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The study of endometriosis and adenomyosis related microbiota in female lower genital tract in Northern Chinese population

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ABSTRACT

Background: Endometriosis is a chronic disease that affects women in reproductive age, and adenomyosis was known as "endometriosis in the uterus". Endometriosis is an immune-dysfunction-related disease, contributing to the diversity of microbiota in the lower genital tract. Endometriosis is also an infection-related disease, the number of bacteria may contribute to some unknown mechanisms. Presently, the feature of microbiota between endometriosis patients and normal people is not fully understood.

Methods: To identify the microbiota differences and features of endometriosis patients, 298 samples from the cervical canal, posterior fornix of the vagina and uterine cavity were analyzed by 16s-rRNA sequencing. Raw data were filtered, analyzed, and visualized. We conducted diversity analysis, statistical data of microbiota abundance, biomarker identification, random forest, and environmental factors analysis.

Results: Alpha diversity was not distinctive in endometriosis and adenomyosis patients. Posterior fornix near cervix was a better sampling location to analyze the dysmenorrhea-related microbiota feature; few dysmenorrhea-related bacteria were identified. Endometrial bacteria is controversial, and the result of 16s-rRNA sequencing was not good enough to conduct further analysis. *Anaerococcus* was a possible biomarker of adenomyosis-endometriosis patients. The identified bacteria were representative only in specific periods during the menstrual cycle. GnRH-a treatment impacted microbiota feature the most compared with other environmental factors.

Conclusion: This study provided us with a new concept of endometriosis and bacteria; different microbiota features may relate to endometriosis. The bacterial involvement should be considered in the future study of endometriosis. New non-invasive diagnosis and therapeutic methods through bacterial medication are prospective.

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1. Introduction

Endometriosis is a chronic gynecological disorder characterized by endometrial tissue outside the uterus and affects 10–15 % of women in reproductive age.[1–4] Endometriosis contributes a lot to pelvic pain, ovarian mass, and infertility.[1,5] In addition, adenomyosis, which is known as "endometriosis of the uterus", an important gynecological disorder and causes severe pelvic pain[6,7], but their interrelations is not fully understood.[6] Immunological disorder, angiogenesis and endocrine are closely related to the development of endometriosis. H.Kobayashi has reported that Neurotrophins (NTs) are overexpressed in endometriosis and encompass nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and NT-3 and NT-4/5, An increased release of proinflammatory cytokines from endometriotic lesions is contribute to the excessive sensory innervation and development of chronic pelvic pain[8]. Immune system dysfunction greatly impacts the development of endometriosis, which involves various types of immune cells and cytokines.[1,9] The feature of the host immunity is also closely related to bacterial vaginosis (BV),[10] previous studies had demonstrated that infection of the genital tract might be associated with endometriosis, lipopolysaccharide regulates the pro-inflammatory response and the growth of endometriosis through the LPS/TLR4 cascade. Besides, the cross-talk between inflammation and ovarian steroids or the stress reaction also was observed in the pelvic peritoneum.[11,12] Menstrual cycle-related taxa are over-represented in endometriosis and adenomyosis patients.[13]

Bacterial vaginosis (BV) is best described as a polybacterial dysbiosis,[14] and it is correlated to immune dysfunction.[15,16] Numbers of evidence suggested that endometriosis was associated with bacterial infection and lipopolysaccharide.[10,16,17] The related bacteria may rise from the vaginal to the uterine cavity, but the exact mechanism is still unrevealed. Human Microbiome Project (HMP) is aimed to recognize the importance of bacterial community in human health.[17] Since the 16s-ribosome RNA sequencing technology was developed, much more bacteria were identified because culture-based technology can only culture finite live bacteria while approximately 1 % of bacteria can be culture.[18] HMP project had been conducted in many different aspects, including urine, fetal-amniotic fluid-maternal host, digestion tract, and human skin[17–24]. Some researchers even performed the gut microbiome analysis in endometriosis mice[25]; however, there is only a few studies on the microbiome of endometriosis or adenomyosis in the female genital tract. *Chen* et al. conducted the random forest models distinguished depletion or enrichment of many bacteria subjected to diseases.[13] The results were relatively debatable for those who had previously demonstrated the possible microbiome features in endometriosis patients. A large cohort study had revealed that BV is positively correlated to endometriosis,[26] but the BV-related Atopobium was found depletion.[27]

It is important to investigate the microbiome in endometriosis and adenomyosis patients. We can alleviate endometriosis by regulating vaginal bacterial if the exact functions of the microbiome are identified in the future.

2. Materials and methods

2.1. Patient selection

This study was approved by Ethics Committee of Peking Union Medical College Hospital (PUMCH), approval No. JS-1532. The outpatients in clinical visit and inpatients who were hospitalized for surgery were included in the study from April 2018 to May 2019. The inclusion criteria include: regular menstrual cycle (28 ± 7 days), age range from 18 to 45-year-old, antibiotic-free within 30 days, no *trans*-vaginal intercourse within 2 days, no douching and *trans*-vaginal medications within 5 days, no cervical treatment within 7 days. The exclusion criteria include: participants with symptomatic BV, cervicitis, pelvic inflammatory disease (PID), any acute systemic inflammation, malignancy, autoimmune disorders, pregnancy, and intrauterine devices, oral-contraceptive treatment in 30 days, during menstruation. Informed consent was obtained from all participants.

149 participants were included in this study, and 298 samples were available totally. Cervical canal (site-A) and posterior fornix (site-B) samples were collected from each participant, and the data of 147 samples from the cervical canal (site-A) and 146 samples from posterior fornix (site-B) were available eventually. 7 samples from the uterine cavity (site-C) were collected, and 5 (71.43 %) of them generated available data successfully (Fig. 1 a). Considering the small sample size and low PCR quality, intra-uterine samples were excluded temporally. 134 participants underwent surgery intervention, with traceable surgery records and pathological reports. While the other 15 outpatients did not undergo surgery intervention, and their final diagnoses were obtained from radiological examinations (magnetic resonance images and ultrasounds).

2.2. Sample collection

Disposable swabs (Jiangsu Tianli Medical Instrument CO., LTD.) were used for cervical sampling canal (site A), and posterior fornix of the vagina (site B); vacuum suck tubes (Jiangsu Tianli Medical

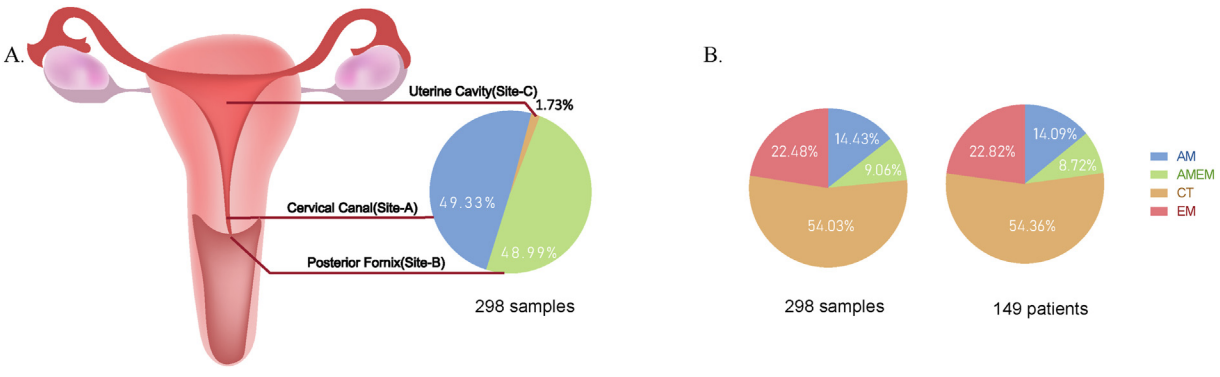

Fig. 1. The exact proportion of sample among each group. (a) The samples from three different locations had been collected: uterine cavity, posterior fornix, and cervical canal. The pie chart shows their proportion in all 298 samples. (b) The proportion of four groups in all 298 samples and 149 participants.

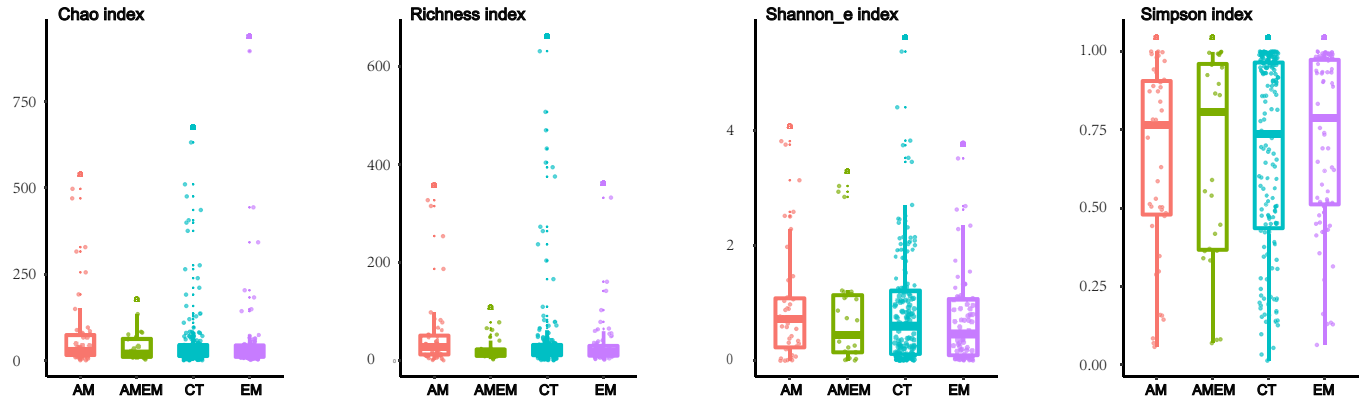

Fig. 2. Alpha diversity
Four types of alpha diversity were analyzed, including Chao index, Richness index (Observed number of OTUs), Shannone index, and Simpson index. For each alpha index, four groups were analyzed, and $p < 0.05$ was identified as statistically significant. The result showed no significance in all groups.

Instrument CO., LTD. Guardking, JDC-II) were used for sampling endometrium (site C). All the samples were then placed in ice and subsequently stored at −80 °C, then transported on dry ice to Annoroad Gene Technology Co. Ltd (Beijing, China) for further analysis.

2.3. 16S ribosomal-RNA gene sequencing

Total genome DNA from samples was extracted using CTAB/SDS method (E.Z.N.A.® soil DNA Kit, Omega Bio Tek, Norcross, GA, U.), according to manufacturer's protocols. DNA concentration and purity were evaluated by NanoDrop 2000 UV vis spectrophotometer (Thermo Scientific, Wilmington, USA). 16S rRNA genes of distinct regions (16SV3–V4) were amplified used specific primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Samples with the bright main strip between 400 and 450bp were selected for further experiments. PCR products were mixed in equidensity ratios. Then, mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Lastly, the library was sequenced on an IluminaHiSeq2500 platform, and 250 bp paired-end reads were generated.

2.4. 16s ribosomal RNA sequence analysis

Raw sequence reads of 16s rRNA gene sequences were filtered and analyzed by vsearch and usearch.[28,29] The amplicon sequence variants (ASV, 100 % cluster) were classified taxonomically through Unoise3[30] and using the Greengenes 16s ribosomal RNA gene reference database. The taxonomic composition of microbial communities was visualized using R and STAMP v.2.1.3, subjected to Benjamini-Hochberg false discovery rate (FDR) correction.

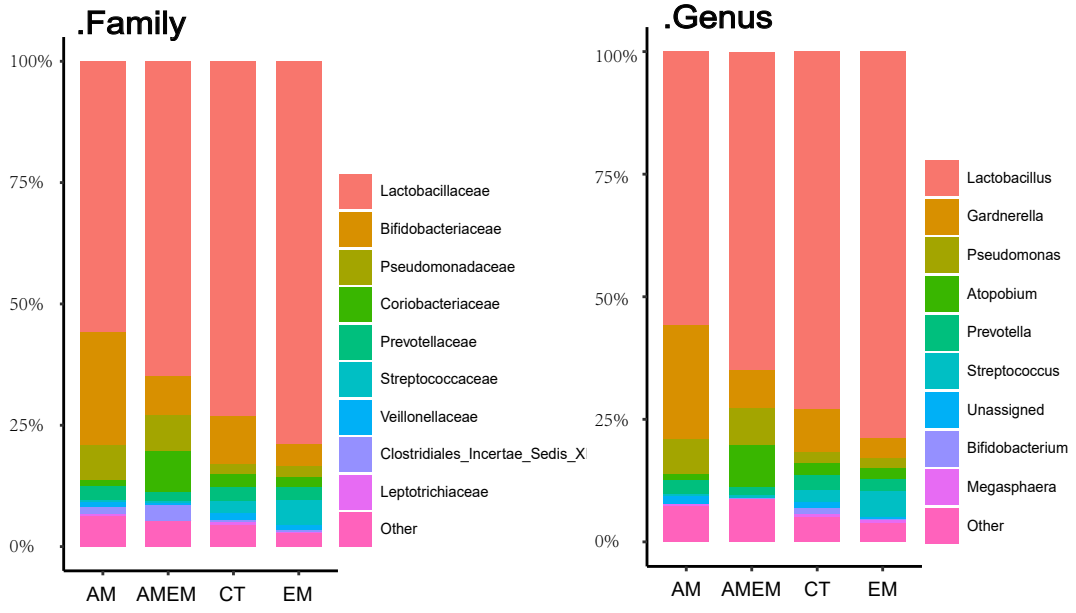

Fig. 3. Beta diversity
Each column was integrated by groups after the normalization of each sample, both Family and Genus level are shown. The top 10 (abundance) OTUs in all groups had been selected, Y-axis represents the proportion by percentage.

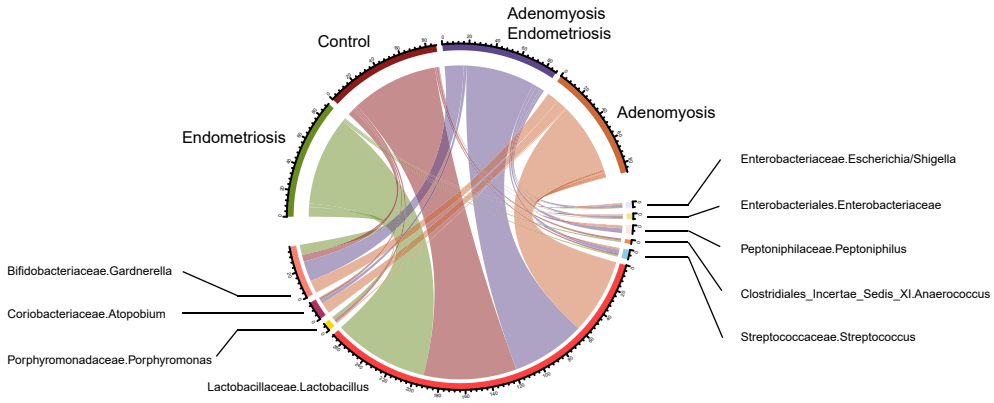

Fig. 4. Circulized plot.
With OTU abundance >0.5 % selection, 9 OTUs were selected in this figure. The upper half part of the circle shows four disease groups and the relative proportion of 9 OTUs. The lower half part of the circle shows the proportion taken by each disease group in each OTU.

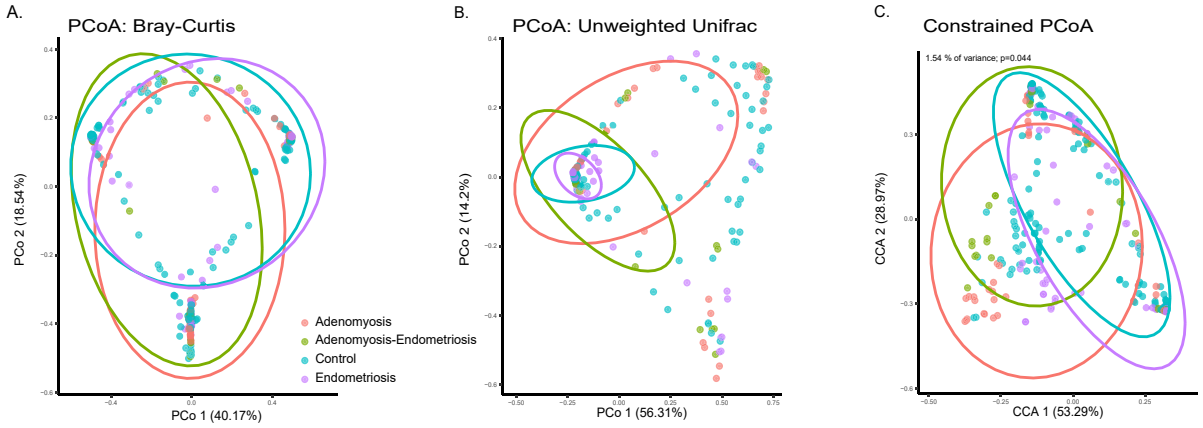

Fig. 5. Principle Co-ordinate Analysis (PCoA)
The percentages of the Y-axis and X-axis provide the degree of explanation. The ellipses represent 95 % CI of each group. (a) The Bray-Curtis distance matrix is a quantitative matrix, and it is used to analyze the feature of abundance. (b) Unweighted-Unifrac distance is a qualitative matrix, and it only considers the presence or absence of a feature. (c) Canonical Correspondence Analysis (CCA) is the matrix including environmental factors and species information. This two-dimensional plane can explain the 1.54 % difference of all samples (1.54 % of the variance, $p = 0.044$). CI: Confidential Interval.

2.5. Principal coordinates analysis (PCoA)

PCoA can simplify the distances of samples from multiple-to two-dimension. Two types of distance matrixes (Bray-Curtis and Unweighted Unifrac) were compared and constrained PCoA (CPCoA) was also performed. These methods were designed to dig out the distinctive distance of each sample group.

2.6. LDA and LEfSe analysis

To identify the distinctive cervical canal microbiota between different groups, linear discriminant analysis (LDA) and LDA Effect Size (LEfSe) method were used to compare the composition of cervical canal microbiota using an online tool (www.ehbio.com). Cladogram, LDA barplot, and proportion histogram were generated.

2.7. Machine learning

Machine learning was emerged as a method to determine how the microbiome can be used to separate samples based on current state or predict future state.[31] Random Forest classification contained the predictive bacterial taxa of endometriosis and adenomyosis with 5 folds cross-validation. Visual Analogue Scale (VAS) and menstrual cycle were studied using Random Forest regression with 10 folds cross validation to find out the important taxa in different phases.

2.8. Environment factor analysis

Environment factor analysis was conducted to identify specific factors that correlate to the feature of microbiota using the Adonis test ($p < 0.05$). We included the number of gravidity and parity, height, weight, body mass index (BMI), days of menstrual period, days of the menstrual cycle, VAS, infertility, gonadotrophin-releasing hormone agonists (GnRH-a) treatment. Variance Partitioning Analysis (VPA) was used to identify the best combination of factors that impact the different features the most.

3. Results

3.1. Demographic

21 (14.09 %) participants were diagnosed adenomyosis without endometriosis, 34 (22.82 %) participants were endometriosis (including ovarian endometriosis, deepDIE, peritoneal type, and other special types) without adenomyosis, 13 (8.72 %) participants

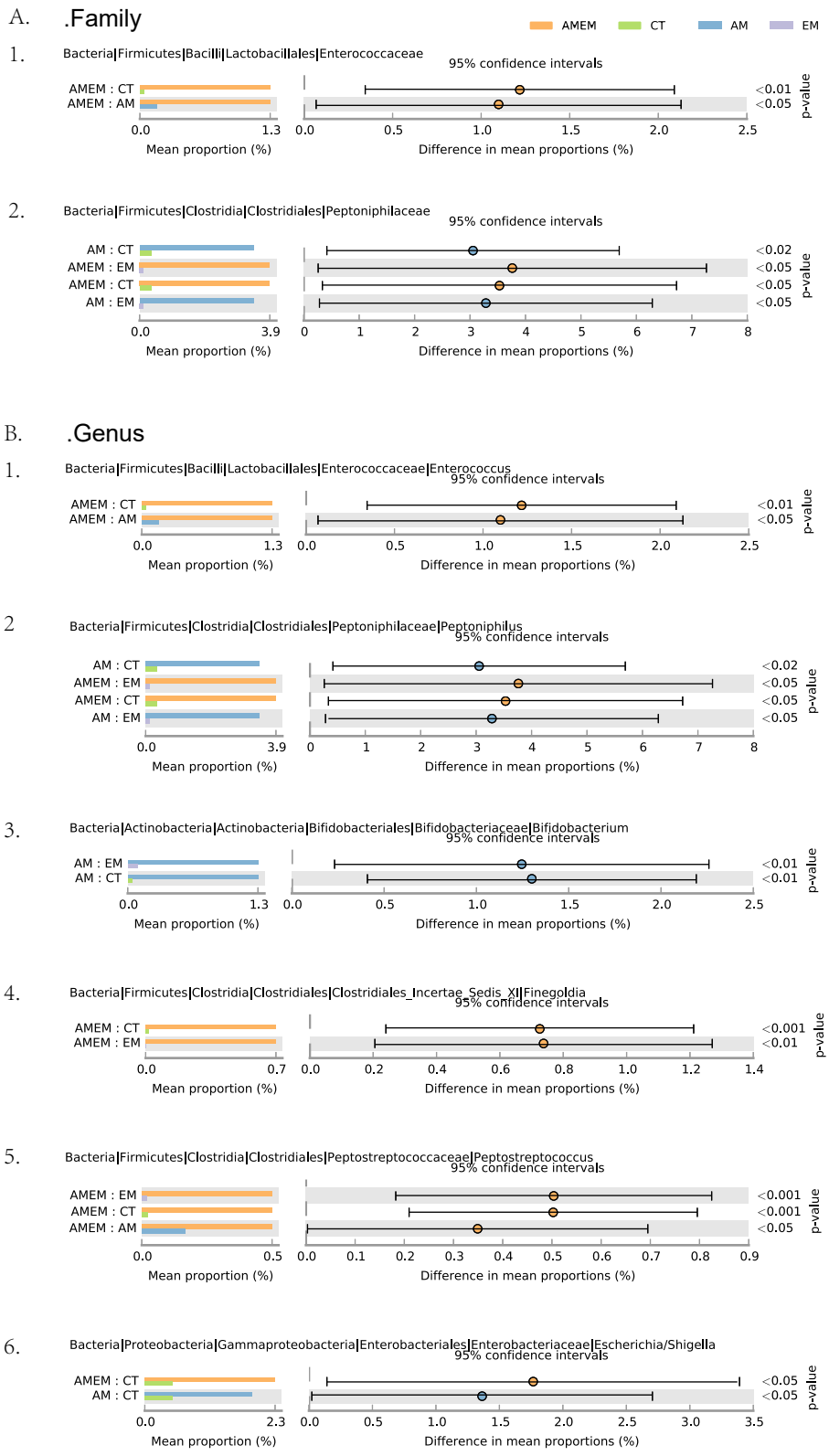

Fig. 6. Beta diversity These histograms show filtered differences with $p < 0.05$, mean proportion, and 95 % confidential interval are displayed in the figure. (a) This column displayed two microbiotas through two-two comparisons among each group at the family level. (b) This column displayed six microbiotas through two-two comparisons among each group in genus level.

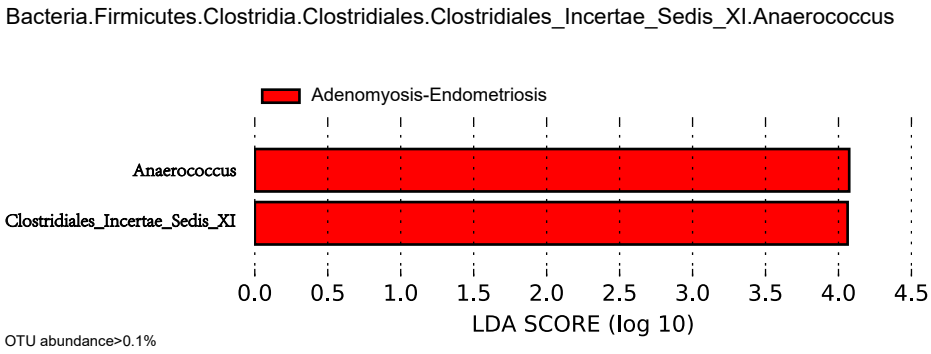

Fig. 7. LEfSe analysis. LEfSe method is used for biomarker discovery. With OTU abundance >0.01 % selection, the analysis emphasized both statistical significance and biological relevance. Effect size thresholds 4 (log 10) and p = 0.05 were used.

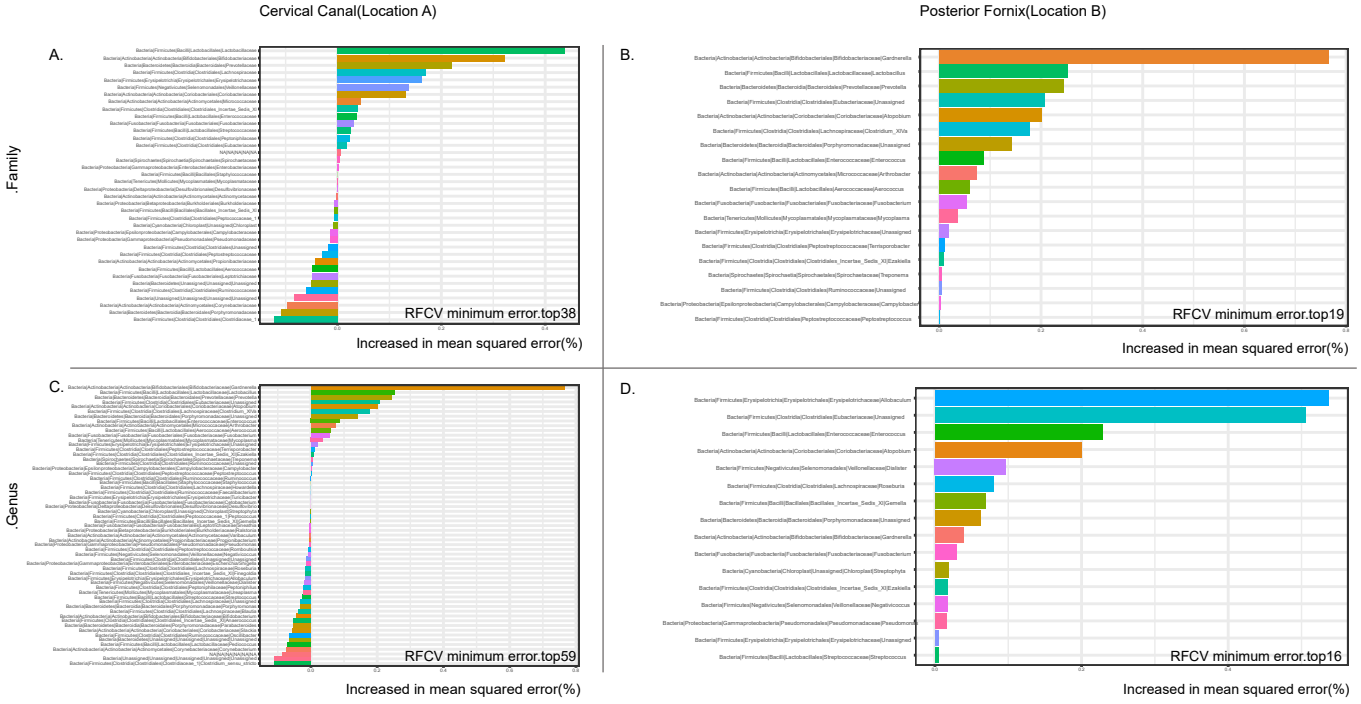

Fig. 8. Random forest regression by VAS Random forest regression by VAS. InMSE represents the feature importance of each microbiota. InMSE: Increased in mean squared error (%) VAS: Visual Analogue Score NA: Not Applicable taxonomy.

were adenomyosis combine with endometriosis, 81 (54.36 %) patients were classified as control group, including infertility, leiomyoma, ovarian borderline tumor and teratoma. In total 298 samples, 67 (22.48 %) samples were endometriosis group (EM), 161 (54.03 %) samples were control group (CT), 27 (9.06 %) samples were adenomyosis-endometriosis group (AMEM) and 43 (14.43 %) samples were adenomyosis group (AM) (Fig. 1 b).

3.2. Microbiota features in group analysis

3.2.1. Microbiota diversity of adenomyosis and endometriosis

The patients were divided into four different groups (AM, AMEM, CT, and EM). The dominant genus of bacteria in the lower genital tract is *Lactobacillus*, including *L.crispatus*, *L.iners, L. jensenii* and *L. gasseri*.[14] Chao index, Richness index, Shannon_e index and Simpson index showed no statistical difference (Fig. 2). The alpha-rarefaction curve was also assessed to validate the depth of sample size (Supplementary Fig. 1). In the beta diversity analysis, the top 10 OTUs (Operational Taxonomy Units) were selected according to abundance and showed in family and genus levels (Fig. 3). AM group shared the largest proportion in *Atopobium*, adenomyosis-endometriosis took the largest proportion in *Gardnerella* (Fig. 4). *Lactobacillus* is still the most dominant OTU as reported,[9,13] but for several cases, *Lactobacillus* only took a small proportion while *Gardnerella*, *Atopobium* or *Prevotella* were significant dominant (Supplementary Fig. 2).

In the Bray-Curtis matrix analysis, AM and AMEM groups were nearly fully overlapped with each other while EM and CT groups were mostly overlapped; four groups of distribution areas with 95 % confidential interval (CI) were almost the same (Fig. 5 a). In the Unweighted-Unifrac matrix, the areas of four groups were not separated distinctively. However, AM groups showed a wider distance than any other groups (Fig. 5 b), and CPCoA almost experienced the same problem (Fig. 5 c).

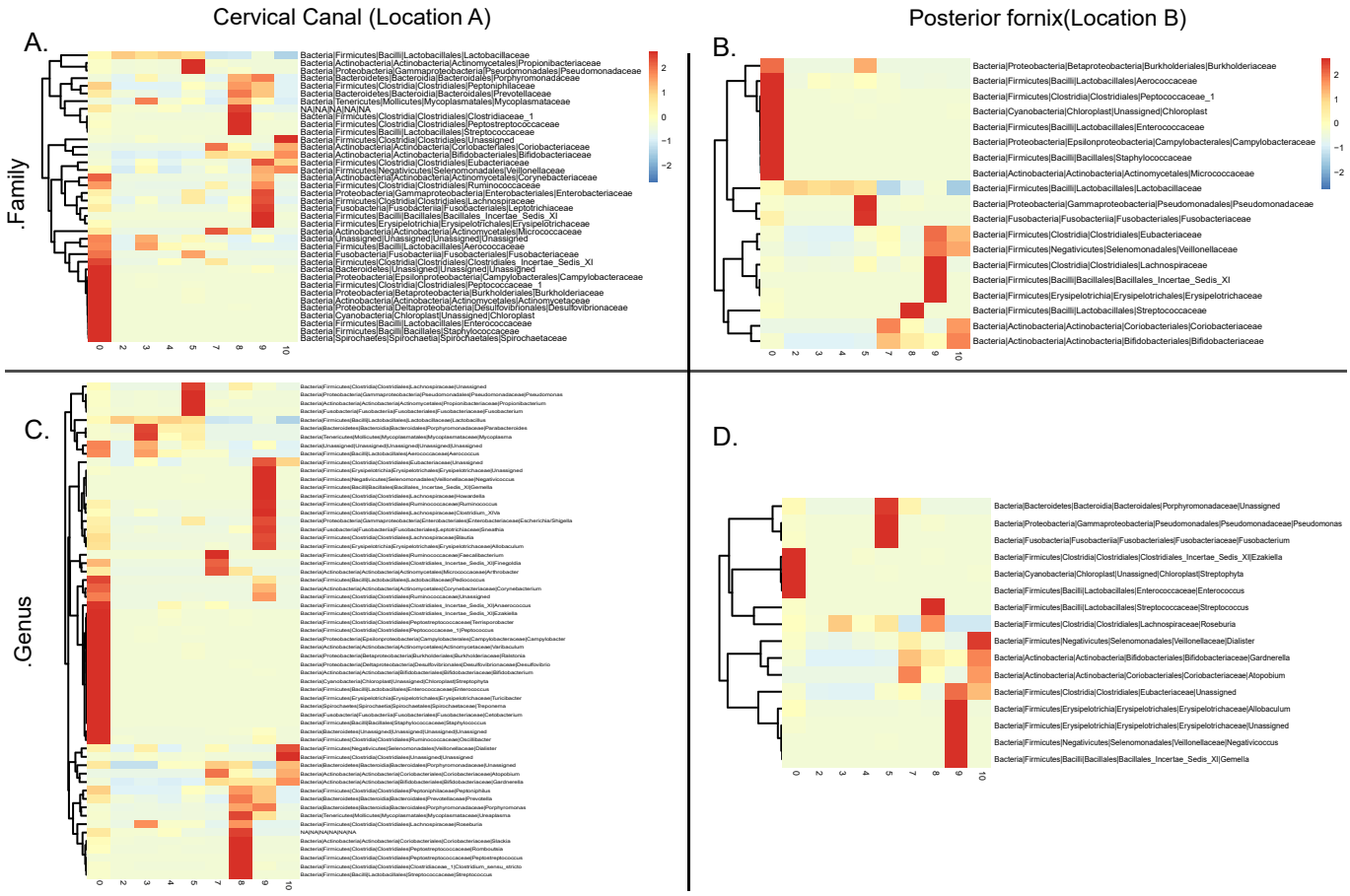

Fig. 9. Random forest regression by VAS Random forest regression by VAS. These four heat maps show the tendency of microbiota change with VAS increase. Blue represents negative feature importance; red represents positive feature importance. The color from blue to red indicates the increasing tendency of feature importance. Y-axis represents microbiotas, and X-axis represents the VAS score. VAS: Visual Analogue Score NA: Not Applicable species.

According to different methods of beta diversity analysis, although the proportion of such OTUs was different in the histogram (Fig. 3 and Supplementary Fig. 2), the groups may still not be separated significantly in the PCoA plot (Fig. 5).

3.3. Distinctive species of different disease groups

The two-two comparison showed significantly distinctive microbiotas ($p < 0.05$). At family level, only two microbiotas showed differences. The proportion of *Peptoniphilaceae* was higher in AM and AMEM (Fig. 6 a 2) and shared almost the same proportion in AM-AMEM and EM-CT comparisons (Supplementary Fig. 3 a 2). *Enterococcaceae* showed no differences between AMEM($n = 27$) and EM($n = 67$), $p = 0.17$. In the genus level, *Bifidobacterium* was significantly higher in AM than any other group. Other microbiotas of *Finegoldia* and *Peptostreptococcus* occupied more proportions in the AMEM group (Fig. 6 b 4,5). *Enterococcus* showed no differences between AMEM($n = 27$) and EM($n = 67$), $p = 0.17$; *Finegoldia* also showed no differences between AMEM($n = 27$) and AM($n = 43$), $p = 0.19$. These histograms only analyzed the different proportions of microbiotas, but it could not find out their importance (Fig. 6). The three-dimensional PCA figure also did not show significant differences in four disease groups (Supplementary Fig. 4).

3.4. Possible biomarker of microbiota in the lower genital tract

At the family level, *Clostridiales_Incertae_Sedis_XI* demonstrated significant distinction with LDA score (log 10) >4.0; in the genus level, *Anaerococcus* exhibited distinctive importance with LDA score (log 10) >4.0 (Fig. 7, Supplementary Fig. 5). The result of proportional differences in beta diversity did not reveal such characteristics at family level, and the relative abundance of *Clostridiales_Incertae_Sedis_XI Anaerococcus* displayed significant individual differences (Supplementary Fig. 6).

3.5. Sample site selection and feature importance of microbiota in dysmenorrhea related study

Previous studies have proved that endometriosis dysmenorrhea is correlated to some imbalanced immune factors, such as IL-6 and IL-8,[32] but the mechanism is still not fully understood. VAS performed random forest regression to figure out the importance and to change tendency of exact microbiota through heat maps. Two sites of sample collection (cervical canal & posterior fornix) were compared with each other. By selecting the OTU number that could minimize the error rate (Supplementary Fig. 7), the corresponding top number of microbiotas were selected, and their feature importance was calculated, respectively (Fig. 8 a-d). Nearly half of the microbiota contributed to negative InMSE (increased in mean squared error %) in site A (Fig. 8 a,c), while all the OTUs at site B showed positive InMSE (Fig. 8 b,d). These result suggested that the posterior fornix could be a better sampling site than the cervical canal to evaluate dysmenorrhea-related microbiota in both family and genus levels.

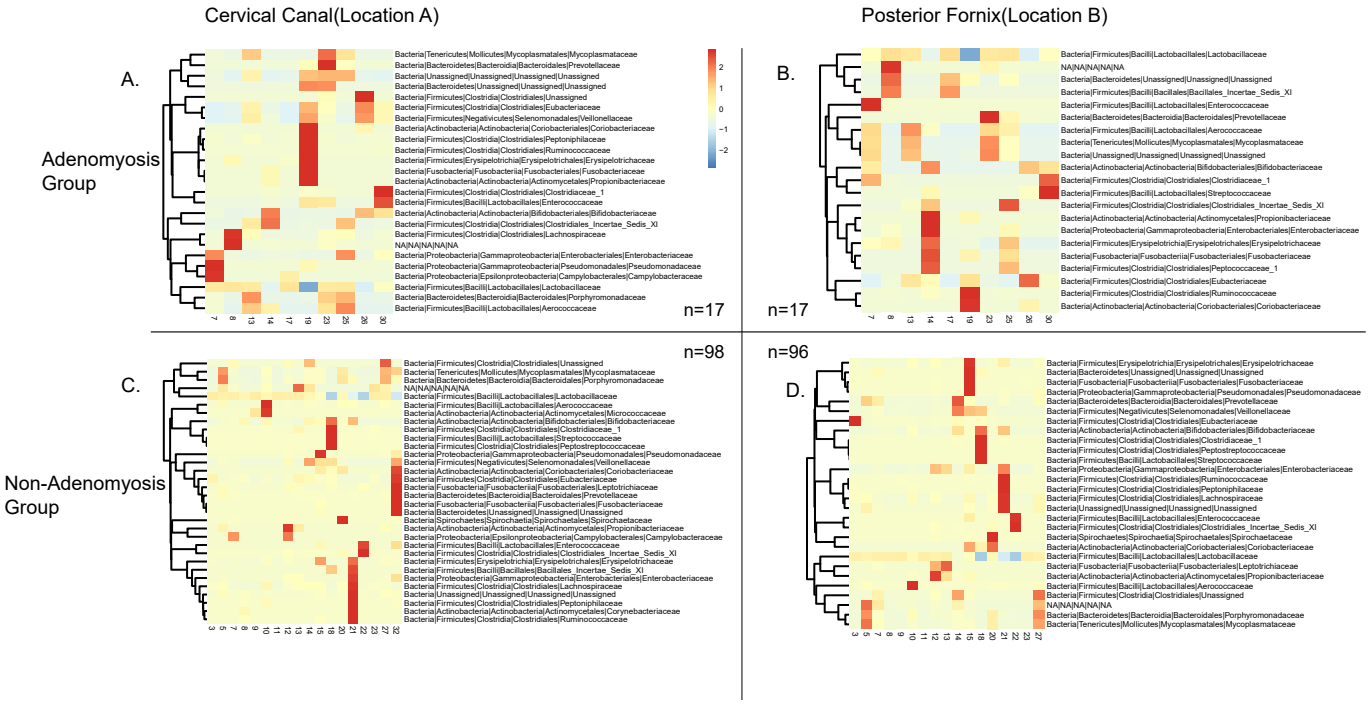

Fig. 10. Random forest regression by menstrual cycle (adenomyosis). Random forest regression by the menstrual cycle. Blue and red represent negative and positive feature importance, respectively. The color from blue to red indicates the increasing tendency of feature importance. This analysis was conducted at the family level. Y-axis represents microbiotas, and X-axis represents the menstrual cycle day of sampling. NA: Not Applicable species GnRH-a: Gonadotrophin Releasing Hormone agonist.

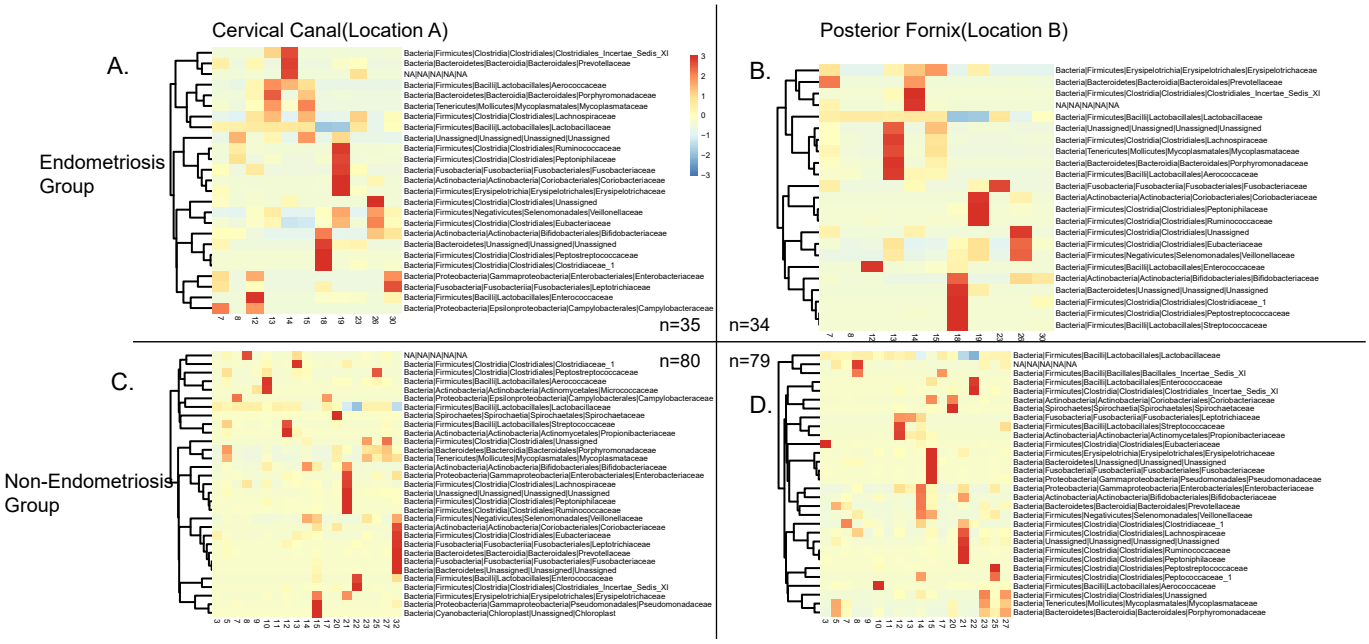

Fig. 11. Random forest regression by menstrual cycle (endometriosis). Random forest regression by the menstrual cycle. Blue and red represent negative and positive feature importance, respectively. The color from blue to red indicates the increasing tendency of feature importance. This analysis was conducted at family level. The right Y-axis represents the cladogram, X-axis represents the menstrual cycle day of sampling. NA: Not Applicable species.

In addition, the important features of the heat map were also generated (Fig. 9 a-d). In the samples at site B, the important features of microbiota changed from upper left to lower right with the increase of VAS (Fig. 9 b,d), suggesting that VAS is associated with the important change of microbiota.

3.6. Menstrual cycle-related microbiota shift in endometriosis and adenomyosis

Random forest regression was also used to analyze the microbiota features during the menstrual cycle. Estrogen is associated

Table 1
Adonis test.

Environment Factors	R^2	*P*
Gravidity	0.00825	0.005 *
Parity	0.01011	0.001*
Height	0.00457	0.157
Weight	0.00788	0.006 *
BMI	0.00569	0.048 *
Menstrual cycle	0.00751	0.006 *
Menstrual period	0.00509	0.085
Visual Analogue Score	0.00762	0.013 *
Infertility	0.00634	0.017 *
GnRHa treatment	0.01410	0.001 *

R^2 represents the degree of explanation, the explanation degree is stronger with the R^2 closer to 1. The R^2 of GnRHa treatment was the largest, it contributed to the feature the most. *P < 0.05 was considered statistically significant.
GnRHa: Gonadotrophin Releasing Hormone.
BMI: Body Mass Index.

with interleukin (IL)[33] as well as macrophages[34]; the change of IL is also correlated to the bacterial micro-environment.[10] The microbiotas that impact the micro-environment the most are shown in Supplementary Figs. 8 and 9. GnRH-a-treated patients were excluded (n = 27). For the adenomyosis patients (n = 35), *Erysipelotrichaceae* was the most important bacteria in the posterior fornix of vagina (Supplementary Figure 8 b). While for the endometriosis patients, *Clostridiaceae* and *Mycoplasmataceae* were the most important ones in the cervical canal and posterior fornix, respectively (Supplementary Figs. 9a–b). Heat maps show the microbiota change within the menstrual cycle (Figs. 10 and 11).

3.7. Environmental factors contributing to the microbiota features

Adonis test showed that eight factors were significantly related to microbiota feature, gravidity, parity, weight, BMI, menstrual cycle, VAS, infertility, and GnRH-a treatment (p < 0.05). GnRH-a was the most important factor that contributes to the feature differences between microbiota (Table 1). In addition, the different combinations of those factors also contributed to the differences to various extent; the VPA result demonstrated that the single factor of GnRH-a was the most relative, while the other combinations were not important as GnRH-a (Fig. 12). CCA showed how those significant factors affected the microbiota features. The vector of GnRH-a was the longest, indicating that GnRH-a treatment is the most effective factor (Fig. 13). Infertility, dysmenorrhea, and the menstrual cycle had similar effects on microbiota features.

4. Discussion

This pilot study focused on the microbiota feature and diagnostic model of endometriosis and adenomyosis. Although the microbiota in uterine cavity has been studied, the results and methods of those studies were miscellaneous; not all researchers were able to obtain a clear conclusion due to potential contaminations or infection.[11–13,18,35] It is still debatable whether the uterine cavity is sterilized, in other words, whether bacterial contamination only occurs in specific situations, such as in hypercontraction phases of the uterus[36] or post-GnRH-a treatment.[37] The PCR results of our samples from the uterine cavity were unsatisfactory, suggesting that some of the uterine cavities were

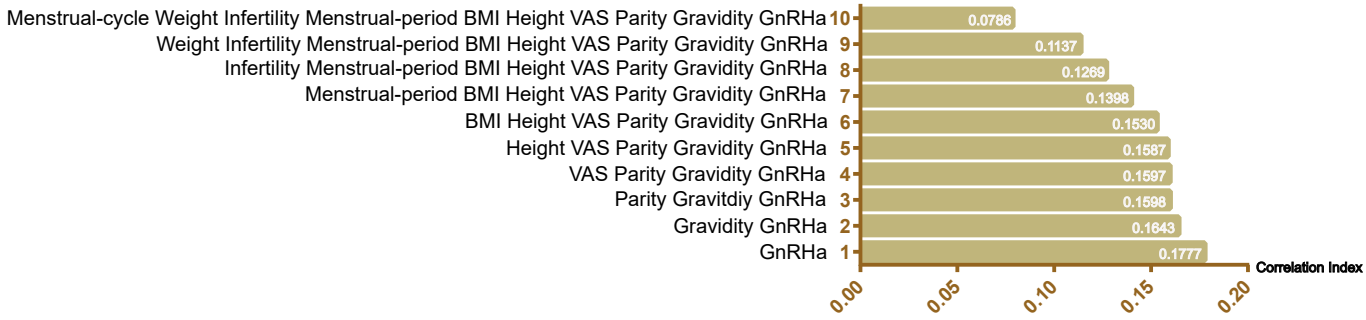

Fig. 12. Variance Partitioning Analysis (VPA). VPA is used to dig out the best combination of factors correlated to the different features the most. The correlation index indicates the impact on the microbiota feature. GnRH-a: Gonadotrophin Releasing Hormone agonist.

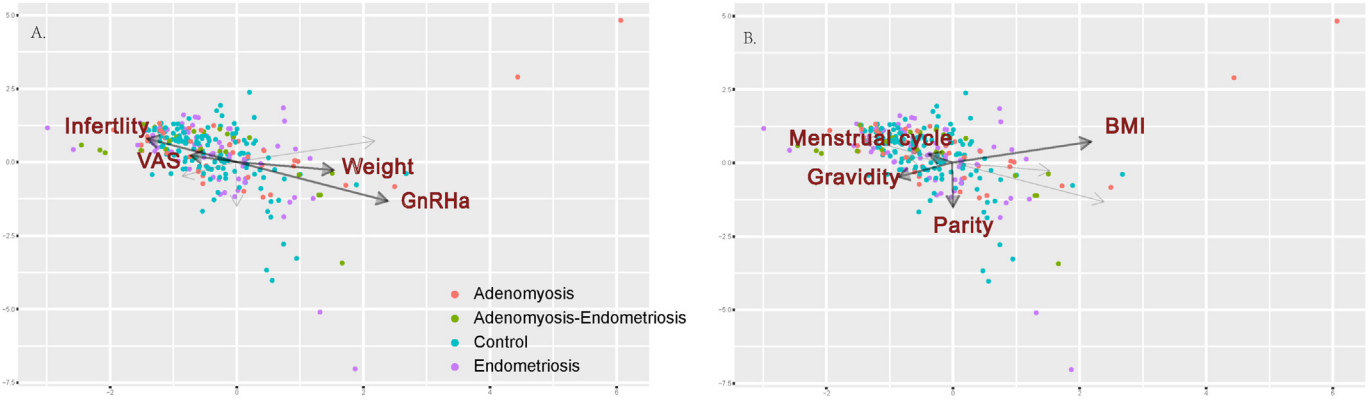

Fig. 13. CCA is a special type of PCoA; containing environmental factors. Because some of the vectors were overlapped, all eight environmental factors (p < 0.05) were displayed in two same coordinate systems. CCA: Canonical Corresponding Analysis; PCoA: Principle Co-ordinate Analysis; VAS: Visual Analogue Score of dysmenorrhea.

bacteria-free, and the real condition and the exact situation can be more complicated. Moreover, the potential contamination cannot be excluded. In addition, due to the limited methods to collect samples, contamination may occur in sampling, as *J.Baker* et al. mentioned.[18] *C.Chen* et al. collected the samples from the upper genital tract during operation, but the intra-operational antibiotic usage may affect the final result of the study.[13]

Since intra-uterine and cervical microbiotas features have not been demonstrated, the microbiotas near the cervix were investigated instead of the endometrium. Two locations were included, i.e., cervical canal and posterior fornix of the vagina. Although these two sites cannot fully represent the whole community in genital tract, they are the closest sites to the endometrium, and these two sites are easily accessible. A better sampling location was expected to proceed with the analysis and minimize the error of microbiota analysis. The investigation on dysmenorrhea by random forest regression identified the importance of the features of microbiota. The cervical canal samples displayed more OTUs with negative impacts, while the posterior fornix samples showed several OTUs with only positive impacts. It proved that posterior fornix is a better sampling location to analyze the microbiota feature in dysmenorrhea investigation.

We divided the participants into four groups, AM, EM, AMEM and CT. It is noteworthy that the different proportions in beta diversity do not completely stand for statistical differences. Only one microbiota, *Clostridiales_Incertae_Sedis_XI Anaerococcus,* was identified as a biomarker in AMEM patients*. Anaerococcus* was not significantly different in statistical analysis ($p > 0.05$), but LeFSe analysis includes comprehensive algorithms of all OTU features, and was identified as a potential biomarker. Genus *Anaerococcus* was first identified in 2001 by Ezaki,[37] a common colonized bacteria in the gut and women's genital tract.[38–40]

In beta diversity, *Atopobium* took more proportion in AMEM patients in our study, compared with the low abundance in AM or EM. *Atopobium* is an endometrial cancer-related bacteria, but whether it can facilitate malignancy from adenomyosis or endometriosis through downstream effect is unknown.[41] In addition, *Atopobium* is also a BV-related bacterium, and sub-clinical infection of BV may affect the microbiota feature analysis. Thus, it may not be a better point of endometriosis compared with *Anaerococcus.* In some other cases, the BV-related bacteria, such as *Atopobium, Gardenerella* and *Prevotella*, can also act as dominant ones in the vagina; similar results were also reported by *F.Polatti* and *K.Murphy* et al.[10,30]

The abundance and functions of microbiota is different between secretory and proliferating phase.[13] Herein, the importance index (InMSE) of microbiota during the whole menstrual cycle were analyzed. Using heat map, each microbiota could be recognized as only important in specific days during the period, while a few bacteria even had a negative importance index. Different study levels and sampling sites also showed different results.

Environmental factors analysis is aimed to find out the possible factors that impact the microbiota features and how they impact the features. Ten factors that have been routinely recorded in medical records were included, but different live styles and other unknown factors were not traceable. Since now there are no quantitative measurement of sexual activity, so we included gravidity and parity as two possible variants to represent sexual activity in this study. Eight environmental factors were statistically significant with $p < 0.05$. GnRH-a treatment was the most critical factor influencing the microbiota feature, followed by BMI and weight, but each led to different feature characteristics of the microbiotas. Infertility, days of menstrual cycle and dysmenorrhea showed similar effectiveness in different extents. For GnRH-a-treated patients, their vaginal environments were similar to postmenopause women, and bacterial contamination can be detected in the uterine cavity and endometriosis cystic fluid.[37] Infertility, menstrual cycle, and GnRH-a treatment are all associated with ovarian function; previous research has also demonstrated that estrogen and macrophages' cross talk contributes to endometriosis dysmenorrhea.[34] These findings indicated that the microbiota feature is correlated to estrogen level or ovarian function. The exact mechanism will be identified in future studies. All considered, the factors only contribute to the feature for less than 2 %, according to the Adonis test, which means that other unknown factors should determine the feature of microbiota for more than 98 %.

5. Conclusion

In this paper, the microbiota feature and differences among endometriosis and adenomyosis patients were discussed. Because there is no valid consensus that the uterine cavity is bacteria-free or not, the microbiotas from the cervical canal and posterior fornix were compared to find out a better sampling site that could represent the feature of diseases. Our findings proved that the posterior fornix of vagina was a better site to analyze dysmenorrhea. A few bacteria were identified as important microbiota in different phases of the menstrual cycle. *Anaerococcus* was defined as the biomarker of adenomyosis-endometriosis patients in both family and genus levels. GnRH-a was the most critical environmental factor that impacts microbiota, while other environmental factors also contributed to the variety of features in different extent. In addition, biomarker identification may contribute to a new method of non-invasive diagnosis, and bacterial transplantation will be a possible therapy in the future.

Authors' contributions

Sample collection: Sikai Chen, Zhiyue Gu, Wen Zhang, Ping Zheng.
Investigation: Sikai Chen.
Data analysis: Sikai Chen.
Data visualization: Chen Sikai, Zhiyue Gu.
Writing of original draft: Sikai Chen, Zhiyue Gu.
Writing-review and editing: Sikai Chen, Shuangzheng Jia, Yi Dai.
Final Approval of manuscript: Jinhua Leng, Yi Dai.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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