ENDOMETRIOSIS

Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method

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Objective: To systematically compare the endometrial microbiota in infertile women with and without chronic endometritis (CE), as diagnosed by a quantitative and reference range-based method.
Design: Case-control observational study.
Setting: University-affiliated hospital.
Patient(s): One hundred and thirty infertile women.
Intervention(s): Endometrial biopsy and fluid (uterine lavage, UL) collected precisely 7 days after LH surge, with plasma cell density (PCD) determined based on Syndecan-1 (CD138)-positive cells in the entire biopsy section and culture-independent massively parallel sequencing of the *16S ribosomal RNA* gene performed on both the CE and non-CE endometrial fluid samples.
Main Outcome Measure(s): Relative abundance of bacterial taxa.
Result(s): Chronic endometriosis was diagnosed if the PCD was above the 95th percentile (>5.15 cells per 10 mm^2) of the reference range in fertile control subjects. With this stringent diagnostic criterion, 12 women (9%) were diagnosed with CE. Sequencing was successfully performed on all endometrial samples obtained by UL) (CE, n = 12; non-CE, n = 118). The median relative abundance of *Lactobacillus* was 1.89% and 80.7% in the CE and non-CE microbiotas, respectively. *Lactobacillus crispatus* was less abundant in the CE microbiota (fold-change, range: 2.10–2.30). Eighteen non-*Lactobacillus* taxa including *Dialister*, *Bifidobacterium*, *Prevotella*, *Gardnerella*, and *Anaerococcus* were more abundant in the CE microbiota (fold-change, 2.10–18.9). Of these, *Anaerococcus* and *Gardnerella* were negatively correlated in relative abundance with *Lactobacillus* (SparCC correlation magnitude, range: 0.142–0.177).
Conclusion(s): Chronic endometriosis was associated with a statistically significantly higher abundance of 18 bacterial taxa in the endometrial cavity.

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Chronic endometritis (CE) is a state of persistent inflammation in the endometrial lining caused by bacterial pathogens. It has been reported to be associated with reproductive failure (1–4). The presence of plasma cells in endometrial stroma has been accepted as the criterion standard to establish a diagnosis of CE (5). Nevertheless, the reported prevalence of CE varies widely from 2.8% (6) to 57% (7) of infertile women. This wide variation may be attributed to the different methods for quantifying plasma cells. Investigators may look for $CD138^+$ plasma cells from a varying number of randomly chosen high-power fields or the entire biopsied specimen (8). Further, there is no consensus on the diagnostic criterion to define what constitutes CE—at least seven such criteria, most of which were arbitrarily chosen, were proposed among the literature reporting the prevalence of CE.

To address these issues, we have developed a new method comprising steps to count all $CD138^+$ cells in the entire section, quantify the area of the examined tissue section, and express the plasma cell density (PCD) as cells per unit area (8). The cell counts and section area were analysed using a well-established computer software for the analysis of scientific images (ImageJ; U.S. National Institutes of Health) (9). The intraobserver and interobserver correlation coefficients of PCD with this new method were considerably higher than the $CD138^+$ cell count per 10 high-power fields in the commonly used method. Notably, we observed that plasma cells also were present in proven fertile women. Thus, we used a new threshold (PCD >5.15 cells per 10 mm^2 of endometrium), which was the 95th percentile of the reference range from a population of fertile women, to define CE (8). The new method and threshold derived from a reference range forms the stringent criterion in defining CE in our current study.

Previously, using microbial culture, investigators have detected *Enterococcus faecalis*, Enterbacteriaceae, *Streptococcus* spp., *Staphylococcus* spp., *Gardnerella vaginalis*, *Mycoplasma* spp., *Neisseria gonorrhoeae*, *Mycoplasma*, *Ureaplasma urealyticum*, and *Chlamydia trachomatis* in the endometrial samples of women with CE (10, 11). Moreno et al. (12) developed a molecular diagnostic tool based on species-specific quantitative polymerase chain reaction (qPCR) assays targeting nine selected bacterial species, and compared this method with three classic diagnostic tools. From the results obtained from 113 and 65 women subjected to qPCR assays and the combined three classic tests, respectively, the investigators illustrated that those nine qPCR assays were 77% accurate in classifying 13 women who had concordant diagnostic results of CE from the classic histology, hysteroscopy, and microbial culture tests.

In this study, we first diagnosed the status of CE in 130 infertile women using our quantitative method and threshold derived from a reference range. Then we profiled the endometrial microbiota by sequencing the *16S ribosomal RNA* (*16S rRNA*) gene of both the CE and non-CE samples. We reasoned that our study would facilitate a clearer interpretation of which bacteria are far more abundant in the CE endometrial cavity compared with the non-CE endometrial cavity.

MATERIALS AND METHODS

Study Cohort

Asymptomatic women with recurrent miscarriages or infertility were recruited from the Assisted Reproductive Technology Unit, Department of Obstetrics and Gynaecology, Prince of Wales Hospital, the Chinese University of Hong Kong. The inclusion criteria were nonsmoking, aged between 24 and 45 years, and having a regular menstrual cycle (25–35 days) and a normal uterus as assessed by ultrasonography and hysterosalpingography. The exclusion criteria were the presence of hydrosalpinx; uterine pathology such as fibroids, endometrial polyps, or intrauterine adhesions; significant medical conditions such as systemic lupus erythematosus; or recent use of antibiotics, estrogen, or progestogen hormone therapy or an intrauterine contraceptive device the previous within 6 months.

Written informed consent was obtained from all participants. Ethics approval was obtained from the institutional review board (Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee, reference No.: 2015.477).

Sample Collection

All samples were collected precisely 7 days after the luteinizing hormone surge (day LH+7). A daily urine dipstick test was performed from day 9 of the menstrual cycle onward to identify the LH surge. Generic precautions were applied to avoid contamination and bacterial growth in all procedures, as detailed in our previous publications (8, 13). The collection of endometrial fluid (uterine lavage, UL) was performed via a double-sheathed catheter (Cook Medical).

After insertion of a sterilized speculum, excessive vaginal secretions were cleansed by cotton buds slightly moistened with saline solution in a downward direction. A double-lumen embryo transfer catheter was inserted into the ectocervix, ensuring its tip did not touch the lower genital tract. The outer sheath of the catheter then was inserted into the junction between the endocervical canal and

the uterine cavity; the sheath protects the inner catheter from bacteria in the endocervix. The inner catheter was then advanced into the uterine cavity for collection of endometrial fluid (UL).

To ensure comprehensive and representative sampling of microbiota, 1.0 mL of collection medium (DNase/RNase-free distilled water; Thermo Fisher Scientific) was injected and withdrawn by gentle suction. We collected two negative controls (one collection medium-only control, and one air swab control) per woman. The samples were stored promptly at −80°C until DNA extraction.

Endometrial scratch was performed after the collection of endometrial fluid (UL). A Pipelle (Prodimed) was inserted into the uterine cavity until it reached the fundus. The inner plunger of the sampler was withdrawn to generate a negative suction. Then the sampler was gradually rotated as it was moved up and down to collect fully the endometrial tissue via the small hole located near the tip of the sampler. The tissue collected was dispensed from the interior of the sampler and rinsed thoroughly for 5 minutes in DNase/RNase-free water. The endometrial tissue was then immersed into 10% neutral buffered formalin for overnight fixation at room temperature and subsequently embedded into paraffin wax for histopathologic assessment.

Definition and Assessment of CE

Chronic endometriosis was diagnosed based on our improved method, which is more quantitative and stringent than existing methods (8). Plasma cells were identified by means of immunohistochemistry staining for Syndecan-1 (CD138). The identification of $CD138^+$ cells was performed manually under the microscope. Image analysis was performed by first scanning the slides at lower magnification, then capturing images (×400) of all the fields of $CD138^+$ cells with the use of a Leica DM6000B system. Then the whole section of each specimen was tile scanned under ×50 magnifications by the same system, which was able to merge separate images into one covering the whole tissue section. Plasma cell density (PCD)—that is, the total number of $CD138^+$ cells per unit area—was calculated based on cell counts and section area as measured using ImageJ software (version 1.51a; U.S. National Institutes of Health). We diagnosed CE when the PCD was higher than the 95th percentile (5.15 $CD138^+$ cells per 10 mm^2 of endometrium) of the normal reference range established from a fertile population.

Genomic DNA Extraction and Massively Parallel Sequencing

Extraction of bacterial genomic DNA was performed according to a published protocol (14). Extracted DNA from all endometrial samples and 16 negative controls (eight collection medium-only controls and eight air swab controls) were PCR amplified using primers targeting the *16S rRNA* gene (V4 region) and sequenced based on a validated protocol on the MiSeq platform (Illumina) (15).

Sequencing Data Analysis

Established bioinformatics pipelines in the open-source software package *mothur* were used to quality-filter and denoise the reads (16). Briefly, read pairs were first assembled into contigs. Contigs with ambiguous bases and length >275 base pairs were filtered. Chimeric sequences were removed using VSEARCH (17). Processed reads of over 98% identity in nucleotide sequence were clustered as a bacterial taxon (operational taxonomic unit, OTU). Taxonomic classification of each OTU was based on aligning its representative sequence to the SILVA rRNA database (release 132) (18). The read counts of each OTU were tabulated for each sample.

To account for differences in sequencing depth, the counts were normalized using either ratio-based method, built-in normalization in linear discriminant analysis (LDA) effect size (LEfSe) (19), Sparse Correlations for Compositional (SparCC) data (20), or the R package *metagenomeSeq* (21) before any statistical analysis. LefSe (19) and metagenomeSeq (21) were used to identify statistically significantly abundant bacterial clades or taxa between two groups. SparCC was used to infer the correlation of relative abundances between two OTUs (20). *P* values were adjusted after multiple testing correction by the false discovery rate method.

RESULTS

Characteristics of Study Population

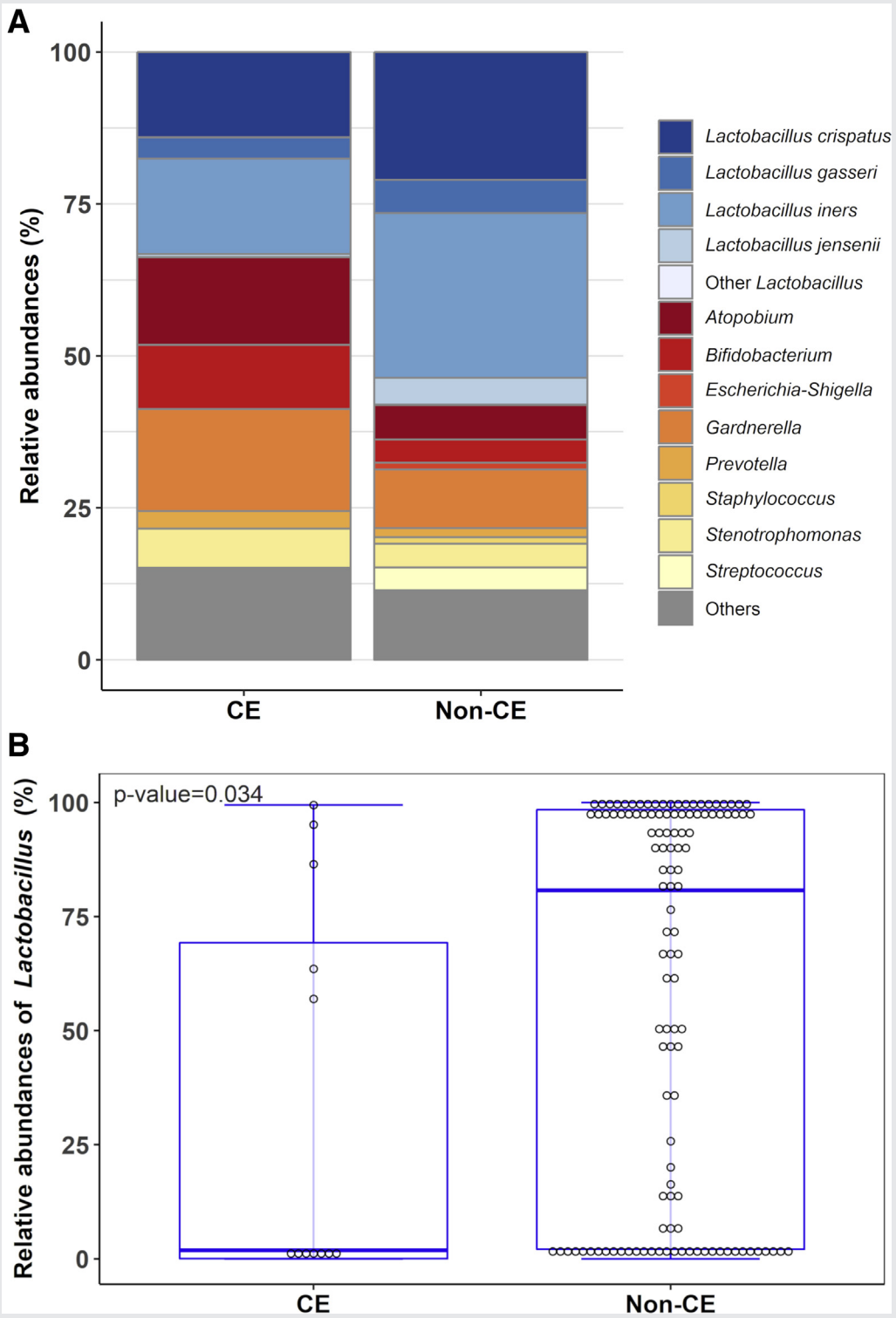
A total of 130 women were recruited. Maternal characteristics are detailed in Supplemental Table 1 (available online). There were no statistically significant differences in maternal age, body mass index, or history of pregnancy between the CE and non-CE groups. Chronic endometriosis was diagnosed in 12 women by use of our stringent criterion of PCD >5.15 per 10 mm^2.

Amplicon Sequencing of the *16S rRNA* Gene

Polymerase chain reaction amplification of the bacterial *16S rRNA* gene and sequencing were successfully performed for all 130 samples. The sequencing data for this study have been deposited in Sequence Read Archive (SRA, study ID PRJNA530321). An average of 10,141 processed reads per sample was achieved, and 12,177 OTUs were detected. Based on the positive control sample (HM-277D, BEI resources), the error rate in our sequencing experiment targeting the V4 hypervariable region was 0.016%, which was comparable to the study that validated this sequencing protocol (15).

We collected one collection medium-only control and one air swab control per woman. According to absorbance measurements made on a spectrophotometer, all negative controls were unlikely to contain DNA (i.e., absorbance at 260 nm [A260] below detection limit or poor A260/A280 ratio). Nevertheless, we attempted sequencing on 16 negative controls (eight of each type of negative controls) with marginally detectable amounts of DNA. Only two air swab controls yielded a mean read count of 9, which was 470 times lower than that of the endometrial samples. No reads were obtained from any of the collection medium-only controls sequenced.

FIGURE 1



Overview of endometrial microbiota in the chronic endometritis (CE) and the non-CE groups. **(A)** The most abundant bacterial genera in the endometrial microbiotas of the two groups. The mean relative abundance of *Lactobacillus* (gradient of *blues*, each representing a species) and non-*Lactobacillus* genera (gradient from deep *red* to pale *yellow*) in each group are shown. Non-*Lactobacillus* genera with a mean abundance of less than 1% were grouped as Others *(grey)*. **(B)**Dot-boxplot of the relative abundance of *Lactobacillus* in the two groups. Each dot is drawn to the relative abundance of *Lactobacillus* in the endometrial microbiota of each participant. The *bold line* within the box is drawn to the median of each group, the *bottom* and *top* of the box to the 25th and 75th percentiles, respectively. The whiskers are drawn to the 10th and 90th percentiles.

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Endometrial Microbiota Composition in CE and Non-CE Groups

The most abundant genus in endometrial microbiotas was *Lactobacillus* (Fig. 1A, gradients of blues combined). The median relative abundance of *Lactobacillus* in endometrial samples in the CE group was 42.7 times lower than in the non-CE group (1.89% vs. 80.7%, Mann-Whitney test, $P=.034$) (see Fig. 1B).

The underlying causes of patients seeking IVF treatment were infertility and recurrent miscarriage. The relative abundance of *Lactobacillus* was not different between these two causes (84.9% vs. 63.3%, Mann-Whitney test, $P=.44$), or between women above or below the median age (36 years) at the time of endometrial sampling (63.0% vs. 83.1%, Mann-Whitney, $P=.62$).

Taxa Associated with CE in the Endometrial Microbiotas

Lactobacillus (up to the Firmicutes phylum) was more abundant in the non-CE microbiota (Fig. 2A and 2B). The Actinobacteria phylum and the Negativicutes class (down to *Dialister* genus) were more abundant in the CE microbiota.

Thirteen OTUs were exclusively detected in the non-CE microbiota but not the CE microbiota (Fig. 3A and 3B, upper panel), including four *Lactobacillus: L. delbrueckii*, *L. coleohominis*, *L. mucosae*, and *L. antri*. *Acinetobacter* was detected only in the CE microbiota (see Fig. 3B, lower panel).

In addition, two OTUs of *L. crispatus* were less abundant (see Fig. 3C, upper panel), and 19 OTUs (18 non-*Lactobacillus* OTUs) were more abundant (lower panel) in the CE microbiota than the non-CE microbiota ($|\log_2$ fold change (LFC)|, range: 1.07–4.24) (Supplemental Table 2, available online). The taxa with increased abundance in the CE endometrial samples included *Dialister* (Otu024), *Bifidobacterium* (Otu008), *Prevotella* (Otu019 and Otu012), *Gardnerella* (Otu085), and *Anaerococcus* (Otu068 and Otu063) (the five greatest LFC).

To explore whether these differentially abundant taxa might be used to classify CE, we calculated a score by dividing the total abundance of the 19 CE-increased taxa (see Fig. 3C, lower panel) by the total abundances of the two CE-decreased taxa (see Fig. 3C, upper panel), and plotted the scores in the CE and non-CE women (Supplemental Fig. 1, available online; Mann-Whitney, $P=.017$). The area under the receiver-operating characteristics curve of the score in classifying CE was 0.710 ($P=.019$) (Supplemental Fig. 2, available online).

Correlation Network of Bacteria in Endometrial Microbiotas

Negative correlations (Fig. 4, red lines) were observed between *L. iners* and each of *Anaerococcus* (Otu0068), *Finegoldia* (Otu0045), *Gardnerella* (Otu0003 and Otu0320), *Polaromonas* (Otu0194), and *Staphylococcus* (Otu0011) (SparCC correlation magnitude, range: $0.142 \rightarrow 0.177$). Also positive correlations (see Fig. 4, blue lines) were observed between pairs of *Lactobacillus* OTUs and between pairs of non-*Lactobacillus* OTUs.

DISCUSSION

Here we present the first study investigating the endometrial microbiome of CE, with all specimens obtained precisely 7 days after the LH surge, a period considered to be a favorable time for embryo implantation. Despite a relatively moderate sample size ($n = 130$), we believe the consistency in the sampling time reduced possible variances due to cyclic changes in the endometrium.

We compared the endometrial microbiota profiles between women with and without CE, defined by our recently reported diagnostic method (8). We examined the entire specimen for $CD138^+$ plasma cells and measured the PCD per unit area. In contrast, many other investigators have examined merely a dozen of randomly chosen high-power fields, which covered a tiny portion of the specimen. Our method took the size of the specimen and the distribution of plasma cells into account.

Previously, we defined CE as PCD >5.15 cells per 10 mm^2, which was the 95th percentile of a fertile population and served to establish a normal reference range (8). Because there is a basal level of PCD in the endometrial stroma of normal fertile women, this threshold is more clinically relevant than the conventional threshold, which defines CE by the presence of any plasma cell in the endometrial stroma. With this stringent threshold, the prevalence of CE in our group of infertile women is 9%, in contrast with that of 43% using the conventional threshold. It appeared that the prevalence of CE reported in many earlier studies could have been overestimated.

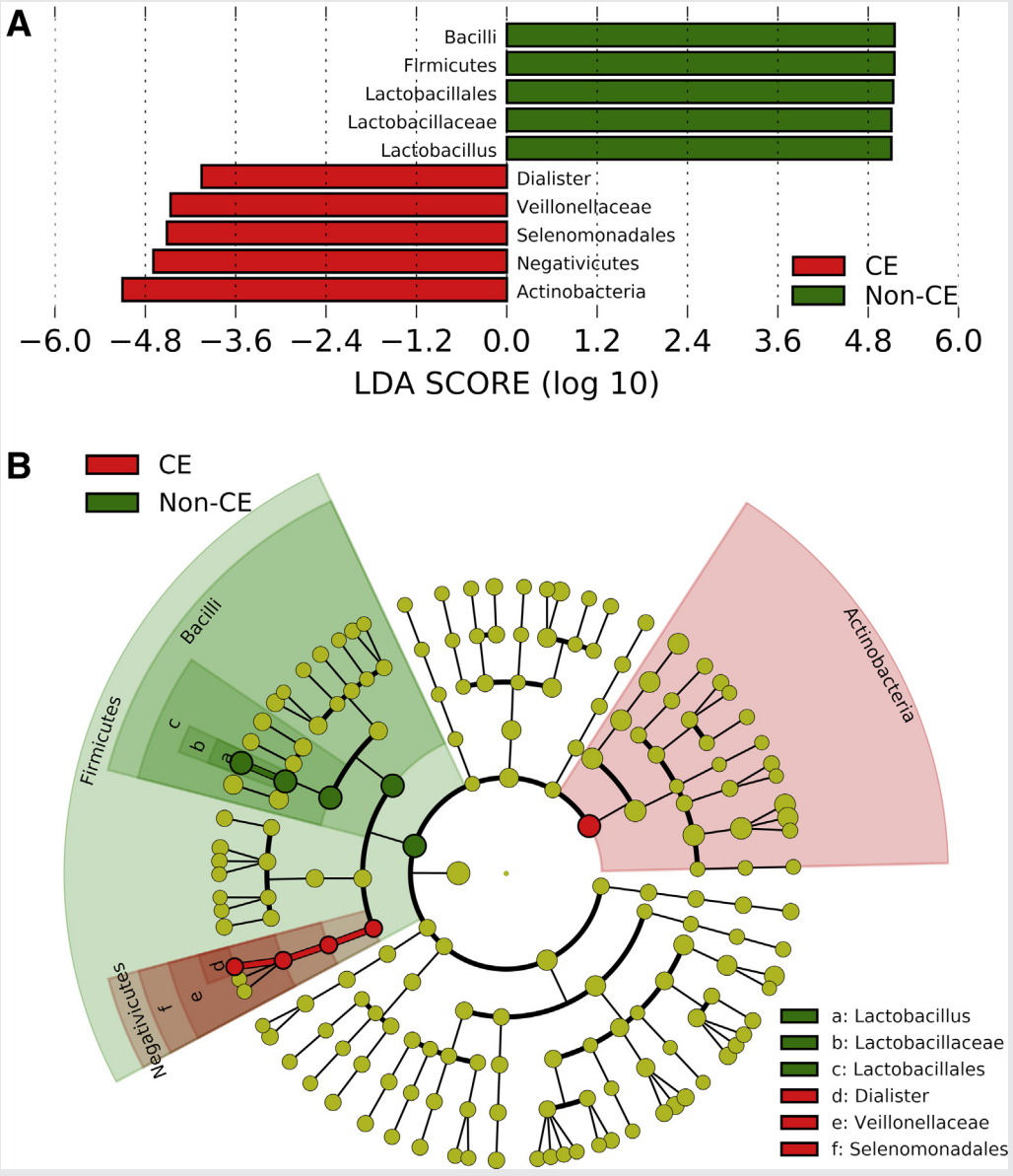
The discrepancy in the prevalence of CE between our study and others may partly due to variations in the genera present in different ethnic groups. Ethnic/racial background has been proven to be one of the strongest associations of both pathways and microbes with clinical metadata (22). In our study, the patients recruited were all of southeastern Chinese origin.

A limitation of our study was the comparison of sequencing results between women with and without CE based solely on histologic criteria. The comparison of sequencing results between women with and without CE based on other diagnostic methods such as microbial culture or hysteroscopy may provide different results.

Although microbial culture does allow the detection of common bacteria that may also be related to CE, it was not applied in our study due to two reasons. First, microbial culture of endometrial secretions in women with CE is often negative. Second, not all microorganisms responsible for CE are culturable. It is estimated that 99% of microbial species resist cultivation due to stringent growth requirements such as optimal combination of nutrients, growth temperatures, and dissolved-oxygen levels, or the need of cocultivation with other key microbial partners (23).

Previously, using generic culture media, Cicinelli et al. (10, 11) detected *Enterococcus faecalis*, Enterbacteriaceae, *Streptococcus* spp., *Staphylococcus* spp., *Gardnerella vaginalis*, *Mycoplasma* spp., *Neisseria gonorrhoeae*, *Mycoplasma*, *Ureaplasma urealyticum*, and *Chlamydia trachomatis* in endometrial samples from women with CE. Other nonculturable bacteria in the CE-positive endometrium

FIGURE 2



Differentially abundant bacterial clades between the chronic endometritis (CE) and the non-CE endometrial microbiotas. **(A)** Linear discriminant analysis (LDA) of the relative abundance of the bacterial clades. The LDA scores estimate the effect size of clades with increased *(red)* or decreased *(green)* abundance between the two groups. **(B)** Taxonomic cladogram of bacteria detected. The first to sixth levels depict the kingdom of Bacteria (innermost), phylum, class, order, family and genus (outermost), respectively. Each detected clade is shown as a small circle, the diameter of which is proportional to its abundance. Any clade with an abundance that was increased or decreased in the CE group is colored as a *red* or *green circle*, respectively. Each differential clade is highlighted in a shade radiating from the concerned circle to a taxonomic label. These labels are arranged from the genus (innermost) to the phylum (outermost) levels.

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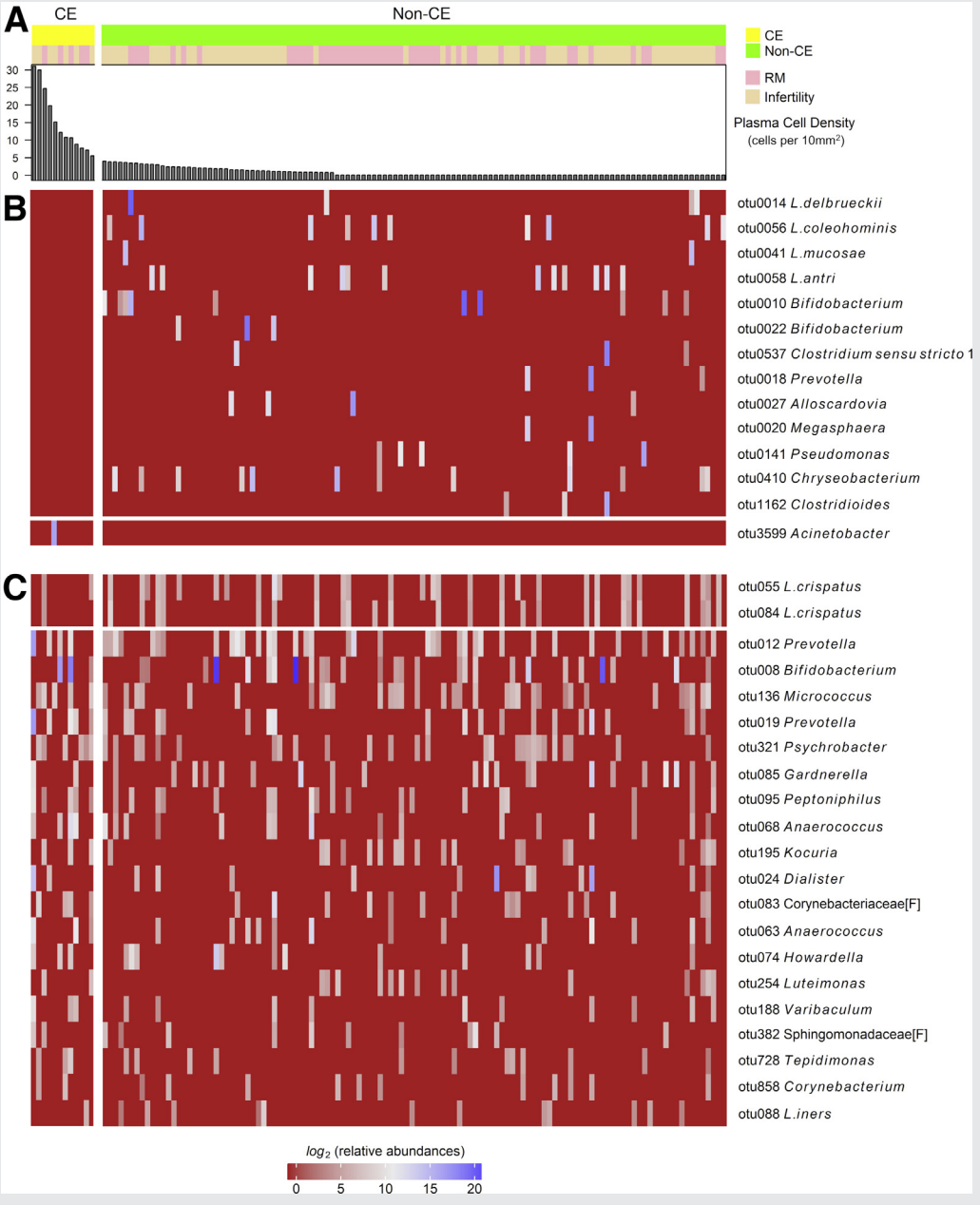
would have gone undetected even though they might be responsible for CE. Like Moreno et al. (12), we opted for a more sensitive, culture-independent approach—namely, *16S rRNA* gene sequencing—to gain a more comprehensive view of the bacteria in the CE endometrial cavity.

In view of the rising concern over the issue of contamination in microbiome studies, we believe the role of negative control is crucial. Potential contaminants might be introduced from the extraction kit, the sampling medium, and the environment, which could lead to false identification of bacteria (24). This could have a strong effect on the validity of the results and the interpretation on clinical relevance of the taxa identified (25, 26). We collected two types of negative controls per patient, which we processed alongside the real endometrial samples. Very few of the negative control samples showed an absorbance spectrum that was characteristic of DNA. Of those samples with marginally detectable levels of DNA, attempted sequencing resulted in

FIGURE 3



Differentially abundant bacterial taxa (operational taxonomic units, OTUs) in the chronic endometritis (CE) and the non-CE endometrial microbiotas. **(A)** Plasma cell density (PCD) in endometrial biopsy. **(B)** *Upper panel:* OTUs absent in the CE group but detected exclusively in the non-CE group. *Lower panel:* OTUs absent in the non-CE group but detected exclusively in the CE group. The $\log_2$ (relative abundance) of an OTU (row) in a woman (column) is shown in each cell according to the color key *(bottom)*. Taxonomic classification of each OTU to the genus level, unless otherwise indicated, is based on the RDP database. **(C)** OTUs with decreased abundances in the CE group compared with the non-CE group (*upper panel*). OTUs with decreased abundance in the non-CE group compared with the CE group. [F], Family level. *L.*, *Lactobacillus*.

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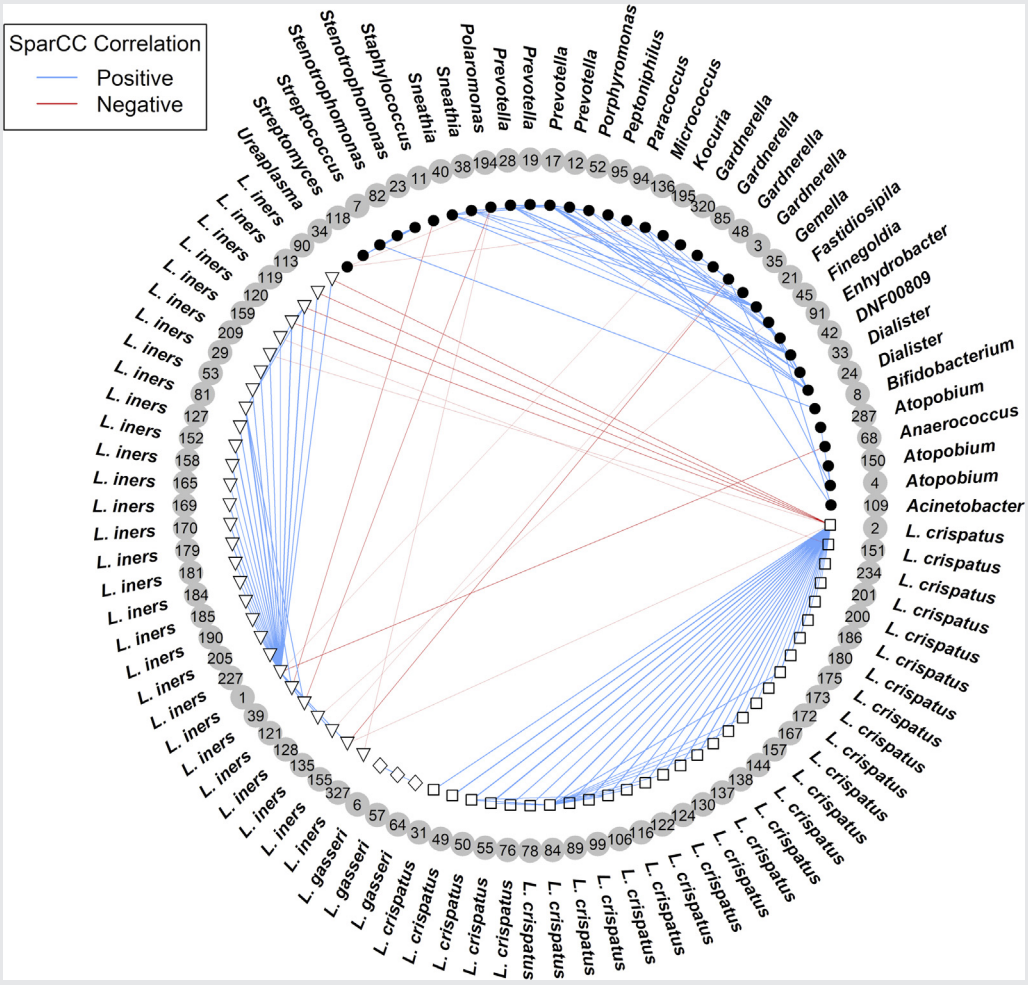
a mean read count 470 times lower than that from the endometrial samples. Such low sequence reads from air swab controls and unsuccessful sequencing in collection-medium controls suggest that contamination in our sequencing data set was minimal, and that the sequencing signals from the endometrial samples were nearly 500-fold higher than the background.

Lactobacillus species are known to inhibit other bacteria by the producing hydrogen peroxide and lactic acid (27–32). We observed negative correlations between *Lactobacillus* and each of *Anaerococcus*, *Finegoldia*, and *Gardnerella*, which were reported to be associated with preterm delivery and bacterial vaginosis (33). This is consistent with previous studies that *Lactobacillus* depletion

FIGURE 4



Correlation of the relative abundance between pairs of bacterial taxa (operational taxonomic units, OTUs) in the chronic endometritis (CE) and the non-CE endometrial microbiotas. Each node represents an OTU with its number shown in *grey circle*. Correlation is inferred by the Sparse Correlations for Compositional (SparCC) data technique. Statistically significant correlations between two OTUs are shown as lines between the nodes. *Blue* and *red colored lines* represent positive and negative correlations, respectively. The weight of each line is proportional to the SparCC correlation magnitude. For clarity, only lines corresponding to positive correlations whose magnitude is greater than 0.3 and negative correlations whose magnitude is greater than 0.14 are drawn. *L.* = *Lactobacillus*.

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and an increased abundance in bacterial vaginosis–associated bacteria are commonly observed (34–36), and that *Lactobacillus* plays a major role as protective bacteria against potential harmful microorganisms (27–32, 37).

Consistent with studies on bacteria detected in the CE endometrial cavity (10, 11), our data suggest that *Gardnerella* was more abundant in the CE endometrial samples than the non-CE samples, and it was negatively correlated in abundance with *Lactobacillus*. Further, our data suggest that *Staphylococcus* was negatively correlated with *Lactobacillus*. However, we observed no statistically significant association of *Streptococcus*, *Mycoplasma*, or *Ureaplasma* with CE, although they were previously detected in the CE endometrial cavity in other studies (10, 11). In other words, besides *Gardnerella* and *Staphylococcus*, the association of 16 non-*Lactobacillus* genera with CE, as identified in our study (see Fig. 3A, upper panel), has not been previously reported.

Of the other CE-associated taxa identified in our study, *Dialister*, *Bifidobacterium*, *Prevotella*, *Anaerococcus*, *L. iners*, Sphingomonadaceae, *Corynebacterium*, *Tepidimonas*, and *Peptoniphilus* were reported to be associated with bacterial vaginosis (34–36, 38–40), preterm birth (33, 37, 41, 42), adenomyosis or endometriosis (43), or female genital tract infection (38, 44–49). *Micrococcus* has been suggested to be an opportunistic pathogen (50) and associated with early embryonic developmental arrest (51).

Additionally, our data suggest that *Finegoldia* and *Polaromonas* were negatively correlated in abundance with *Lactobacillus*. It is interesting that *Finegoldia* has been reported to be associated with infection (52, 53). *Polaromonas* has been detected in some women with the CE diagnosis, but its

statistical association with CE was not mentioned (12). Descriptions of the above taxa are detailed in Supplemental Table 3 (available online).

Of note, the microbiome signature comprising the 19 CE-increased and two CE-decreased bacteria may potentially be useful for classifying CE—it correctly classified the CE and non-CE status in 71% of these 130 women. Validation using an independent cohort will facilitate estimation of sensitivity and specificity of this signature in classifying CE.

Moreno et al. (12) introduced molecular microbiology methods to the diagnosis of CE and compared the results with three classic methods, including histology, hysteroscopy, and microbial culture tests. Among 13 subjects with concordant CE diagnosed by the three classic methods, they examined the samples by reverse-transcription PCR and sequencing. They found that the microbiome results using next-generation sequencing were concordant with reverse-transcription PCR in 92% of cases. The investigators concluded that, compared with the commonly used histology, hysteroscopy, and microbial culture diagnostic methods, reverse-transcription PCR can effectively detect and quantify bacterial DNA from CE-causing pathogens in endometrial samples, thus providing an improved method of diagnosis of CE.

In contrast, we employed our new histologic diagnostic criterion to define CE and sequenced the endometrial microbiota of 118 non-CE women for comparison with the microbiota of the 12 women with CE. Our sequencing data set allowed us to perform statistical tests for differential abundance and draw a more robust conclusion. According to our literature search, our data set of massively parallel sequencing–based endometrial microbiome on 130 infertile women, presented in this report, comprises the largest sample size for studies of its kind. We reason that our 130-women endometrial microbiome data set, a stringent diagnostic criterion for diagnosing CE and statistical tests on differential abundance, contributes additional knowledge to the understanding of CE.

CONCLUSION

In this study, we have identified bacteria that were differentially abundant in the endometrial microbiota of women with or without CE, as diagnosed using a more quantitative method and a more stringent threshold. This includes *Dialister*, *Bifidobacterium*, *Prevotella*, *Gardnerella*, and *Anaerococcus*, which were more abundant in the endometrial microbiota of women with CE than in the microbiota of women who do not have CE. Further studies are needed to confirm the reproducibility and prognostic value of these CE-associated bacteria in the diagnosis and targeted therapy of CE.

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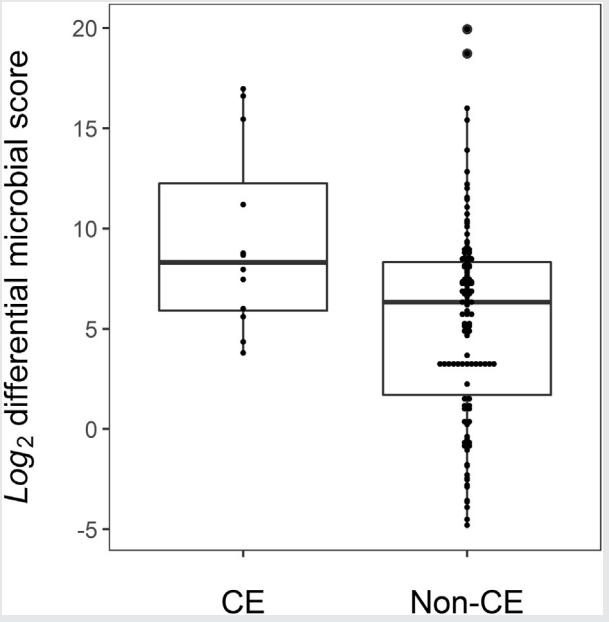
SUPPLEMENTAL FIGURE 1



Differential microbial score based on abundance ratio of selected bacteria in the endometrial cavity. Differentially abundant bacterial taxa in the CE samples compared with the non-CE samples were identified. For each sample, total abundance of the CE-increased taxa, and total abundance of the CE-decreased taxa were calculated. The ratio between the total abundance of the CE-increased taxa and that of the CE-decreased taxa is expressed as a microbial score in the $\log_2$ scale and plotted. Mann-Whitney P=.017.

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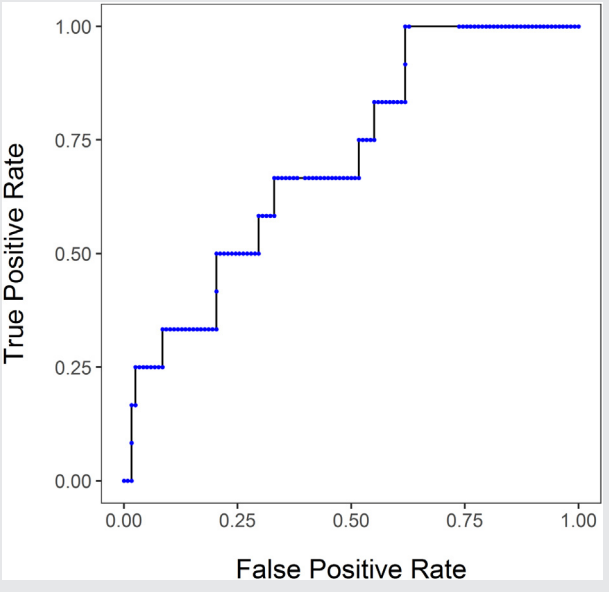
SUPPLEMENTAL FIGURE 2



Receiver-operating characteristics curve of the differential microbial score in classifying CE and non-CE women.

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