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Characterization of the vaginal and endometrial microbiome in patients with chronic endometritis

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ABSTRACT

Objective: To compare the endometrial and vaginal microbiome of women with and without chronic endometritis.

Study design: A cohort study with 60 patients undergoing assisted reproductive treatment with their own or donated gametes was undertaken. Vaginal and endometrial samples were taken in the cycle prior to embryo transfer. The endometrial and vaginal microbiome was analysed by mass sequencing of the V3V4 region of 16S rRNA gene. Bioinformatics analysis was performed using QIIME2 and MicrobiomeAnalyst packages. Alpha diversity, beta diversity and taxonomic characterization were compared between samples that tested positive and negative for chronic endometritis on CD138 immunohistochemistry.

Results: Different bacterial communities were detected when vaginal and endometrial samples were analysed in patients with and without endometritis diagnosed using CD138 immunohistochemistry. In patients with endometritis, a higher alpha-diversity index was found in vaginal samples (p = 0.15 for the Shannon index) and significant differences were found in endometrial samples (p = 0.01 for the Shannon index). In the beta-diversity analysis, no significant differences were observed between the groups with and without endometritis. Vaginal and endometrial samples from women with endometritis showed a microbiome pattern that was not dominated by *Lactobacillus* spp. Relative abundance analysis identified *Ralstonia* and *Gardnerella* spp. in endometrial samples, and *Streptoccoccus* and *Ureaplasma* spp. in vaginal samples of patients diagnosed with chronic endometritis on CD138 immunohistochemistry. When comparing endometrial and vaginal samples diagnosed with endometritis on CD138 immunohistochemistry, both alpha diversity (p = 0.06 for the Shannon index and p = 0.08 for the Simpson index) and beta diversity (p < 0.001) showed significant differences. *Lactobacillus* spp. (p = 3.76E-4), *Ralstonia* spp. (p = 8.19E-4), *Delftia* spp. (p = 0.004) and *Anaerobacillus* spp. (p = 0.004) were identified in these sample groups.

Conclusion: These results demonstrate the existence of a characteristic vaginal and endometrial microbiota in patients with chronic endometritis. Different genera and species were identified in patients with and without chronic endometritis depending on whether the sample was endometrial or vaginal. There is a clear relationship between changes in the vaginal microbiome and chronic endometritis. The microbiota is a continuum throughout the female reproductive tract, so study of the vaginal microbiota could be useful for the diagnosis of diseases of the upper reproductive tract, such as chronic endometritis.

Introduction

Chronic endometritis (CE) is a disease characterized by persistent inflammation of the endometrial lining. Clinically, CE can be asymptomatic or may present subtle symptoms, so it is likely to be underdiagnosed in the general population. The prevalence of CE in infertile patients, especially among those suffering from implantation failure, ranges from 14% to 58% [1]. Recently, two single-centred studies in fertility clinics in Italy [2] and China (Hong Kong) [3] have shown that CE may be correlated with an altered endometrial microbiome. However, the causality between CE and reproductive failure is yet to be established. It is well

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known that the microbiome may be affected by ethnicity. Therefore, this study was undertaken to investigate this topic in a Spanish fertility clinic [4].

Diagnosis of CE is based on hysteroscopy of the uterine cavity and endometrial biopsy with histological analysis of endometrial stromal plasma cells. There is a great deal of controversy regarding the diagnosis of CE as different techniques have different sensitivity and specificity. In a study by Chen et al., the rate of CE detection with CD138 immunohistochemical staining was 27.96% in infertile patients [5]. However, Fan et al. found no obvious differences between two different CD138 + cell quantification methods with prevalence of 31.2% [6]. Zargar et al. used two methods for CE diagnosis – hysteroscopy and immunohistochemistry with CD138 – in the diagnosis of recurrent implantation failure (23.4%, 21.3%) and repeated pregnancy loss (36.8%, 31.6%) after in-vitro fertilization [7]. Liu et al. reported that, because a small number of plasma cells may be present in the endometrium of fertile subjects, CD138 improves the accuracy of CE diagnosis, and the prevalence of CE in women with reproductive impairment was only 10% [8].

Histopathological evaluation by immunohistochemistry for plasmacyte marker CD138 (also known as sindecan-1, a proteoglycan of transmembrane-type heparan sulfate) is currently the most reliable and sensitive diagnostic method for CE [9]. Endometrial-positive CD138 appears to be a negative prognostic indicator for patients who have experienced repeated implantation failure [5]. Microbiome analysis based on subunit 16S rRNA sequencing can enable rapid identification of pathogenic microorganisms associated with CE. These new approaches provide information on the relationship between endometritis and microorganisms responsible for unfavourable conditions in the uterine cavity [10].

The main bacteria at vaginal and endometrial level are *Lactobacillus* spp.; these bacteria produce lactic acid that maintains the acidic pH of the vagina and acts as a barrier against pathogens. Micro-organisms frequently detected in endometrium with CE are common bacteria, such as *Mycoplasma* spp., *Ureaplasma* spp. and *Gardnerella* spp. [11]. Microbial analysis based on sequencing of the 16S rRNA gene has identified cultivable and non-cultivable pathogenic microorganisms associated with CE.

Abnormal endometrial microbiota has been associated with implantation failure and pregnancy loss [12]. Evaluation of the endometrial microbiome is important as a future tool to improve reproductive outcomes in infertile patients with CE [13]. Study of the vaginal microbiota in these patients is important due to its capacity to colonize the endometrium.

This study aimed to advance CE research. Classic diagnostic methods of CE and immunohistochemistry with CD138 for the diagnosis of CE were analysed using the vaginal and endometrial microbiome by sequencing the bacterial 16S rRNA gene.

Materials and methods

Design and study population

This was a pilot study involving 60 patients and 120 samples. The study population consisted of patients from the Instituto Bernabeu Fertility Clinic, Alicante, Spain who underwent transfer of frozen euploid embryos between May 2017 and May 2019. Preimplantation genetic testing of aneuploidy (PGT-A) was performed at blastocyst stage (Veriseq, Illumina, San Diego, CA, USA). PGT-A is recommended in cases of advanced maternal age (>38 years), recurrent implantation failure, repeated pregnancy loss and severe male factor infertility. The inclusion criteria were: age between 18 and 50 years; own or donated oocytes; and use of intracytoplasmic sperm injection to generate embryos. In addition, included patients did not receive any antibiotics for 3 months preceding fertility treatment. The exclusion criteria were uterine malformation, untreated hydrosalpinx, or known implantation failure factors.

Collection of vaginal and endometrial samples

Vaginal and endometrial samples were taken during the secretory phase of the cycle preceding in-vitro fertilization treatment. A dry swab was used to collect vaginal samples from the bottom of the back sac by direct visualization with a vaginal speculum and in the lithotomy position. Endometrial samples were collected using a Tao Brush IUMC Endometrial Sampler. Samples were stored at −80 °C until processing.

Analysis of vaginal and endometrial samples

Conventional anatomical analysis

Tissues were fixed in neutral buffered formalin and embedded in paraffin wax for histological assessment. Samples were cut into 4-μm slices and stained with haematoxylin and eosin. The criterion for pathological diagnosis of CE was the number of plasma cells in the endometrial stroma.

Evaluation of CD138 immunohistochemistry

For immunohistochemical identification of plasma cells, antibody for CD138 [CD138/syndecan-1 (B-A38)] was applied in all cases using an ultraView Universal DAB Detection Kit on a BenchMark GX Instrument (Ventana, Roche, Basel, Switzerland). Staining quantification was performed by two observers using a multihead BX50 microscope (Olympus, Shinjuku, Japan). Results were expressed as the number of positive cells per five high-power fields. Cases with at least two stained cells were considered positive for CE.

Metagenomics

The 16S rRNA gene was studied from vaginal and endometrial samples. Sample analysis was carried out at Instituto Bernabeu Biotech.

Microbial DNA extraction

DNA extraction was performed using the PureLink Microbiome DNA Purification Kit (ThermoFisher, Waltham, MA, USA). DNA was quantified using a Qubit 2.0 Kit (ThermoFisher). DNA was stored at −20 °C for further analysis.

Amplification of V3V4 region of 16S rRNA gene

Polymerase chain reaction (PCR) amplification of the V3V4 variable region of the 16S rRNA gene was performed with Taq DNA polymerase (2x KAPA HiFi HotStart, Roche) in the presence of dNTP, and oligonucleotides 357F and 806R at 1 μM and 100 ng of DNA, with a final volume of 25 μl. PCR was carried out in a thermocycler (Verity, Applied Biosystems, Foster City, CA, USA). PCR products were displayed in 1% agarose gel (449 bp). Amplification products were stored at −20 °C for sequencing.

Sequencing the V3V4 region of 16S rRNA gene

The V3V4 amplicon was purified and a library was generated with indexes of each sample using a Nextera XT Sequencing Kit (Illumina). After purification of the library, samples were quantified using Qubit 2.0. Samples were diluted to 4 nM, mixed and prepared for sequencing. The final concentration of the library was 15 pM. The library was sequenced using Miseq Reagent Kit v3 (Illumina) reagents. Miseq (Illumina) was used as the sequencer and metagenomics was used as the workflow.

Bioinformatics analysis of the sequences

The primary analysis of sequences obtained from sequencing consisted of demultiplexing using MiSeqReporter (Illumina) software. Unindexed paired endpoint sequences were exported from Miseq to continue their analysis in FASTA format.

Bioinformatics analysis of sequences was performed using QIIME2 [14,15] with Deblur (trim-length 450). SILVA was used for taxonomic characterization. In addition, MicrobiomeAnalyst [16] and Bioconductor Phyloseq [17] were used for further data analysis. Sequences were grouped into operational taxonomic units (OTUs) with a similarity percentage of 97%. To study microbial diversity, analysis was performed at 1000 sequences per sample for different alpha-diversity indexes (Shannon and Simpson indexes). Alpha diversity measures the richness of a species in a given community. The Shannon index determines the species biodiversity of a community. The Simpson index is a quantitative measure of species in a community, and considers the phylogenetic relations between the individuals. Beta diversity analysis was performed by calculating the UniFrac index. UniFrac is a measure of beta diversity that uses phylogenetic information to compare samples belonging to two groups of interest. The results were displayed with QIIME2 using graphs generated by principal coordinates analysis (PCoA) obtained with EMPEROR. Taxonomic mapping used a classification based on filtering of the 99_otus sequence from the SILVA database to the V3V4 region. Sequences obtained were assigned to at least one genus. Finally, specific analysis was performed for each taxon according to the results obtained.

Statistical analysis

Microbiome patterns were compared for vaginal and endometrial samples between groups with and without endometritis using double-input tables. Chi-squared test was used to determine the distribution of qualitative variables. Parametric Student's *t*-test or non-parametric Mann–Whitney *U* test was used to determine the distribution of quantitative variables. Raw *p*-values were adjusted for multiple testing based on the false discovery rate method.

Results

Description of the variables studied

Sixty patients were originally recruited in this study, and 54 were included in the analysis. One hundred and twenty samples were obtained, and 108 samples were analysed. In total, 194 OTU and 210,594 sequences were identified in all analysed samples (3899 average reading per sample). Fig. 1 shows the study flow. Thirty patients were CD138-positive (at least two stained cells) and were considered positive for CE. The rate of CE detection by CD138 immunohistochemical staining was 55.5% in infertile patients. Sociodemographic characteristics and prior clinical outcomes of patients included in the study are detailed in Table 1.

Vaginal and endometrial microbial patterns in diagnosis of CE

This study investigated differences in the microbiome pattern in samples which had undergone conventional anatomopathological analysis with haematoxylin and eosin to determine chronic endometritis. In terms of alpha diversity, no differences in the Shannon index (p = 0.98) and Simpson index (p = 0.99) (Fig. 2a) were found between endometrial samples, classified previously into three categories: positive, negative and probable. The relative frequencies of the most abundant genera according to histological diagnosis are shown in Fig. 2b.

Differences in the microbiome pattern in samples tested were studied using CD138 immunohistochemistry. In analysis of the endometrial microbiome for alpha diversity, significant differences

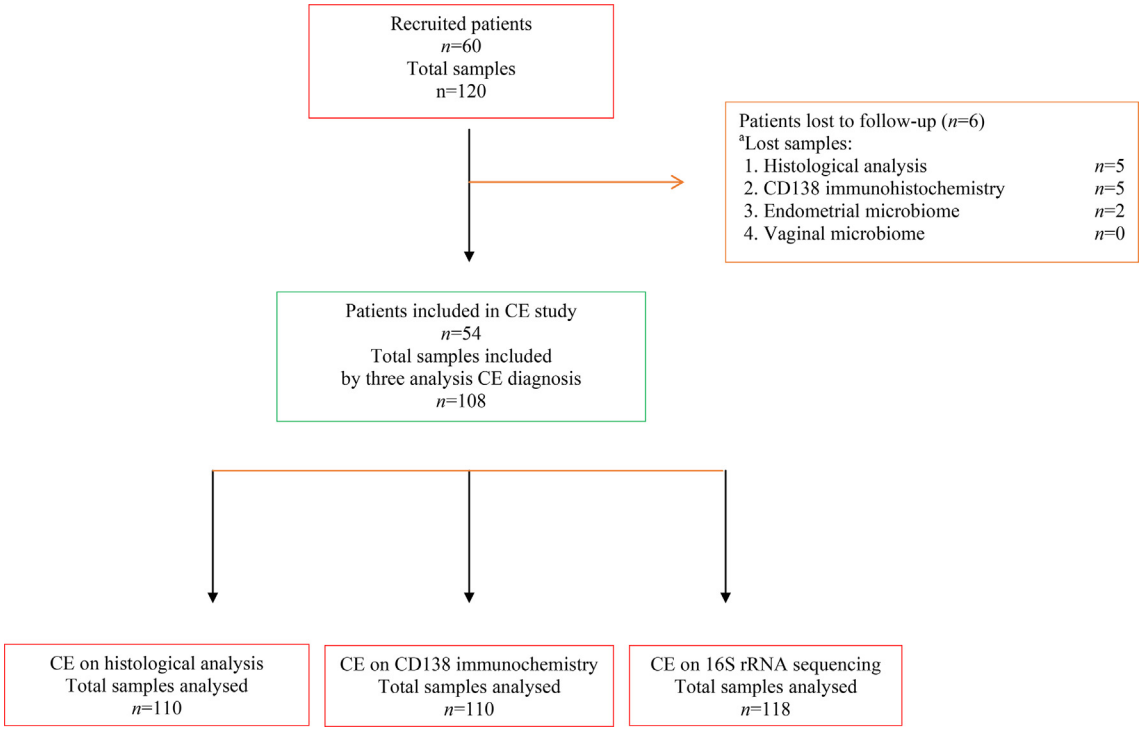

Fig. 1. Diagram of the study flow and distribution of the population investigated. Comparative diagnosis of chronic endometritis (CE) performed by three analysis: Histological analysis, CD138 immunohistochemistry and 16S rRNA gene sequencing (endometrial and vaginal microbiome). [a]Lost samples overlapped in the four categories and corresponded to six patients. There were insufficient samples to amplify and sequence.

Table 1
Sociodemographic characteristics and clinical outcomes of patients included in the study.

	Total
n	60
Age (years)	39.2
Weight (kg)	67.25
Height (m)	1.57
Tobacco user (%)	13.8
Previous pregnancies (%)	50
Previous miscarriages (%)	58
No. of previous miscarriages	2.5
Previous treatments (%)	74

were found between the Shannon index (p = 0.011) and the Simpson index (p = 0.018) (Fig. 3a). The vaginal microbiome showed differences in alpha diversity with the Shannon index (p = 0.15) and the Simpson index (p = 0.22), although these differences did not reach significance (Fig. 3c). In terms of beta diversity, no clear pattern of separation was observed between the groups with positive or negative CD138 results, as shown in the graphs generated by PCoA for endometrial (Fig. 3b) and vaginal samples (Fig. 3d).

Regarding taxonomic characterization, Fig. 4a shows taxonomic diversity in endometrial samples. *Lactobacillus* was the most common genus (85.5%), followed by *Gardnerella* (3%), *Dialister* (2.5%) and *Burkholderia* (2.5%). In vaginal samples (Fig. 4b), *Lactobacillus* (95%) was the most common genus, followed by *Streptococcus* (2.7%), *Escherichia* (1%) and *Prevotella* (0.5%).

Microbiome profiles show different genera and species in the endometrial (Fig. 5a) and vaginal (Fig. 5b) samples with respect to CD138 diagnosis. Regarding the relative frequencies of the most abundant genera, significant differences were observed in vaginal and endometrial samples between samples that had positive and negative CD138 results (Table 2). The univariant of relative abundance of different genera was significant for *Lactobacillus* (p = 0.02), *Ralstonia* (p = 0.04) and *Gardnerella* (p = 0.05), and a trend was observed for *Anaerobacillus* (p = 0.08), *Streptococcus* (p = 0.08) and *Burkholderia* (p = 0.13) for endometrial samples from CE patients with endometritis (Fig. 5a). In the univariant analysis according to genera, significant differences were found for *Streptococcus* (p = 0.03) and *Ureaplasma* (p = 0.09) in vaginal samples of patients with chronic endometritis.

Fig. 6 shows a positive correlation between *Gardnerella* and *Anaerobacillus*, *Bacillus* and *Ralstonia* in endometrial samples. On other hand, a negative correlation was found between *Gardnerella* and *Dialister*, *Delftia*, *Burkholderia*, *Streptococcus* and *Lactobacillus*. Thus, eight genera were correlated with *Gardnerella*. Samples from women who did not have chronic endometritis had a higher proportion of *Lactobacillus* spp., while women who had chronic endometritis had a higher proportion of *Gardnerella* spp.

Finally, the microbiome patterns of the endometrium and vagina were compared in samples that were CD138 positive (Fig. 7). In terms of alpha diversity, near significant differences were found between the endometrial and vaginal samples for the Shannon index (p = 0.06) and the Simpson index (p = 0.08) (Fig. 7a). For beta diversity (Fig. 7b), a clear pattern of separation was observed between the endometrial and vagina samples, as seen in the graph generated by PCoA (p < 0.001). Fig. 7c shows the profile of the microbiome with different genera present in endometrial and vaginal samples.

Discussion

The results of this study show that the vaginal and endometrial microbiome, analysed by the V3V4 region of 16S rRNA gene, is associated with the diagnosis of chronic endometritis, as the profiles observed differed between samples from patients with and without CE using the CD138 marker for diagnosis. Profiles not dominated by *Lactobacillus* spp. were associated with, and a relationship was found between the diversity of the vaginal and endometrial microbiome and CE.

Many studies have highlighted the diagnostic value of CD138 for CE, risk factors for the pathogenesis of CE, and the effect of CE on pregnancy. All of them have concluded that immunohistochemistry with CD138 can improve the diagnostic rate for CE [7].

In the present study, alpha diversity analysis showed low values associated with the diagnosis of CD138-positive endometritis. Women with CE showed significantly more alpha diversity than women who did not have CE. In other studies that analysed the vaginal microbiome by gestational week, lower rates of diversity were reported in patients with ongoing pregnancies [18]. Therefore, the fact that patients with CE have greater alpha diversity aligns with the fact that an embryo is less likely to implant and develop into an ongoing pregnancy in these patients [19].

With regard to beta diversity, some authors have observed differences between in beta diversity between pregnant and non-pregnant women. A normal pregnancy is characterized by a microbiome that has low diversity and high stability [20].

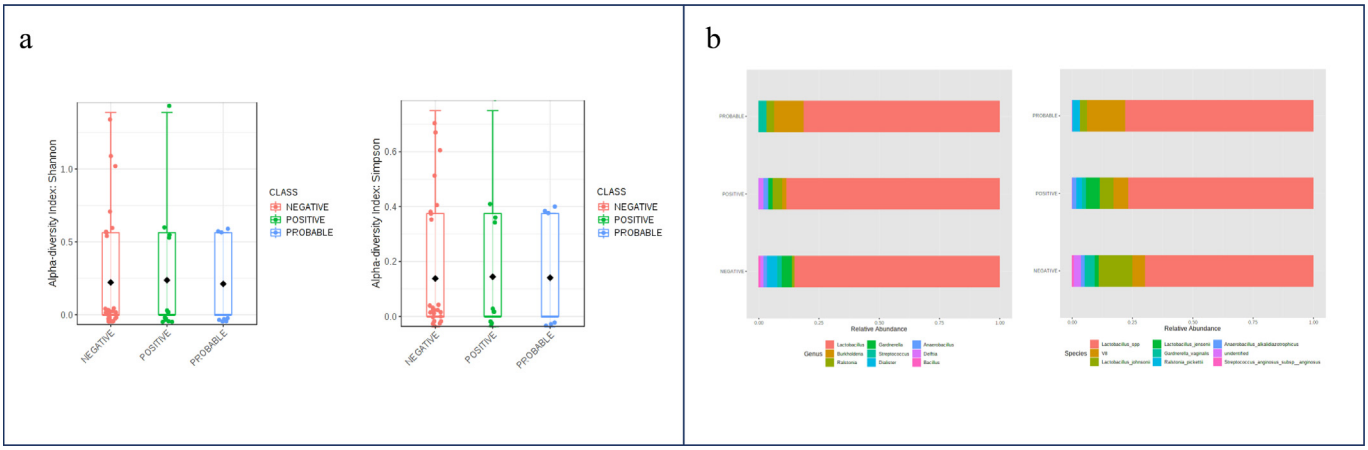

Fig. 2. Comparison of alpha-diversity values in histological diagnostic groups. (a) Comparative analysis of the Shannon diversity index (p = 0.98) and the Simpson diversity index (p = 0.99) for positive, probable and negative histological diagnostic groups in endometrial samples. (b) Bar chart of the relative frequency of the most abundant genera and species of histological diagnostic groups in endometrial samples.

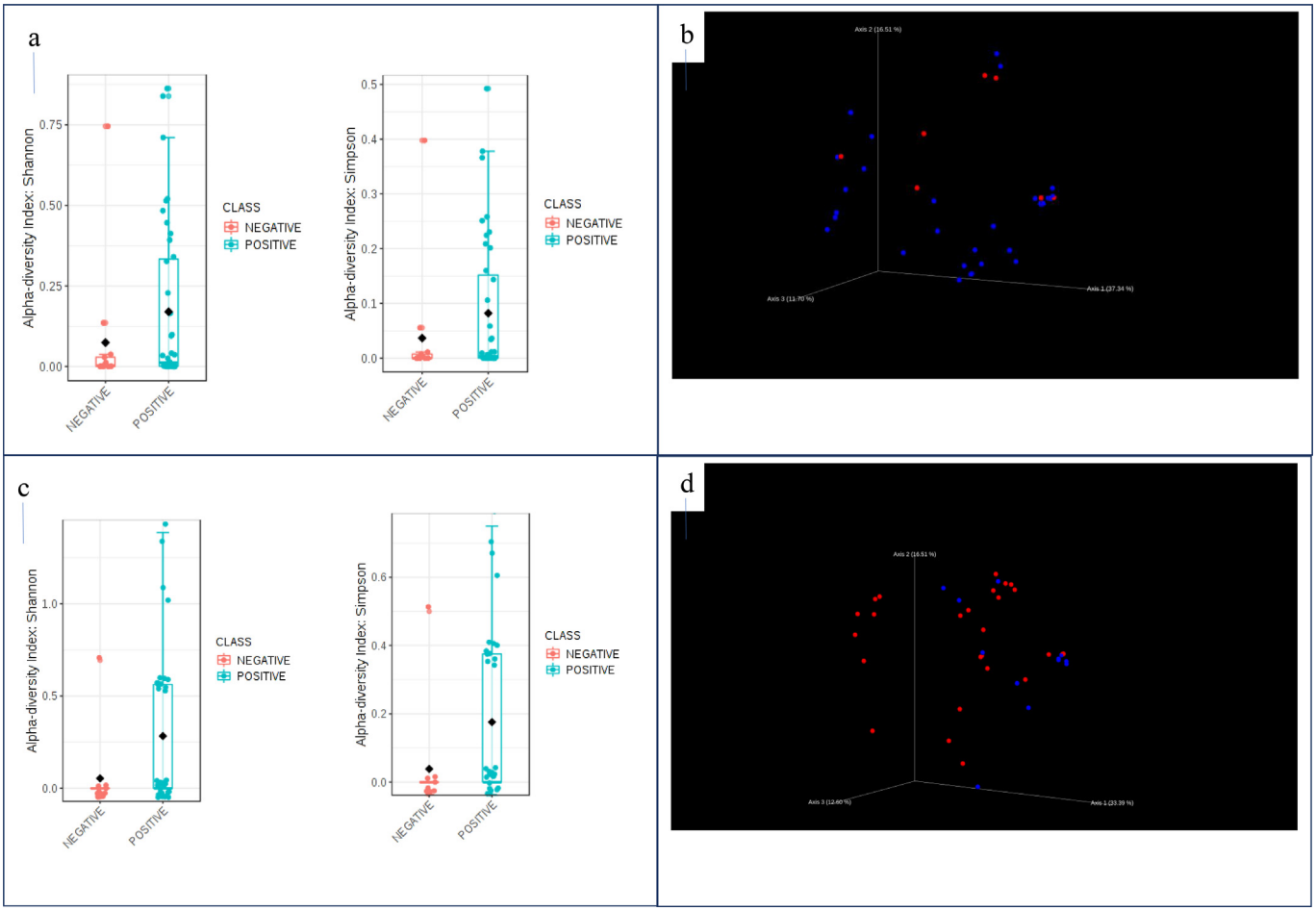

Fig. 3. (a) Comparison of alpha-diversity values for positive and negative results on CD138 immunohistochemistry, Shannon diversity index ($p = 0.15$) and Simpson diversity index ($p = 0.22$) in endometrial samples. (b) Comparison of beta-diversity values for positive (red) and negative (blue) CD138 results on endometrial samples. (c) Comparison of alpha-diversity values for CD138 immunohistochemistry, Shannon diversity index ($p = 0.011$) and Simpson diversity index ($p = 0.018$) in vaginal samples. (d) Principal coordinates analysis for positive (red) and negative (blue) CD138 results in vaginal samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

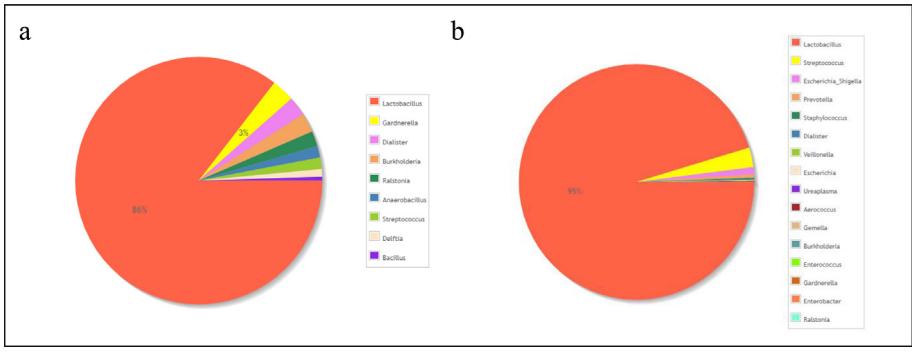

Fig. 4. (a) Taxonomic diversity by genus in samples included in this study. Pie chart showing the relative frequency of the most abundant species in endometrial samples on CD138 immunohistochemistry. (b) Pie chart showing the relative frequency of the most abundant species in vaginal samples on CD138 immunohistochemistry.

Taxonomic characterization showed unequivocal dominance of the genus *Lactobacillus* in the vaginal and endometrial microbiome. However, a surprising aspect was the finding that different genera were found in endometrial and vaginal samples.

Comparison of the relative abundance of genera provided more data on the relationship between the microbiome and chronic endometritis. On one hand, samples dominated by *Lactobacillus* spp. and showing less diversity were characterized by being negative for CE. Endometrial samples that were not dominated by *Lactobacillus* spp. and contained *Ralstonia* spp. and *Gardnerella* spp., and vaginal samples that contained *Streptococcus* spp. and *Ureaplasma* spp. as well as *Lactobacillus* spp. were positive for CE. Moreover, a negative correlation was observed between *Lactobacillus* spp. and *Gardnerella* spp., *Anaerobacillus* spp.,

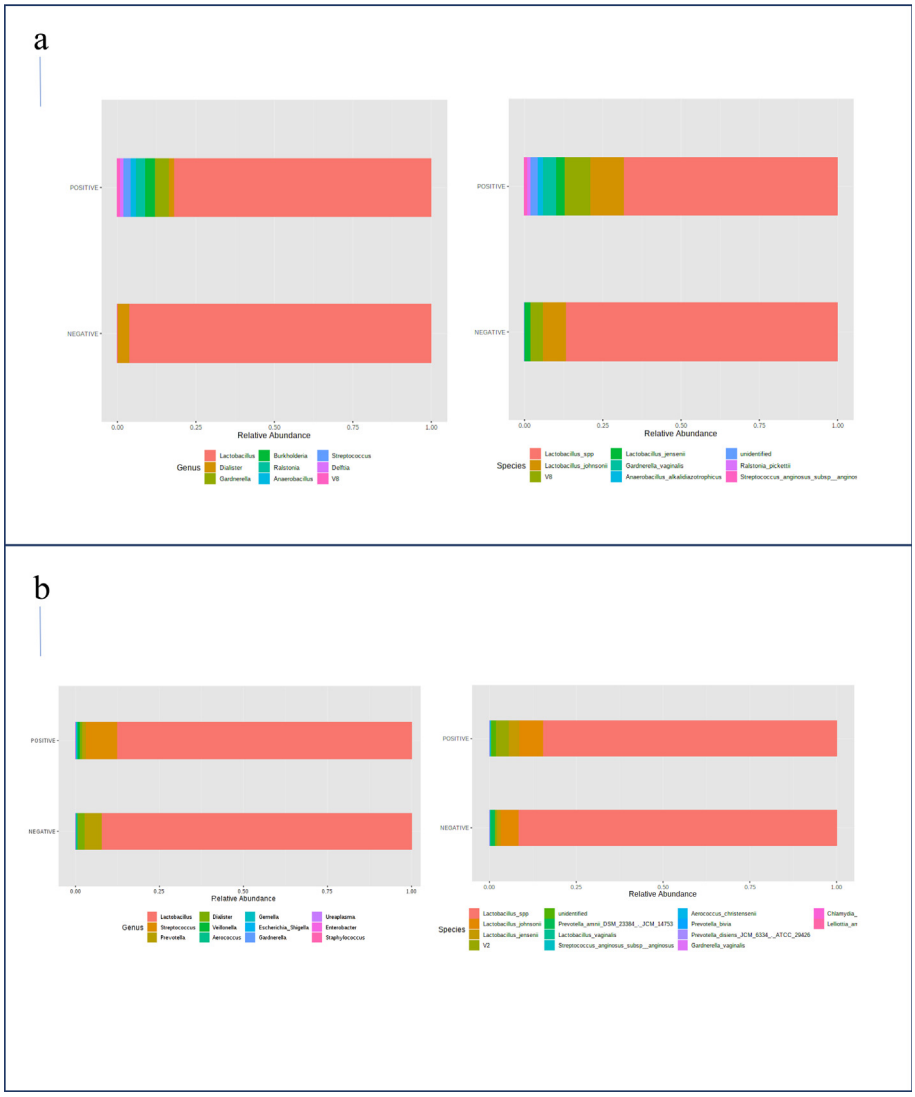

Fig. 5. (a) Bar chart of the relative frequency of the most abundant genera and species in endometrial samples on CD138 immunohistochemistry. (b) Bar chart of the relative frequency of the most abundant genera and species in vaginal samples on CD138 immunohistochemistry.

Table 2
Differences in the genus present in microbiome profiles in endometrial and vaginal samples that were negative or positive on CD138 immunohistochemistry.

	Genus	CD138-negative (%)	CD138-positive (%)	*p*-value
Endometrium	*Anaerobacillus*	0.00	2.03	0.08
	Burkholderia	0.00	3.38	0.13
	Delftia	0.00	1.35	0.16
	Dialister	3.85	2.03	0.68
	Lactobacillus	96.15	81.76	0.02
	Ralstonia	0.00	2.70	0.04
	Gardnerella	0.00	4.05	0.05
	Streptococcus	0.00	2.03	0.08
Vagina	*Aerococcus*	0.38	0.09	0.41
	Dialister	2.18	0.68	0.70
	Escherichia	0.00	0.17	0.59
	Gardnerella	0.13	0.04	0.43
	Klebsiella	0.00	0.04	0.59
	Lactobacillus	92.05	87.44	0.26
	Prevotella	5.13	0.98	0.87
	Staphylococcus	0.00	0.04	0.59
	Streptococcus	0.00	9.44	0.03
	Ureaplasma	0.13	0.00	0.09
	Veillonella	0.00	0.68	0.43

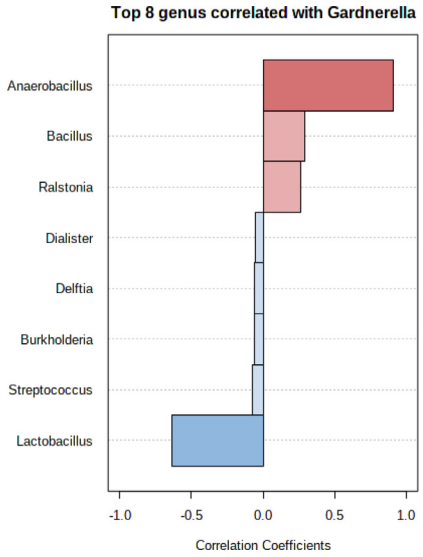

Fig. 6. Univariate analysis: correlation coefficients showing the most abundant species in endometrial samples. Analysis of correlation with *Gardnerella* spp. (p = 0.0059) and *Anaerobacillus* spp. (p = 0.0748). Top eight genera correlated with *Gardnerella* spp.

Bacillus spp. and *Ralstonia* spp. in endometrial samples; these genera are known to be associated with bacterial vaginosis and preterm birth.

In accordance with other studies that have detected bacteria in the endometrial cavity of patients with CE [3], the present study found that *Ralstonia* spp. and *Gardnerella* spp. were more abundant in endometrial samples from CE patients with endometritis, and *Streptococcus* spp. and *Ureaplasma* spp. were more abundant in vaginal samples from patients with endometritis, and this was negatively correlated with the abundance of *Lactobacillus spp*. The microbiota in women with bacterial vaginosis is significantly altered compared with the normal healthy state, in which the microbiota is dominated by *Lactobacillus* spp. In both bacterial vaginosis and chronic endometritis, *Lactobacillus* spp. are substituted by anaerobic species, including *Gardnerella* and *Prevotella* spp. [21].

Next-generation sequencing is more accurate than quantitative PCR as it allows amplification of all bacterial species, showing the enormous diversity of the vaginal or endometrial microbiome in CE patients with endometritis. Molecular microbiology and metagenomics are diagnostic tools that allow identification of cultivable and non-cultivable endometrial pathogens associated with CE [2].

Franasiak et al. used microbial analysis to investigate the relationship between endometritis and unknown infectious conditions in the uterine cavity [22]. New approaches have the potential to shed light on this relationship [9].

With the use of next-generation sequencing, the endometrial microbiome is being characterized in a more detailed manner. However, there is no consensus regarding whether an altered microbiome is the cause or the effect of upper gynaecological tract disease [23].

The study of endometrial samples is essential, but it is also necessary to study vaginal samples as studies have shown that the microbiota differs within the reproductive tract. This could be useful for the detection of common diseases in the upper reproductive tract [24].

Studies have suggested that the uterine microbiota is likely to reflect bacterial invaders, rather than a resident population that contributes to health and homeostasis. An altered microbiome pattern could be predictive of disease, and this dysbiosis could lead to negative outcomes for reproductive function [25].

This study has a novel approach to the diagnosis of CE, and confirmed that alterations in the microbiome, including changes in the domination of *Lactobacillus* spp. in the female reproductive tract, are associated with chronic endometritis. Future studies are needed to confirm the reproducibility and prognostic value of bacteria associated with chronic endometritis.

Conclusions

This study found that patients with chronic endometritis have a characteristic endometrial and vaginal microbiome. The microbiome profile of chronic endometritis is not dominated by *Lactobacillus* spp.; *Streptoccoccus* spp. and *Ureaplasma* spp. were also observed in vaginal samples, and *Ralstonia* spp. and *Gardnerella* spp. were also observed in endometrial samples. Further studies should be conducted to confirm these findings, and to determine the role of antibiotic and/or probiotic treatment for normalization of the microbiome pattern and its consequences in terms of clinical outcome.

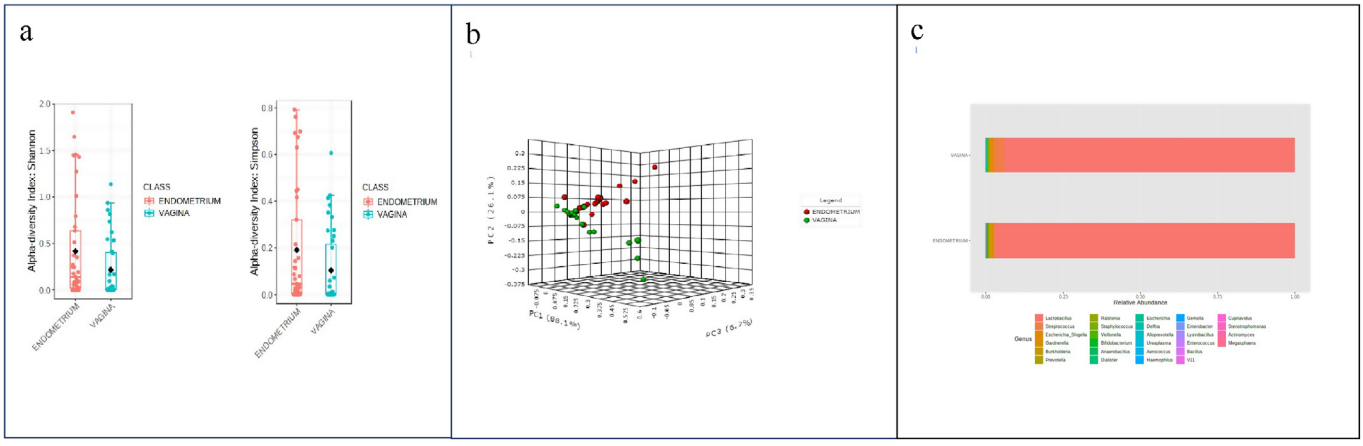

Fig. 7. (a) Comparison of alpha-diversity values for endometrial and vaginal samples that tested positive on CD138 immunohistochemistry, Shannon diversity index (p = 0.06) and Simpson diversity index (p = 0.08). (b) Comparison of beta-diversity values for endometrial (red) and vaginal (blue) samples on CD138 immunohistochemistry (p < 0.001). (c) Bar chart of relative frequency of the most abundant genera in endometrial and vaginal samples on CD138 immunohistochemistry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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