Comparative Analysis of the Vaginal Microbiome Between women with Polycystic ovary syndrome and Healthy women: A Large-sample cross-sectional study

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Abstract

Abstract Objectives To investigate the vaginal microbiome (VMB) of a large sample size of polycystic ovary syndrome (PCOS) patients using 16S ribosomal RNA (16S rRNA) sequencing. Design A cross-sectional study Setting Ji Nan, China Sample A total of 1,446 subjects were recruited (PCOS, n=713, the controls, n=733). Methods Vaginal swabs were analyzed using 16S rRNA gene sequencing. Main outcome measures The microbiome diversity and composition of the PCOS group and control group were compared. In the PCOS prediction model, microbial interaction networks and functions prediction were investigated. Results The PCOS group had a higher alpha diversity in the VMB than controls ($P<0.05$), while higher intra-group variability was observed in PCOS ($P<0.05$). At the genus level, the proportion of Lactobacillus in the PCOS group decreased, while the proportion of Gardnerella and Ureaplasma increased (FDR<0.2). Gardnerella vaginalis, Prevotella buccalis, and Prevotella timonensis were identified as differential species and were strongly associated with blood parameters of PCOS. The VMB interaction network indicated that Prevotella and Lactobacillus may be key drivers in the PCOS group. Overall, 55 differentially predicted genes were found between PCOS and controls (FDR<0.25). Conclusions The PCOS group had a higher diversity in the vaginal microbiome and showed an enhanced level of heterogeneity. The proportion of Lactobacillus in PCOS group decreased, while the proportions of Gardnerella and Ureaplasma increased. These results warrant further research that can validate the correlation between PCOS and VMB.

Introduction

Polycystic ovary syndrome (PCOS) is a leading endocrine cause of female infertility characterized by oligo-amenorrhea or ovulatory dysfunction (OA), hyperandrogenism (HA), and polycystic ovarian morphology

(PCOM)[1]. The global prevalence of PCOS is estimated to be 5-15% based on the diagnostic criteria applied[2]. PCOS can also lead to metabolic manifestations such as obesity, hyperinsulinemia/insulin resistance (IR), and an increase in the risk of developing type 2 diabetes (T2D)[3, 4] and coronary heart disease[5]. One of the most widely used diagnostic criteria for PCOS is the Rotterdam criteria[6], which requires the presence of at least two of above three cardinal traits. This results in four different phenotypes: phenotype A: HA + OA + PCOM; phenotype B: HA + OA; phenotype C: HA + PCOM; phenotype D: OA+ PCOM, which diversifies the presentation of PCOS.

Despite its prominent impact on female reproductive and metabolic health, the etiology of PCOS remains unclear. Although attention has been focused on key genetic factors during the past decade[2], recent studies on PCOS intestinal microbiota have suggested a novel environmental-related disease mechanism[7, 8]. Apart from the gut, another important microbial niche in women is the vagina. Dysbiosis of the microbiome in the lower female reproductive tract has been implicated in a variety of diseases, such as preterm birth[9], sexually transmitted infections [10, 11], pelvic inflammatory disease[12], and gynecological cancers[13, 14]. Recent studies have also begun to explore the role of the vaginal microbiome (VMB) in PCOS. However, due to a limited sample size and complex confounding factors of the selected cohort, studies that used 16S ribosomal RNA (16s rRNA) gene sequencing techniques to compare women diagnosed with PCOS and healthy controls have led to different results regarding the diversity and composition of the vaginal microbiota[15, 16]. Furthermore, endocrine and metabolic characteristics vary among different PCOS subsets[17], but discrepancies in vaginal microbial PCOS subtypes have rarely been clarified.

In this study, we analyzed the vaginal microbial characteristics of 1,446 subjects using high-throughput sequencing to determine the microbial characteristics, bacterial community interactions, as well as functional prediction of the vaginal microbiota and different subsets related to PCOS.

METHODS

Study Participants

In this study, 1,446 subjects (713 in the PCOS group and 733 in the control group) were recruited from the Center for Reproductive Medicine, Shandong University, China, from March to September 2019. Basic demographic, socio-economic and life habits related characteristics were assessed using questionnaires, while the income level, vaginal douching frequency, and the frequency of sexual activity was matched between women with PCOS and healthy controls. PCOS was mainly diagnosed using Chinese Guidelines for the Diagnosis of PCOS[18] based on modified Rotterdam Criteria[6]. PCOS diagnosis requires the presence of oligomenorrhea (menstrual cycle that lasts longer than 35 days) or irregular uterine bleeding, combined with either clinical/biochemical signs of hyperandrogenism or a polycystic ovary indicated using ultrasonography (either more than twelve follicles (2–9 mm in diameter) and/or increased ovarian volume (>10 mm^3) in each ovary). Women who were diagnosed with congenital adrenal hyperplasia, testosterone-secreting tumors, Cushing's syndrome, or other causes that result in excess testosterone were excluded. The subjects included in the control group had regular menstrual cycles (21-35 days) and no clinical or biochemical evidence of hyperandrogenemia. The exclusion criteria was antibiotic usage within one month of swab sampling, vaginal douching or vaginal medication within one week of sampling, irritation around the genital area or abnormal vaginal discharge within one week of sampling, menstruation, and sexual intercourse within 48 hours. In addition, the PCOS group was divided into PA (PCOS phenotype A) subgroup (OA+HA+PCOM, n=171) and PD (PCOS phenotype D) subgroup (OA+PCOM, n= 542). The study was approved by the Institutional Review Board of Reproductive Medicine, Shandong University. Written informed consent was obtained from all participants enrolled in the study.

Laboratory Measurements

Serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), estradiol (E_2), Anti-Müllerian hormone (AMH), fasting insulin and fasting plasma glucose were determined, as previously reported[19]. To define insulin resistance, homeostasis model assessment (HOMA-IR) was made using the equation: fasting glucose (mmol/L) × fasting insulin (mIU/L)/22.5.

Vaginal swab preprocessing

Vaginal swabs collection was performed by two gynecologists following a strict sampling protocol. In brief, the participants assumed a lithotomy position and the gynecologist used a sterile swab to press against the posterior fornix after a sterile speculum was inserted into the vagina. After sampling, the swab head with vaginal discharge was immediately cut using a sterile surgical scissor and placed in a sterile tube. The sample was then placed in dry ice and transferred to a -80°C freezer within 6 hours for subsequent 16S rRNA gene sequencing.

DNA extraction and 16S rRNA amplicon sequencing

DNA was extracted from the vaginal samples using the Magnetic Soil and Stool DNA Kit (Tiangen Biotech, Beijing), following the manufacturer's instructions. The DNA samples were stored at -80degC. Then, the V1-V2 region of the 16S rRNA gene was amplified using the 27F and 338R primers. Processing of raw sequencing data was performed on a Illumina Novaseq platform. A coverage depth of at least 50,000 reads was generated in each sample. The median sequencing read depth was 58,457 reads. Data was processed using USEARCH (version 11). Reads of an inferior quality (MaxEE less than 1.00) and length less than 300 bp were removed before further analysis. We detected Chimeras using the gold database of UCHIME2[20]. The taxonomy of each amplicon sequence variant (ASV) was generated using UNOSIE3. Representative sequences were annotated using the Ribosomal Database Project (RDP) classifier and the RDP database (rdp_16s_v16_sp.fa)[21] with a confidence threshold of 0.8. Overall, 126,966,654 reads and 4,250 ASVs were obtained.

Vaginal microbiome diversity and composition analysis

Alpha diversity indicators, including the Chao1 richness estimator, Shannon Wiener diversity index, and Simpson diversity index were determined using the 'vegan' R package (v2.5). The correlation between alpha-diversity and other variables were evaluated using Spearman's rank correlation. The p-value was adjusted for multiple tests using the Benjamin – Hochberg method[22] , which is controlled by the False Discovery Rate (FDR). FDR values of <0.2 were considered to indicate statistical significance. The overall microbiota composition was analyzed based on Bray Curtis dissimilarity.

Random Forest classification

ASVs with a relative abundance of over 0.001% were added to the Random Forest classification model. The presence of this feature was confirmed using the Boruta algorithm[23] and was selected as important species for classification and was accordingly included in the final model.

Co-occurrence network of VMB

ASVs present in less than 10% of samples were excluded from the network analysis. Significant correlations between the relative abundance of ASVs were evaluated using Sparse Correlations for compositional data algorithm implemented in the SparCC python module (Friedman and Alm, 2012), while the corresponding networks were plotted using the R package, 'igraph'. Only correlations with an absolute correlation greater than 0.15 and an adjusted p-value of less than 0.05 were plotted.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

ASVs with a relative abundance much more than 0.001% were included and high abundance ASV taxonomy was mapped based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the R package, 'Tax4Fun;. The Wilcoxon test was performed to detect biomarkers of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that differed significantly between the PCOS group and the control group (FDR<0.25).

Statistical Analysis

The Wilcoxon rank-sum test was used to analyze clinical characteristics between the groups. Alpha diversity was indicated by the Shannon index, Chao1 index and Simpson index, through the Wilcoxon rank-sum test.

The β-diversity index compared between groups were directly presented using the Adonis nonparametric test. The Spearman correlation test was used to assess the relevance between clinical indicators and ASVs.

RESULTS

1. Clinical parameters of polycystic ovary syndrome and control participants

We summarized the baseline characteristics of each group of participants (Table 1). Compared with the control group, the PCOS group had higher levels of BMI, LH, E_2, AMH and T ($P<0.05$), while the highest levels of AMH, LH, and total testosterone was found in the PA group ($P<0.05$). As for the lipid metabolism profile, the levels of low-density lipoprotein (LDL), triglycerides (TG) and total cholesterol (TC) were significantly higher in the PCOS group than in the control group ($P<0.05$) (Supplementary Table 1).

2. The diversity and intra-group heterogeneity of the VMB was higher in PCOS patients

At the genus level, the proportion of *Lactobacillus* reduced (FDR¡0.2) and the proportion of *Gardnerella* and*Ureaplasma* increased in the PCOS group (FDR¡0.2) (Figure 1a), compared with the control group. At the species level, we found that *U. parvum* , *G. vaginalis* , *A. baumannii* , *P. buccalis* ,*P. timonensis* , and *P. acnes* were more abundant in the PCOS group, while the abundance of *L. Jesenia* ,*L. iners, B. breve,* and *L. pontis* were significantly depleted (FDR¡0.2) (Figure 1b).

The increase of the Shannon index and decrease of the Simpson index indicated that the VMB in the PCOS group had higher diversity than that of the control group ($P<0.05$) (Figure 2a,2b). The Chao 1 index and Shannon index of the PD subgroup was also higher than that of the control group ($P<0.05$)(Figure 2c,2d). However, there was no significant difference in diversity between the PA subgroup and the control group ($P>0.05$). The PCoA analysis indicated that there was no significant difference in the VMB structure between groups(Figure 3a,3b) ($P>0.05$). PCOS group had higher intragroup variation compared with control group ($P<0.05$) (Figure 3c)

We constructed a random forest model for PCOS. The feature confirmed by the Boruta algorithm23 was selected as an important species for classification accuracy. We could accurately distinguish PCOS patients from healthy controls, as indicated by the area under the receiver operating curve (AUC), which had a maximum value to 0.8 (Figure 4)

3. **Correlations between VMB and clinical indicators**

A total of 35 bacterial species from an overlap set of differential species between PCOS and controls and significant species in model of random forest model were used to analyze the correlation with clinical indicators. *G. vaginalis* was positively correlated with serum level of AMH, E_2, and P ($p<0.05$). AMH, LH and T showed the highest positive correlation strength with*U.parvum* and *A. baumannii* , but a negative correlation with *Prevotella* . In addition, HDL and TG levels were associated with the abundance of *L. acidophilus* , *P.buccali* and *U. parvum* (Figure 5).

4. *Lactobacillus crispatus* **and *Prevotella timonensis* drove changes in PCOS vaginal microbiota co-occurrence network**

Two networks were separately constructed for the PCOS and control groups. The topology of the two networks were similar. In both groups module 1 and 2 mostly contained *L. crispatus* , and *L.iners,* which showed a negative correlation with each other (Figure 6a,6b). The largest was module 3 in the two groups and was mainly composed of potential vaginal pathogens, including *G. vaginalis, P. bivia, P. timonensis, P. amnii, P. buccalis,P. disiens, A. vaginae, D. micraerophilus, S. sanguinegens,* and

A. christensenii.

Additionally, we observed that there were 43 connector nodes in both networks. The common connectors in both groups included*L.crispatus* ,*L. gasseri* , *G. vaginalis* and several other *Prevotella* . Connectors that were unique to the PCOS network, such as *L. iners* , *P. timonensis* , and*A. vaginae* , showed increased connectivity and may have had an impact on the strength of the network structure (Figure 6c,6d).

Additionally, we identified other important nodes that drove network shifts, which are indicated as larger red dots (Figure 7a). Mapping of the annotated species results showed that *L. crispatus* and*P. timonensis* were the key bacteria species that were involved in driving vaginal microbial interaction network changes in the PCOS group (Figure 7b).

5. **KEGG Functional Analysis**

We predicted the function of the vaginal microbiota using KO (Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous group) using the high abundance of vaginal bacteria. Microbiota functional pathways were particularly enriched the in metabolism of other amino acid in the control group (Figure 8a). Microbial differential functional genes in the lower reproductive tract of the PCOS patients were mainly enriched in the biosynthesis of cofactors, membrane protein, biosynthesis of secondary metabolites and cofactors, ABC transporters, biotin metabolism, and fatty acid biosynthesis and metabolism. The other 39 genes in the control group were enriched in peptide/nickel transport system permease proteins, phenylalanine, tyrosine, and tryptophan biosynthesis , fructose and mannose metabolism, butanoate metabolism, amino and nucleotide sugar metabolism (Figure 8b).

DISCUSSION

Main findings

This is the largest cross-sectional study conducted based on 16S rRNA sequencing that explored differences in VMB composition between the PCOS and control groups. Compared with the control group, we observed increased diversity and intra-group variability of the VMB in PCOS, including a significant declination in the abundance of*Lactobacillus* and the enrichment of potential pathogens, including *U . parvum* , *G. vaginalis* , *P. buccalis, P. timonensis,* and *A. baumanni* . Additionally, serum levels of LH, T, and AMH were closely associated with *U.parvum, A. baumannii,* and *P. buccalis* . We also found that opportunistic pathogens, such as *L. crispus, P. timonensis,* and *P. buccalis* contributed to alter the vaginal microbiome in PCOS and was identified as key bacteria, which drive changes in the vaginal microbial interaction network in patients with PCOS.

Strengths

Previous studies on the PCOS VMB were conducted using smaller sample sizes ($n < 100$) [15, 16, 24, 25] compared with our study ($n = 1614$). Our study was robust for the detection of differences in the vaginal microbiome between women with PCOS and healthy women. Microbiome–host interactions and functional predictions were analyzed in this study.

Limitations

A random forest model was conducted to classify PCOS, which showed the high accuracy of bacteria biomarkers for PCOS patients in Ji Nan (AUC=0.8), which indicated the significant association between PCOS and aberrant VMB. The ability of our model to distinguish PCOS has not been verified in other populations.

We excluded clinical symptoms, such as abnormal leucorrhea, pruritus vulva, and vaginal congestion at the time of sampling. Only asymptomatic case-control differences were observed and the VMB difference between symptomatic PCOS and controls was not yet determined. Therefore, we lack studies on symptomatic PCOS patients.

Interpretation

Since PCOS patients are characterized by irregular menstrual cycles and sex hormone disorders, while many studies have indicated that the diversity of vaginal bacterial species increases along with the decrease in the content of *Lactobacillus* [15, 16, 24]. We also found a significant decrease in the content of*Lactobacillus* and was significantly enriched in potential pathogens, such as *U. parvum, G. vaginalis, P. buccalis* ,*P. timonensis,* and *A. baumanni,* in PCOS patients. After *lactobacilli* spp. dominance was destroyed, *G.vaginalis* become the dominant bacterium in the vaginal biome[15, 16, 24]. *G. vaginalis* is closely associated with the

pathogenesis of bacterial vaginosis[26] and in combination with *U.parvum* further increases the risk of spontaneous preterm birth[27]. Further, the abundance of *P. buccalis* and *P.timonesis* were inversely associated with *Lactobacillus*abundance, which maintains vaginal homeostasis[28, 29]. *P . timonensis* abundance was associated with mucosal inflammation[30] and cervical intraepithelial neoplasia[31]. Additionally, *A. baumanniii*can cause preterm delivery and chorioamnionitis during pregnancy[32], as well as infection in vaginally delivered infants[33]. In conclusion, the reduction in*Lactobacillus* abundance in PCOS patients leads to changes in vaginal pH and destruction of the immune barrier[34], making it easier for potentially vaginal commensal anaerobic bacteria, such as *G. vaginalis, U. parvum, Prevotella* spp*., A. baumannii* to colonize and reproduce.

These potential vaginal pathogens mentioned above cause disease at a greater frequency and severity and can potentially result in BV, miscarriage, preterm birth, pre-cancerous, and cancerous cervical lesions[8, 35-38]. In addition, *B. breve* was a subdominant group of the vaginal microbiota in the VMB of healthy indivuals[39, 40] but its abundance was depleted in the PCOS group. Giordani et al.[39] found that the oral administration of *B. breve* as a probiotic bacteria can prevent urogenital infections. Since PCOS is a common cause of infertility and increases the risk of abortion, fetal arrest, and preterm birth[15], we suspect that there is a correlation between altered compositions of the VMB and adverse reproductive outcomes in PCOS patients. Therefore, the adverse pregnancy prognosis of PCOS patients may not be related only to the disease itself but also to their vaginal microbiological status, which should not be neglected either. Modifying the composition of vaginal microorganisms may be a novel method of clinical treatment for clinicians to improve the clinical pregnancy outcome in patients with PCOS. Nevertheless, we cannot ignore the role of endocrine disorders in PCOS, as they may play a role in impacting the diversity and composition of the vaginal microbiota. There may be a mutual causal relationship between PCOS and vaginal microbiota.

Intriguingly, the within-group variability was significantly higher in the PCOS group than in the control group. PCOS phenotype A patients showed slightly lower vaginal microbiota diversity compared with PCOS phenotype D patients. Serum levels of testosterone, LH, and AMH in PCOS may contribute to this discrepancy in findings. First, a high level of testosterone is associated with an elevated abundance of*Lactobacillus* [24]. Some researchers have also demonstrated that testosterone can alter the gut microbiota and reduce alpha-diversity[7, 8, 41]. Second, this study indicates that AMH is the biochemical marker that is most closely with the vaginal microbiota of PCOS women. Third, serum levels of T, LH and AMH were positively correlated with *U. parvum, A. baumanniii*abundances, but negatively correlated with the abundance of *P. buccalis* , suggesting that the abundance of vaginal microflora was affected. Therefore, a greater degree of variation was observed in PCOS samples but the exact mechanism involved needs to be further investigated.

We performed the validation of a PCOS prediction model (AUC, 0.8). We conducted in-group verification and obtained satisfactory results. In future, we will need to use independent samples to verify our model. Our model was able to accurately distinguish PCOS patients from healthy controls. The dysbiotic vaginal microbiome captured by the classifier offered further evidence for the identification of PCOS-associated microbial composition and indicated that those bacteria were highly correlated with PCOS.

Furthermore, we explored microbiome community interactions between the two groups. In the VMB network, module 1 and module 2 showed that*L. crispatus* and *L. iners* were clustered together, and indicated a negative correlation between *L. crispatus* and *L. iners.*The results were consistent with the results of many previous studies[42, 43]. Module 3 clustered potential pathogens, including *G. vaginalis* , *P. bivia,* and *A. vaginae* .*A. vaginae* and *P. bivia* can be incorporated as they influence gene expression in *G. vaginalis*biofilms[44.] *Gardnerella spp* . can produce metabolite amino acids that are used by *Prevotella spp.* as nutrients to produce ammonia, which in turn is used by*Gardnerella* spp[45] . Indeed, bacterial genera clustered in modules had similar requirements different vaginal micro environments. Next, the network analyses revealed that the unique connectors of the PCOS group were*L.iners* , *A.christensenii* ,*A. vaginae, P.timonensis, A. omnicolens,* and *Sneath sanguinegens* , which have been reported as potential pathogen involved in BV, miscarriage, preterm birth, pre-cancerous, and cancerous cervical lesions[8, 35-38]. We further found that*L.crispus* , *P. timonensis, and P. buccalis* can be identified as key bacteria that drive changes in the vaginal microbial interaction network in PCOS patients. These key bacteria are potential threats for the development of PCOS

and we can facilitate the conversion of the vaginal microbial interactions network back to normal by restoring normal levels of these bacteria, by using techniques such as vaginal microflora transplantation (VMT) [46]and Prebiotics[40], which provide a natural protection by restoring vaginal homeostasis.

Finally, we predicted the function of high-abundance vaginal bacteria, while the metabolic pathways involved and microbiome influence to the host were investigated in our study. In addition, a higher concentration of activated peptide/nickel transport system permease proteins that are associated with quorum sensing (QS), which is a regulatory system that allows bacteria to share information on cell density and can adjust gene expression accordingly[47], was found in the control group. Mixed species of bacteria communities can influence and be influenced by the activities of QS or other neighboring species[48]. PCOS showed a low expression of QS or a lack of intercommunity regulation. These differential functional pathways further suggest a possible pathogenic mechanism exerted by vaginal flora in PCOS patients.

Conclusion:

This research was based on a cross-sectional study with a large sample size to analyze the structure of VMB in PCOS. The VMB of PCOS patients showed a reduction in the abundance of *Lactobacillus,* but increases in the abundance of potential pathogenic bacteria, such as*U. parvum* , *A.baumannii* , *Prevotellaspp* ., and *G. vaginalis.* There were interactions between serum levels of testosterone, AMH, LH, and changes in the VMB.*G. vaginalis* , *U. parvum* , *P. L.crispus* , *P. timonensis,* and *P. buccalis* were identified as key bacteria that drive changes in the vaginal microbial interaction network of PCOS patients. Due to the complex etiology of PCOS, the way forward for VMB research may not be as straightforward as it seems. However, the results of this study not only enhance our understanding of the PCOS vaginal microbiome but also provide a basis for future research on the potential mechanism by which pathogenic bacteria are involved in PCOS vaginal microbial imbalance and to develop relevant methods of treatment.

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Authors' contributions

C. J., L. Q. and H. Z. contributed to design of the study and drafted the manuscript; C. J., L. Q., T. S. , Q. S. , Z.L. and Y.C. did the experiment and were involved in the analysis of data. C. J., L. Q. X. L., G. X., J. W., T.H., L.Y., J.S., F.Z., F.L., Y.Z., Y.H., Y.P., Y.L., Z.Y., H.C., Z.Z., S.Z., Y.F., Y.Z., and Q.Y. collected the samples . C. J., L. Q. and H. Z. revised the article. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate.

This study was approved by the Institutional Review Board of Center for Reproductive Medicine of Shandong University(2019LSZ14). All the enrolled subjects gave a written consent.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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Figure legend

Fig 1. Composition of the vaginal microbiota in two groups. The mean percentage of relative abundances at the phylum (a) , genus (b) in two groups.

Fig 2. By shannoon index (a)and simposon index (b),box plots present the vaginal microbiome diversity between PCOS and control group; comparation among the control gorup,PA group, PD group by shannoon index(c) and Chao 1 index(d).PA:OD+HA+PCOM;PD:OD+PCOM.OD: ovulatory dysfunction ;HA: hyperandrogenism ;PCOM: polycystic ovarian morphology.* p¡0.05

Fig 3. β-diversity of microbiome was performed using PCoA analysis: (a) comparaion between pcos and control (b).comparation among three groups ; (c) box plot present within-group median BC distance.*** P¡0.001

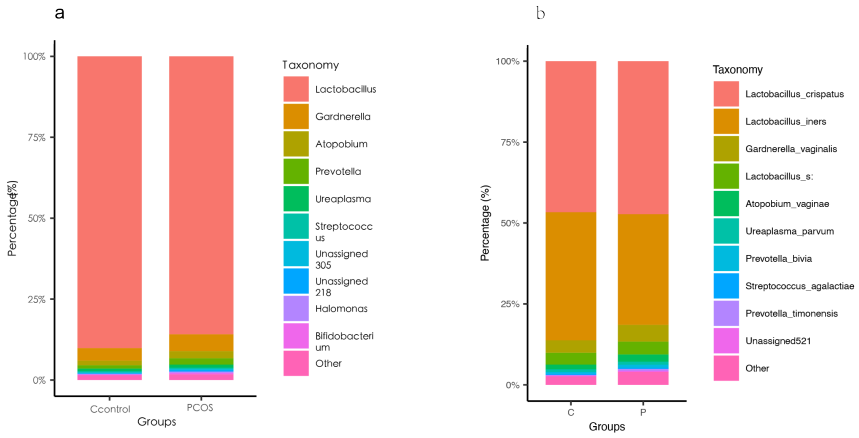
Fig.4 Feature selection analysis (Boruta algorithm) to identify the taxa important to the classification of PCOS(a). ROC curve based on the taxa that were selected as confirmed species by Boruta algorithmb(b).

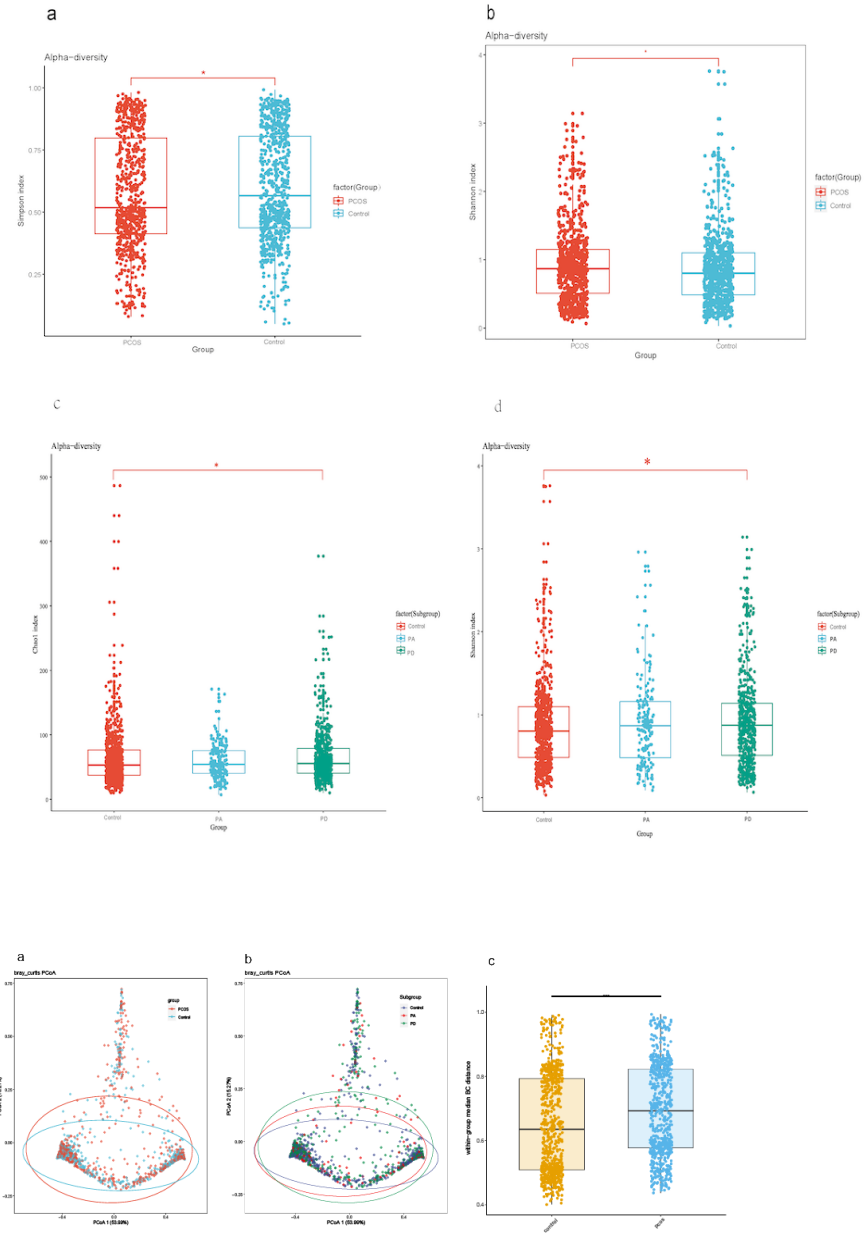
Fig 5. Comparation in control group and PCOS group , heat map of Spearman's correlation analysis between the intersected different vaginal microbiome and the clinical indices.(*p ¡0.05,**P¡0.01,***P¡0.001)

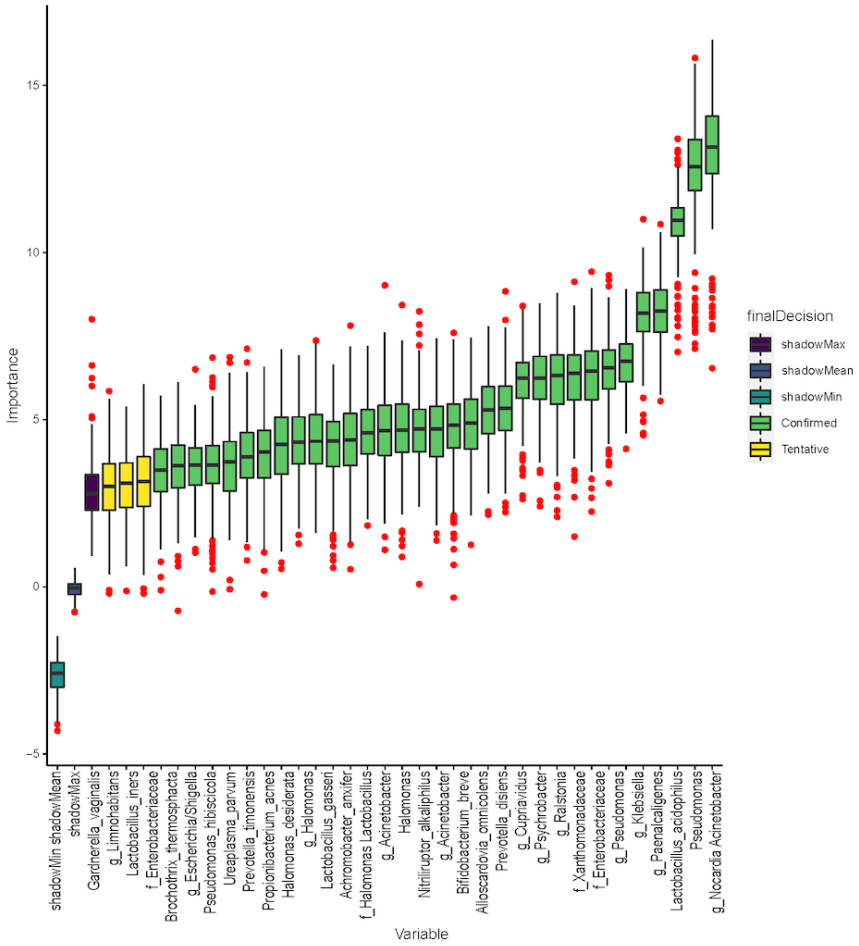
Fig 6. Microbiota community structure was evaluated by networks of ASVs using (SparCC) . Each node represents an ASV, and the identified ASV is annotated with its species, lines show the connection among species. The top five modules in the network in terms of module size were colored. Numbers of different colored nodes represent the relative content of the microbiota composition.(ASV noted in Suppl table). Scatter diagrams show the connectivity among and within modules in PCOS(c)and in Ccontrol(d), nodes represent each specie.

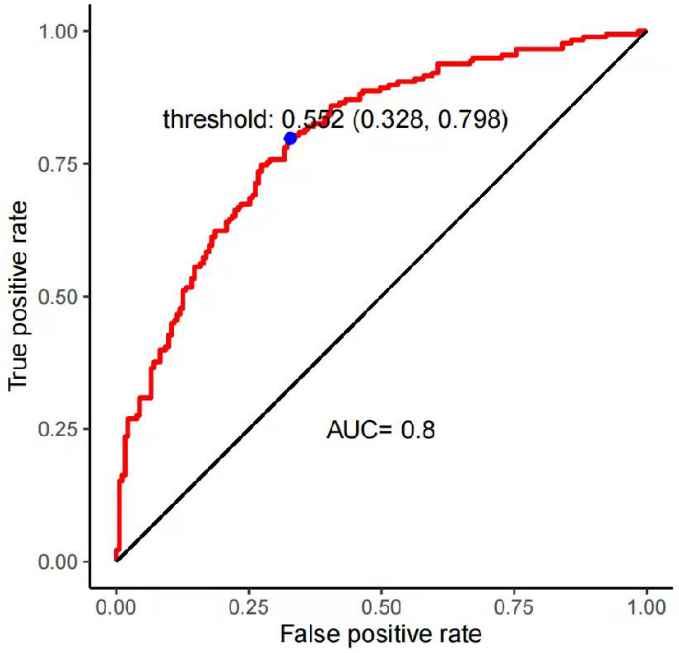
Fig7. (a) The plot shows the most common sub-network between the control and PCOS network. All nodes belonging to a same community are randomly assigned a similar color. Nodes which are big and red are particularly important 'drivers' . Red edges are present only in PCOS, Green edge are present only in control and Blue are present in both.(b) The Community shuffle plot enables one to understand the extent to which the identified communities in the control and case networks are similar using a heatmap. Similar (or conserved) communities are shown starting with a blue (deep blue indicated most similar community pairs) gradient in the heatmap while dissimilar ones are shown as green gradient (deep green indicates most dissimilar pairs). The values in the cells are calculated as the intersecting set count between the sets of node contents of the two compared communities (vertical axis as 'control' and horizontal axis as 'case'). More community splits (from 'Control' to 'Case') represents increased community shuffling. Hence, plots having less shuffling will show less horizontal splits (in the blocks of the matrix) and individual blocks will have a higher cell values.

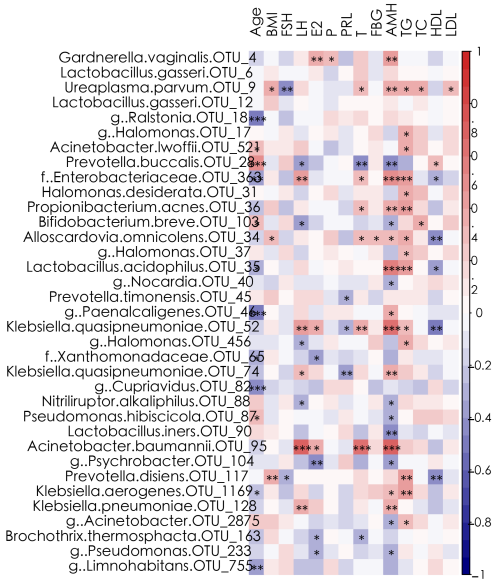
Fig 8. Functions influenced by PCOS (n = cases, controls). (a)KEGG gene enriched in the samples with and without PCOS. The pathways were detect by Wilcoxon test.(* P¡0.05) ◦ (b)The pathways were arranged by unsupervised hierarchical clustering.

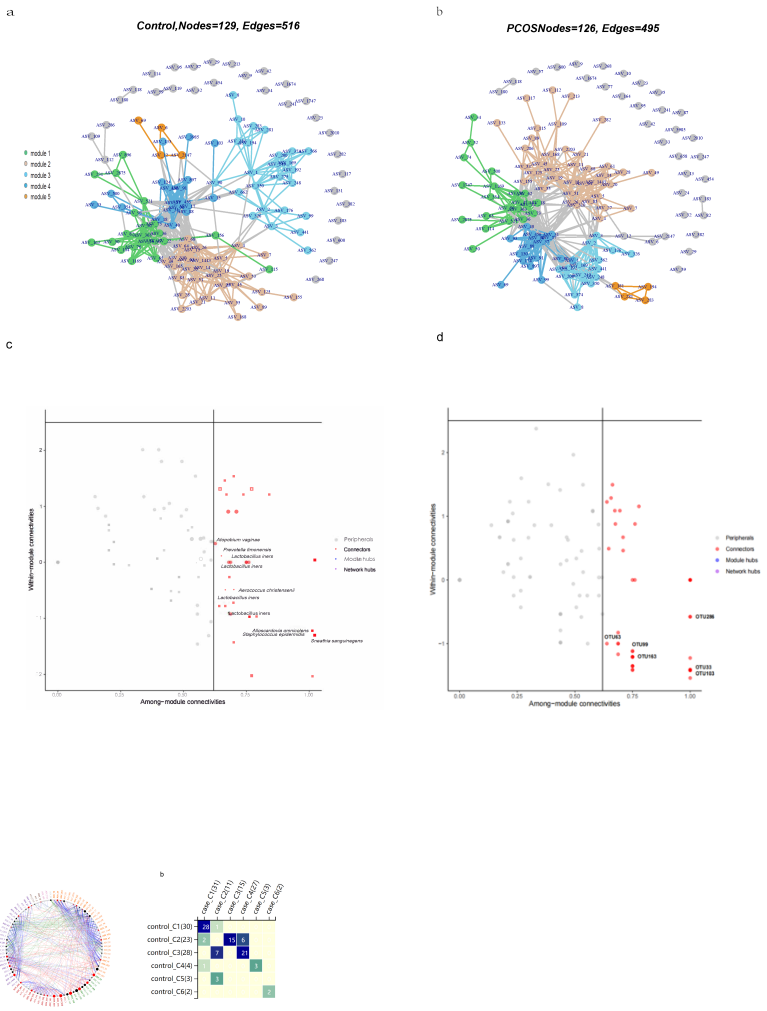


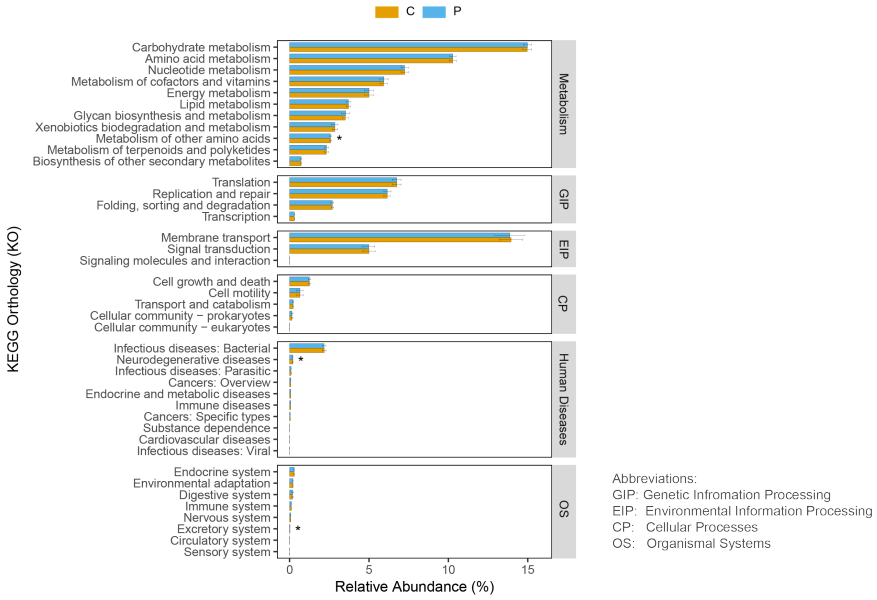


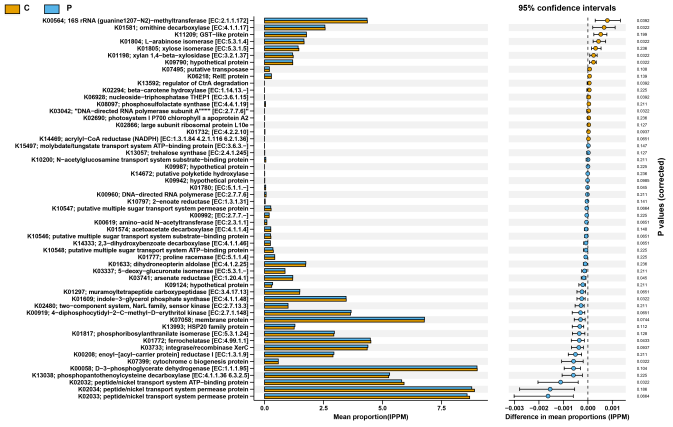












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