.

Conflict of interest

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

References

Aitchison J. The statistical analysis of compositional data. *J R Statist Soc B Methodol* 1982;**44**:139–160.

Aitchison J, Barceló-Vidal C, Martín-Fernández JA, Pawlowsky-Glahn V. Logratio analysis and compositional distance. *Math Geol* 2000;**32**:271–275.

Allan CA, McLachlan RI. Androgens and obesity. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:224–232.

Allen NG, Edupuganti L, Edwards DJ, Jimenez NR, Buck GA, Jefferson KK, Strauss JF, Wickham EP, Fettweis JM; Vaginal Microbiome Consortium. The vaginal microbiome in women of reproductive age with healthy weight versus overweight/obesity. *Obesity (Silver Spring)* 2022;**30**:142–152.

Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;**26**:32–46.

Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists, or invaders? *Front Immunol* 2018;**9**:208.

Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update* 2018;**24**:393–415.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;**37**:852–857.

Bruni V, Capozzi A, Lello S. The role of genetics, epigenetics and lifestyle in polycystic ovary syndrome development: the state of the art. *Reprod Sci* 2022;**29**:668–679.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–583.

Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, Albert AY, van Schalkwyk J, Reid G, Hemmingsen SM *et al.* Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome* 2014;**2**:23.

Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, Li F, Yu X, Feng Q, Wang Z *et al.* The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun* 2017;**8**:875.

Chen H, Chen T, Giudici P, Chen F. Vinegar functions on health: constituents, sources, and formation mechanisms. *Compr Rev Food Sci Food Saf* 2016;**15**:1124–1138.

Chen P, Jia L, Zhou Y, Guo Y, Fang C, Li T. Interaction between endometrial microbiota and host gene regulation in recurrent implantation failure. *J Assist Reprod Genet* 2022;**39**:2169–2178.

Cicinelli E, De Ziegler D, Nicoletti R, Colafiglio G, Saliani N, Resta L, Rizzi D, De Vito D. Chronic endometritis: correlation among hysteroscopic, histologic, and bacteriologic findings in a prospective trial with 2190 consecutive office hysteroscopies. *Fertil Steril* 2008;**89**:677–684.

Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J Clin Endocrinol Metab* 1981;**53**:58–68.

Franks S, Hardy K. What causes anovulation in polycystic ovary syndrome? *Curr Opin Endocr Metab Res* 2020;**12**:59–65.

Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X, Koenig SSK, Fu L, Ma Z (, Zhou X *et al.* Temporal dynamics of the human vaginal microbiota. *Sci Transl Med* 2012;**4**:132ra52.

Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome datasets are compositional: and this is not optional. *Front Microbiol* 2017;**8**:2224.

Häkkinen MR, Murtola T, Voutilainen R, Poutanen M, Linnanen T, Koskivuori J, Lakka T, Jääskeläinen J, Auriola S. Simultaneous analysis by LC–MS/MS of 22 ketosteroids with hydroxylamine derivatization and underivatized estradiol from human plasma, serum and prostate tissue. *J Pharm Biomed Anal* 2019;**164**:642–652.

Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wolner-Hanssen P, Holmes KK. Hydrogen peroxide—producing lactobacilli and acquisition of vaginal infections. *J Infect Dis* 1996;**174**:1058–1063.

Hernandes C, Silveira P, Rodrigues Sereia AF, Christoff AP, Mendes H, Valter de Oliveira LF, Podgaec S. Microbiome profile of deep endometriosis patients: comparison of vaginal fluid, endometrium and lesion. *Diagnostics* 2020;**10**:163.

Hlebowicz J, Darwiche G, Björgell O, Almér L-O. Effect of apple cider vinegar on delayed gastric emptying in patients with type 1 diabetes mellitus: a pilot study. *BMC Gastroenterol* 2007;**7**:46.

Hong X, Qin P, Huang K, Ding X, Ma J, Xuan Y, Zhu X, Peng D, Wang B. Association between polycystic ovary syndrome and the vaginal microbiome: A case-control study. *Clin Endocrinol (Oxf)* 2020;**93**:52–60.

Hsu M-I. Changes in the PCOS phenotype with age. *Steroids* 2013;**78**:761–766.

Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, Vo KC, Caughey AB, Hilton JF, Davis RW *et al.* Diversity of the Vaginal Microbiome Correlates With Preterm Birth. *Reprod Sci* 2014;**21**:32–40.

Kitaya K, Takeuchi T, Mizuta S, Matsubayashi H, Ishikawa T. Endometritis: new time, new concepts. *Fertil Steril* 2018;**110**:344–350.

Krog MC, Hugerth LW, Fransson E, Bashir Z, Nyboe Andersen A, Edfeldt G, Engstrand L, Schuppe-Koistinen I, Nielsen HS. The healthy female microbiome across body sites: effect of hormonal contraceptives and the menstrual cycle. *Hum Reprod* 2022;**37**:1525–1543.

Kyono K, Hashimoto T, Nagai Y, Sakuraba Y. Analysis of endometrial microbiota by 16S ribosomal RNA gene sequencing among infertile patients: a single-center pilot study. *Reprod Med Biol* 2018;**17**:297–306.

Lee S, Tejesvi MV, Hurskainen E, Aasmets O, Plaza-Díaz J, Franks S, Morin-Papunen L, Tapanainen JS, Ruuska TS, Altmäe S *et al.* Gut bacteriome and mood disorders in women with PCOS. *Human Reproduction* 2024;**39**:1291–1302.

Liu Y, Ko EY-L, Wong KK-W, Chen X, Cheung W-C, Law TS-M, Chung JP-W, Tsui SK-W, Li T-C, Chim SS-C. Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method. *Fertil Steril* 2019;**112**:707–717.e1.

Lu C, Wang H, Yang J, Zhang X, Chen Y, Feng R, Qian Y. Changes in vaginal microbiome diversity in women with polycystic ovary syndrome. *Front Cell Infect Microbiol* 2021;**11**:755741.

Lüll K, Arffman RK, Sola-Leyva A, Molina NM, Aasmets O, Herzig K-H, Plaza-Díaz J, Franks S, Morin-Papunen L, Tapanainen JS *et al.* The gut microbiome in polycystic ovary syndrome and its association with metabolic traits. *J Clin Endocrinol Metab* 2021;**106**:858–871.

Lüll K, Org E. Uterine microbiome: does the sampling technique matter? *Semin Reprod Med* 2023;**41**:144–150.

Lykke MR, Becher N, Haahr T, Boedtkjer E, Jensen JS, Uldbjerg N. Vaginal, cervical and uterine pH in women with normal and abnormal vaginal microbiota. *Pathogens* 2021;**10**:90.

Medina-Bastidas D, Camacho-Arroyo I, García-Gómez E. Current findings in endometrial microbiome: impact on uterine diseases. *Reproduction* 2022;**163**:R81–R96.

Molina N, Sola-Leyva A, Saez-Lara M, Plaza-Diaz J, Tubić-Pavlović A, Romero B, Clavero A, Mozas-Moreno J, Fontes J, Altmäe S. New opportunities for endometrial health by modifying uterine microbial composition: present or future? *Biomolecules* 2020;**10**:593.

Molina NM, Sola-Leyva A, Haahr T, Aghajanova L, Laudanski P, Castilla JA, Altmäe S. Analysing endometrial microbiome: methodological considerations and recommendations for good practice. *Hum Reprod* 2021;**36**:859–879.

Moran LJ, Norman RJ, Teede HJ. Metabolic risk in PCOS: phenotype and adiposity impact. *Trends Endocrinol Metab* 2015;**26**:136–143.

O'Callaghan JL, Turner R, Dekker Nitert M, Barrett HL, Clifton V, Pelzer ES. Re-assessing microbiomes in the low-biomass reproductive niche. *BJOG* 2020;**127**:147–158.

Ogawa N, Satsu H, Watanabe H, Fukaya M, Tsukamoto Y, Miyamoto Y, Shimizu M. Acetic acid suppresses the increase in disaccharidase activity that occurs during culture of Caco-2 cells. *J Nutr* 2000;**130**:507–513.

Osmancevic A, Daka B, Michos ED, Trimpou P, Allison M. The association between inflammation, testosterone and SHBG in men: a cross-sectional multi-ethnic study of atherosclerosis. *Clin Endocrinol (Oxf)* 2023;**99**:190–197.

Palomba S, de Wilde MA, Falbo A, Koster MPH, La Sala GB, Fauser BCJM. Pregnancy complications in women with polycystic ovary syndrome. *Hum Reprod Update* 2015;**21**:575–592.

Quinn TP, Gordon-Rodriguez E, Erb IA. A Critique of differential abundance analysis, and advocacy for an alternative. *arXiv*. 2021;2104.07266. preprint: not peer reviewed.

Sakakibara S, Yamauchi T, Oshima Y, Tsukamoto Y, Kadowaki T. Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-A(y) mice. *Biochem Biophys Res Commun* 2006;**344**:597–604.

Shen L, Zhang W, Yuan Y, Zhu W, Shang A. Vaginal microecological characteristics of women in different physiological and pathological period. *Front Cell Infect Microbiol* 2022;**12**:959793.

Skarin A, Sylwan J. Vaginal lactobacilli inhibiting growth of *gardnerella vaginalis, mobiluncus* and other bacterial species cultured from vaginal content of women with bacterial vaginosis. *Acta Pathol Microbiol Immunol Scand B* 1986;**94**:399–403.

Sola-Leyva A, Andrés-León E, Molina NM, Terron-Camero LC, Plaza-Díaz J, Sáez-Lara MJ, Gonzalvo MC, Sánchez R, Ruíz S, Martínez L *et al.* Mapping the entire functionally active endometrial microbiota. *Hum Reprod* 2021;**36**:1021–1031.

Sola-Leyva A, Pérez-Prieto I, Molina NM, Vargas E, Ruiz-Durán S, Leonés-Baños I, Canha-Gouveia A, Altmäe S. Microbial composition across body sites in polycystic ovary syndrome: a systematic review and meta-analysis. *Reprod Biomed Online* 2023;**47**:129–150.

Song SD, Acharya KD, Zhu JE, Deveney CM, Walther-Antonio MRS, Tetel MJ, Chia N. Daily vaginal microbiota fluctuations associated with natural hormonal cycle, contraceptives, diet, and exercise. *mSphere* 2020;**5**. https://doi.org/10.1128/msphere.00593-20

Stener-Victorin E, Teede H, Norman RJ, Legro R, Goodarzi MO, Dokras A, Laven J, Hoeger K, Piltonen TT. Polycystic ovary syndrome. *Nat Rev Dis Primers* 2024;**10**:27.

Tao X, Franasiak JM, Zhan Y, Scott RT, Rajchel J, Bedard J, Newby R, Scott RT, Treff NR, Chu T. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: next generation sequencing (NGS) analysis of the 16S ribosomal gene. *Hum Microb J* 2017;**3**:15–21.

Teede HJ, Tay CT, Laven JJE, Dokras A, Moran LJ, Piltonen TT, Costello MF, Boivin J, Redman LM, Boyle JA *et al.* Recommendations from the 2023 international evidence-based guideline for the assessment and management of polycystic ovary syndrome. *J Clin Endocrinol Metab* 2023;**189**:G43–G64.

Tu Y, Zheng G, Ding G, Wu Y, Xi J, Ge Y, Gu H, Wang Y, Sheng J, Liu X *et al.* Comparative analysis of lower genital tract microbiome between PCOS and healthy women. *Front Physiol* 2020;**11**:635088.

Wu J, Ning Y, Tan L, Chen Y, Huang X, Zhuo Y. Characteristics of the vaginal microbiome in women with premature ovarian insufficiency. *J Ovarian Res* 2021;**14**:172.

Xu J, Bian G, Zheng M, Lu G, Chan W, Li W, Yang K, Chen Z, Du Y. Fertility factors affect the vaginal microbiome in women of reproductive age. *American J Rep Immunol* 2020;**83**:e13220.

Yan Z, Zheng Z, Xia T, Ni Z, Dou Y, Liu X. Causal relationship between gut microbiome and sex hormone-binding globulin: a bidirectional two-sample Mendelian randomization study. *Am J Reprod Immunol* 2024;**91**:e13824.

Yang X, Gilman-Sachs A, Kwak-Kim J. Ovarian and endometrial immunity during the ovarian cycle. *J Reprod Immunol* 2019;**133**:7–14.

Human Reproduction, 2025, 00, 1–32
https://doi.org/10.1093/humrep/deae270
Original Article