Changes in the vaginal microbiota associated with primary ovarian failure

CURRENT STATUS: Under Review

BMC Microbiology  •  BMC Series

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Subject Areas

General Microbiology Obstetrics & Gynecology

Keywords

vaginal microbiota, primary ovarian failure, 16S rRNA, pathogenesis, female reproductive tract
Abstract

Background: Primary ovary failure (POF) is defined as follicular failure in women of reproductive age. Although many factors are speculated to contribute to the occurrence of POF, the exact aetiology is unclear. Alterations in the microbiome of women with POF are poorly studied;

Methods: This study investigated the vaginal microbiota of 22 patients with POF and 29 healthy individuals. High-throughput Illumina Miseq sequencing targeting the V3-V4 region of the 16S ribosomal RNA (rRNA) gene was used to evaluate the relationships between vaginal flora and clinical characteristics of POF.

Results: Different from before, we found that the diversity and richness of the vaginal flora of patients with POF was significantly different from that of healthy controls. Comparison of the flora of patients with POF with that of menopausal women revealed that the relative abundance of Lactobacillus was significantly reduced in the latter, and women with reduced relative abundance of Lactobacillus in microbiota community decreased the pregnancy success rate at term. The result suggests the present study enabled identification of microbiota associated with POF, further investigations of differences in the microbiota in the context of POF will improve our understanding of the pathogenesis of the disease which involve modification of the vaginal microbiota.

Conclusion: The present study identified the microbiota associated with POF. Further investigations on the differences in the microbiota in the context of POF will improve our understanding of the pathogenesis of the disease which involves modification of the vaginal microbiota.

Introduction

Primary ovarian failure (POF) is defined as failure of ovarian function in women below the age of 40. Clinically, the condition is characterised by amenorrhea lasting 4 months or more, accompanied by oestrogen deficiency and elevated levels of gonadotropin[1, 2]. The prevalence of POF is 1% in the women before <40 years of age [3, 4], and the incidence is increasing. Development of POF is associated with particular autosomal gene defects, autoimmune dysfunction, infection, andiatrogenic factors. Smoking, drinking, and nutritional factors may influence the age at which menopause occurs[2]. However, in nearly half the cases of POF, the causal factors are not clear. Furthermore, the clinical manifestation differs between individuals, the most serious being gonadal dysplasia secondary to infertility—only 5% of women with POF can conceive naturally. Patients with POF can also experience comorbidities including osteoporosis, dyslipidaemia, blood pressure fluctuations, and cardiovascular disease[5]. The clinical recommendations are to undergo in vitro fertilisation-embryo transfer (IVF-ET) with egg donation on the basis of hormone-replacement therapy (HRT)[6]. The complexity of POF and the decreasing age of patients experiencing POF means that research into the underlying mechanism of the disease is of high importance.

There is tons of evidence indicating that the vaginal microbiota of women of childbearing age mainly comprises Lactobacillus species[7]. Prevotella, Atopobium, and Gardnerella spp. are associated with bacterial vaginosis (BV) and their presence in the vaginal microbiota may lead to preterm birth[8]. A number of studies have found that a high incidence of BV in women undergoing IVF, and reported this condition to be associated with infertility[9, 10]. Women with a high relative abundance of Gardnerella and Atopobium spp in the vaginal microbiota have been reported to have poor IVF outcomes in terms of pregnancy[11]. When the vaginal microbiota is altered, the production of lactic acid will be changed, potentially leading to an increase in the secretion of inflammatory factors such as interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF)-α which activate the immune system and cause the body to be in a chronic inflammatory state. This affects the success rate of pregnancy[12, 13], highlighting the important roles of vaginal microbiota in the reproductive tract microbiome and maintenance of reproductive tract health in females[14].

Primary ovarian failure has been shown to have an autoimmune component. Proposed mechanisms of POF have suggested that viral, genetic, or other environmental stimuli may induce the expression of major
histocompatibility complex (MHC) Class I (I) and class II (II) antigens in granulosa cells. These antigens are recognised by ovarian T cells which respond by secreting lymphokines to stimulate macrophages to secrete more interferon (INF)-γ which further increases expression of MHC in ovarian granulosa cells triggering humoral and cell-mediated autoimmune responses including secretion of IL-1 from macrophages and lymphocytes and acceleration of follicular atresia[15]. However, the vaginal epithelium has many innate immune protection mechanisms including the presence of tight junctions, antimicrobial peptides (AMPs), and mucus. In addition, immune cells such as γ- and δ-T cells, dendritic cells (DC), and macrophages are present below and between the epithelial cell layer of the vagina[16].

Class II antigen expression can be induced in patients with POF, and the in vitro expression of these antigens in granulosa cells is enhanced by the addition of IFN-γ to cell culture[17]. Vaginal microbiota has also been linked to female infertility via its effect on the concentration of various inflammatory factors in plasma. Compared with women with normal fertility, the vaginal lavage fluid of infertile women was found to have increased levels of inflammatory factors such as TNF-α and IFN-γ, and decreased levels of IL-6 and IL-10[18]. A number of studies have also shown that the proliferation of Gardnerella vaginalis associated with inflammatory response can be inhibited by Lactobacillus. By activating TLR-2 on the surface of monocytic THP-1 cells, G. vaginalis activates NF-kB to induce the secretion of large amounts of TNF-α[19, 20]. Similar increases in TNF-α levels have also been reported from a study on a transgenic rat model of POF[21]. Therefore, it is important to study the vaginal microbiota of women with POF during the development of the disease.

We present the first study to use 16S rRNA gene sequencing to investigate the microbiota communities of the vaginal microbiota of patients with POF in comparison with healthy women. Furthermore, we analysed the relationship between vaginal microbiota and clinical characteristics of POF.

### Methods

#### Study cohort and sample collection

We recruited 22 patients aged 20–40 years who visited the Reproductive Hospital affiliated to Shandong University for premature ovarian failure from June to August 2018. According to the diagnostic criteria for POF[22], patients reported a previously regular menstrual cycle with the cessation of menstruation for at least four menstrual cycles and blood follicle-stimulating hormone (FSH) level of 40 IU/L for more than one month. All patients with POF have received hormone replacement therapy for more than three consecutive months, but serum hormone levels still met the diagnostic criteria for POF. We also recruited 29 healthy volunteers for the control group. These participants were selected according to normal menstrual cycles and regulatory factors (FSH of 10 IU/L, anti-Mullerian hormone (AMH) of ≤2 IU/L). Exclusion criteria for both groups were antibiotic treatment within three months prior to enrollment, liver or kidney dysfunction, surgical resection of one side of the ovary, previous smoking history, and vaginal medication in the past three days. In addition, this study included 50 women with standard post-menopausal data for at least 1 year and exclude those with other organic lesions. This study and all its protocol were approved by the Reproductive Ethics Committee of Ren Ji Hospital affiliated to Shanghai Jiao Tong University School of Medicine (approval number: 2018072610). All participants gave informed consent and voluntarily signed informed consent.

The outpatient doctor at the department of gynaecology collected vaginal secretions from the vaginal posterior fornix using a sterile cotton swab according to standard clinical practice. Samples were treated by adding 750 μl of PowerSoil®-htp Bead Solution (MO BIO Laboratories, Inc. Carlsbad, CA, USA; catalogue number 12955-12-BS), then stored at −80 °C until analysis. Samples were collected from the posterior vaginal fornix and were stored in duplicate.

#### Laboratory measurements

Baseline blood samples were collected and stored at −80 °C until measurement. Serum AMH, FSH, luteinising hormone (LH), estradiol (E2), and thyroid stimulating hormone (TSH) were tested used enzyme-linked immunosorbertent assay (ELISA) in the laboratory at the Affiliated Reproductive Hospital of Shandong University.
Extraction of DNA and 16S rRNA amplicon sequencing

We isolated DNA from vaginal samples and assessed the DNA quality using a Thermo NanoDrop 2000 UV spectrophotometer and electrophoresis on 1% agarose gel to assess integrity and size. The 16S rRNA gene was amplified using the universal primers U341F 5′- CCTACGGGRSGCAGCAG -3′ and U806R 5′- GGACTACVGGGTATCCTAATC -3′ targeting the V3-V4 hypervariable regions. All quantified amplicons were pooled to equalise concentrations prior to sequencing using the Illumina MiSeq system (Illumina Inc., CA, USA). Library construction and sequencing were conducted at the Realbio Genomics Institute (Shanghai, China). Reads obtained by paired-end sequencing were merged into a sequence by the Pandaseq 2.9[23] to obtain long reads of the hypervariable region. Then we use the our home-made program to perform the following processing on the stitched Reads to obtain Clean Reads: remove the Reads with an average quality value of less than 20; remove the Reads with more than 3 N-based bases and Reads range is 220 ~ 500nt. To facilitate downstream microbial diversity analysis, long reads were clustered into operational taxonomic units (OTUs). Usearch was used to cluster reads at a similarity of 0.97, then Chimeric sequences were removed to obtain OTUs[24], each of which was considered to represent a single taxon. We rarified our OTU table to 27,420 per sample, to minimize spurious effects of effort on downstream diversity analyses. The most abundant s sequence was selected from each OTU as a representative sequence for that OTU and was submitted to the Ribosomal Database Project database[25] to obtain the annotation. The annotated sequences were classified for each OTU and the composition of flora analysed at the level of the phylum, class, order, family, and genus. The bioinformatics pipeline QIIME1.9.1[26] was used to calculate alpha diversity, carry out beta diversity analysis and to create the corresponding dilution curves. Alpha diversity represents the richness and evenness of microbial communities, including the observed species, Chao1, Shannon, and Simpson indices, as well as the phylogenetic diversity whole tree index. Beta diversity analysis is used to identify differences between different samples. To further investigate differences in taxon diversity between samples, we calculated the unweighted and weighted UniFrac distances for beta diversity analysis using the OTU table and phylogenetic tree. We generated ordination plots using principal coordinate analysis as implemented in R 3.5.1. Principal coordinate analysis (PCoA) was then performed, and linear discriminant analysis (LDA) effect size (LEfSe1.0) analysis[27] (which is often used to identify the presence and effect size of region-specific OTUs among different groups) was used to determine the microbiota associated with POF by comparing the flora of the POF and control groups[28]. The organisms which demonstrated the differences between groups most comprehensively were identified different organisms using an LDA score cut-off of 2.0.

Redundancy analysis

Redundancy analysis (RDA) is a multivariate direct gradient analysis method based on the development of corresponding analysis. Corresponding analysis is combined with multiple regression analysis and each step is calculated considering environmental factors. This analysis is based on a linear model and is mainly used to investigate the relationship between microflora and environmental factors. We draw network diagrams to reveal the important relationship between species and environmental factors by R cytoscape[29].

Random forest classification

Random forest classification is a tree-based algorithm which requires simulation and iteration, and is utilised in machine learning. In general, random forests randomly generate hundreds to thousands of classification trees, and select the tree with the highest degree of repetition as the final result. Based on the real category and prediction probability of the sample, receiver operating characteristic (ROC) curves can be generated, and the area under the curve (AUC) calculated to evaluate the model.

Functional inference of 16S data

Functional annotation analysis was performed based on PICRUSt2.0 (Phylogenetic Sitos by Unobserved States) [30]. It is a software that predicts functional abundance based d on 16S rRNA genes sequence data and other marker gene reference sequence databases (covering KO, EC, COG, MetaCyc, PFAM, TIGRFAM database, etc.), predict the macro genomic functional composition. Accurately, it is first necessary to standardize the number of genus of the original 16S sequencing data, as the number of 16S copies contained by different genus bacteria is
not the same. The 16S genus composition information is then obtained by mapping the genogenic functional gene composition of the constructed sequenced genome to obtain the predictive functional results.

Statistical analyses

Data analysis was performed using SPSS 23.0 statistical software. Normally distributed data sets were compared using an independent-samples t test. Non-normally distributed data were compared using the Wilcoxon signed-rank test function of the R language stats package. Continuous data are presented as mean ± standard deviation. Differences were considered statistically significant when $P < 0.05$. Correlation analysis was carried out using Spearman’s rank correlation coefficient.

Results

Demographic and clinical characteristics of the study population

We enrolled 22 women with POF and 29 healthy controls for analysis. The clinical characteristics of the two groups are shown in Table 1. Among patients with POF, the mean age was 30.50 ± 3.17 years, body mass index (BMI) was 22.34 ± 3.32, waist-to-hip ratio was 0.83 ± 0.04. Among the healthy control group, the mean age was 29.79 ± 3.99 years, BMI was 23.47 ± 3.51, and waist-to-hip ratio was 0.84 ± 0.06. The age, BMI, and waist-to-hip ratio were not significantly different between the two groups ($P > 0.05$), while AMH and E2 were significantly lower in the POF group ($P < 0.001$). Levels of FSH and LH were higher among patients with POF compared with control ($P < 0.001$ and $P < 0.01$, respectively). Among menopausal women, the mean age was 57.96 ± 6.57 years, BMI was 23.57 ± 3.21, and menopause had been experienced for at least 1 year.

Microbial profiling

The mean community diversity indexes (alpha diversity, including Chao1, observed species, Shannon, and Simpson indices) were significantly higher in the POF group compared with the control group (Figure 1A, Supplementary Figure 2, $P < 0.01$). Beta diversity was also significantly different between groups according to the weighted UniFrac phylogenetic distance matrices (analysis of similarities, $R = 0.175$, $P = 0.002$), and showed in PCoA plots (Figure 1B and C). Thus, the vaginal microbiota of the POF group was significantly different to that of the control group. The detailed 16S rRNA raw sequence data were available in the NCBI Sequence Read Archive (SRA) under accession number SRP594533.

Abundance of taxa in the two groups

By LEfSe analysis, we identified 51 genera-discriminative features (Figure 2A, LDA > 2, $P < 0.05$). Comparison of vaginal microbiota by the Mann-Whitney U test revealed 51 taxa that were differentially abundant between the groups ($P < 0.05$); the species of the top 20 are shown in Figure 2B. The agreement of results of the two analytical methods indicates the stability of the vaginal microbiological data.

The relative abundance of bacterial taxonomic groups at the genus level showed that ten genera including Gardnerella, Prevotella, Bacteroides, Sneathia, Dialister, and Anaerococcus were abundant in the POF group. Only Lactobacillus was found to be abundant in the control group (Supplementary Table 1). The Lactobacillus members are mainly grouped into two species: L. gallinarum was the most abundant, followed by L. iners and L. jensenii (Supplementary Table 2) in the control group. However, as our observation in women with POF, the relative abundance of L. iners increased as the colonization of L. gallinarum and L. jensenii dropped (Figure 2C).

A Spearman heatmap was constructed to identify correlations among the above-mentioned genera, which revealed Lactobacillus to be negatively correlated with other genera (Figure 2D). Lactobacillus is a probiotic which plays an important role in human health[31, 32]. Thus, studying the interactions of this genus with other genera could provide insight into the functions of these species in the development of POF.

Analyses of correlations between reproductive-related clinical indicators and vaginal flora
Redundancy analysis was used to produce a two-dimensional sorting map relating vaginal flora to reproductive-related clinical indicators. Serum FSH and LH levels showed the greatest association with female vaginal flora, and E2 had a significant effect. *Gardnerella* and *Prevotella* were positively correlated with serum FSH and LH, and negatively correlated with E2. *Lactobacillus* in the vagina were positively correlated with E2 and negatively correlated with FSH and LH. Similar to E2, AMH was positively correlated with *Lactobacillus* and negatively correlated with *Gardnerella* and *Prevotella* (Figure 3A). Subsequently, we analyzed the correlation between the *Lactobacillus* members and clinical indicators. The relative abundance of *L. gallinarum* was negatively corrected with FSHLH and P level. Meanwhile, the abundance of  *L. gallinarum* was positively related to E2 level and there was a significant difference (P<0.05). In addition, the relative abundance of *L. iners* was negatively related to E2 level. That means *L. gallinarum* and *L. iners* play a weighted part in female reproductive health (Figure 3B).

Next, the predictive model from the random forests system was based on the vaginal flora profile including the taxon, taxon that exhibited significantly different abundances at the genus level from Wilcoxon rank-sum test. We identified 34 genera that could be used to predict occurrence of POF with the random forests model (Figure 4A). A mean classification error of 0.382 was achieved, and the AUC was 0.841 (95% confidence interval [CI]: 0.618-1, sensitivity: 71.4%, specificity: 100%, cut-off rate: 43.2%; Figure 4B).

**Functional alterations in the vaginal flora**

Then, we analyse the metabolic pathways of the two groups of subjects. The predicted genome database has been greatly expanded such that in addition to metabolic pathways, related enzymes, genes and other information can be obtained. LEfSe analysis identified seventeen KO identifiers enriched in women with POF patients including K02014(iron complex outermembrane receptor protein)K07497(putative transposase),K00123(formate dehydrogenase major subunit),K00799glutathione S-transferase,K01223(6-phospho-beta-glucosidase),etc.(Figure 5A).

Figure 5B shows the results of Metacyc, the predictive functions performed using PICRUSt2.0. The pathways that were significantly enriched in POF were gondoate biosynthesis, fatty acid elongation – saturated, palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate), superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass, superpathway of tetrahydrofolate biosynthesis and salvage, pyridoxal 5'-phosphate biosynthesis I, superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis, D-galactarate degradation I and so on. Whereas the microbial functions related to aerobic respiration I (cytochrome c), myo-, chiro- and scillo-inositol degradation, adenosine nucleotides degradation II, superpathway of L-serine and glycine biosynthesis I were higher in the vaginal microbiota of the POF group(Supplemental Table 3).

**Comparison of vaginal flora in the case of premature ovarian failure or menopause**

Finally, we compared the vaginal microbial composition of patients with POF and menopausal women. The high abundance of *Lactobacillus, Gardnerella*, and *Prevotella* was confirmed from comparison of vaginal microbiota of women with POF and menopausal individuals. However, the flora of menopausal women exhibited increased diversity (Figure 6A and B). Differential species analysis showed *Lactobacillus* to be less abundant among menopausal women than those with POF (Figure 6C).

**Discussion**

In recent years, a wealth of evidence has been published supporting the significant contribution of cervicovaginal microbiota to genitourinary and reproductive health outcomes[33]. It was first found that the microbial taxonomic composition differs between women with POF and healthy individuals. We found the vaginal microbiota to be increasingly diverse with increased species richness in the case of POF, and a significant shift in overall microbial diversity was observed. However, a previous cross-sectional study of microbiota failed to identify obvious differences between individuals in terms of vaginal microbiota diversity[34, 35]. The strength of our study lies in the comprehensive description of microbial communities associated with POF achieved through the use of 16S rRNA sequencing; particularly, the association with clinical characteristics of POF; and the utilisation of predictive models to identify bacterial taxa that are differentially expressed in POF.
Previous studies on the vaginal microbiota in the case of POF have mainly involved amine test (or the Whiff test) [34], whereas our study focused on differences at the level of the genus. One of the most attractive features of 16S rRNA gene sequence informatics is the potential for genus identification[36]. Dysbiosis of the vaginal microbiota was characterised by the altered abundance of 34 genera in POF. The combination of these 34 associated taxa were able to discriminate patients with POF from the control group with high accuracy. We noted that vaginal-microbiota-based analysis displayed a similar predictive ability for the disease as the classifier based on POF-associated genetic variants (which had an AUC of 0.841, sensitivity of 71.4%, specificity of 100%, and cut-off rate of 43.2%), implying that the microbial signature that we identified could represent a powerful tool for the prediction of POF. Our results of the changes in the relative abundance of a particular genus in terms of POF confirm that *Lactobacillus* is the dominant vaginal genus. Their dominance in the vaginal niche indicates that *L. iners* and *L. gallinarum* are the dominant facultative anaerobes of the genital tract, which is supported by their presence in most women[37-39].

In women with higher levels of basal FSH and lower levels of basal E2, there were fewer *Lactobacillus* in the vagina than the control group. Eade et al. evaluated the presence of *Lactobacillus* spp in confluent monolayers of endocervical, ectocervical, and vaginal epithelial cells and they found that the majority of *Lactobacillus* caused a significant decrease in the expression of AMPs, although *Lactobacillus* increased in the vaginal[40]. The expression of AMPs, which include cathelicidins, defensins, can also promote IL-22 secretion and thus prevent autoimmune disease[41]. A previous study has suggested that *Lactobacillus* can reinforce the mononuclear phagocytic response by inducing production of the autophagy-promoting factors[42]. Studies have also shown that inflammatory ageing and the autoimmune response are closely related to POF[15, 43]. Our results suggest that reduced colonization of *Lactobacillus* may accelerate the development of POF disease through the induction of immune responses by some inflammatory factors.

Moreover, our study found for the first time that the relative abundance of *L. gallinarum* in the vagina were correlated with the FSH, E2 and AMH levels. FSH and AMH levels had previously been deemed determined the ovarian reserve[44, 45]. And we showed that while *L. gallinarum* was positively correlated to E2 level, *L. iners* was negatively related to E2 level, which was consistent with our clinical changes. That means *L. gallinarum* and *L. iners* were the predominant might be connected with the decline of ovarian function.

As previously studied, hormonal changes cause menstruation and menopause, result in a drastic decreases in *Lactobacillus* in the vaginal microbiota. In this case, infections by *Gardnerella* vaginalis (GV) are increased 8. GV has a significant role in vaginal immunity. In fact, the overgrowth of anaerobic species during menopause can increase the release of the immune molecules such as NF-κB TNFαCOX-2, iNOS [46]. Our result also found that the higher level of genera *Prevotella*,

*Gardnerella*. Abnormal vaginal microbiota may have adverse effect on the pregnancy. We observed the negative correlation between anaerobic species and *Lactobacillus*. These bacteria exploit the same class of environmental resources in a similar way and are defined as an ecological “guild”. Guild members do not necessarily share taxonomic similarities, but they adapt to the changing environment to co-exist to affect female reproductive function by altering the concentration of inflammatory factors.

Our functional analysis showed that the pathways involved in glycolipid metabolism, energy synthesis were related to POF[47]. However, little is known about the relationship between POF patients and metabolic pathway from the research of POF disease in recent years. With the numerous results of KOs, we proposed a hypothesis of underlying mechanisms regarding how imbalanced vaginal microbiota participated in the pathological progress of POF (Supplemental Figure 3). According to the hypothesis, enriched KOs convey that POF patients harbored a disrupted inflammation condition. Phosphatidylglycerol, phosphate, L-methionine, pyruvate ,whose metabolic enzymes were enriched in the POF group, were known to be related to inflammation and Class II antigen[48, 49]. In turn, inflammation promotes the production of acetyl-CoA. Secondly, overproduction of pyruvate could lead to cell apoptosis to mediate mitochondrial dysfunction due to a higher glucose environment[50]. DNA damage activated the mitochondrial apoptosis pathway through oxidative stress and therefore resulted in reproductive dysfunction[51]. That suggests that further exploration need to be done about the metabolic pathways. In our experiment, the glycolipid metabolism and some metabolic enzymes were enriched in the POF group. Additionally, the mTOR signaling pathway metabolic pathway enriched in the Control group, which were
connected with Autophagy. Further research is required to deepen our understanding of this subject.

When we compared the vaginal microbiota of menopausal women with that of women with POF, we found that although the three most abundant genera were the same, the relative abundance of *Lactobacillus* is reduced in the case of menopause, which supports previous research[52]. It is well known that menopause and POF have similar clinical manifestations. Menopause is a natural physiological phenomenon caused by age, but POF is mostly related to genetics and immunity. Our results further validate the important role of relative abundance in *Lactobacillus* during the development of ovarian insufficiency.

In conclusion, our study provides a clear link between POF and vaginal community type 4. Future treatments for POF may, therefore, target the reproductive tract microbiota and involve probiotic treatment to slow follicular atresia, which may improve the success rate of IVF. However, the present study had several limitations which should be addressed in future studies. First, the sample size was small and we couldn’t trace the date of the POF diagnosed. Second, we could not clarify detailed roles of specific constituents of the vaginal microbiota in the pathogenesis of POF. Third, the inclusion of a disease verification model could reveal more accurate information related to the composition of the microbiome and its functions. Therefore, future studies involving larger study populations and animal models are needed in order to explore potential mechanisms underlying the association of the vaginal microbiota with POF. Genomics represents a potential approach to elucidate associations between the vaginal microbiota and disease, and analysis of the gut microbiota may help to explain other pathologies and improve many aspects of prevention and treatment.

Conclusion

The result reveals for the first time that there are differences in the reproductive tract flora of women with premature ovarian failure, confirming that *Lactobacillus* plays a vital role in female reproductive health. We suggest that *Lactobacillus* may affect women’s ovarian function through inflammation and Mitochondrial dysfunction, etc. Its in-depth research in the future provides new possibilities for the treatment of Primary ovarian insufficiency.

Declarations

Ethics approval and consent to participate

This study and all its protocol were approved by the Reproductive Ethics Committee of Ren Ji Hospital affiliated to Shanghai Jiao Tong University School of Medicine (approval number: 2018072610).

Consent for publication

Not applicable.

Availability of data and materials

The detailed 16S rRNA raw sequence data were available in the NCBI Sequence Read Archive (SRA) under accession number SRP594533.

Competing interests

The authors have no conflict of interest to disclose.

Funding

This work was partly supported by grants from the National Key Research and Development Program of China (No. 2017YFC1001002 and No. 2018YFC1003202), the National Natural Science Foundation (No.81671414, 81490743 and 81971343), the Chinese University of Hong Kong-Shanghai Jiao Tong University Joint Research
Collaboration Fund (4750357) and Shanghai Commission of Science and Technology (No.17DZ2271100).

Author contributions

Juan Wang and Yanzhi Du conceived and designed the study. Juan Wang, jieying Xu and Yingying Qin collected and provided patient specimens and related information. Juan Wang, jieying Xu, Qixin Han, Gang Lu, Wai-Yee Chan, Weiwei Chu, and Yanzhi Du analyzed the data. Juan Wang and Yanzhi Du drafted and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgements

Not applicable.

Authors’ Information

Not applicable.

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Supplementary File Captions

Supplemental table 1. Genera abundance

Supplemental table 2. Per-sample Lactobacilli species abundance

Supplemental table 3. MetaCyc annotation

Supplemental table 4. EC annotation

Supplementary Figure 1. Diagram of the number of clean reads randomly selected from a sample, showing species diversity within a single sample. One curve represents the sample, The curve tends to be flat, indicating an adequate amount of sequencing data.

Supplementary Figure 2. The abscissa indicates sample grouping, and the ordinate indicates the alpha diversity index value under different groupings. a: The Chao1 index was used to estimate the total number of OTUs contained within a sample. b: Observed Indicates the actual number of OTU observations. c: A greater Simpson value higher microbial diversity. Key: *0.01<p<0.05 indicates a significant difference, **p<0.01 indicates an extremely significant difference; “NS” indicates no significant difference.

Supplementary Figure 3. This diagram stands for a hypothesis regarding the possible mechanisms underlying relationship between vaginal microbiota abundance and pathological changes of POF. Gray text boxes denote enriched microbes, product, or pathway in POF patients. Red text boxes denote the pathological changes and complications in POF patients.

Table 1

Clinical information of patients
|                                | Primary ovary failure | Control group | P value |
|--------------------------------|-----------------------|---------------|---------|
| Number of subjects            | 22                    | 29            |         |
| Age (year)                    | 30.50±3.17            | 29.79±3.99    | >0.05   |
| Waist to hip ratio (cm)       | 0.83±0.04             | 0.84±0.06     | >0.05   |
| BMI (kg/m^2)                  | 22.34±3.32            | 23.47±3.51    | >0.05   |
| AMH (pmol/L)                  | 0.06±0.03             | 4.31±2.25     | <0.001  |
| FSH (IU/L)                    | 75.28±27.60           | 5.82±1.22     | <0.001  |
| LH (IU/L)                     | 42.23±16.00           | 4.68±1.51     | <0.001  |
| E2 (pg/ml)                    | 17.47±19.75           | 35.56±17.19   | <0.01   |

Data shown as mean±SD. BMI, body mass index (kg/m²); Calculated using two independent samples T test.

**Figures**

A. Alpha diff boxplot

B. Weighted Unifrac Rank
Figure 1

Comparison of diversity and shift of vaginal flora composition of females with POF and healthy controls. A: The abscissa indicates the sample grouping, and the ordinate indicates the alpha diversity index value under different groupings. A greater Shannon value indicates higher diversity. B: Beta diversity analysis is used to compare species diversity between each sample. The abscissa represents all samples (between) and each group, and the ordinate represents the rank of the Unifrac distance. R>0 indicates that the between-group difference is greater than the within-group difference; R<0 indicates that within-group difference is greater than the between-group difference. P < 0.05 was considered as statistically significant. C: Horizontal and vertical coordinates represent the first and second main coordinates, respectively. Percentages indicate the contribution rate of the corresponding main coordinate to the sample difference, and the P value is the test p value of the corresponding main coordinate. The points represent the respective samples, different colours represent different groups. The horizontal box diagram illustrates the distribution of values of different groups on the first principal coordinate; the vertical box diagram illustrates the distribution of values of different groups on the second principal coordinate.
Figure 2
Comparison vaginal flora phylotype between groups A: Species that were able to classify significant differences between patients and groups. B: The abscissa is the name, the ordinate is the value of the log of relative
abundance, and different colours represent different groups. Species that were abundant in at least one group are not displayed. C: Species abundance map of the two groups. D: The correlation coefficient between top 30 most abundant species at all levels of classification. Right blue indicates a positive correlation and red indicates a negative correlation. Darker colour indicates a stronger correlation between the species. The species prefixes "k_", "p_", "c_", "o_", and "f_" on the left indicate that the species are annotated to the boundaries, gates, classes, orders, and subjects.
Figure 3

Coloured triangles represent sample groups in different environments or under different conditions. A: red: POF group, blue: control group; arrows represent different environmental factors; an acute angle between arrows indicates a positive correlation, a negative correlation is indicated by an obtuse angle. The length of the solid line of the environmental factor indicates the impact of the factor. Dotted lines pointing to the type of bacteria indicate the corresponding genus level. B: Heat map of Spearman correlation analysis between environmental factors and species. The abscissa indicates environmental factors, and the ordinate indicates species. The depth
of the colour visually shows the correlation between the species and the environmental factors. When $P < 0.05$, “+” marks the significant. when $P < 0.01$, “*” marks significance.
The predictive model based on genus-level abundance taxa using a random forests model A: The difference in contributions of different species enabled groups A and B to be distinguished; B: The ROC curve of a random forest model was constructed based on the sorted different species, where the abscissa is 1-specificity and the ordinate is sensitivity. When the area under the curve (AUC is 0.5-0.7, the accuracy is low; when AUC is 0.7-0.9, there is certain accuracy; when AUC is above 0.9, the accuracy is high). Larger AUC indicates better model prediction effect.
Figure 5

Functional predictions of vaginal flora of the POF and control groups A: The abscissa is the log value obtained after KO has a significant effect in different groupings through LDA, the threshold for LDA was 2. Different colours represent that the EC is enriched in different groups of samples. B: The abscissa is the log value obtained after MetaCyc_pathway has a significant effect in different groupings through LDA, the threshold for LDA was 2. Different colours represent that the MetaCyc_pathway is enriched in different groups of samples.
Figure 6

Species abundance map between POF with menopause A: Phylum level barplot; B: Genus level barplot; C: Species of significant differences between POF and menopause.

**Supplementary Files**

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

- SupplementaryFigure3.tif
- SupplementaryFigure1.tif
- SupplementaryFigure2.tif
- SupplementaryTable1.xlsx
- SupplementaryTable4.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx