Characterization of the vaginal microbiota of women with premature ovarian insufficiency

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Research

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35 Abstract

36 **Background:** Premature ovarian insufficiency (POI) is a complex reproductive endocrine
37 disease that can affect multiple systems. It is highly heterogeneous in etiology and the exact
38 etiology remains unclear. Infectious factors may be related to POI, but researches on the
39 alterations of microbiome in POI patients are scarce.

40 **Results:** Vaginal swabs were collected from 52 POI patients (29 for sequencing, 23 for
41 validation) and 46 healthy individuals of comparable age (26 for sequencing, 20 for
42 validation). 16S rRNA gene sequencing targeting the V3-V4 hypervariable regions was
43 performed to evaluate the alterations of vaginal microbiota in POI patients. The relative
44 abundance of *Actinobacteria* (23.34% vs 10.65%, $P=0.017$), *Atopobium* (11.11% vs 0.01%, P
45 < 0.001), and *Gardnerella* (8.05% vs 3.14%, $P=0.002$) were significantly increased in POI
46 patients, while *Bifidobacterium* (3.95% vs 7.44%, $P=0.017$) was significantly decreased.
47 Cluster analysis of dominant strains showed that the proportion of POI patients whose
48 predominant bacteria were not *Lactobacillus* was increased than the control group, but the
49 difference was not statistically significant (8/29 vs 2/26, $P=0.105$). More interestingly, these
50 changes in vaginal microbiota were significantly correlated with declined ovarian function in
51 POI patients, including decreased ovarian reserve, ovarian endocrine disruption, and
52 symptoms of perimenopausal syndrome. *Actinobacteria*, *Atopobium*, and *Gardnerella*
53 appeared to be detrimental to ovarian function, while *Bifidobacterium* seemed to be beneficial.

54 **Conclusions:** The present study revealed the correlation between vaginal microbiota and POI,
55 fill the gap in the field of microbial and POI research and provide a new research strategy for
56 POI. But the causal relationship is still unclear, and we could not clarify detailed roles of
57 specific constituents of the vaginal microbiota in the pathogenesis of POI. Therefore, future
58 studies are needed to explore potential mechanisms underlying the association of vaginal
59 microbiota and POI.

60 **Keywords:** Premature ovarian insufficiency, Vaginal microbiota, 16S rRNA gene sequencing,
61 Ovarian reserve, Ovarian function.

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76 **Background**

77 Premature ovarian insufficiency (POI) is a clinical syndrome defined by loss of ovarian
78 activity before 40 years old. Clinically, POI is characterized by menstrual disturbance
79 (oligomenorrhea or amenorrhea) with raised gonadotrophins and low estradiol¹. According to
80 the epidemiological data, the prevalence of POI is approximately 1%, population
81 characteristics such as ethnicity may affect the prevalence¹⁻³. Recently, it is believed that the
82 morbidity is increasing, the reported morbidity may be lower than it actually is, and the age of
83 onset tends to be younger. There is no effective method to predict the occurrence of POI now,
84 and when patients reach the diagnostic criteria, their ovarian function is often nearly complete
85 failure.

86 POI is a complex disease that can affect multiple systems. The clinical manifestation differs
87 between individuals, mainly including menstrual disturbance, infertility and symptoms of
88 perimenopausal syndrome¹. Besides, POI patients may experience complications such as
89 cardiovascular disease and osteoporosis, mainly due to the lack of estrogen^{4,5}. A diagnosis of
90 POI also has a significant negative impact on psychological wellbeing and quality of life^{1,6}.
91 Therefore, early prevention, early detection and early intervention to reduce the risk of
92 long-term complications and other systemically related diseases of POI are very important.
93 The clinically recommended treatments are hormone replacement therapy (HRT) and in vitro
94 fertilization-embryo transfer (IVF-ET) with eggs donation based on HRT¹. However, for those
95 with HRT contraindication or no source of donated eggs, they often suffer from the disease
96 due to the absence of effective alternative therapies.

97 POI is highly heterogeneous in etiology and the exact etiology remains unclear. Although a
98 wide spectrum of causes has been considered, including genetic^{2,7-9}, autoimmune^{3,10},
99 iatrogenic¹¹⁻¹³ or environmental pollutants^{14,15}. However, as many as half of patients are
100 diagnosed with idiopathic POI because the causal factors are unknown. The complexity of

POI, the increasing morbidity, the decreasing age of onset, and the lacking of early warning and treatment methods all indicate that it is of great importance to study POI.

The vaginal microbiota accounts for about 9% of the total human microbiota and remains under-studied despite its importance for female health and future generations¹⁶⁻¹⁹. The bacteria inhabiting the human vagina are thought to be the first line of defense against vaginal infection as a result of both the competitive exclusion and direct killing of other, pathogenic microbes^{16,20,21}. Disruptions of normal vaginal microbiota have long been linked to pelvic inflammatory disease²², miscarriages²³, and prematurity^{24,25}. The composition of vaginal microbiota is quite dynamic, it is influenced to a large extent by factors like age, ethnicity, genetics, sexual activity, diet, hygiene status, and gynecological or reproductive status of women^{17,20,26-28}. Apart from these, the previous studies have also found considerable changes in the vaginal microbiota of postmenopausal women²⁹⁻³². *Lactobacilli* is the dominant bacteria in the vaginal of most women, while it dramatically decreases in postmenopausal women, with the increase of non-lactic acid-producing bacteria, and the diversity of vaginal microbiota also increases³². Changes in vaginal microbiota lead to reduced lactic acid production and an increasing in the secretion of inflammatory factors such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-8, which activate the immune system and cause the body to be in a chronic inflammatory state³³⁻³⁶. These findings suggest that vaginal microbiota is very important for maintaining female reproductive health and there is a relationship between vaginal microbiota and ovarian function.

Menopause is a sign of ovarian aging. As a pathological ovarian aging, the reproductive endocrine status of POI patients is similar to that of postmenopausal women^{1,37}, while whether their vaginal microbiota will undergo similar changes is still unknown and remains to be explored. In addition, inflammation and autoimmunity are important factors that affect ovarian function and promote the occurrence and development of POI^{3,38}. Moreover, cross-sectional studies have also found that the levels of TNF- α , IL-1 α , IL-1 β , IL-6 in the serum and (or) follicular fluid of POI patients are significantly higher than those in healthy

controls^{39,40}. And the levels of these cytokines have significant correlations with sex hormones. Considering the important role of vaginal microbiota in maintaining female reproductive health, as well as the potential connection between POI and chronic inflammation caused by changes of vaginal microbiota, it is of great importance to explore the change pattern of vaginal microbiota in POI patients.

The purpose of this case-control study was to draw the characteristic spectrum of vaginal microbiota in POI patients by comparing with healthy women of comparable age, and to analyze the correlation between changes in vaginal microbiota and the declined ovarian function. Through this research, we want to lay a foundation for further in-depth exploration of the etiology and pathogenesis of POI, and provide new research strategies for early detection and treatment of POI.

Methods

Study population and sample collection

This was a case-control study to describe the characterization of the vaginal microbiota of POI patients. POI is defined as oligo/amenorrhea for at least 4 months before 40 years old, and an elevated follicle stimulating hormone (FSH) level > 25 IU/L on two occasions > 4 weeks apart¹. Women younger than 40 years old with regular menstruation and normal ovarian function were included as the control group. Exclusion criteria were as follows: (1) women during pregnancy or lactation, (2) women who have taken exogenous sex hormones in the past three months, (3) women who have received antibiotics, probiotics, prebiotics, intravaginal drugs, etc. in the last month, (4) women who are suffering from serious organic diseases and requiring long-term medical treatment, (5) women who are participating in other clinical trials.

This study was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All participants agreed to join the study and signed an informed consent form. From April 2018 to May 2019, a total of 52 eligible POI patients and 46 controls were included in the Menopause & Ovarian Aging Specialty Clinic of Tongji Hospital. Participants' basic clinical characteristics data were obtained from questionnaires and electronic medical records by experienced interviewers. The information included in the analysis included demographic characteristics, lifestyle, reproductive history, menstrual history, serum basal sex hormone levels (days 2-5 of the menstrual cycle for controls), Kupperman index (KMI) scale, Hospital anxiety and depression (HAD) scale.

Vaginal swab samples were collected for each participant by experienced gynecologists. All samples were obtained in compliance with clinical ethics regulations, and the principle of sterility was strictly observed during the sampling process. Samples from 29 POI patients and 26 controls were used for 16S rRNA gene sequencing, while samples from the other 23 POI patients and 20 controls were used to verify the sequencing results by quantitative polymerase chain reaction (qPCR). All samples were stored at -80°C until assayed. Specimens from all participants were processed similarly in terms of sample collection, storage, DNA extraction, library preparation and sequencing.

DNA extractions

Genomic DNA was extracted from archived vaginal swab specimens using the E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer's instructions. The reagent which was designed to uncover DNA from trace amounts of sample has been shown to be effective for the preparation of DNA of most bacteria. Nuclear-free water was used for blank. The total DNA was eluted in 50 µl of elution buffer and stored at - 80°C until measurement in the PCR by LC-Bio Technology Co., Ltd, Hang Zhou, Zhejiang Province, China.

PCR amplification and 16S rRNA gene sequencing

The V3-V4 hypervariable regions of the bacterial small-subunit (16S) rRNA gene was amplified with slightly modified versions of primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3')⁴¹. The 5' ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. PCR amplification was performed in a total volume of 25 µl reaction mixture containing 50 ng of template DNA, 12.5 µl PCR premix, 2.5 µl of each primer, and PCR-grade water to adjust the volume. The PCR conditions to amplify the bacterial 16S fragments consisted of an initial denaturation at 98°C for 30 seconds, 32 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 45 seconds, and then a final extension at 72°C for 10 minutes. The PCR products were confirmed with 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water, instead of a sample solution, was used to exclude the possibility of false-positive PCR results as a negative control. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on NovaSeq PE250 runs platform.

Bioinformatics analysis

Samples were sequenced on an Illumina NovaSeq platform according to the manufacturer's recommendations, provided by LC-Bio. Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (v1.2.8)⁴². Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4)⁴³. After dereplication using DADA2, we obtained feature table and feature sequence^{44,45}. Alpha

diversity and beta diversity were calculated by normalized to the same sequences randomly. Then according to SILVA (release 132) classifier, feature abundance was normalized using relative abundance of each sample.

Alpha diversity was applied in analyzing complexity of species diversity for a sample through five indices, including Observed-otus, Chao1, Shannon, Simpson, Goods_coverage, and all these indices in our samples were calculated with QIIME 2⁴⁶. The Shannon and Simpson indices provide a quantitative measure of species diversity (richness and evenness), whereas the Observed-otus and Chao1 indices provide a quantitative measure of species richness. Goods_coverage index refers to the microbial coverage and actually reflects whether the sequencing results represent the true situation of the sample. Beta diversity were evaluated by QIIME2, the graphs were drawn by R package. Blast was used for sequence alignment, and the feature sequences were annotated with SILVA (release 132) and NT-16S database for each representative sequence. A cluster analysis and a LEfSe analysis were conducted to compare differences in the composition of vaginal microbiota. Other diagrams were implemented using the R package (v3.5.2). Bioinformatic analysis was performed using the OmicStudio tools at <http://www.omicstudio.cn/tool>.

Quantitative PCR and data analysis

Quantitative PCR reactions were performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) in samples from the validation population to further verify the results of 16S rRNA gene sequencing. All qPCR reactions contained 10 µl of 2× SYBR Green Master Mix, 2 µl of primers (final concentration 10 pm/µl), 500 ng of the DNA template. The primer sequences were listed in Supplementary table 1. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 65°C for 30 s, extension at 72°C for 30s, and a fluorescence acquisition step at 72°C for 10 min. DNAs extracted from vaginal samples were amplified in triplicate, and the mean values were used for statistical

analysis. Amplification and detection were performed on an ABI 7900HT Fast Real Time PCR system (Applied Biosystems). The delta threshold cycle (ΔC_t) value was calculated and used to compare the differences between groups in the abundance of the flora. The higher the ΔC_t value, the lower the abundance of the flora in the sample.

Statistical analysis

Shapiro-Wilk test was conducted to test the distribution types of continuous variables. Continuous variables conforming to normal distribution were presented as mean $\pm$ SD (standard deviation) and compared by group using the Student's *t* test, while variables with skew distribution were expressed as median (interquartile, IQR) or median (range) and compared using the nonparametric Wilcoxon rank sum test. Categorical variables were expressed as frequencies (percentages, %) and compared using the chi-square test. KMI scores ranging from 0-6, 7-15, and > 16 were used to rate the degree of severity as none, mild and moderate to severe, respectively⁴⁷. HAD scores ranging from 0-7, and > 7 represented normal and anxiety/depression, respectively⁴⁸. Spearman correlation was used to analyze the correlation between vaginal microbiota and clinical indicators related to ovarian reserve, ovarian endocrine function, and symptoms of perimenopausal syndrome. Meanwhile, for each important phylum or genus with significant differences, we constructed the received operating characteristic (ROC) curve and computed the area under the curve (AUC) value. A two-tailed $P < 0.05$ was considered statistically significant. All data were analyzed using IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the study population

The age range of the 55 participants (29 POI patients, 26 controls) whose samples were used for 16S rRNA gene sequencing was 17-39 years old. Among POI patients, the median age

was 36 (IQR: 28-38.5), the mean body mass index (BMI) was 21.48 ± 3.02 (Table 1). Among the controls, the median age was 33 (IQR: 28.75-38.25), the mean BMI was 20.89 ± 2.39 . There were no significant differences between the two groups in age and BMI, as did other demographic characteristics, lifestyle, reproductive history, and HAD scores ($P > 0.05$) (Table 1, Supplementary table 2). Whereas, the age of menarche in the POI group was significantly lower than that of the controls ($P=0.022$). Moreover, FSH and luteinizing hormone (LH) were significantly higher in the POI group ($P < 0.001$). Serum levels of testosterone (T), estradiol (E2), anti-mullerian hormone (AMH), and inhibin B were lower among POI patients than the controls ($P < 0.001$, $P=0.001$, $P < 0.001$, $P < 0.001$, respectively). Levels of prolactin (PRL) and progesterone (PRG) were not significantly different between the two groups ($P > 0.05$). These differences were basically in line with the clinical phenotype and pathophysiological characteristics of POI.

Differences in the vaginal microbiota diversity between POI patients and the controls

No significant difference was found in the alpha diversity of vaginal microbiota between POI patients and the controls (Supplementary Fig. 1 A-E). Whereas, beta diversity was significantly different between the two groups according to the Anosim (analysis of similarities, $R=0.083$, $P=0.01$) and showed in PCA (principal component analysis) plots (Supplementary Fig. 1F).

Alterations in the composition of vaginal microbiota in POI patients

The dominant vaginal bacteria in both POI patients and the controls were *Firmucutes* at the phylum level and *Lactobacillus* at the genus level (Supplementary Fig. 2A, E). The top five bacterial genera in relative abundance in all samples were *Lactobacillus*, *Atopobium*, *Gardnerella*, *Streptococcus*, and *Bifidobacterium*, respectively belonging to *Firmucutes* and *Actinobacteria* (Fig. 1A). Moreover, at the phylum level, POI patients had a significantly higher abundance of *Actinobacteria* (23.34% vs 10.65%, $P=0.017$), while the abundance of *Firmucutes* was lower, but it was not statistically significant (72.34% vs 83.99%, $P=0.162$).

(Fig. 1B). At the genus level, the relative abundance of *Atopobium* and *Gardnerella* were significantly increased in POI patients (11.11% vs 0.01%, $P < 0.001$; 8.05% vs 3.14%, $P=0.002$), while the relative abundance of *Bifidobacterium* was significantly decreased (3.95% vs 7.44%, $P=0.017$). The relative abundance of *Lactobacillus* was decreased in POI patients but showed no significant difference between the two groups (62.98% vs 79.54%, $P=0.08$) (Fig. 1C).

At the species level, hierarchical clustering of bacterial community composition data showed that the 55 vaginal samples could be assigned to six distinct clusters based on differences in the composition of relative abundances of bacterial taxa (Fig. 2A). The average proportion of *Lactobacillus crispatus* in cluster A ($N=15$) was 95.89% (95% CI: 93.92%, 97.86%), while the average proportion of *Lactobacillus iners* in cluster B ($N=16$) was 92.09% (95%CI: 87.83%, 96.35%), and that of *Lactobacillus* sp. L-YJ in cluster C ($N=8$) was 54.47% (95%CI: 46.33%, 62.61%). The communities in cluster D ($N=2$) and E ($N=4$) were dominated by uncultured *Lactobacillus* sp. and *Bifidobacterium*, respectively. Finally, communities in cluster F ($N=10$) exhibited greater diversity and evenness and included several codominant taxa, including *Atopobium*, *Streptococcus*, *Gardnerella*, and *Haemophilus*. *Lactobacillus* was still the dominant strain in most of the population (19/29 in the POI group, 22/26 in the control group), while the proportion of cluster F in the POI group (8/29) was higher than that in the control group (2/26), but the differences were both not statistically significant ($P=0.119$, $P=0.105$) (Fig. 2B).

To identify the specific and key bacterial taxa associated with POI, LEfSe analysis was performed to generate the cladogram of the vaginal microbiota (Fig. 3B). At the phylum level, *Actinobacteria* was significantly enriched in POI patients (Fig. 3A). At the genus level, *Atopobium* and *Gardnerella* were dramatically enriched in POI patients, whereas *Bifidobacterium* was significantly enriched in the controls. According to the LEfSe analysis, these were all key phylotypes differentiating vaginal microbiota of POI patients and controls, and might be potential microbiological markers for discriminating patients with POI.

Collectively, these differences in the variation of vaginal microbial composition revealed a correlation between vaginal microbiota and POI.

Identification of key bacterial taxa by qPCR

Quantitative PCR was performed to detect the above key bacterial taxa in samples from the validation population, including *Firmucutes*, *Actinobacteria*, *Lactobacillus*, *Bifidobacterium*, *Gardnerella vaginalis*, and *Atopobium vaginae*, and further verified the results of 16S rRNA gene sequencing. The abundance of *Actinobacteria*, *Gardnerella vaginalis*, and *Atopobium vaginae* were significantly enriched in the POI group ($P=0.001$, $P=0.007$, $P=0.010$, respectively), and no statistically difference was found in *Firmucutes* ($P > 0.05$), in accordance with the results obtained by 16S rRNA gene sequencing (Supplementary Fig. 3). *Lactobacillus* was significantly decreased in POI patients ($P=0.006$), and no statistically difference was found in *Bifidobacterium* ($P > 0.05$), while the sequencing results showed that *Lactobacillus* was decreased in POI patients but not statistically significant, and *Bifidobacterium* was significantly decreased.

Analyses of correlations between vaginal microbiota and clinical indicators related to ovarian reserve, ovarian endocrine function, and symptoms of perimenopausal syndrome

The association between vaginal microbiological characteristics (alpha diversity indices, relative abundance of bacterial taxa) and clinical indicators related to ovarian reserve (AMH, inhibin B), ovarian endocrine function (FSH, LH, PRL, PRG, T, E2), and symptoms of perimenopausal syndrome (KMI score) were investigated. No significant correlation was found between alpha diversity and these clinical indicators ($P > 0.05$) (Supplementary Fig. 4), as were the relative abundances of *Firmucutes*, *Lactobacillus*, and *Streptococcus* (Fig. 4). The relative abundances of *Actinobacteria*, *Gardnerella*, and *Atopobium* were all significantly negatively correlated with serum AMH and inhibin B levels, which were usually decreased in

POI patients, while significantly positive correlations were found in *Bifidobacterium*. The relative abundances of *Actinobacteria*, *Gardnerella*, and *Atopobium* were all significantly positively correlated with serum FSH and LH levels, which were usually increased dramatically in POI patients, while *Bifidobacterium* was significantly negatively correlated with serum FSH levels. E2 is one of the most important steroid hormones secreted by the ovaries, and its level is significantly reduced in POI patients. The relative abundance of *Atopobium* was significantly negatively correlated with serum E2 levels, while *Bifidobacterium* was significantly positively correlated with serum E2 levels. The KMI score reflects the severity of the symptoms of perimenopausal syndrome. We found that the relative abundances of *Gardnerella* and *Atopobium* were all significantly positively correlated with the KMI score. Collectively, the increase of *Gardnerella* and *Atopobium* and the decrease of *Bifidobacterium* in vaginal microbiota were significantly correlated with declined ovarian reserve, endocrine disruption, and symptoms of perimenopausal syndrome in POI patients.

The potential of key bacterial taxa in the vagina to predict POI

For key bacterial taxa with significant differences in relative abundance between the two groups, including *Actinobacteria*, *Gardnerella*, *Atopobium* and *Bifidobacterium*, ROC curves were constructed and AUC values were calculated to assess their potential to predict POI. The accuracies of *Gardnerella* and *Atopobium* in predicting POI were considered acceptable, the corresponding AUC values were 0.732 and 0.759, respectively, which were both greater than 0.7⁴⁹ (Fig. 5). However, the accuracies of *Actinobacteria* and *Bifidobacterium* were very low, with AUC values of 0.685 and 0.678, respectively, which were both less than 0.7⁴⁹. Our results indicated that the accuracy of a single bacterial taxa for predicting POI was limited, and more other relevant indicators may need to be combined to provide improved early detection of POI.

Discussion

Principal findings of the study

In this case-control study, with 16S rRNA gene high-throughput sequencing technology, we found that the relative abundance of *Actinobacteria*, *Atopobium*, and *Gardnerella* were significantly increased in POI patients, while *Bifidobacterium* was significantly decreased. Moreover, these changes in vaginal microbiota were significantly correlated with declined ovarian reserve, endocrine disruption, and symptoms of perimenopausal syndrome in POI patients. These bacterial taxa may be potential new biomarkers for POI and related to the occurrence and development of POI, but further researches and explorations are needed.

Interpretation of the findings

POI is highly heterogeneous in etiology and the exact etiology remains unclear. Infectious factors may also be related to POI, such as mumps virus, human immunodeficiency virus (HIV), varicella zoster virus, and shigella⁵⁰. While, there are only case reports or epidemiological data, lacking of etiological mechanism research. Due to the limitations of traditional culture-based techniques, our current findings may be just the tip of the iceberg. Besides, most of these reports focus on extraneous virous infection, while it is still unclear whether the disturbance of organism microbiota, such as vaginal microbiota, the most important microbiota of the female reproductive system, are related to POI. Under this circumstance, we carried out this research, hoping to contribute to this field.

In recent years, a large number of researches have shown that the vaginal microbiota plays an important role in maintaining the health of female reproductive system^{16,27,51-57}. Although other anaerobic and aerobic bacteria colonize the vagina, *Lactobacillus* is the dominant species in most women^{32,58}. The relative abundance of *Lactobacillus* is significantly reduced in postmenopausal women^{17,59}, and a similar change has been observed in women with

premature ovarian failure (POF)³³. In our study, we also observed a decrease in *Lactobacillus* in POI patients, but the difference was not statistically significant. With the in-depth study of the etiology and the accumulation of clinical cases, doctors and researchers have gradually realized that ovarian aging is a group of diseases with diverse clinical manifestations, complex etiology, and progressive development, including the hidden stage, the biochemical abnormal stage and the clinical abnormal stage, and menopause, POF and POI represent different stages in the progression of ovarian aging^{1,37,60,61}. POI is a disease state that occurs when ovarian function declines to a certain stage before the age of 40. To a certain extent, it can reflect the diversity and progress of ovarian aging. However, menopause and POF can only represent the terminal stage of ovarian failure and cannot reflect the progression of the disease. In the definition of POI and POF, the diagnostic threshold of FSH is different. The diagnostic criteria of POF is FSH > 40 IU/L, while the diagnostic criteria of POI is FSH > 25 IU/L. The latter lowers the diagnostic threshold of FSH in order to identify POI patients as early as possible to achieve the purpose of early diagnosis and early treatment. Therefore, in the following analysis, we divided the POI patients into two groups according to the level of FSH and then conducted a subgroup analysis. We found that POI patients with FSH levels of 25-40 IU/L compared with the control group, the relative abundance of *Lactobacillus* was lower but not statistically significant, while the relative abundance of *Lactobacillus* in POI patients with FSH $\geq$ 40 IU/L was significantly lower than that of the control group (58.77% vs 79.54%, $P=0.032$) (Supplementary Fig. 5C). Our results suggest that the abundance of vaginal *Lactobacillus* may gradually decrease during the decline of ovarian function, and further studies are needed in the future to explore whether changes in the vaginal microbiota of patients are progressing gradually like ovarian aging.

The bacteria inhabiting the human vagina are thought to be the first line of defense against vaginal infections²⁰. *Lactobacillus* inhibit binding of other bacteria to epithelial cells and produce substances, including lactic acid and hydrogen peroxide, that directly kill or inhibit the growth of other microorganisms^{16,21,62}. Intracellular lactic acid is an epigenetic regulator of gene activity, lactic acid blocks histone deacetylases, thereby enhancing gene transcription

and DNA repair⁶³. Another novel property of lactic acid is its ability to induce autophagy in epithelial cells to degrade intracellular aged or defective organelles, dysfunctional proteins as well as bacteria, viruses, protozoa, and their components, and promote homeostasis⁶⁴⁻⁶⁶. More importantly, the ability of *Lactobacillus* to inhibit infection without inducing inflammation can even inhibit the release of proinflammatory factors when the innate immunity is activated²¹. However, epigenetics, autophagy and inflammation are closely related to the occurrence and development of POI, and the reduction of *Lactobacillus* in the vagina of POI patients may induce the occurrence of POI and accelerate the development of it through the above-mentioned mechanisms, but further in-depth studies are essential to verify this conjecture^{38,67,68}.

We found that the relative abundances of *Atopobium* and *Gardnerella*, which both belonged to *Actinobacteria*, were significantly increased in POI patients, and were significantly correlated with declined ovarian reserve, endocrine disruption, and symptoms of perimenopausal syndrome in POI patients. Whereas, subgroup analysis only found significant differences between POI patients with FSH ≥ 40 IU/L and the control group (15.28% vs 0.01%, $P < 0.001$; 11.08% vs 3.14%, $P=0.002$) (Supplementary Fig. 5E, F). *Gardnerella vaginalis* and *Atopobium vaginae* are anaerobic bacteria, which are one of the most common pathogens causing bacterial vaginitis. The excessive growth of *Gardnerella vaginalis* can promote the release of TNF- α , IL-6, and IL-8³⁴⁻³⁶. In vitro experiments have also found that it can activate the NLRP3 inflammasome signaling pathway⁶⁹. *Atopobium vaginae* has also been found to be related to gene mutations related to the TLR2 signaling pathway, which is one of the key pathways of inflammation⁷⁰. The increase of *Gardnerella vaginalis* and *Atopobium vaginae* in POI patients may enhance the inflammatory response through a variety of mechanisms, leading to a chronic inflammatory state, thereby affecting the function of ovaries.

The relative abundance of *Bifidobacterium* was dramatically decreased in POI patients. *Bifidobacterium* is mainly colonized in the human intestines, and only a small amount is colonized in the vagina. As a kind of probiotic, it has the effects of anti-inflammatory,

improving immune function, and resisting oxidative damage⁷¹⁻⁷³. Clinical studies have found that *Bifidobacterium* can improve the metabolism and cardiovascular function of postmenopausal women⁷⁴⁻⁷⁶. Studies have also found that *Bifidobacterium* can regulate the secretion of sex hormones in polycystic ovarian syndrome patients and significantly reduce serum LH levels and LH/FSH ratio⁷⁷. There is no research on probiotics in POI patients currently. Whether probiotics such as *Bifidobacterium* can delay the development of POI or contribute to the treatment of POI is still unknown and remains to be explored in the future. Moreover, due to the influence of human internal factors and environmental factors, the individual differences in the use of probiotics are relatively large. The mainstream trend in the future is personalized probiotic intervention strategies. This is also true for POI patients. Individualized probiotic intervention studies need to be carried out according to the patient's own situation.

Strengths and limitations

The strength of our study lies in the comprehensive description of vaginal bacterial communities associated with POI archived through 16S rRNA gene sequencing. We identified bacterial taxa that were enriched in POI patients and evaluated their value in predicting POI. Particularly, the correlations between the vaginal microbiota and the clinical characteristics, mainly ovarian reserve and ovarian endocrine function indices, were analyzed. However, the limitations of the current study also merit careful consideration. We only focused on changes in vaginal microbiota in POI patients, but did not study the microbiota in other parts of the body, such as gut, mouth, skin, etc. Furthermore, our results can only suggest that there is a correlation between vaginal microbiota and POI, but the causal relationship between them is still unclear, and we could not clarify detailed roles of specific constituents of the vaginal microbiota in the pathogenesis of POI. Therefore, future studies involving larger and wider study populations, prospective cohort, and animal models are needed to more comprehensively describe the composition of vaginal microbiota under different stages of ovarian aging and explore potential mechanisms underlying the association of the vaginal

microbiota and POI. Researches on the gut microbiota of POI patients may also help to clarify the role of microbiota in POI and improve many aspects of prevention and treatment.

Conclusions

In summary, our study found that the relative abundance of *Actinobacteria*, *Atopobium*, and *Gardnerella* were significantly increased in POI patients, while *Bifidobacterium* was significantly decreased. Moreover, these changes in vaginal microbiota were significantly correlated with declined ovarian reserve, endocrine disruption, and symptoms of perimenopausal syndrome in POI patients. Our study revealed the correlation between vaginal microbiota and POI, filled the gap in the field of microbial and POI research and provided a new research strategy for POI. But the causal relationship is still unclear, and we could not clarify detailed roles of specific constituents of the vaginal microbiota in the pathogenesis of POI. Therefore, future studies are needed to explore potential mechanisms underlying the association of vaginal microbiota and POI.

Additional files

Additional file 1: Table S1. Primer sequences for quantitative PCR.

Additional file 2: Table S2. Clinical information of participants whose samples were used for 16S rRNA gene sequencing (demographics, lifestyle, menstrual and reproductive history, HAD score).

Additional file 3: Table S3. Phylum abundance.

Additional file 4: Table S4. Genus abundance.

Additional file 5: Table S5. Species abundance.

482 **Additional file 6: Table S6.** Dominant bacterial taxa and its abundance in each sample.

483 **Additional file 7: Figure S1.** Comparison of vaginal microbiota diversity of women with
484 POI and healthy controls. A-E. Comparison of alpha diversity index Observed-otus, Chao 1
485 index, Shannon index, Simpson index and Goods_coverage. F. Beta diversity was
486 significantly different between the two groups according to the Anosim (analysis of
487 similarities, $R=0.083$, $P=0.01$) and showed in PCA (principal component analysis) plots.

488 **Additional file 8: Figure S2.** Composition of the vaginal microbiota of POI patients and the
489 controls. Relative abundance was shown at the phylum (A), class (B), order (C), family (D),
490 genus (E) and species (F) levels.

491 **Additional file 9: Figure S3.** qPCR was used to validate the key bacterial taxa in samples
492 from the validation population. $P < 0.05$ was considered as statistically significant. * $P < 0.05$,
493 ** $P < 0.01$.

494 **Additional file 10: Figure S4.** No spearman correlation was found between alpha diversity
495 indices of vaginal microbiota and clinical indicators related to ovarian reserve, ovarian
496 endocrine function, and symptoms of perimenopausal syndrome. AMH, anti-mullerian
497 hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; PRG,
498 progesterone; T, testosterone; E2, estradiol.

499 **Additional file 11: Figure S5.** Subgroup comparison of the relative abundance of vaginal
500 microbiota based on serum FSH levels. $P < 0.05$ was considered as statistically significant.
501 *Comparison between POI patients with FSH levels of 25-40 IU/L and the control group, * P
502 < 0.05 , ** $P < 0.01$. #Comparison between POI patients with $FSH \geq 40$ IU/L and the control
503 group, # $P < 0.05$, ## $P < 0.01$. FSH, follicle stimulating hormone.

504 **Abbreviations**

505 POI: premature ovarian insufficiency; HRT: hormone replacement therapy; IVF-ET: in vitro
506 fertilization-embryo transfer; HIV: human immunodeficiency virus; TNF: tumor necrosis
507 factor; IL: interleukin; FSH: follicle stimulating hormone; KMI: Kupperman index; HAD:
508 hospital anxiety and depression; qPCR: quantitative polymerase chain reaction; LEfSe: linear
509 discriminant analysis effect size; SD: standard deviation; IQR: interquartile; ROC: receiver
510 operating characteristic; AUC: area under the curve; BMI: body mass index; LH: luteinizing
511 hormone; T: testosterone; E2: estradiol; AMH: anti-müllerian hormone; PRL: prolactin; PRG:
512 progesterone; PCA: principal component analysis; POF: premature ovarian failure.

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520 NO.82001498).

521 **Availability of data and materials**

522 Raw sequence data files for the samples described in this study were deposited to the NCBI
523 Sequence Read Archive. The data that support the findings of this study are available from the
524 corresponding authors upon reasonable request.

525 **Authors' contributions**

526 WJY, ZJJ and WSX conceived and designed the study. WJY, FYZ, YW, YSZ and LAY
527 recruited participants and collected their vaginal samples and related information. WJY and
528 ZJJ analyzed the data. WJY and WSX drafted and revised the paper. All authors reviewed the
529 results and approved the final version of the manuscript.

530 **Competing interest**

531 The authors declare that they have no competing interests.

532 **Ethics approval**

533 This study and all its protocol were approved by the Ethical Committee of Tongji Hospital,
534 Tongji Medical College, Huazhong University of Science and Technology. All participants
535 agreed to join the study and signed an informed consent form.

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Figure legends

Figure 1. Comparison of vaginal microbiota composition of women with POI and healthy controls. A. Sankey plot showed the top five bacterial genera in relative abundance in all samples, the width of the line indicated the relative abundance of the corresponding bacteria in each group. B, C. Differences in relative abundance at phylum and genus levels. $P < 0.05$ was considered as statistically significant. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Comparison of dominant vaginal bacterial taxa. A. Cluster analysis of vaginal bacterial communities found in 55 participants. B. Number of women in cluster A-F in the two groups.

Figure 3. Comparison of vaginal microbiota phylotype between groups. A. Histogram of the LDA scores for differentially abundant bacterial taxa. B. Taxonomic representation of statistically and biologically consistent differences between the two groups displayed by a cladogram. LDA, linear discriminant analysis.

Figure 4. Spearman correlation between the relative abundance of vaginal microbiota and clinical indicators related to ovarian reserve, ovarian endocrine function, and symptoms of perimenopausal syndrome. AMH, anti-mullerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; PRG, progesterone; T, testosterone; E2, estradiol. $P < 0.05$ was considered as statistically significant. * $P < 0.05$, ** $P < 0.01$.

Figure 5. ROC curves were constructed and AUC values were calculated to assess the potential of key bacterial taxa to predict POI. A. The AUC was 0.685 for *Actinobacteria*, $P=0.019$. B. The AUC was 0.732 for *Gardnerella*, $P=0.003$. C. The AUC was 0.759 for *Atopobium*, $P=0.001$. D. The AUC was 0.678 for *Bifidobacterium*, $P=0.023$. ROC, received operating characteristic; AUC, area under the curve.

Figures

Figure 1

Comparison of vaginal microbiota composition of women with POI and healthy controls A. Sankey plot showed the top five bacterial genera in relative abundance in all samples , the width of the line indicated the relative abundance of the corresponding bacteria in each group. B , C . Differences in relative abundance at phylum and genus level s . $P \leq 0.05$ was considered as statistically significant. $P \leq 0.05$, $P \leq 0.01$.

Figure 2

Comparison of dominant vaginal bacterial taxa. A. Cluster analysis of vaginal bacterial communities found in 55 participants B. Number of women in cluster A-F in the two groups.

Figure 3

Comparison of vaginal microbiota phylotype between groups. A. Histogram of the LDA scores for differentially abundant bacterial taxa. B. Taxonomic representation of statistically and biologically consistent differences between the two groups displayed by a cladogram. L D A, linear discriminant analysis

Figure 4

Spearman correlation between the relative abundance of vaginal microbiota and clinical indicators related to ovarian reserve, ovarian endocrine function and symptoms of perimenopausal syndrome. AMH, antimullerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; PRG, progesterone; T, testosterone; E2, estradiol. $P \leq 0.05$ was considered as statistically significant. $P \leq 0.05$, $P \leq 0.01$.

Figure 5

ROC curves were constructed and AUC values were calculated to assess the potential of key bacterial taxa to predict POI. A. The AUC was 0.685 for Actinobacteria, $P = 0.019$. B. The AUC was 0.732 for Gardnerella, $P = 0.003$. C. The AUC was 0.759 for Atopobium, $P = 0.001$. D. The AUC was 0.678 for Bifidobacterium, $P = 0.023$. ROC, receiver operating characteristic; AUC, area under the curve

Supplementary Files

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- [Supplementarytable1.doc](#)
- [Supplementarytable2.doc](#)
- [Figures.pdf](#)
- [Supplementarytable3Phylumabundance.xlsx](#)
- [Supplementarytable4Genusabundance.xlsx](#)
- [Supplementarytable5Speciesabundance.xlsx](#)
- [Supplementarytable6Dominantbacterialtaxa.xlsx](#)