

## ORIGINAL STUDY

# Endometrial microbiota from endometrial cancer and paired pericancer tissues in postmenopausal women: differences and clinical relevance

Lili Wang, MD,<sup>1</sup> Jiaolin Yang, MD,<sup>1</sup> Huancheng Su, MD,<sup>1</sup> Liuming Shi, MD,<sup>2</sup> Bangtao Chen, MD, PhD,<sup>3</sup> and Sanyuan Zhang, MD, PhD<sup>1</sup>

### Abstract

**Objective:** The incidence of postmenopausal endometrial cancer (EC) is rising, and the uterine microbiota has recently been suggested to be an etiology of EC. However, the differences in microbiota profiles in paired EC and the adjacent non-EC endometrium, and the functional microbiota of clinical relevance remain largely unknown. Therefore, we examined the differences in microbiota profiles between EC and non-EC endometrium and investigated their clinical relevance to EC.

**Methods:** Twenty-eight EC-affected postmenopausal women undergoing hysterectomy were enrolled. Endometrial microbiome from paired EC and adjacent non-EC tissue samples were detected using 16S rRNA sequencing, and the data were analyzed using R language software.

**Results:** The  $\alpha$  diversity and evenness of the endometrial bacterial community significantly increased in EC tissues than those in pericancer tissues ( $P < 0.05$  for all variables). *Lactobacillus* and *Gardnerella* were the main bacterial genera present in both EC and adjacent non-EC-involving endometrium, whereas *Prevotella*, *Atopobium*, *Anaerococcus*, *Dialister*, *Porphyromonas*, and *Peptoniphilus* were more commonly enriched in the EC endometrium (corrected  $P < 0.05$  for all variables). Finally, the abundance of some observed endometrial bacteria was associated with clinical aspects, particularly the vaginal pH, vaginal *Lactobacillus* abundance, and EC clinical stage.

**Conclusions:** Paired EC and adjacent non-EC endometrium harbor different endometrial microbiota, and the functional bacteria residing in the endometrium are clinically relevant but require further investigation.

**Key Words:** 16S rRNA sequencing – Clinical relevance – Endometrial cancer – Tissue microbiota.

Endometrial cancer (EC), which originates in the endometrium, has become the most common gynecological tumor in developed or high-income countries because of the increase in risk factors, such as obesity.<sup>1,2</sup> Histopathological studies suggest that endometrioid adenocarcinoma with estrogen dependence (type I EC) is the most frequent subtype.<sup>3</sup> The removal of the uterus with or without uterine appendages through surgery followed by adjuvant therapy is the primary treatment strategy.<sup>4</sup> Recently, several studies have focused on eluci-

dating the mechanisms underlying endometrial carcinogenesis by identifying potential causative factors and validating them at the molecular or cellular level.<sup>5,6</sup> To date, the disparate roles of host genetic alterations, hereditary predisposition, and identified environmental factors for EC development, including obesity, diabetes, inflammation, gonadal hormones, and menopausal status, have advanced the understanding of EC's biological heterogeneity.<sup>1,2</sup> Despite these advances, further studies are necessary to identify previously unrecognized tumorigenic mechanisms.

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From the <sup>1</sup>Department of Gynaecology, The First Hospital of Shanxi Medical University, Taiyuan, China; <sup>2</sup>Department of Vascular Surgery, The First Hospital of Shanxi Medical University, Taiyuan, China; and <sup>3</sup>Department of Dermatology, Chongqing University Three Gorges Hospital, School of Medicine, Chongqing University, Chongqing, China.

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Address correspondence to: Sanyuan Zhang, MD, PhD, Department of Gynaecology, The First Hospital of Shanxi Medical University, No.85, Jiefang South Road, Yingze District, Taiyuan 030001, Shanxi Province, China. E-mail: zsyprofessor2022@163.com

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Emerging evidence demonstrates that the uterus harbors its own microbiota, which can modify the uterine functions in health and disease.<sup>7</sup> Through next-generation sequencing techniques, several cross-sectional studies have presented and compared endometrial microbiota profiles in healthy women and women with EC.<sup>8-11</sup> The endometrial microbial community exhibits considerable heterogeneity, with contributing factors including procedures for obtaining endometrial samples, gonadal hormones, menopausal status, and vaginal microbial communities.<sup>12,13</sup> However, the uterine microbial composition and its role in EC progression or development remain poorly elucidated. In particular, differences in tissue microbiota from EC and paired pericancer (PC) tissues have not yet been addressed.

In this cohort study, we compared the tissue microbiota in EC and paired PC tissues in postmenopausal women using 16S rRNA sequencing and attempted to clarify its correlation with clinicopathological EC profiles. We hope that our results will provide definitive evidence for preventing EC occurrence and contribute to improving related therapy by modifying the uterine microbial composition.

## METHODS

### Participant Enrollment

This investigation was conducted in the Department of Obstetrics and Gynecology of a comprehensive tertiary hospital in Taiyuan, China, from October 2020 to December 2021. Menopausal women histopathologically diagnosed with endometrioid adenocarcinoma and who underwent hysterectomy using the open surgical approach were enrolled voluntarily. The exclusion criteria were as follows: (a) nonmenopausal women; (b) individuals with current autoimmune diseases and gastrointestinal disorders, or a history of gastrointestinal surgery; (c) individuals with a history of genital tract infection and/or application of any antimicrobial treatments to the genital area within the past 3 months; (d) individuals receiving preoperative chemotherapy or radiotherapy; (e) use of systemic antibiotics, corticosteroids, or any other immunosuppressive therapy in the past 6 months; (f) smokers with smoking index >400; and (g) drinkers with daily ethanol intake  $\geq 20$  g in the past 5 years or  $\geq 80$  g in the past 2 weeks. The study was approved by the Medical Ethics Committee of the First Hospital of Shanxi Medical University, and written informed consent was obtained from all participants during recruitment.

### Participant Profiles

A structured questionnaire was used to acquire data on general demographic variables (age, age of menarche and menopause, ethnicity, height, and weight), surgical history, previous or current medications, smoking habits, and alcohol intake. A worksheet was used to collect clinicopathological profiles including diabetes, fertility, number of abortions, intrauterine device (IUD) use, vaginal pH, vaginal *Lactobacillus* abundance, estrogen level, necrosis in EC, clinical stage, and EC differentiation grade, from electronic medical records. Specifically, semi-quantitative vaginal *Lactobacillus* determination (*Lactobacillus* presence in average five to eight microscopic fields) was performed under an oil microscope: 0, no *Lactobacillus*; 1+, 1 *Lactobacillus*; 2+, 2 to 5 *Lactobacillus*; 3+, 6 to 30 *Lactobacillus*;

and 4+, >30 *Lactobacillus*. Histopathological assessment of the EC differentiation degree was conducted referring to the International Federation of Gynecology and Obstetrics grading system of EC. Briefly, an endometrial glandular component area corresponding to >95%, 50% to 94%, and <50% neoplasm was classified as well-, moderate-, poor-differentiated EC, respectively, with corresponding cellular atypia.<sup>14</sup>

### Tissue Sample Processing, DNA Extraction, and Sequencing

Once removed, any necrotic or eroded uterine surface tissue was immediately scraped using sterile scalpels before macrodissecting the residual endometrium and cancer tissues. In detail, the visible cancerous tissue and normal tissue 2 cm away from the cancerous tissue were defined as EC and PC groups (confirmed by histopathology), respectively. Notably, the tissue specimens obtained were rinsed three to five times in sterile saline (0.85% NaCl) for removing the microbiota with unstable adhesions or tissue fluid adherence. To homogenize the samples, the tissues collected in a sterile plastic cup were first frozen in liquid nitrogen for 24 hours and then stored at  $-80^{\circ}\text{C}$  until DNA extraction. DNeasy PowerSoil kit (Qiagen, Hilden, Germany) was used to isolate bacterial DNA from the endometrial samples according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and 1.0% agarose gel electrophoresis were used to determine the DNA concentration and integrity, respectively. The entire process was conducted by an experienced gynecologist and a laboratory technician under strict aseptic procedures. In addition, a negative control group including six tubes of prepared diethyl pyrocarbonate water was set to avoid potential bacterial contamination of molecular biology reagents. The processes of DNA extraction from water specimens to polymerase chain reaction (PCR) were completely synchronized with those from tissues. Our PCR assays failed to obtain positive results from the negative control group.

To analyze the bacterial colonies in tissue, the V3-V4 region of the 16S rRNA gene was amplified using PCR with bacterial universal primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3'). All PCRs were performed in triplicate using a 50  $\mu\text{L}$  mixture containing 1  $\times$  Phanta Flash Master Mix (#P520; Vazyme, Wuhan, China), 10  $\mu\text{M}$  primers, and 100 ng template DNA. The amplification conditions were as follows:  $98^{\circ}\text{C}$  for 30 seconds (1 cycle); followed by  $98^{\circ}\text{C}$  for 10 seconds,  $56^{\circ}\text{C}$  for 5 seconds,  $72^{\circ}\text{C}$  for 5 seconds (30 cycles); and  $72^{\circ}\text{C}$  for 1 minute (1 cycle). The amplicons were then recovered from 2% agarose gels and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, San Francisco, CA) and quantified using QuantiFluor-ST (Promega, Madison, WI) according to corresponding protocols. According to the instruction, the purified amplicons were pooled in equimolar and paired-end sequenced ( $2 \times 300$ ) on an Illumina MiSeq platform (Illumina, San Diego, CA). The raw reads were deposited into the NCBI Sequence Read Archive database (accession number, PRJNA847034).

### Bioinformatic Analysis and Statistical Tests

Paired-end raw reads with overlap were merged to tags, and tags were clustered to operational taxonomic units (OTUs) at

97% sequence similarity using UPARSE (version 7.1, <http://drive5.com/uparse/>). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier Algorithm (<http://rdp.cme.msu.edu/>) against the Silva (SSU128) 16S rRNA database, with 70% confidence threshold. QIIME and R packages (v3.2.0) from the free online Majorbio I-Sanger Cloud Platform ([www.i-sanger.com](http://www.i-sanger.com)) were used to analyze the sequencing data. Species diversity in a single sample was determined by calculating the  $\alpha$  diversity indices (Shannon and Simpson index), evenness indices (Shannon even index), and richness estimators (Sobs and Chao index) at OTU and genus level using Mothur v. 1.30.2. The differences in these indices between the groups were further assessed using the Wilcoxon rank-sum test.  $\beta$  Diversity, representing the microbial community heterogeneity between groups, was first shown on the principal coordinates analysis (PCoA) conducted using the R package at the genus level. Then, rank of distance, representing the microbial distribution difference between groups, was determined using the analysis of similarities package in R-vegan. Statistically, significant differences in the relative abundance of taxa were determined by Wilcoxon rank-sum tests, whereas linear discriminant analysis was used for assessing taxa responsible for the differences between the groups. Pearson correlation was calculated between the differential abundance at the genus level and clinical characteristics using the VGAM package. Before the correlation analysis, the variance inflation factor (VIF) was analyzed to assess the existing multicollinearity between clinical factors, and the factors with VIF >10 were usually removed because of their high multicollinearity. The continuous and categorical variables are presented as mean  $\pm$  SD and percentage (%), respectively. The  $\chi^2$  test or Student *t* test was conducted to compare the demographic information and clinical characteristics between the two groups.  $P < 0.05$  (two sides) was accepted as the cutoff for statistical significance, and a false discovery rate was considered for correcting the *P* value.

**RESULTS**

**Participant Characteristics**

During the time of study, 134 women were diagnosed with EC and underwent hysterectomy in our department. After excluding cases with nonmenopausal status (57 cases) and the other exclusion conditions mentioned before (36 cases), 41 EC samples with corresponding PC samples were further used for bacterial DNA extraction. However, bacterial DNA was undetectable in 3 EC tissues, 6 PC tissues, and 4 EC with PC tissues; therefore, only 28 cases were included. The demographic and clinical characteristics of the included participants are summarized in Table 1. Specifically, 3 women had a nonhormone-containing IUD in place during surgery, and 14 women had a history of IUD use (the average interval between the time of IUD removal and the time of surgery was  $4.88 \pm 4.38$  years).

**Sequencing Data**

We obtained total 9,331,057 raw reads with 2,332,764,250 bases from the 56 tissue samples. After quality filtering and trimming, 171,488 (ranging from 81,218 to 299,622; median,

**TABLE 1.** Demographic and clinical characteristics of the 28 included participants

Items	Valuations
Age, y	60.41 $\pm$ 5.22
Age of menarche, y	14.20 $\pm$ 1.31
Age of menopause, y	54.36 $\pm$ 2.69
BMI, kg/m <sup>2</sup>	25.68 $\pm$ 1.97
Diabetes, n (%)	13 (46.43)
Childless, n (%)	5 (17.86)
No. abortions (-/1/2)	8/13/7
IUD history or presence, n (%)	17 (60.71)
Vaginal pH	4.76 $\pm$ 1.06
Vaginal <i>Lactobacillus</i> (0/1+/2+/3+/4+), n	4/11/4/8/1
Estrogen level, pg/mL	23.51 $\pm$ 10.49
Necrosis in EC, n (%)	7 (25.00)
Clinical stage (I/II/III), <sup>a</sup> n	14/12/2
EC differentiation (well/moderate/poor), n	11/10/7

Data are presented as mean  $\pm$  SD unless otherwise is indicated. BMI, body mass index; EC, endometrial cancer; IUD, intrauterine device; pH, pondus hydrogenii.  
<sup>a</sup>Staging by International Federation of Gynecology and Obstetrics.

168,918) valid sequences with a mean length of 422 bp and 161,763 (ranging from 89,791 to 211,375; median, 164,717) valid sequences with a mean length of 426 bp were screened from 28 tissue samples in EC and PC cohorts, respectively. The reads from the two cohorts involved 1,472 OTUs, including 25 phyla, 60 classes, 127 orders, 204 families, 367 genera, and 547 species. The value of the good's coverage estimator was 99.99%. Clear asymptotes observed in refraction curve analyses with Shannon or Sobs index at the OTU level indicate a near-complete sampling of the bacterial communities.

**Bacterial Diversity Analysis**

The EC group had higher index values for Sobs and Chao at the OTU and genus levels than the PC group (Table 2), demonstrating increased bacterial community richness in the EC tissues (corrected  $P > 0.05$  for all variables). In comparison with those in the PC group, the Simpson estimator and index values for Shannon or Shannoneven at OTU and genus levels in the EC group were remarkably lower and higher, respectively (corrected  $P < 0.01$  for all variables), which implies increased  $\alpha$  diversity and evenness of the endometrial bacterial community in EC tissues. At the genus level, distinguished cohorts were visualized by hierarchical clustering tree, and  $\beta$  diversity analyzed using the PCoA based on unweighted UniFrac metrics also showed significant distinct clusters among the two cohorts ( $R = 0.149$ ,  $P = 0.001$ , Fig. 1A, B).

**Overall Distribution of Endometrial Microbiota**

The groups shared 157 genera, with 178 and 32 unique genera detected in the EC and PC groups, respectively. More than 65% endometrial bacteria in both groups belonged to the phyla *Firmicutes* and *Actinobacteriota* (Fig. 2A). The genera *Lactobacillus* (85.24%) and *Gardnerella* (12.26%) occupied the vast majority of the bacteria in the PC tissues, whereas the top eight genera identified in EC tissues were *Lactobacillus* (53.79%), *Gardnerella* (12.19%), *Prevotella* (8.79%), *Atopobium* (3.58%), *Bifidobacterium* (3.48%), *Streptococcus* (3.15%), *Sneathia* (2.08%), and *Proteus* (1.36%) (Fig. 2B).

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**TABLE 2.**  $\alpha$  Diversity analysis in the two cohorts at OTU and genus levels

	EC (OTU)	PC (OTU)	EC (Genus)	PC (Genus)
Sobs	118.82 ± 128.41 <sup>a</sup>	57.11 ± 29.94	45.85 ± 33.81 <sup>b</sup>	24.85 ± 8.09
Chao	154.34 ± 117.72 <sup>a</sup>	113.84 ± 63.25	57.62 ± 36.63 <sup>a</sup>	41.81 ± 34.64
Shannon	1.17 ± 0.94 <sup>b</sup>	0.42 ± 0.36	0.84 ± 0.89 <sup>b</sup>	0.15 ± 0.23
Simpson	0.54 ± 0.32 <sup>b</sup>	0.77 ± 0.22	0.66 ± 0.35 <sup>b</sup>	0.91 ± 0.16
Shannoneven	0.25 ± 0.19 <sup>b</sup>	0.11 ± 0.01	0.21 ± 0.22 <sup>b</sup>	0.05 ± 0.07
Good coverage	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00

Wilcox rank-sum test was used for statistical differences between the groups. EC, endometrial cancer; OTU, operational taxonomic units; PC, pericancer.

<sup>a</sup>Corrected  $P < 0.05$ .

<sup>b</sup>Corrected  $P < 0.01$ .

### Community Structure Differences

At the phylum level, EC tissues had significantly lower relative abundance of *Firmicutes* ( $64.27 \pm 38.91$  vs  $87.51 \pm 26.85$ , corrected  $P < 0.05$ ) and higher relative abundance of *Bacteroidota* ( $9.49 \pm 17.19$  vs  $0.01 \pm 0.03$ , corrected  $P < 0.000$ ), *Fusobacteriota* ( $2.15 \pm 6.05$  vs  $0.002 \pm 0.004$ , corrected  $P < 0.05$ ), and *Campilobacterota* ( $0.07 \pm 0.21$  vs  $0.005 \pm 0.001$ , corrected  $P < 0.05$ ) than the PC tissues. Differences in the relative abundance of the top 20 genera in the endometrial microbiota in the two cohorts were further analyzed using the Wilcox rank-sum test. The results showed that *Prevotella* ( $8.79 \pm 16.69$  vs  $0.01 \pm 0.03$ , corrected  $P < 0.000$ ), *Atopobium* ( $5.58 \pm 6.49$  vs  $0.003 \pm 0.007$ , corrected  $P < 0.01$ ), *Anaerococcus* ( $1.18 \pm 3.64$  vs  $0.009 \pm 0.017$ , corrected  $P < 0.01$ ), *Dialister* ( $0.59 \pm 0.97$  vs  $0.002 \pm 0.007$ , corrected  $P < 0.000$ ), *Porphyromonas* ( $0.57 \pm 1.69$  vs  $0.005 \pm 0.000$ , corrected  $P < 0.000$ ), and *Peptoniphilus* ( $0.56 \pm 1.35$  vs  $0.004 \pm 0.006$ , corrected  $P < 0.000$ ) abundance significantly increased in the EC cohort than that in the PC cohort (Fig. 3).

In addition, the application of the linear discriminant analysis effect size method identified 31 features with significantly different abundance from the phylum to genus levels between the EC and PC cohorts (linear discriminant analysis score  $>3$ ,  $P < 0.05$ ; Fig. 4A). Specifically, the endometrial microbiota of EC tissues was differently enriched in the genera *Prevotella*,

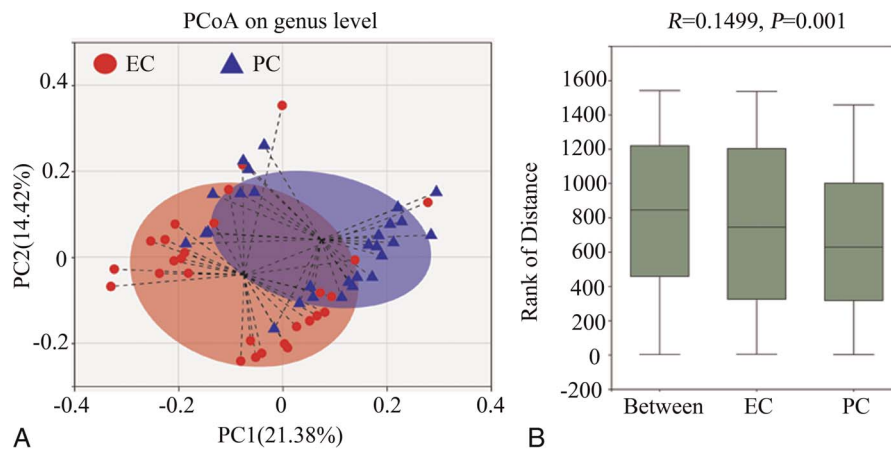
*Atopobium*, *Peptostreptococcus*, *Anaerococcus*, *Fastidiosipila*, *Finexgoldia*, *DNF00809*, *Dialister*, *Peptoniphilus*, *Porphyromonas*, *Anaeroglobus*, and *Criibacterium* ( $P < 0.05$  for all variables), whereas that of PC tissues was enriched only in genera *Lactobacillus* ( $P < 0.05$ ; Fig. 4B).

### Relationship with Clinical Characteristics

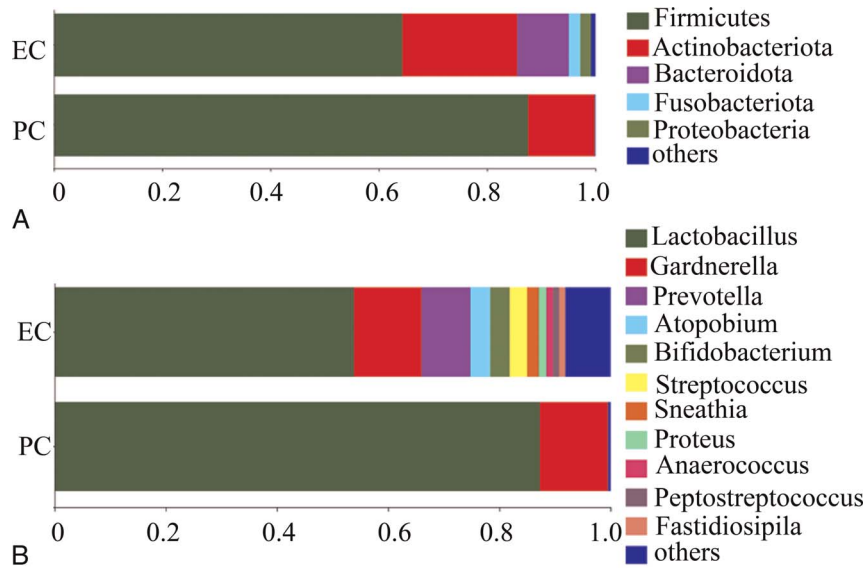
Finally, we analyzed the relationship between the observed genera in the participants and the clinical characteristics. A clinical indicator with VIF  $>10$  was considered to influence subsequent correlation analysis because of the existence of multicollinearity between clinical factors. The VIF of age during enrollment, menarche age, menopause age, body mass index, diabetes status, childless status, number of abortions, IUD use, vaginal pH, vaginal *Lactobacillus* abundance, estrogen level, necrosis in EC, pathological stage, and EC differentiation were 4.75, 2.13, 4.11, 1.51, 1.78, 2.71, 1.81, 1.27, 2.51, 2.27, 1.96, 1.95, 1.57, and 1.71, respectively. As the correlation heatmap graph demonstrated (Fig. 5), vaginal pH is moderately positively and negatively correlated with the abundance of the endometrial genera *Lactobacillus* ( $R = 0.540$ ,  $P < 0.01$ ) and *Atopobium* ( $R = -0.491$ ,  $P < 0.01$ ), respectively. Vaginal *Lactobacillus* abundance is strongly positively associated with endometrial genera *Prevotella* ( $R = 0.603$ ,  $P < 0.001$ ); moderately with *Gardnerella* ( $R = 0.487$ ,  $P < 0.01$ ), *Atopobium* ( $R = 0.533$ ,  $P < 0.01$ ) and *Dialister* ( $R = 0.555$ ,  $P < 0.01$ ); and strongly negatively with *Lactobacillus* ( $R = -0.785$ ,  $P < 0.001$ ) abundance. In addition, endometrial genera *Gardnerella* ( $R = 0.661$ ,  $P < 0.001$ ), *Atopobium* ( $R = 0.782$ ,  $P < 0.001$ ), *Sneathia* ( $R = 0.518$ ,  $P < 0.01$ ), and *Fastidiosipila* ( $R = 0.488$ ,  $P < 0.01$ ) abundance was strongly-to-moderately positively correlated with EC clinical stage.

### DISCUSSION

The incidence of EC is rising, and the estimated cumulative risk of developing EC by the age of 75 years is 1%. Endometrial cancer, the fifth most common cancer in women, is the 14th leading cause of death in cancers.<sup>1,2,15</sup> Clarifying the potential causative events and pathophysiology of EC is a continuing



**FIG. 1.**  $\beta$  Diversity analysis of the gut microbial structure. At the genus level, (A) principal co-ordinate analysis and (B) comparison of distance rank by analysis of similarities were performed as specified in the Methods. EC, endometrial cancer; PC, pericancer; PCoA, principal coordinates analysis.

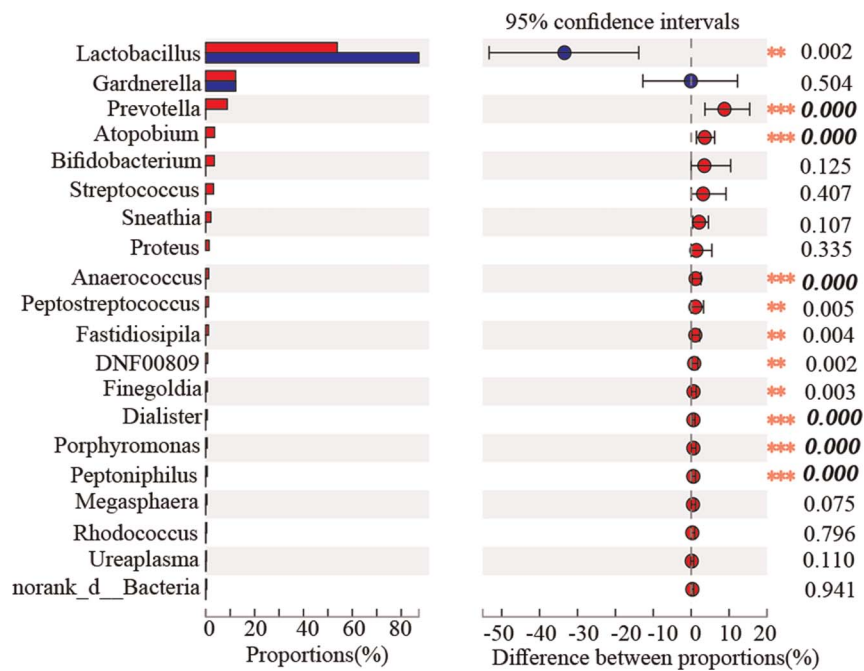


**FIG. 2.** Composition and relative proportion of endometrial bacteria in the two separate tissues at (A) phylum and (B) genus levels. EC, endometrial cancer; PC, pericancer.

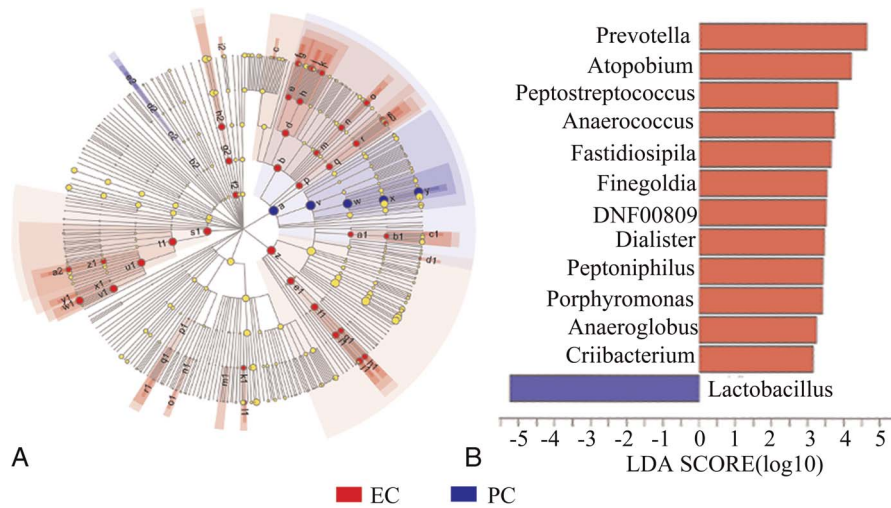
need, and efforts are in progress. Available evidence has shown that only 10% to 20% EC cases can be attributed to host genetic alterations or hereditary factors.<sup>8</sup> However, all EC cases are likely to be the combined result from genetic and environmental factors, such as long-term estrogen exposure, early menarche, late-onset menopause, nulliparity, and obesity.<sup>16</sup> The microbiota is significantly associated with a wide range of diseases. The effect of gut microbiota dysbiosis on gastrointestinal tumors has been extensively explored, and it is supposed to affect EC carcinogenesis through regulating the risk factors for EC occurrence, including estrogen metabolism, obesity, and gut–vaginal microbiome

axis.<sup>17-19</sup> However, the relationship between endometrial bacteria and EC development is of greater concern than that between gut microbiota and EC development because tissue microbiota may directly affect tumor behavior.<sup>20,21</sup> About tissue microbiota, more enigmas wait to be urgently clarified.

The uterus was long considered to be sterile, but current knowledge suggests that the uterus in healthy or diseased conditions harbors its own microbiota.<sup>22-25</sup> Endometrial microbiota has been preliminarily proven to be involved in several gynecological disorders, including chronic endometritis, infertility, endometriosis, dysfunctional menstrual bleeding, and EC.<sup>26-31</sup> Indeed, many



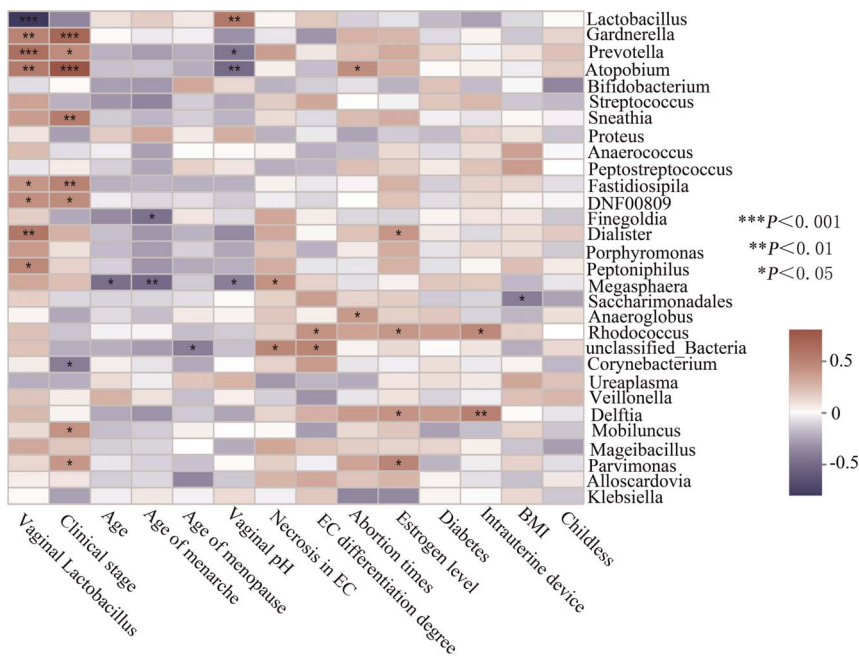
**FIG. 3.** Wilcoxon rank-sum test bar plot at genus level. The bolded and italicized values indicate corrected  $P < 0.05$ .



**FIG. 4.** Differently abundant taxa identified using LEfSe analysis among cohorts. (A) Distribution of differently abundant taxa at the phylum (the innermost circle), class (the second circle), order (the third circle), family (the fourth circle), and genus (the outermost circle) levels between the EC and PC cohorts. The effect of the microbial taxa significantly enriched in the EC and PC cohorts on the differences between the cohorts are expressed as red and blue solid circles, respectively. Large solid circles indicate a high degree of influence of the species on the intergroup. Light yellow circles indicate that the microbial taxa had no significant effects on the differences between groups. (B) Linear discriminant analysis showing the impact of different species on the difference between EC and PC cohorts and visualization of only taxa meeting an LDA  $\geq 3$ . EC, endometrial cancer; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; PC, pericancer.

clinical factors may independently influence the endometrial microbiome structure. Of particular relevance, postmenopausal status is the main driver of an EC-associated polymicrobial network.<sup>32</sup> This study is the first attempt to characterize the tissue microbiota from EC and paired PC tissues in postmenopausal women and determine its correlation with clinicopathological EC profiles. The endometrial microbiota profiles in EC participants and individuals with benign uterine lesions have been recently characterized,<sup>10,11</sup> and decreased  $\alpha$  diversity of the bacterial community

was supposed to be of great importance for EC development. However, in this study, the increased  $\alpha$  diversity and evenness of endometrial bacterial community in the EC tissues compared with those in the adjacent nontumor endometrial tissues further underlines some unclear connections between endometrial microbiota shift and EC progression. The analysis of  $\beta$  diversity measured with spatial distance among EC and corresponding PC tissues using PCoA showed that the samples from different locations in uterine tissues could be clustered independently,



**FIG. 5.** Correlation analysis of endometrial bacteria and related clinical indicators. BMI, body mass index; EC, endometrial cancer.

which is to be expected given that they are tumor and non-tumor-affected samples.

Several studies also uncovered potential alterations in endometrial microbiota composition and function in EC tissues.<sup>8-11</sup> The genera *Anaerostipes*, *Dialister*, *Peptoniphilus*, *Ruminococcus*, *Anaerotruncus*, *Bacteroides*, *Arthrospira*, *Atopobium*, and *Porphyromonas* were significantly enriched in EC tissues than those in endometrial hyperplasia tissues; moreover, relevance analysis showed that the coexistence of *Atopobium* and *Porphyromonas* with vaginal pH >4.5 was strongly associated with EC severity.<sup>8</sup> In China, a study enrolling endometrial tissue samples from 30 EC participants and 10 healthy individuals showed that *Pelomonas*, *Prevotella*, *Nocardioides*, and *Muribaculum* were enriched and *Oscillibacter* was not enriched in the EC endometrium. Of particular interest, the increasing abundance of endometrial *Prevotella* was positively correlated with elevated serum D-dimer and fibrin degradation products, which implies a high tumor burden.<sup>10</sup> Compared with that in benign uterine lesions, the genera *Micrococcus* was also more abundant in EC tissues and positively correlated with endometrial interleukin 6 and interleukin 17 messenger RNA levels, highlighting the clear role of microbiota-inflammation crosstalk in endometrium for EC development.<sup>11</sup> Chen et al<sup>9</sup> recently observed that the abundance of 17 bacterial species in EC tissues is significantly different than that in the normal endometrium, and further interaction analysis suggested the involvement of the functionally activated endometrial bacteria in the tumor metabolic process, including the 6-sulfo-sialyl Lewis x epitope and *N*-acetyl- $\beta$ -glucosaminyl, as well as contributions to tumor migration. Differences in the number of participants, sexual and physical habits, menopausal status, contamination of endometrial samples, and methodological considerations, such as PCR primers, reference database, and analysis software used among studies, may lead to partially inconsistent conclusions.<sup>33</sup>

To minimize the heterogeneity among samples, paired EC and adjacent non-EC endometrium were chosen in this study. In contrast to the case of gut bacteria, endometrial bacteria were detectable in both EC and paired PC tissues in only 68.29% (28 of 41) enrolled cases, which highlights that bacterial invasion of or residency in the endometrium tissue may only occur under specific conditions. Figure 4 showed the increased richness of the genera *Prevotella*, *Atopobium*, *Anaerococcus*, *Dialister*, *Porphyromonas*, and *Peptoniphilus* in the EC endometrium, which partly corroborates the endometrial microbiota composition observed in previous findings.<sup>8-11</sup> The result suggests that uncovering the variability of endometrial flora by subclassifying the endometrium according to EC affection is warranted. The distributions and function of endometrium-resident microbiota remain largely unknown. The tumor-resident intracellular microbiota was recently proved to promote metastatic colonization in breast cancer.<sup>34</sup> Moreover, the endometrial *Porphyromonas* presence has been suggested to be the most predictive microbial marker of EC in high-risk, asymptomatic women. Crooks et al<sup>35</sup> have presented evidence for intracellular invasion of *Porphyromonas* in EC cells in vitro, which revealed the possible activities and distributions of the uterine microbiome,

underscoring the potential role of intracellular *Porphyromonas* in EC progression. However, it is noteworthy that the living bacteria, short bacterial DNA fragments, or microbial components may act synergistically. Furthermore, the origin of endometrium-resident microbiota has not been established yet. The evidence presented by the correlation analysis showed that the abundance of endometrial genera *Lactobacillus* was strongly inversely linked with that of vaginal *Lactobacillus* and moderately positively with vaginal pH, which may lend some support to the supposed bacterial redistribution hypothesis that *Lactobacillus* in the lower reproductive tract may partially migrate and colonize the endometrium, which should be further studied in future studies focusing on the clarification of bacterial transmission. Based on this assumption, the EC clinical stage was demonstrated to be strongly-to-moderately positive with endometrial non-*Lactobacillus* abundance, which may clue that non-*Lactobacillus* species easily colonize the disrupted tissue with EC progression.

The limitations of the current study include the following: (1) the causal relationships among specific endometrial bacteria and EC, particularly whether EC predisposes an area of the uterus to microbial invasion/colonization or the endometrium-resident microbiota predisposes the endometrium to EC occurrence or progression, are not adequately explained as with all case-controlled clinical studies; (2) isolation of bacteria invasive in the endometrium or adherent to the endometrial surface by removing endometrium luminal surface was unsuccessful because the cancer-unaffected endometrium from the postmenopausal uterus is very thin; (3) within-subject verification of specific endometrial bacteria in another newly enrolled cohort of larger study populations from multicenter is lacking, including the women with EC in clinical stages III and IV, if the bottlenecks in specimens acquisition are overcome is promising; (4) uterine microbial composition should be modified in clinical settings to confirm the possibilities of bacterial dysbiosis correction for EC interventions; and (5) premenopausal or perimenopausal women were not included in this study.

## CONCLUSIONS

Overall, this study indicates that EC and adjacent EC-unaffected endometrium in postmenopausal individuals have significantly different microbiota, and some observed bacteria are strongly clinically relevant to the tumor's biological behavior.

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