Microbiome in Women with Endometriosis and the *In Vitro* Effects of *Lactobacillus reuteri* on Human Endometrium

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ABSTRACT

Microbiome in Women with Endometriosis and the *In Vitro* Effects of *Lactobacillus reuteri* on Human Endometrium

Background:

Endometriosis (EMS) is a chronic inflammatory condition affecting approximately 10% of women of reproductive age. Recent research suggests that the human microbiome may play a crucial role in the development and progression of EMS due to its influence on immunomodulation and estrogen metabolism. However, the relationship between the microbiome and EMS remains incompletely understood. This study aims to evaluate microbiome composition differences in the reproductive organs, including the vagina, endometrium (EM), and peritoneal fluid (PF), between women with EMS and those without the disease. Additionally, the potential impact of *Lactobacillus reuteri (L. reuteri)* on EM was investigated through *in vitro* co-culture experiments.

Methods:

A total of 41 patients were included in the study, with samples taken from the vagina, EM, and PF. The composition of the microbiome was analyzed using 16S rRNA gene sequencing, targeting the V3 and V4 regions. Additionally, western blot analysis was conducted to identify proteins exhibiting differential expression during the co-culture of endometrial cells with *L. reuteri*, and this process was repeated following the addition of estradiol-17-glucuronide (E2G) to the culture medium. To assess estrogen metabolism mediated by *L. reuteri*, β-glucuronidase concentrations were measured using ELISA, while estradiol, E2G, and the estradiol/E2G ratio were evaluated using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results:

When comparing the overall microbiome composition between women with endometriosis and controls in the vagina, endometrium, and peritoneal fluid, no significant differences were observed in alpha and beta diversity indices between the two groups. However, when analyzing the relative abundance of operational taxonomic units and conducting LEfSe analysis, several bacteria exhibited significant differences between the two groups. Among various bacteria, *L. reuteri* was the only

species that showed significant differences in both the vagina and endometrium, demonstrating relatively large differences compared to other species.

The analysis of microbiome composition across the menstrual cycle revealed no changes at the genus or species levels in the EM and vagina. In contrast, a total of 60 genera and 76 species exhibited statistically significant differences in mean abundance in the PF during the follicular and luteal phases.

When EM cells were co-cultured with *L. reuteri* at a multiplicity of infection of 1, western blot analysis revealed no significant alterations in endometriosis-related protein expression after 24 hours of co-culture. However, upon the addition of estradiol-17-glucuronide (E2G) to the culture medium, BAX/Bcl-2 expression decreased significantly compared to cells cultured with E2G alone without *L. reuteri*, while p-NF-κB expression was significantly increased only in the co-culture containing both *L. reuteri* and E2G.

While estrogen receptor expression was not affected by the presence of *L. reuteri*, the expression of progesterone receptors α and β significantly decreased in EM cells co-cultured with both *L. reuteri* and E2G. To confirm estrogen metabolism mediated by *L. reuteri*, β-glucuronidase levels measured by ELISA significantly increased after 24 hours of co-culture, however, the estradiol/E2G ratio determined by LC-MS/MS remained unchanged, indicating that the conversion of E2G to estradiol was not facilitated.

Conclusions:

In conclusion, this study analyzed microbiome composition among the vagina, endometrium, and peritoneal fluid of women with endometriosis compared to controls. While *L. reuteri* is known as a beneficial bacterium, *in vitro* findings of this study suggests a potential shift towards anti-apoptotic conditions, which could inadvertently support EMS development by promoting cell survival. Future studies should explore the interactions of multiple bacterial species and varying hormonal conditions to better represent *in vivo* environments.

Key words :
Endometriosis, Microbiota, Microbiome, *Lactobacillus reuteri*, 16S rRNA Sequencing, Estrogens/metabolism, β-glucuronidase, Estradiol, Co-Culture Techniques.

1. INTRODUCTION

Endometriosis (EMS) is an inflammatory condition characterized by the abnormal growth of endometrial (EM) tissue outside the uterus, impacting approximately 10% women of reproductive age globally [1,2]. EMS commonly infiltrates pelvic organs such as the ovaries and pelvic peritoneum; however, implants can also be found throughout the abdomen, such as on the bladder and bowel, resulting in painful symptoms such as dysmenorrhea, chronic pelvic pain, and dyspareunia [3]. EMS is commonly attributed to the reflux of menstrual blood into the pelvis via the fallopian tubes. Considering that endometriosis affects only approximately 10% of women, whereas retrograde menstruation of endometrial tissue mixed with blood is routinely observed in nearly all ovulatory women[4], it is improbable that this phenomenon is the sole cause of the disease. While this reflux is believed to contribute to the development of EMS, the precise factors influencing its onset remain unclear.

Recent studies have reported a relationship between the human microbiome and various diseases, including EMS. The microbiome encompasses all the genetic material of the microbes—bacteria, fungi, viruses, and archaea—that reside within the host and regulate numerous physiological functions[5]. The influence of the microbiome on immunomodulation and the development of various inflammatory diseases is well established[6]. Additionally, the microbiome is known to affect estrogen metabolism, while estrogen itself influences gut microbiota[7]. Specifically, β-glucuronidase secreted by various microbiota can enhance the estrogenic effects of estrogen metabolites by deconjugating conjugated estrogen. Given that EMS is an estrogen-dependent inflammatory disease[8], the microbiome's influence on immunomodulation and estrogen metabolism may play a significant role in its development[7].

Previous studies have documented microbiome dysbiosis in the reproductive organs and gut of women with EMS; however, the precise role of the microbiome in the disease remains unclear. Although Muraoka et al. reported that Fusobacterium infection in endometrial cells activates transforming growth factor-β (TGF-β) signaling, leading to the transition of quiescent fibroblasts into transgelin (TAGLN)-positive myofibroblasts with enhanced proliferative, adhesive, and migratory capabilities both *in vitro* and *in vivo*[9], the specific mechanisms by which the microbiome contributes to the disease remain largely unexplored.

In this study, we aim to evaluate the differences in the microbiome of the reproductive organs—

the vagina, endometrium, and pelvic peritoneum—between women with EMS and controls. Furthermore, *Lactobacillus reuteri* (*L. reuteri*), which showed the most significant difference in composition compared to the control group in the vagina and endometrium of women with EMS, was co-cultured *in vitro* with EM cells obtained from women without the disease. The potential impact of *L. reuteri* on EMS was assessed by evaluating the direct cellular responses of EM cells to interactions with *L. reuteri*.

2. METHODS

This manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work. The Institutional Review board of Hospital approved this study (4-2019-0302, 3-2020-0418).

2.1. Microbiome analysis using next-generation sequencing (NGS) in women with endometriosis: study population and sample collection

Forty-five premenopausal women, aged 20 to 45 years, with regular menstrual cycles, were scheduled for surgery due to suspected EMS based on radiological findings (ultrasound or MRI). Patients scheduled for surgery for dermoid cysts, without evidence of EMS or adenomyosis, served as the control group.

Women were excluded from the study if they did not receive a post-operative diagnosis of EMS or dermoid cyst, had a history of female hormone therapy, probiotics, antibiotics, or immunosuppressants within the past two months, had a history of previous abdominal surgery or pelvic inflammatory disease, or were pregnant. Additionally, women with malignant tumors, autoimmune diseases, or a BMI greater than 30 kg/m^2 were also excluded.

Specimen were collected from the vagina and endometrium of the patient in the lithotomy position, immediately before skin preparation and prior to surgery. Vaginal and EM swabs were collected following the insertion of a sterile vaginal speculum using OMNIgene® vaginal device (DNA Genotek Inc. Ottawa, Canada). Two separate swabs and collection tubes were used for vaginal and EM samples. When collecting samples from endometrium, care was taken to avoid the swab touching the cervical walls. Both samples were immediately transferred to −80 °C to be stored in an upright position until DNA extraction.

To obtain peritoneal fluid (PF), the patient's skin was prepared, a 1 cm incision was made in

the umbilicus, and a Glove Port (Meditech Inframed Corp., Seoul, South Korea) was inserted through the incision. After the endoscope entered the abdominal cavity, if body fluid was present in the posterior cul-de-sac, samples were collected aseptically. If no body fluid was present, samples were not collected. A minimum of 5 mL fresh peritoneal fluid was collected in a 15 mL Falcon tube and rapidly transferred to −80 °C to be stored in an upright position until DNA extraction. Preoperative prophylactic antibiotics were administered after sample collection.

To assess potential differences in microbiome composition across menstrual cycle phases, the menstrual cycle phase at the time of sample collection—either follicular (proliferative) or luteal (secretory)—was determined in some patients. A gynecological ultrasound was performed within 24 hours prior to surgery to confirm the menstrual cycle phase[10].

2.2. DNA extraction

All samples were subsequently transported on dry ice to Macrogen, Inc. (Seoul, South Korea) for DNA extraction. The DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) was employed for DNA extraction, with minor modifications to the manufacturer's protocol to accommodate our specific sample types. The procedure commenced with the addition of 800 µL of CD1 solution to a PowerBead Tube, followed by the introduction of the sample. The tube was briefly vortexed to ensure proper mixing, then secured horizontally on a vortex adapter and subjected to vortexing at 1500 rpm for 15 minutes. Post-vortexing, the mixture was centrifuged at 15,000g for 1 minute to pellet any debris. The supernatant, approximately 500-600 µL, was carefully transferred to a clean 2 mL microcentrifuge tube, to which 200 µL of the refrigerated CD2 solution was added. After vortexing the mixture for 5 seconds, it was centrifuged again at 15,000g for 1 minute. Without disturbing the pellet, 700 µL of the supernatant was transferred to another clean 2 mL microcentrifuge tube, followed by the addition of 600 µL of CD3 solution and another brief vortexing for 5 seconds. The resulting mixture was loaded onto an MB spin column in 650 µL increments, each followed by centrifugation at 15,000g for 1 minute, with the flow-through being discarded after each spin until the entire sample had been processed. The column was then washed with 500 µL of EA solution, centrifuged at 15,000g for 1 minute, and the flow-through discarded. This was followed by another wash with 500 µL of C5 solution, also centrifuged at 15,000g for 1 minute, with the flow-through discarded. To ensure complete removal of any residual wash buffer, the column was subjected to a final centrifugation at 16,000g for 2 minutes. The spin column was then transferred to a new 1.5 mL Eppendorf tube, and 32 µL of C6 solution was added directly to

the center of the column membrane. After incubating for at least 3 minutes, the tube was centrifuged at 14,000 rpm for 1 minute to elute the purified DNA.

2.3. Library construction and sequencing

Sequencing libraries were prepared following the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 regions of the 16S rRNA gene. The input genomic DNA (gDNA) at 2 ng or 10 ng was PCR amplified using a 5x reaction buffer, 1 mM dNTP mix, 500 nM each of the universal forward and reverse PCR primers, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The first PCR cycling conditions were as follows: an initial heat activation at 95°C for 3 minutes, followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final extension at 72°C for 5 minutes. The universal primer pair with Illumina adapter overhang sequences used for the first amplification was:

16S Amplicon PCR Forward Primer

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'

16S Amplicon PCR Reverse Primer

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

The first PCR product was purified using AMPure beads (Agencourt Bioscience, Beverly, MA). Following purification, 10 μL of the first PCR product was subjected to a second round of PCR for final library construction, which included indexing using Nextera XT Indexed Primers. The second PCR was performed under the same conditions as the first PCR, but with only 10 cycles. The resulting PCR product was again purified using AMPure beads. The final purified product was quantified using qPCR, following the qPCR Quantification Protocol Guide (KAPA Library Quantification Kits for Illumina Sequencing platforms), and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Sequencing was then performed using the MiSeq™ platform (Illumina, San Diego, USA).

2.4. Operational Taxonomic Unit (OTU) analysis

After sequencing was completed, MiSeq raw data was classified by sample using the index sequence, and FASTQ files were created for each sample. The adapter sequence was removed using the fastp program[11], and error correction was performed on the region where the two reads overlapped. Paired-end data separated for each sample were assembled into one sequence using FLASH (1.2.11)[12]. If the assembled sequence was less than 400 bp or greater than 500 bp in length, it was removed.

The obtained sequences were processed using CD-HIT-OTU[13], an OTU analysis program based on CD-HIT-EST, to eliminate low-quality, ambiguous, and chimeric sequences identified as potential sequencing errors. Subsequently, sequences exhibiting ≥97% similarity were clustered into OTUs at the species level.

For the representative sequence of each OTU, BLASTN (v2.9.0+)[14] was conducted against the NCBI 16S Microbial Reference database, and taxonomic assignment was based on the organism information of the subject with the highest sequence similarity. Taxonomy was not assigned if the query coverage of the best database match was below 85% or if the identity of the aligned region was less than 85%.

Comparative analyses of microbial community composition were performed using QIIME (v1.9)[15] based on the abundance and taxonomic classification of the identified OTUs. Species diversity and evenness within the microbial communities were evaluated using the Shannon Index and Inverse Simpson Index, while alpha diversity was assessed through Chao1 estimates. Beta diversity, representing differences in microbial composition among sample groups, was calculated using Weighted and Unweighted UniFrac distances. Relationships among samples were visualized using Principal Coordinates Analysis (PCoA) and an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram[15,16]. Additionally, a Venn diagram was generated to compare the presence or absence of microorganisms across groups.

2.5. Isolation and primary culture of endometrial cells

Fresh eutopic EM cells were extracted from women without EMS. The EM tissue samples were finely minced and subsequently incubated for 2 h in PBS containing 2.0 mg/mL collagenase type I (Gibco, Waltham, MA, USA) in a humidified incubator maintained at 37 °C and 5% CO2. Following incubation, the cells were collected by filtering through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA). The cell culture medium consisted of Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Cytiva, Marlborough, MA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 2% penicillin-streptomycin (P/S; Cytiva, Marlborough, MA, USA). The cells were re-suspended in the culture medium and incubated at 37 °C and 5% CO2. For passaging, when the cells reached 80–90% confluence, 2 mL of 0.25% trypsin-ethylenediamine tetraacetic acid (Gibco, Waltham, MA, USA) was added to digest the cells for 5 min in the incubator. Then, 1 mL of culture medium was added to stop the digestion. The primary cultured cells from passage 3–5 were used for subsequent experiments.

2.6. *In vitro* co-culture of endometrial cells with *Lactobacillus reuteri* : CCK-8 assay

L. reuteri was obtained from the Korean Collection for Type Cultures (KCTC 3594; NCBI Genome UID: GCA_000010005.1; JGI Genome ID: Gp0131284). *L. reuteri* was prepared for co-culture with EM cells by cultivation in MRS broth (MB Cell, Seoul, South Korea) at 37℃ for 48 hours. After cultivation, the bacteria were collected by centrifugation at 3800 g for 10 minutes, washed once with PBS, resuspended in PBS, and stored in aliquots at -80℃. The number of colony-forming units (CFU)/ml in the aliquots was determined by plate counting on MRS agar (MB Cell, Seoul, South Korea) after thawing. For co-culture with EM cells, aliquots of *Lactobacilli* were thawed and diluted in DMEM/F-12 medium without antibiotics at a multiplicity of infection (MOI) of 1. EM cells cultured in medium without bacteria served as controls. To assess the influence on cell viability, *L.reuteri* were co-cultured with EM cells for up to 24 h.

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). EM cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 hours. Subsequently, 100 μL of DMEM containing 0.5% FBS, with MOI of 1, 5, or 10, or without *L. reuteri*, was added to the wells and incubated for the specified treatment durations. After incubation, 20 μL of CCK-8 solution was added to each well. The plate was covered and incubated in the dark at 37°C for 1–3 hours. Optical density (OD) at 450 nm was measured using a VersaMax microplate reader (Molecular Devices, San Jose, CA, USA).

2.7. Addition of estradiol-17-glucuronide to *in vitro* co-culture of endometrial cells with *Lactobacillus reuteri*

β-Estradiol 17-(β-D-glucuronide) sodium salt (E2G) was obtained as a solid (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in DMSO to achieve a final concentration of 10 mM. *In vitro* assays were performed in a total reaction volume of 50 μl. Each reaction contained 5 μl of uronate dehydrogenase (1 μM final concentration), 5 μl of the enzyme (final concentration of 30 nM), 10 μl of NAD+ (final concentration of 2 mM), and 30 μl of E2G (final concentration of 500 μM), all diluted in assay buffer containing 50 mM HEPES and 50 mM NaCl, with pH varied as required[17].

2.8. Protein isolation and western blot

Proteins were extracted using radioimmunoprecipitation assay buffer (RIPA buffer; Thermo Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Protein concentrations were measured using the bicinchoninic acid assay kit (Thermo Scientific, USA). Equal amounts of protein (20 μg) were mixed with 5X sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Biosesang, Seongnam, Korea) and heated at 95 °C for 5 min. The protein samples were separated via SDS-PAGE on a 10% gel and then transferred onto polyvinylidene fluoride membranes (PVDF; Merck, Darmstadt, Germany). Membranes were blocked with 5% non-fat skim milk in Tris-buffered saline solution (10 mM Tris-HCl [pH 7.4] and 0.5 M NaCl) with Tween-20 (0.1% v/v) at 20 °C for 1 h. The membranes were incubated with the following specific primary antibodies overnight at 4 °C: Phospho-Nrf2 (Ser40) (1:500, Invitrogen, Carlsbad, CA, USA), Nrf2 (E5F1A) (1:250, Cell Signaling Technology, Danvers, MA, USA), Phospho-p44/42 mitogen-activated protein kinases (MAPK) (p-ERK 1/2; 1:500, Cell Signaling Technology, Danvers, MA, USA), Phospho-p53 (p-p53; 1:1000, Santa Cruz, Dallas, TX, USA), Phospho-nuclear factor (NF)-κB p65 (1:1000, Cell Signaling Technology, Danvers, MA, USA), Phospho-c-Jun (p-c-jun; 1:1000, Danvers, MA, USA), Caspase 3 (1:200, Santa Cruz, Dallas, TX, USA), B-cell lymphoma 2 (Bcl-2) (1:500) Santa Cruz, Dallas, TX, USA), Bcl-2 Associated X-protein (BAX) (1:500, Santa Cruz, Dallas, TX, USA), estrogen receptor (ER)-α (1:1000, Cell Signaling Technology, Danvers, MA, USA), ER-β (1:1000, Santa Cruz, Dallas, TX, USA Progesterone Receptor (AB-52) (1:200, Santa Cruz, Dallas, TX, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000, Santa Cruz, Dallas, TX, USA). The membranes were then incubated with goat anti-mouse IgG (H+L) or anti-rabbit IgG (H+L) secondary antibody (1:3000, Thermo Scientific, Waltham, MA, USA) for 1 h at room temperature. Detection was performed with Super Signal West Pico Plus Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) solution and imaged in a chemiluminescence imaging system (Image Quant LAS 4000; General Electric, Chicago, IL, USA). The bands were quantified by densitometry using Image J software (NIH, Bethesda, Maryland, USA).

2.9. ELISA-based quantification of β-glucuronidase

Supernatants from the co-culture of EM cells with isolated *L.reuteri* were analyzed for β-glucuronidase concentrations using an ELISA method. Concentrations were measured after 24 hours of co-culture using the QuantiChrom™ β-Glucuronidase Assay Kit (BioAssay Systems, Hayward, CA, USA) following the manufacturer's instructions. Samples were diluted within the detection

range of the assay. To remove EM cells and bacteria, cell culture supernatants were centrifuged at 2000 g for 5 minutes after harvesting. The supernatants were then stored at -20°C until measurement. Each experiment was performed in duplicate using EM cells from four different donors. β-Glucuronidase concentrations in the supernatants were determined using a VersaMax microplate reader (Molecular Devices, San Jose, CA, USA) by comparing the results to standard curves.

2.10. Measurement of estrogen metabolites using LC-MS/MS system: extraction of estrogens and their glucuronide conjugates

Supernatants from the co-culture of EM cells with isolated *L.reuteri* were analyzed for estradiol and E2G concentrations using Liquid Chromatography with tandem mass spectrometry system (LC-MS/MS system). 200 µL sample was extracted twice using 500 µL of methyl tert-butyl ether (MTBE) with estrone-$^{13}C_3$ (2 ng/mL) as internal standard for non-conjugated estrogen. The organic phase was used to extract estradiol, while the aqueous phase was used for E2G.

The combined organic phases were concentrated to dryness using a speed vacuum concentrator. For dansyl derivatization, 50 µL of dansyl chloride (1 mg/mL in acetone) and 50 µL of 0.1 M sodium bicarbonate buffer (pH 10.4) were added to the dried residue. The mixture was incubated at 60°C for 5 minutes and then cooled to room temperature. The derivatized sample was diluted 1:1 with methanol and filtered through 0.2 µm nylon filters. The final solution was transferred to inserts placed in LC analysis vials.

To extract the glucuronide, 800 µL of acetonitrile with estradiol-$^{13}C_3$ (50 ng/mL) as internal standard for conjugated estrogen was added to the aqueous phase. The mixture was vortexed for 1 minute and then centrifuged at 15,294 × g for 10 minutes at 4°C. The supernatant was transferred into LC analysis vials.

2.11. Instrumental analysis utilizing LC-MS/MS system

Estradiol and its glucuronide conjugate were analyzed using an LC-MS/MS system (LCMS-8060, Shimadzu, Kyoto, Japan). A reversed-phase Dikma Navigatorsil C18 analytical column (2.7 µm particle size, 100 × 2.1 mm) was employed for chromatographic separation. The column temperature was maintained at 40°C. The mobile phases were as follows: mobile phase A (5 mM ammonium formate + 5% acetonitrile in water) and mobile phase B (5 mM ammonium formate + 95% acetonitrile in water). The elution gradient program was: 0% B (0-3 min), 0-100% B (3-18 min), 100% B (18-23 min), 100-0% B (23-25 min), and 0% B (25-30 min). The flow rate and

injection volume were set to 0.2 mL/min and 5 μL, respectively. Optimal LC-MS/MS parameters were as follows: nebulizing gas flow, 3 L/min; drying gas flow, 10 L/min; and heating gas flow, 10 L/min. The MS system was operated with the following temperatures: DL temperature, 250°C; interface temperature, 350°C; and heat block temperature, 400°C. The multiple reaction monitoring (MRM) transitions are detailed in Table 1.

Table 1. Multiple reaction monitoring transitions for estrogen compounds.

#	Compound name	Dansylated	Ion species	Precursor ion	Quantifier ion (Collision energy, V)	Qualifier ion (Collision energy, V)
1	Estradiol-glucuronide	No	[M-H]-	447	85 (26)	271 (30)
2	Estradiol-$^{13}C_3$	No	[M+H]+	509	171 (32)	156 (52)
3	Estradiol	Yes	[M+H]+	506	171 (30)	156 (50)
4	Estrone-$^{13}C_3$	Yes	[M+H]+	507	171 (34)	156 (51)

2.12. Statistical analysis

Microbiome analysis using NGS involved statistical comparisons of species diversity (diversity indices) and microbial community composition (relative abundance) across different sites (vagina, endometrium, and peritoneal fluid) in both the EMS and control groups. The Kruskal-Wallis rank sum test[18] was used for comparisons among three groups, and the Wilcoxon rank-sum test[19,20] was employed for comparisons between two groups. Additionally, to analyze microbial community composition (relative abundance) across the groups, a Linear discriminant Effect Size (LEfSe) analysis[21] was performed to identify microorganisms that exhibited significant differences between the comparison groups, with the magnitude of these differences expressed through the LDA score. To examine the effect of the menstrual cycle phase (follicular or luteal) on microbial community composition (relative abundance) of vaginal, EM, and PF samples, a two-way analysis of variance(ANOVA) was conducted[22,23]. Mean abundance values for each microbiome genus and species group were compared between samples obtained during the follicular and luteal phases. Differences with p-values less than 0.05 after FDR correction were considered statistically significant. For comparisons of samples from the same body area across different phases, p-values

were derived using the Tukey HSD test.

The results of the *in vitro* experiments are presented as the mean ± standard deviation (SD). The data were checked to determine whether they met the requirements for a normal distribution using the Shapiro-Wilk test. Continuous variables were compared using the Student's t test or Mann-Whitney U test where appropriate. SPSS® v.27.0 (IBM®, Armonk, NY, USA) and GraphPad Prism program (GraphPad Software Inc, San Diego, CA, USA) were used for statistical analyses. All statistical analyses were visualized using GraphPad Prism program. A p-value <0.05 was considered statistically significant.

3. RESULTS

3.1. Microbiome composition in genital tract of women with endometriosis and controls

A total of 45 patients were enrolled in the study; however, 4 samples were excluded from the analysis due to poor data quality during NGS library construction process. Consequently, the study was conducted using samples from 41 patients. Baseline characteristics of participants are presented in Table 2. A Venn diagram (Figure 1.) illustrates the number of microbiome species found in the vagina, endometrium, and PF of women with EMS or dermoid cysts (control).

A total of 239 microbiome species were detected in the vaginal samples of this study. Among these, 211 species were identified in women with EMS, and 103 species were identified in the control group, with 75 species shared between the two groups. In the endometrium, a total of 1,077 microbiome species were detected, with 755 species identified in women with EMS and 643 species in the control group; the two groups shared 321 common species. In the PF, 1,433 microbiome species were detected. Of these, 775 species were identified in women with EMS, and 1,104 species were identified in the control group, with 446 species shared between the two groups. Meanwhile, the mean sequence count per sample was 43,396 for vaginal samples, 44,265 for endometrium, and 30,295 for PF samples. In summary, microbial abundance was higher in the vagina and endometrium, while microbiome diversiy increased progressively from the vagina to the endometrium and FP.

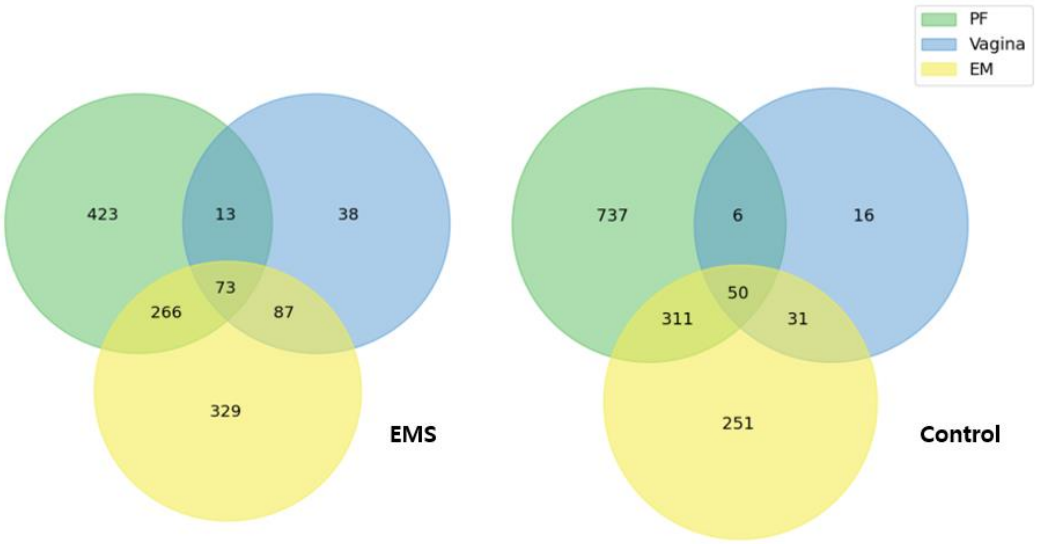


Figure1. Number of microbiome species identified in the vagina, endometrium, and peritoneal fluid (PF) of women with endometriosis (EMS) and controls.

Table 2. Baseline characteristics of the study population.

	Endometriosis group (n=27)	**Control group (n=14)**	***P*-value**
Age, years	32.43±4.38	32.36±4.61	0.413
Height, cm	161.71±4.45	160.79±4.22	0.479
Weight, kg	54.12±7.22	53.07±6.22	0.321
Size of endometrioma, cm	5.14±3.12	-	
Revised ASRM classification, N (%)			
I, II	1 (3.70)	-	
III	16 (59.26)	-	
IV	10 (37.04)	-	
*Revised ASRM classification total score	29 (25-108)	-	

The values are expressed as the mean ± standard deviation

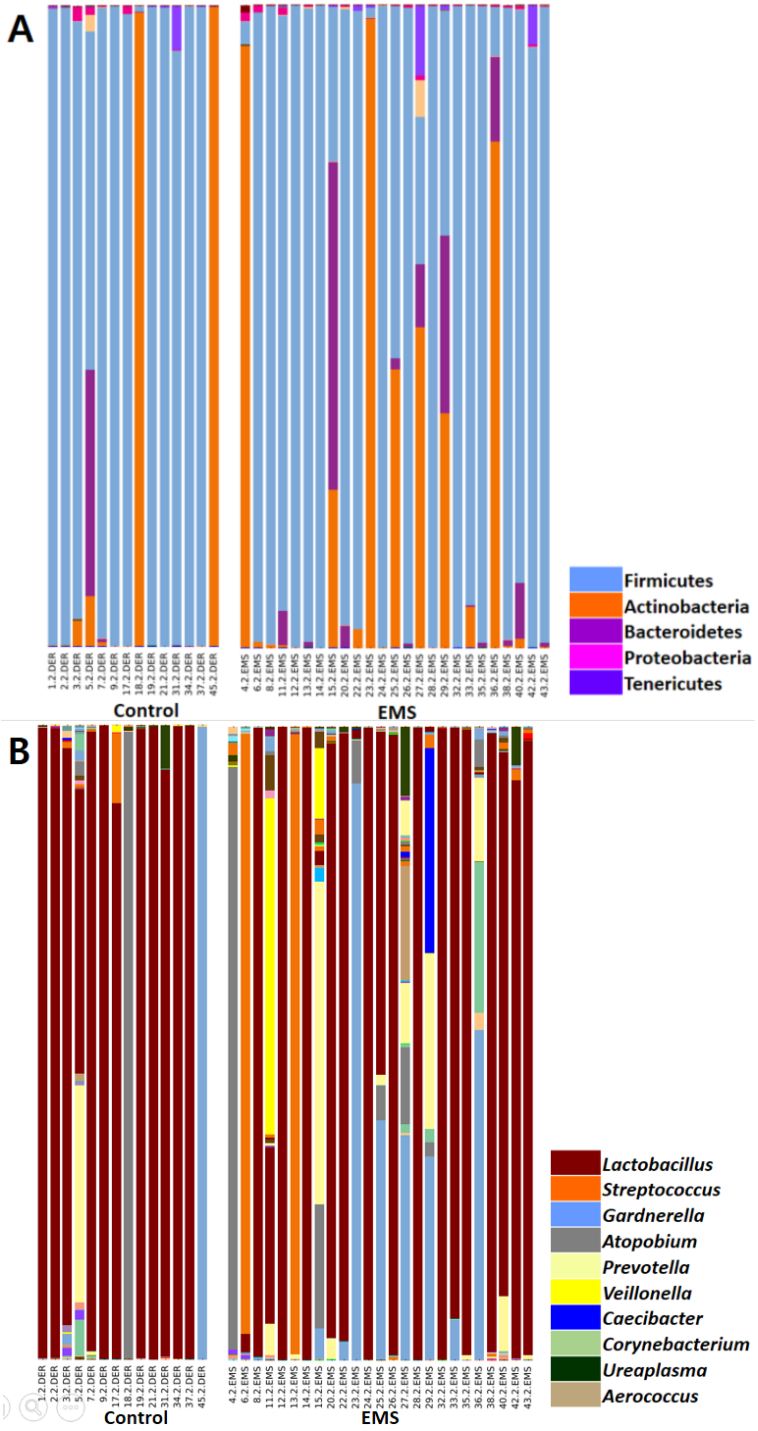
3.2. Vaginal microbiome composition of women with endometriosis and controls

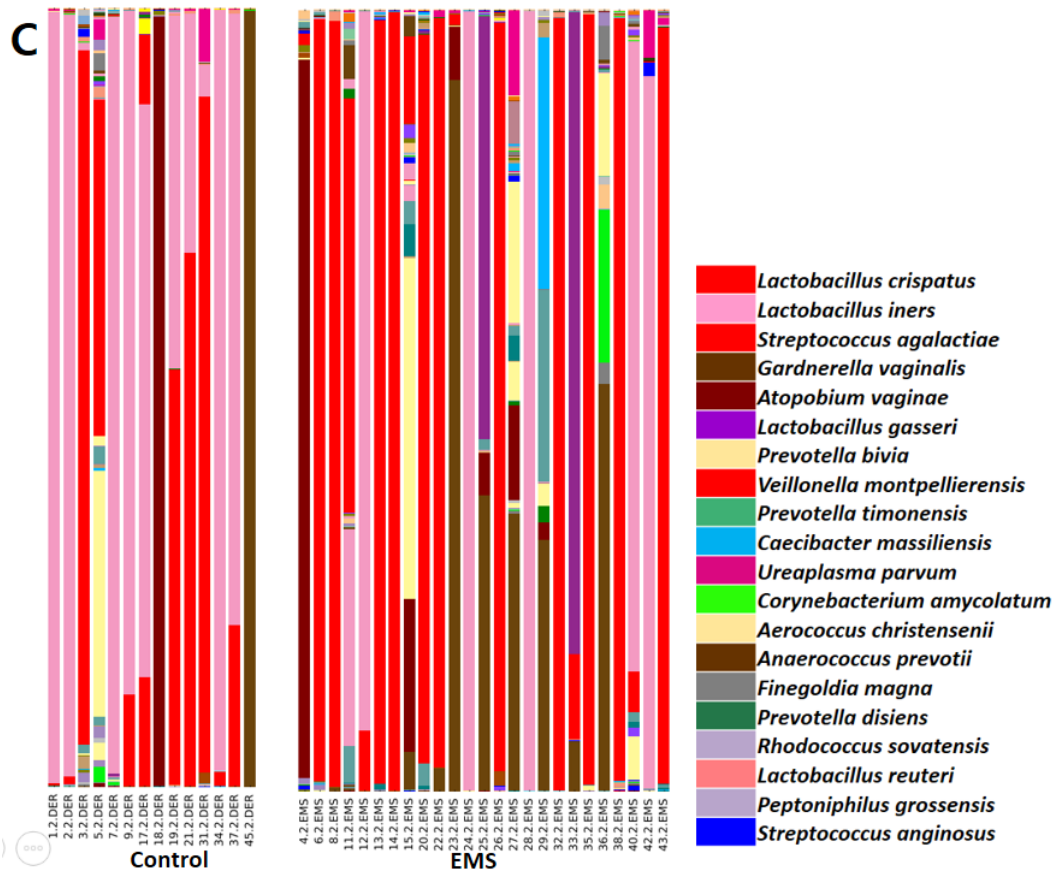
Figure2 illustrates the composition of the vaginal microbiome at the phylum, genus and species levels in women with EMS compared to the controls. At the phylum level, the most abundant taxa, in descending order, were Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Tenericutes (Figure 2A). At the genus level, the most abundant taxa, in descending order, were *Lactobacillus, Streptococcus, Gardnerella, Atopobium*, and *Prevotella* (Figure 2B). At the species level, the most commonly identified species, in descending order, were *Lactobacillus crispatus, Lactobacillus iners, Streptococcus agalactiae, Gardnerella vaginalis, Atopobium vaginae,* and *Lactobacillus gasseri* (Figure 2C).

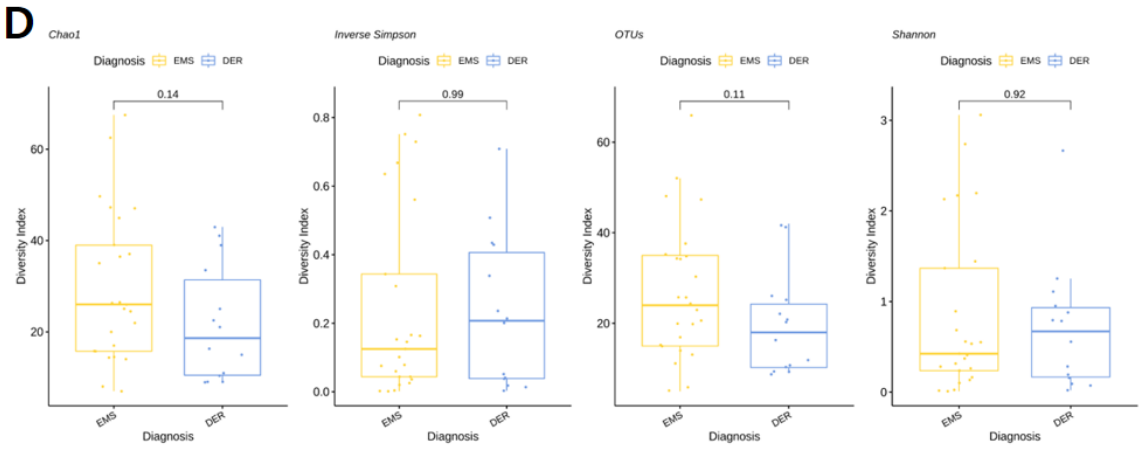
Differences in vaginal microbiome composition between the EMS group and the control group were compared using α-diversity and β-diversity metrics. Analysis of α-diversity, as measured by the Shannon Index, Inverse Simpson Index, and Chao1 values, revealed no significant differences between the EMS group and the control group (Figure2D). Additionally, β-diversity assessments using PCoA analysis and UPGMA tree results also indicated no significant differences between the EMS and control groups (Figure 2E and 2F).

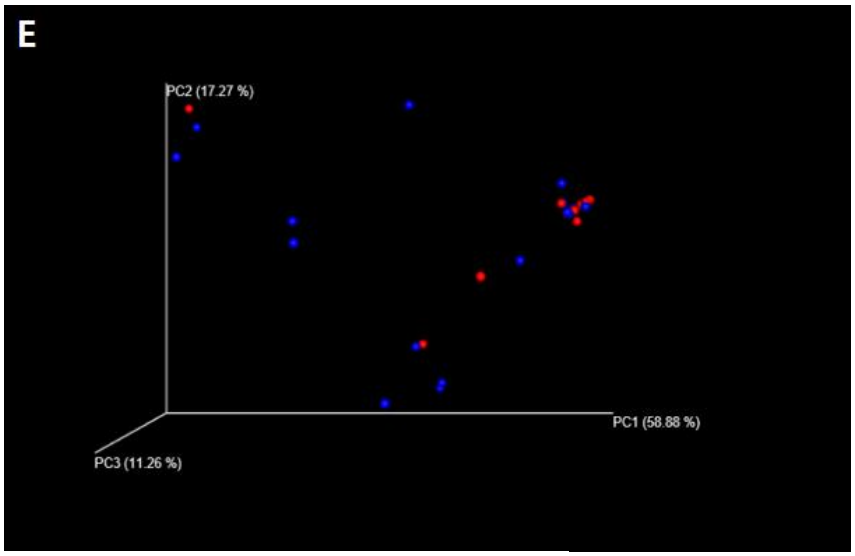
Based on microbial relative abundance, the Wilcoxon Rank Sum Test was employed to identify genera and species with significant differences in microbiome composition between women with EMS and the control group. At the genus level, Sutterella was found to be more prevalent in the vagina of women with EMS ($p=0.021$). At the species level, four species—*Dialister micraerophilus, Porphyromonas bennonis, Lactobacillus iners, and Lactobacillus reuteri*—were identified, revealing significant differences between the two groups (Table3).

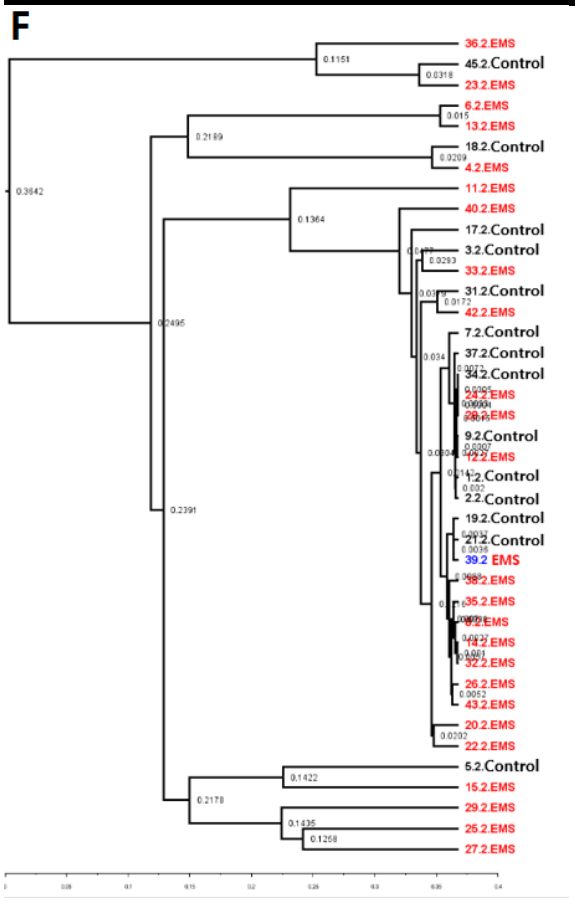
LEfSe analysis was subsequently conducted to compare the microbiome composition between the EMS group and the control group, in order to identify specific microbial taxa that were differentially abundant and to determine which species were most likely contributing to the observed differences between the two groups. The results are presented in the cladogram (Figure 2G) and LDA score (Figure 2H). Similarly, the LEfSe analysis revealed significant differences in the genus Sutterella and four species—*Dialister micraerophilus, Porphyromonas bennonis, Lactobacillus iners, and Lactobacillus reuteri*— between the two groups.











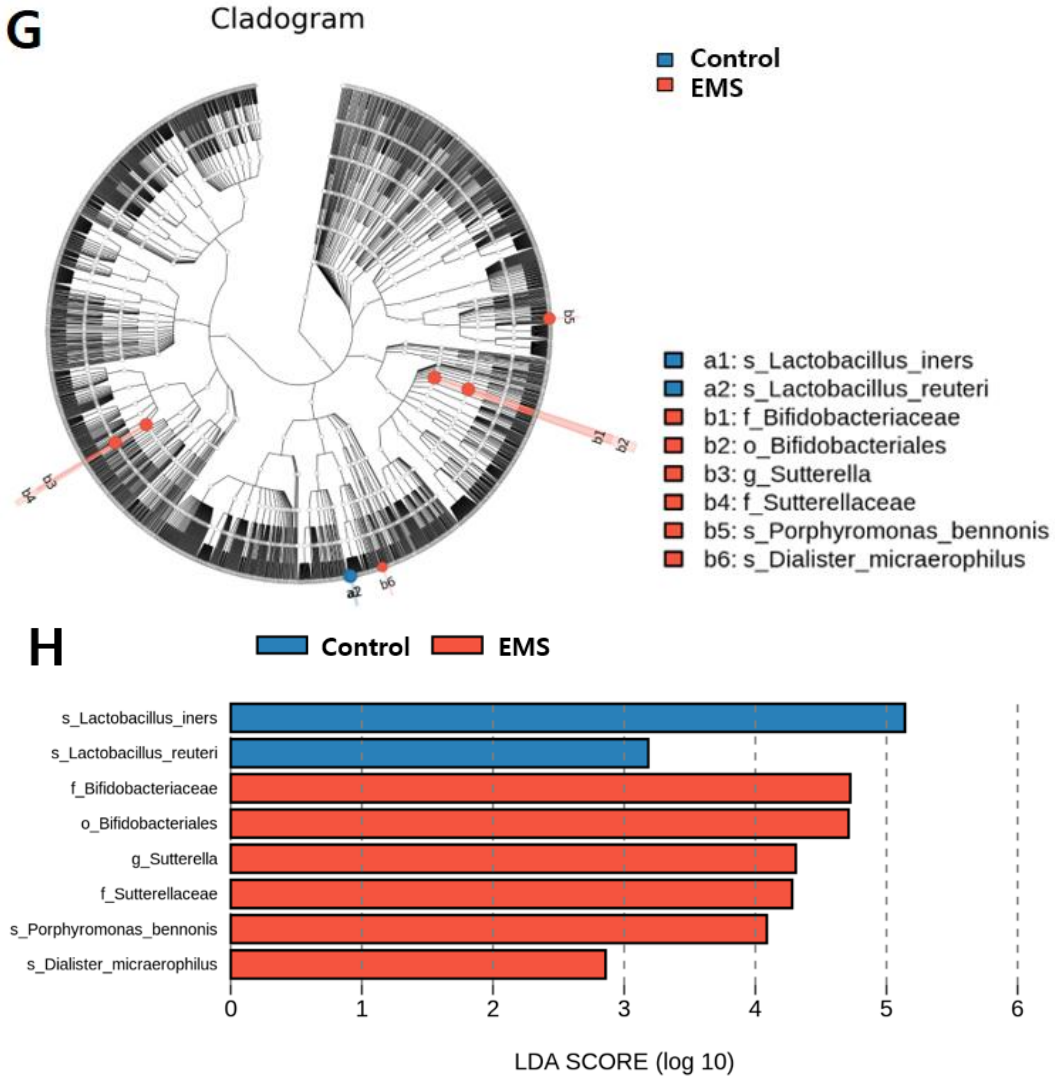


Figure2. Microbiome composition in vaginal samples of women with endometriosis (EMS) and controls. (A) Phylum level. (B) Genus level. (C) Species level. (D) α-Diversity comparison between women with EMS and controls: Shannon Index, Inverse Simpson Index, and Chao1 values. (E) β-Diversity of OTUs between women with EMS and control women: Principal Coordinate Analysis (PCoA) 3D plots. (F) β-Diversity of OTUs between women with EMS and control women: UPGMA tree analysis of the relative abundance at the species level. (G) Cladogram of Linear discriminant analysis Effect Size (LEfSe) analysis of the microbiome in women with EMS and control women. (H) Linear discriminant analysis (LDA) score plot of bacterial taxa with scores higher than 2. Bacterial taxa enriched in women with EMS are indicated in red, while those enriched in the control group are shown in blue.

Table 3. Differences in species-level microbiome composition between women with endometriosis (EMS) and control subjects.

Site	Species	The group in which the species is abundant.	*P*-value
Vagina	*Dialister micraerophilus*	EMS	0.0089
	Porphyromonas bennonis	EMS	0.034
	Lactobacillus iners	Control	0.015
	Lactobacillus reuteri	Control	0.0085
Endometrium	*Acidibrevibacterium fodinaquatile*	Control	0.047
	Actinomadura rifamycini	Control	0.047
	Aeromicrobium panaciterrae	Control	0.047
	Afifella pfennigii	Control	0.047
	Altererythrobacter rigui	Control	0.047
	Arenimonas subflava	Control	0.047
	Campylobacter ureolyticus	EMS	0.041
	Chryseobacterium greenlandense	Control	0.0033
	Corynebacterium tuberculostearicum	EMS	0.02
	Desulfonatronum alkalitolerans	Control	0.047
	Desulfovibrio piger	Control	0.047
	Dongia mobilis	Control	0.047
	Edaphobacter dinghuensis	Control	0.047
	Howardella ureilytica	EMS	0.026
	Insolitispirillum peregrinum	Control	0.047
	Lactobacillus reuteri	Control	0.0061
	Lysobacter dokdonensis	Control	0.047
	Lysobacter niabensis	Control	0.047
	Mycobacterium marinum	Control	0.047
	Natranaerovirga pectinivora	Control	0.047
	Nocardioides alpinus	Control	0.047
	Pelobacter carbinolicus	Control	0.047
	Povalibacter uvarum	Control	0.047
	Prevotella timonensis	EMS	0.018
	Pseudoduganella violaceinigra	Control	0.047
	Pseudoflavonifractor capillosus	Control	0.047
	Pseudoxanthomonas wuyuanensis	Control	0.047
	Rhodanobacter glycinis	Control	0.013
	Smithella propionica	Control	0.047
	Sphingobium mellinum	Control	0.047
	Streptomyces aomiensis	Control	0.047
	Streptomyces chlorus	Control	0.047
Peritoneal fluid	*Thauera chlorobenzoica*	EMS	0.026

3.3. Endometrial microbiome composition of women with endometriosis and controls

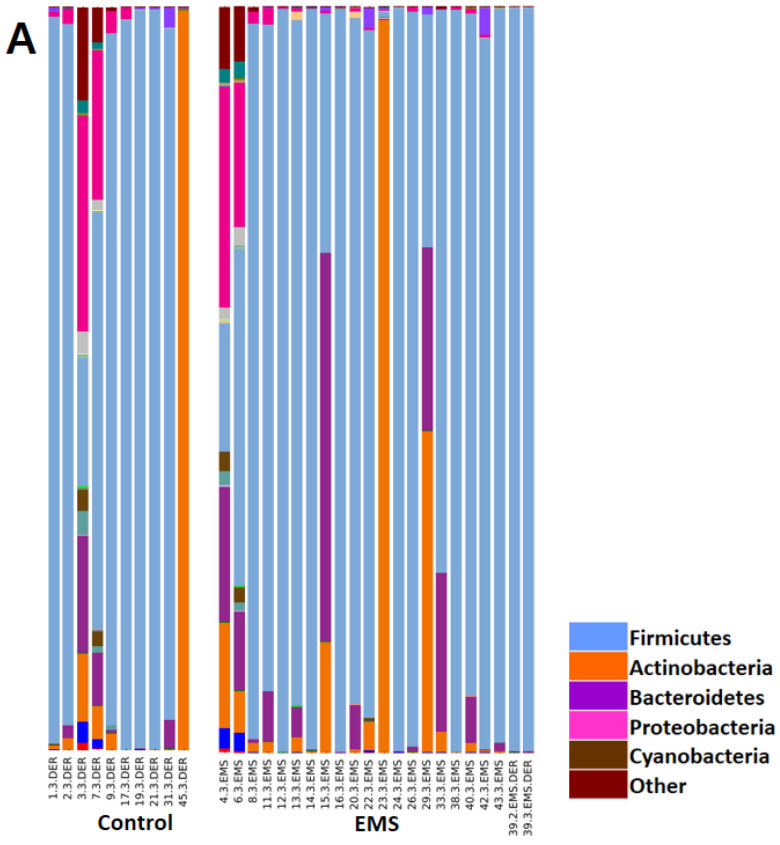
Figure3 illustrates the composition of the EM microbiome at phylum, genus and species levels in women with EMS and control participants. At the phylum level, the most abundant taxa, in descending order, were Firmicutes, Actinobacteria, and Bacteroidetes (Figure 3A). At the genus level, the most abundant taxa, in descending order, were *Lactobacillus, Gardnerella*, and *Streptococcus* (Figure 3B). At the species level, the most commonly identified species, in descending order, were *Lactobacillus iners, Lactobacillus crispatus, Gardnerella vaginalis, Streptococcus agalactiae,* and *Lactobacillus gasseri* (Figure 3C).

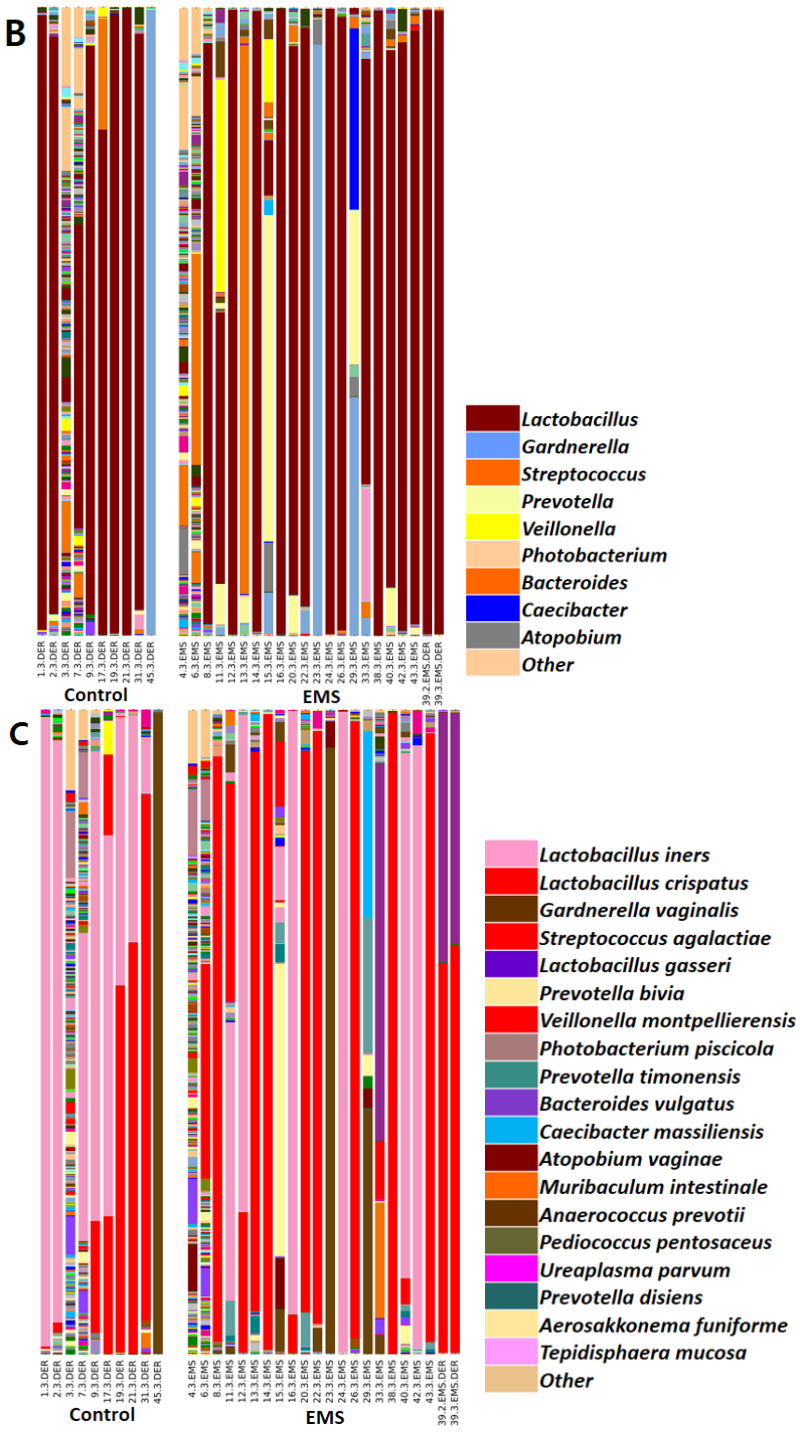
Differences in EM microbiome composition between the EMS group and the control group were compared using α-diversity and β-diversity metrics. Analysis of α-diversity, as measured by the Shannon Index, Inverse Simpson Index, and Chao1 values, revealed no significant differences between the EMS group and the control group (Figure3D). Additionally, β-diversity assessments using PCoA analysis and UPGMA tree results also indicated no significant differences between the EMS and control groups (Figure 3E and 3F).

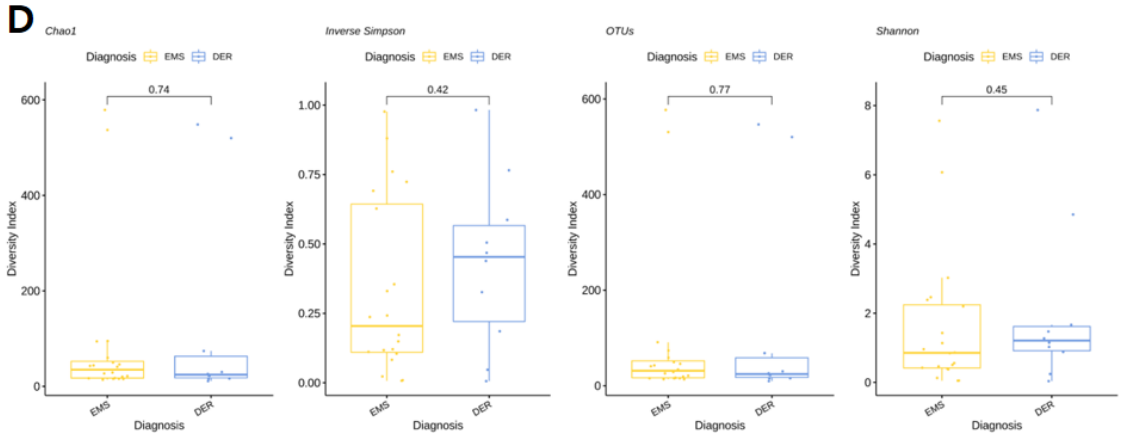
Based on the relative abundance of OTUs, species with significant differences in EM microbiome composition between women with EMS and the control group were identified. A total of 32 species differed between the groups, with 4 species being more abundant in the endometrium of women with EMS, and 28 species being more abundant in the endometrium of women in the control group. Women with EMS had a higher prevalence of species such as *Campylobacter ureolyticus, Corynebacterium tuberculostearicum, Howardella ureilytica,* and *Prevotella timonensis*. In contrast, the control group had 28 species that were significantly more abundant, with *Chryseobacterium greenlandense, Lactobacillus reuteri,* and *Rhodanobacter glycinis* showing the most pronounced differences compared to women with EMS (Table 3).

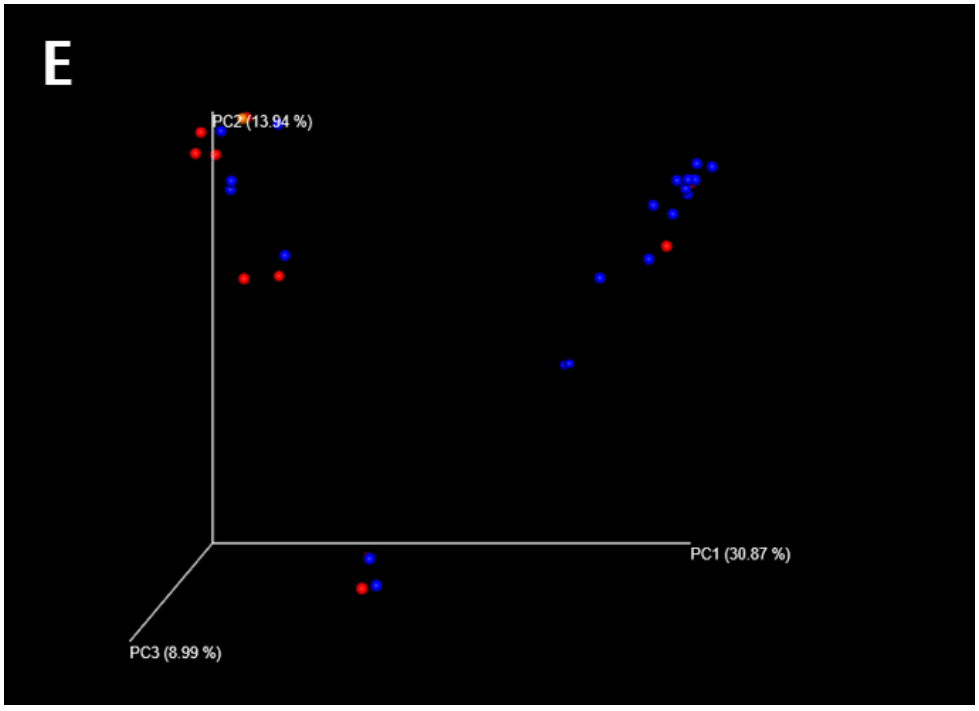
The results of LEfSe analysis are presented in the cladogram (Figure 3G) and LDA score (Figure 3H). The results of the LEfSe analysis indicated that genera such as *Novosphingobium*, *Chryseobacterium*, *Cloacibacterium*, *Rhodanobacter*, *Smithella*, *Afifella*, *Pelobacter*, *Altererythrobacter*, *Actinomadura*, *Selenomonas*, *Achromobacter*, *Sporosarcina*, *Cupriavidus*, *Acidovorax*, *Povalibacter*, *Acidibrevibacterium*, *Rubellimicrobium*, and *Blastococcus* were more abundant in the control group, while *Campylobacter* and *Howardella* were more abundant in women

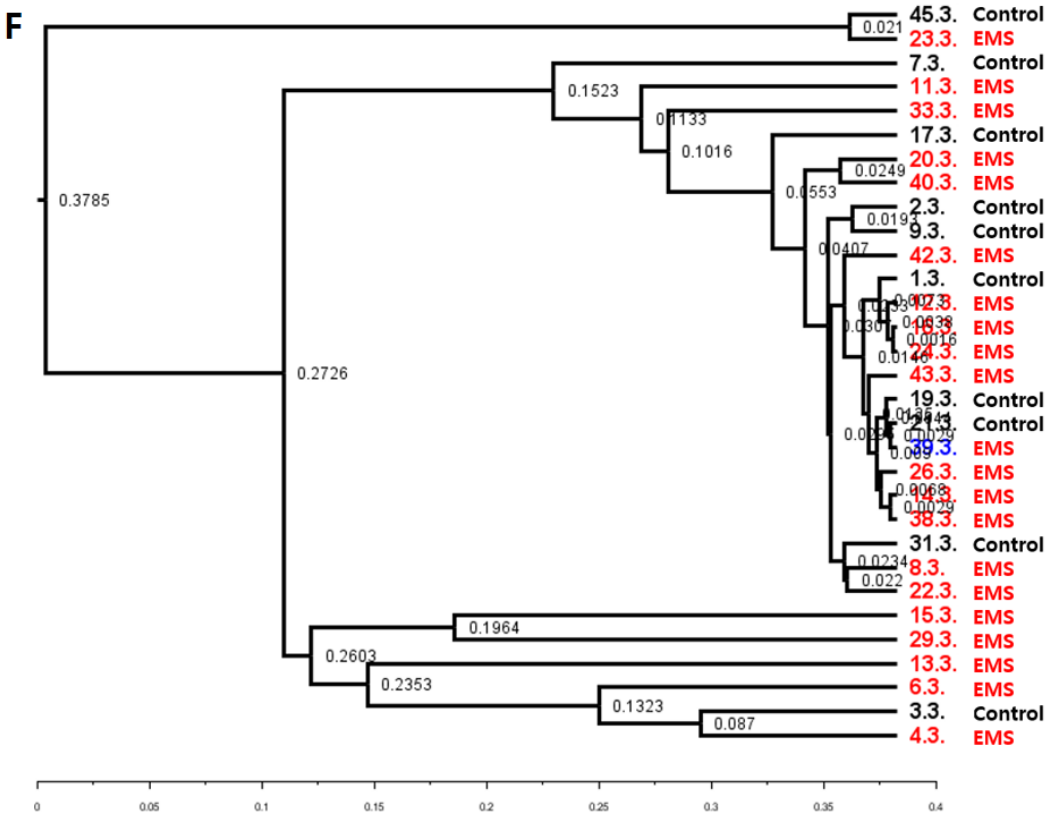
with EMS. At the species level, *Pseudoflavonifractor capillosus*, *Lactobacillus reuteri*, *Dongia mobilis*, *Smithella propionica*, *Afifella pfennigii*, *Streptomyces aomiensis*, *Streptomyces chlorus*, *Aeromicrobium panaciterrae*, *Chryseobacterium greenlandense*, *Pseudoduganella violaceinigra*, *Rhodanobacter glycinis*, *Pseudoxanthomonas wuyuanensis*, *Desulfonatronum alkalitolerans*, *Sphingobium mellinum*, *Nocardioides alpinus*, *Actinomadura rifamycini*, *Arenimonas subflava*, *Povalibacter uvarum*, and *Acidibrevibacterium fodinaquatiled* were more abundant in the control group, whereas *Prevotella timonensis*, *Campylobacter ureolyticus*, *Corynebacterium tuberculostearicum*, and *Howardella ureilytica* were more prevalent in women with EMS.

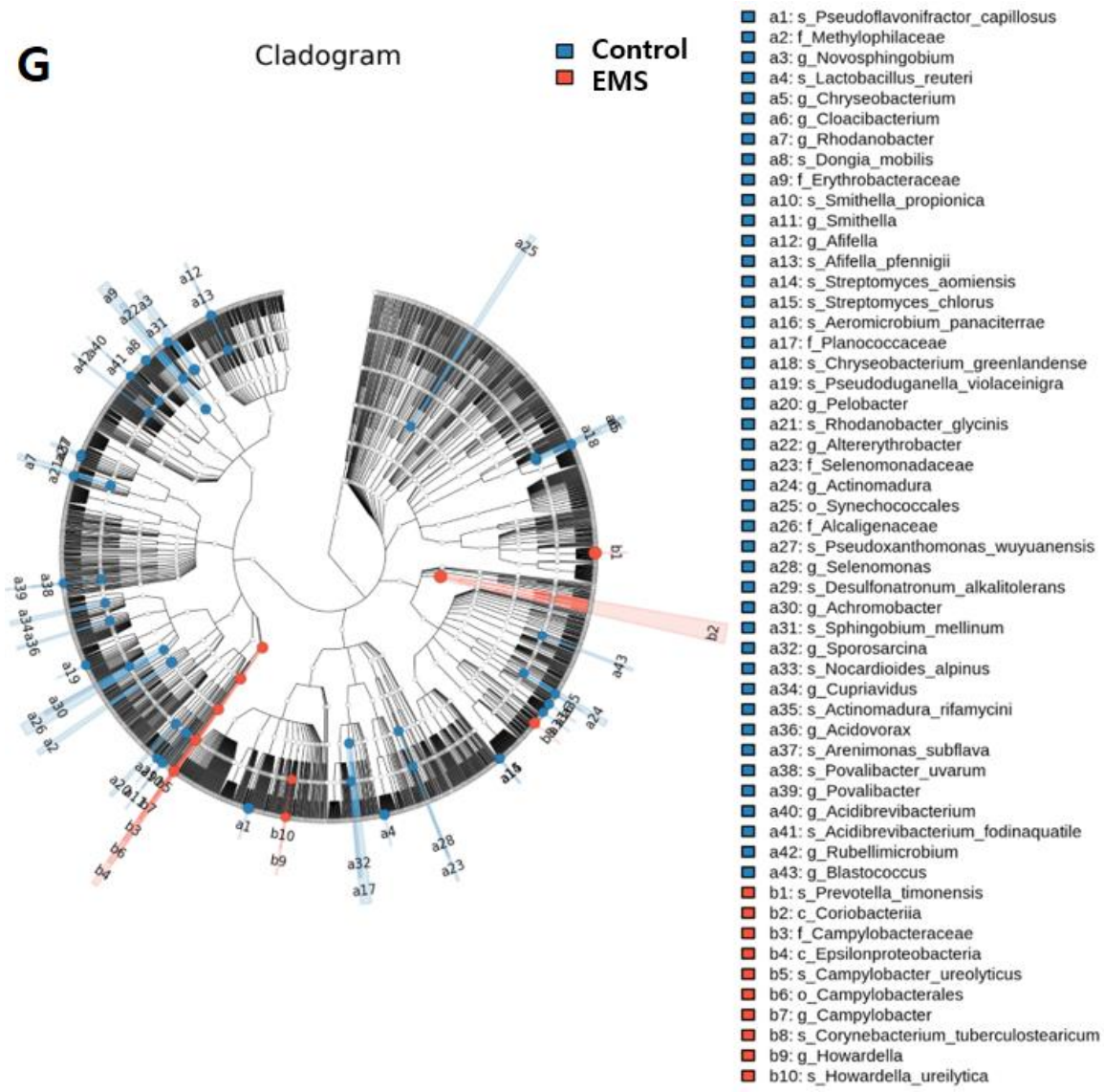












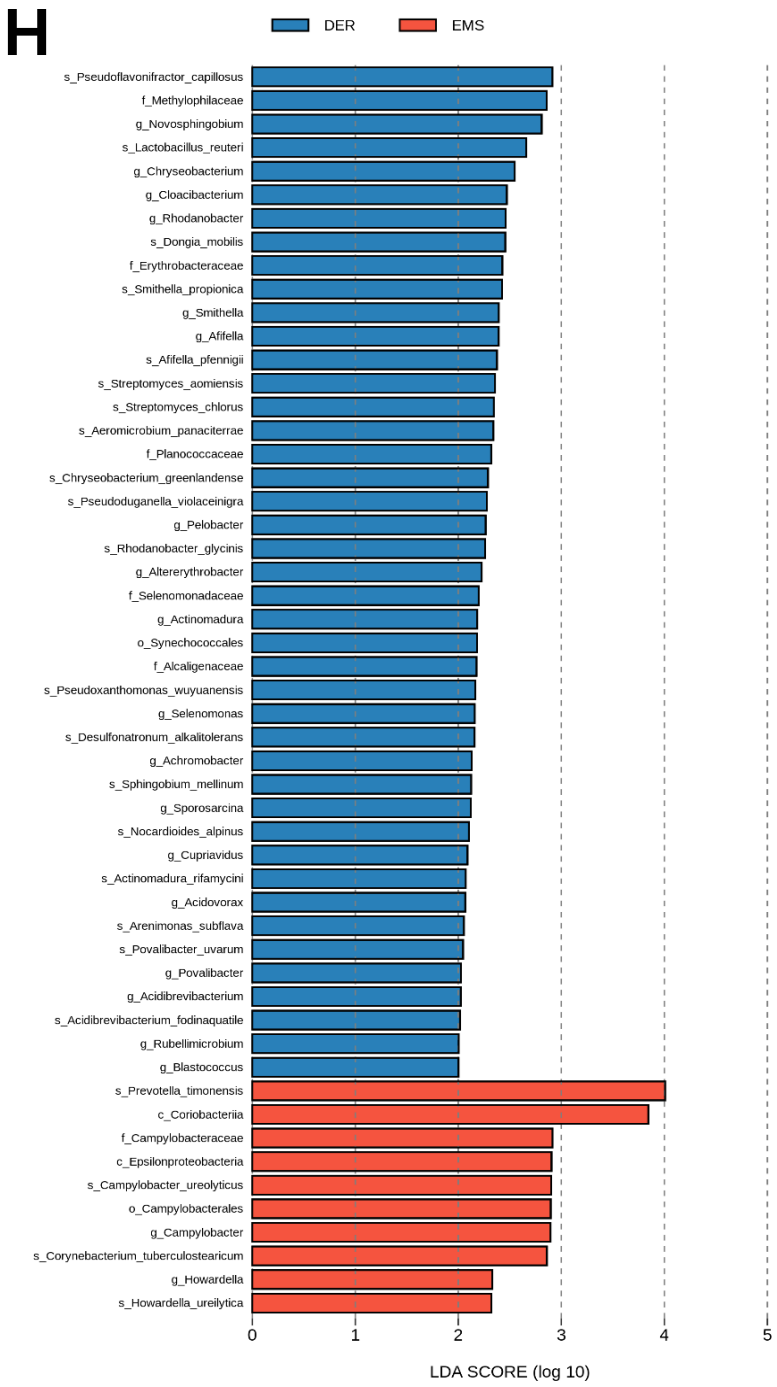


Figure 3. Microbiome composition in endometrial samples of women with endometriosis (EMS) and controls. (A) Phylum level. (B) Genus level. (C) Species level. (D) α-Diversity comparison between women with EMS and control women: Shannon Index, Inverse Simpson Index, and Chao1 values. (E) β-Diversity of OTUs between women with EMS and control women: Principal Coordinate Analysis (PCoA) 3D plots. (F) β-Diversity of OTUs between women with EMS and control women: UPGMA tree analysis of the relative abundance at the species level. (G) Cladogram of Linear discriminant analysis Effect Size (LEfSe) analysis of the endometrial microbiome in women with EMS and control women. (H) Linear discriminant analysis (LDA) score plot of bacterial taxa with scores higher than 2. Bacterial taxa enriched in women with EMS are indicated in red, while those enriched in the control group are shown in blue.

3.4. Microbiome composition in peritoneal fluid of women with endometriosis and controls

Figure 4 presents the composition of the peritoneal microbiome at phylum, genus and species levels in women with EMS compared to the control group. At the phylum level, Proteobacteria, Firmicutes, and Bacteroidetes were found to be the most abundant (Figure 4A). At the genus level, *Photobacterium, Bacteroides, and Lactobacillus* were the most abundant (Figure 4B). At the species level, the most commonly identified species, in descending order, were *Photobacterium piscicola, Bacteroides vulgatus, Lactobacillus aviarius, Pediococcus pentosaceus,* and *Aerosakkonema funiforme* (Figure 4C).

Differences in peritoneal microbiome composition between the EMS group and the control group were compared using α-diversity and β-diversity metrics. Analysis of α-diversity, as measured by the Shannon Index, Inverse Simpson Index, and Chao1 values, revealed no significant differences between the EMS group and the control group (Figure4D). Additionally, β-diversity assessments using PCoA analysis and UPGMA tree results also indicated no significant differences between the EMS and control groups (Figure 4E and 4F).

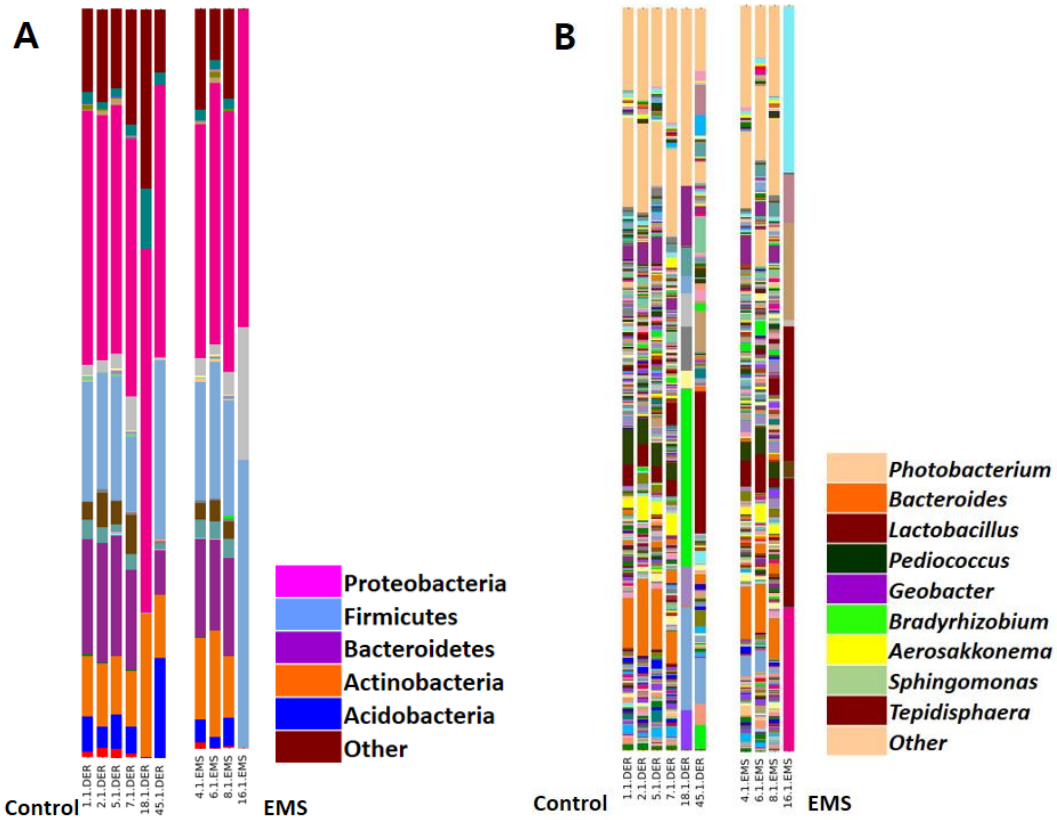
Species with significant differences in the microbiome composition of peritoneal fluid were identified by analyzing the relative abundance of OTUs between women with EMS and the control group, and the species *Thauera chlorobenzoica* exhibited significant differences in the peritoneal fluid between the EMS and control groups (Table3)

The results of LEfSe analysis are presented in the cladogram (Figure 4G) and LDA score

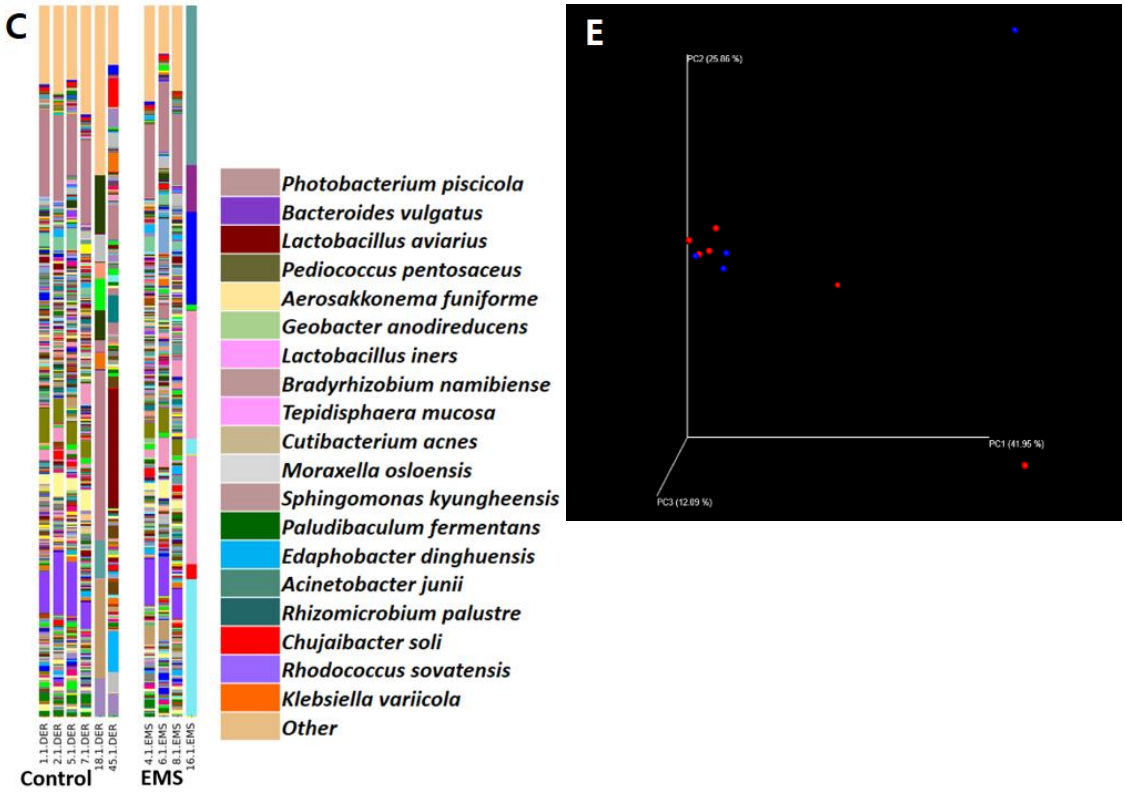
(Figure 4H). The results of the LEfSe analysis indicated that species such as *Lactobacillus brevis, Nitrospira japonica, Flavobacterium aquaticum*, and *Thauera chlorobenzoica* were more prevalent in women with EMS. At the genus level, no genera exhibited significant differences between women with EMS and the control group.

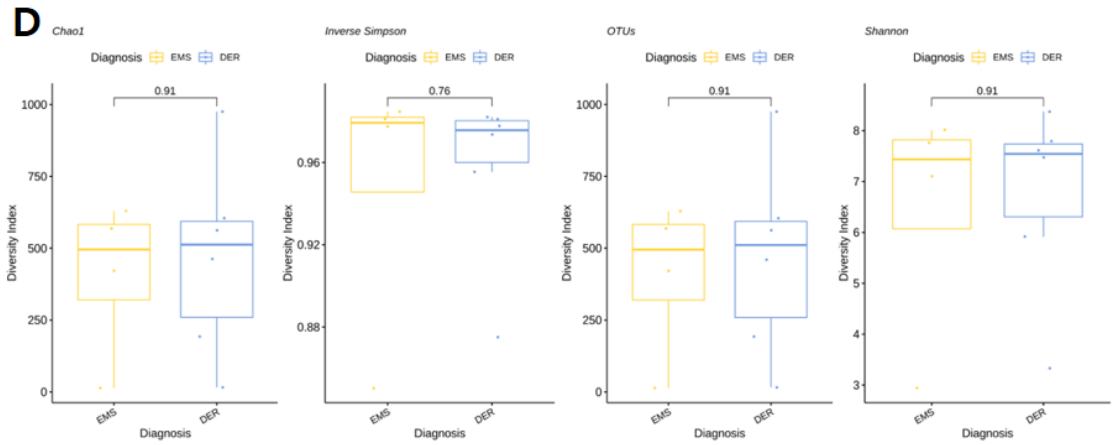
3.5. Microbiome composition throughout the menstrual cycle

We analyzed differences in microbiome composition according to the menstrual cycle in the vagina, endometrium, and PF at both the genus and species levels using a two-way ANOVA. The samples included in the analysis consisted of 6 vaginal samples from the follicular phase and 6 from the luteal phase, as well as 5 EM samples from the follicular phase and 5 from the luteal phase. Additionally, there were 3 PF samples from the follicular phase and 3 from the luteal phase. In the endometrium and vagina, no genera or species exhibited variations related to the menstrual cycle. In contrast, a total of 60 genera and 76 species exhibited statistically significant ($p<0.05$) differences in the mean abundance of the corresponding species present in the PF during the follicular and luteal



phases (Table 4).





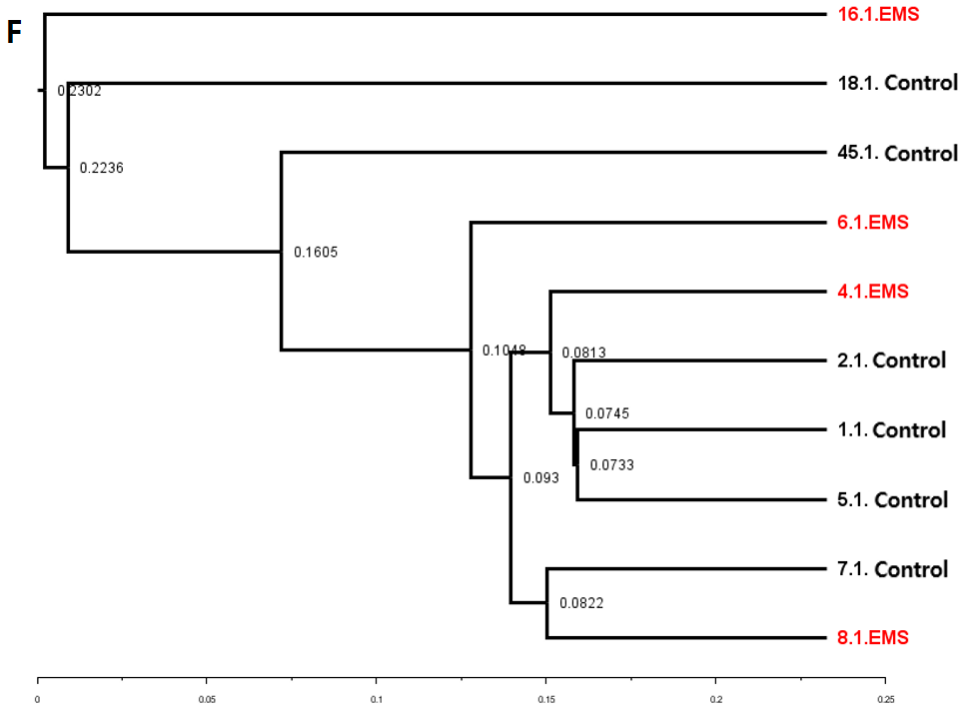


Figure 4. Microbiome composition in peritoneal fluid of women with endometriosis (EMS) and controls. (A) Phylum level. (B) Genus level. (C) Species level. (D) α-Diversity comparison between women with EMS and control women: Shannon Index, Inverse Simpson Index, and Chao1 values. (E) β-Diversity of OTUs between women with EMS and control women: Principal Coordinate Analysis (PCoA) 3D plots. (F) β-Diversity of OTUs between women with EMS and control women: UPGMA tree analysis of the relative abundance at the species level. (G) Cladogram of Linear

discriminant analysis Effect Size (LEfSe) analysis of the peritoneal fluid microbiome in women with EMS and control women. (H) Linear discriminant analysis (LDA) score plot of bacterial taxa with scores higher than 2. Bacterial taxa enriched in women with EMS are indicated in red, while those enriched in the control group are shown in blue.

Table 4. Bacterial taxa exhibiting differences in peritoneal fluid according to the menstrual cycle

Genus
Acetatifactor, Acidothermus, Actinoplanes, Aeromicrobium, Aerosakkonema, Aquisphaera, Asticcacaulis, Azoarcus, Azohydromonas, Bacillus, Bythopirellula, Cephalothrix, Cloacibacillus, Cohnella, Conexibacter, Coprobacillus, Enterocloster, Eubacterium, Faecalibacterium, Flavitalea, Geobacter, Granulibacter, Hydrogenispora, Ignavibacterium, Labilithrix, Lactococcus, Lacunisphaera, Metaprevotella, Methanothrix, Methylibium, Minicystis, Mitsuaria, Mucispirillum, Nakamurella, Niabella, Nioella, Nitrosospira, Nordella, Ohtaekwangia, Patulibacter, Pedobacter, Pirellula, Planifilum, Ramlibacter, Roseiarcus, Salinibacterium, Schlesneria, Solibacillus, Stanieria, Steroidobacter, Streptomyces, Sutterella, Syntrophococcus, Tangfeifania, Thermostilla, Thermotalea, Thioprofundum, Turicibacter, Viridibacterium, Xenophilus
Species
Acetatifactor muris, Acidothermus cellulolyticus, Actinoplanes friuliensis, Aerosakkonema funiforme, Aquisphaera giovannonii, Asticcacaulis biprosthecium, Azohydromonas ureilytica, Azospirillum brasilense, Bacillus oceanisediminis, Bacillus wiedmannii, Bacteroides caccae, Bacteroides vulgatus, Bdellovibrio exovorus, Brevitalea deliciosa, Bythopirellula goksoyri, Cephalothrix komarekiana, Chondromyces apiculatus, Cloacibacillus porcorum, Conexibacter arvalis, Conexibacter stalactiti, Coprobacillus cateniformis, Enterocloster asparagiformis, Faecalibacterium prausnitzii, Faecalicatena contorta, Faecalicatena orotica, Flavitalea antarctica, Flavobacterium tyrosinilyticum, Gemmatimonas aurantiaca, Geobacter anodireducens, Granulibacter bethesdensis, Hydrogenispora ethanolica, Ignavibacterium album, Labilithrix luteola, Lacunisphaera anatis, Lysobacter caseinilyticus, Lysobacter niabensis, Lysobacter niastensis, Marinobacter hydrocarbonoclasticus, Massilia arvi, Massilia aurea, Metaprevotella massiliensis, Methanothrix soehngenii, Methylibium petroleiphilum, Microvirga calopogonii, Minicystis rosea, Mitsuaria noduli, Mucispirillum schaedleri, Niastella yeongjuensis, Nioella nitratireducens, Nitrosospira multiformis, Nordella oligomobilis, Ohtaekwangia koreensis, Ohtaekwangia kribbensis, Parabacteroides distasonis, Paraburkholderia lacunae, Phenylobacterium hankyongense, Pirellula staleyi, Prevotella buccalis, Prevotella loescheii, Pseudomonas balearica, Rhodoplanes tepidicaeni, Roseiarcus fermentans, Salinibacterium xinjiangense, Schlesneria paludicola, Solibacillus kalamii, Stanieria cyanosphaera, Streptacidiphilus griseoplanus, Streptomyces vulcanius, Syntrophococcus sucromutans, Tangfeifania diversioriginum, Tetrasphaera elongata, Thermostilla marina, Thermotalea metallivorans, Turicibacter sanguinis, Viridibacterium curvum, Xenophilus arseniciresistens

3.6. *In vitro* effects of *Lactobacillus reuteri* on human endometrium: optimizing co-culture conditions using CCK-8 assay

CCK-8 assay was used to assess the viability of EM cells co-cultured with *Lactobacillus reuteri* at different multiplicities of infection (MOI = 1, 5, and 10), and cell viability was measured at 6 and 24 hours post-co-culture (Figure5A). The results demonstrated that during the 6-hour co-culture, cell viability remained relatively stable across all tested MOIs. However, after 24 hours, cell viability gradually decreased at MOI > 1, with a significant reduction observed at MOI = 10.

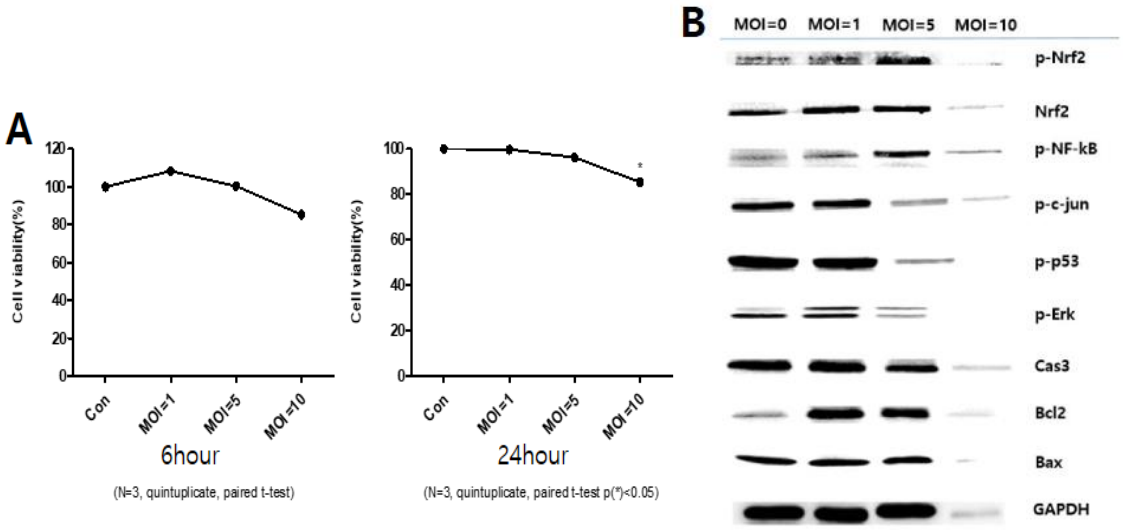


Figure5. Optimizing co-culture conditions of *Lactobacillus reuteri (L. reuteri).* (A) Viability of EM cells co-cultured with *L. reuteri* at MOI 1, 5, and 10 at 6 hours (left) and 24 hours (right) using the CCK-8 assay. (B) Protein expression of EM cells after 24-hour co-culture with *L. reuteri* at MOI 1, 5, and 10. (N=3, quintuplicate, p(*)<0.05)

Following a 24-hour co-culture of *L. reuteri* and EM cells, western blot analysis was performed to examine several key genes associated with endometriosis in EM cells. The findings mirrored those from the CCK-8 assay. As the MOI increased to 1, 5, and 10, protein expression showed a marked decline, particularly when MOI exceeded 1 (Figure5B).

3.7. Protein expression in endometrial cells co-cultured with *Lactobacillus reuteri*

Expression levels of other endometriosis-associated genes showed non-significant changes after 24 hours, including a moderate increase in Nrf2, BAX/Bcl-2, phosphorylated p53 (p-p53), phosphorylated ERK (p-ERK), and Caspase-3 (Cas-3), as well as a moderate decrease in the phosphorylated Nrf2/Nrf2 ratio (p-Nrf2/Nrf2), phosphorylated c-Jun (p-c-Jun), and phosphorylated

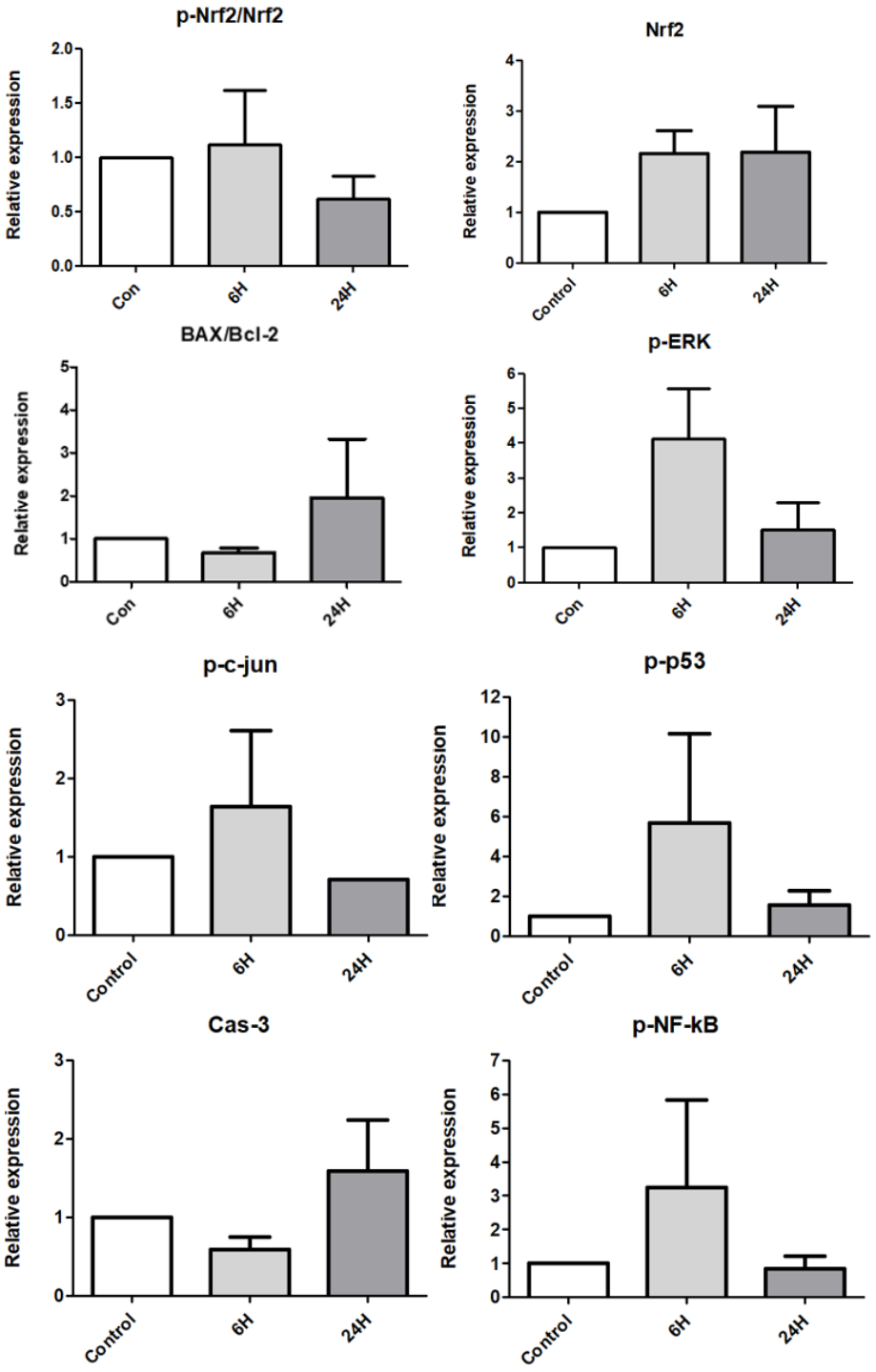


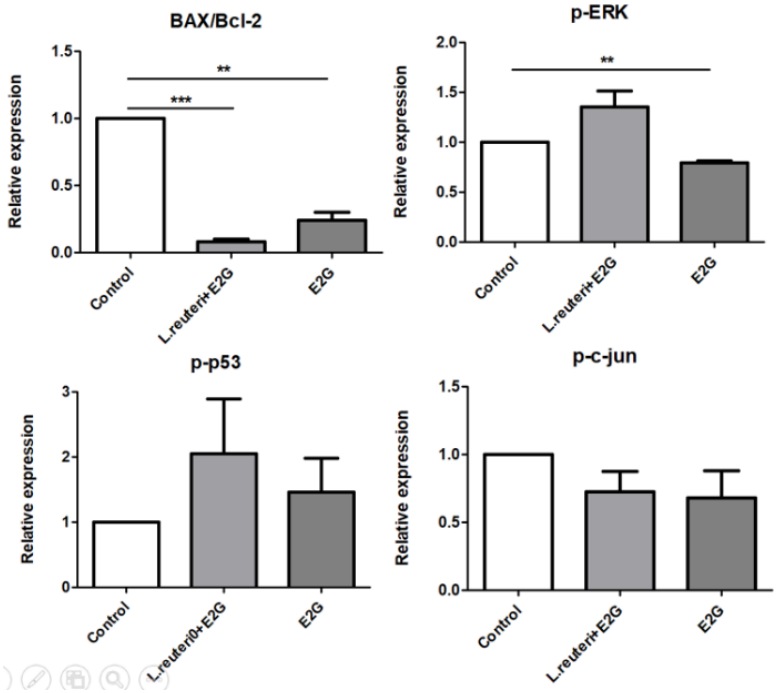
Figure 6. Protein expression of endometriosis-associated genes in EM cells co-cultured with *Lactobacillus reuteri.* (N=5, MOI=1, p(*)<0.05)

NF-κB (p-NF-κB).

3.8. Protein expression in endometrial cells co-cultured with *Lactobacillus reuteri* and estradiol-17-glucuronide

To simulate the follicular phase, E2G was added to EM cells co-cultured with *L. reuteri* for 24 hours (Figure 7). BAX/Bcl-2 expression was further reduced in EM cells co-cultured with *L. reuteri* and E2G compared to EM cells cultured with E2G alone. Additionally, p-NF-κB was significantly increased only in the co-culture of EM cells with *L. reuteri* and E2G.

The expression of p-ERK and the p-Nrf2/Nrf2 ratio also increased relative to EM cells, although these changes did not reach statistical significance. While Cas-3 expression was significantly reduced compared to the control group, there was no statistically significant difference when compared to EM cells cultured with E2G.



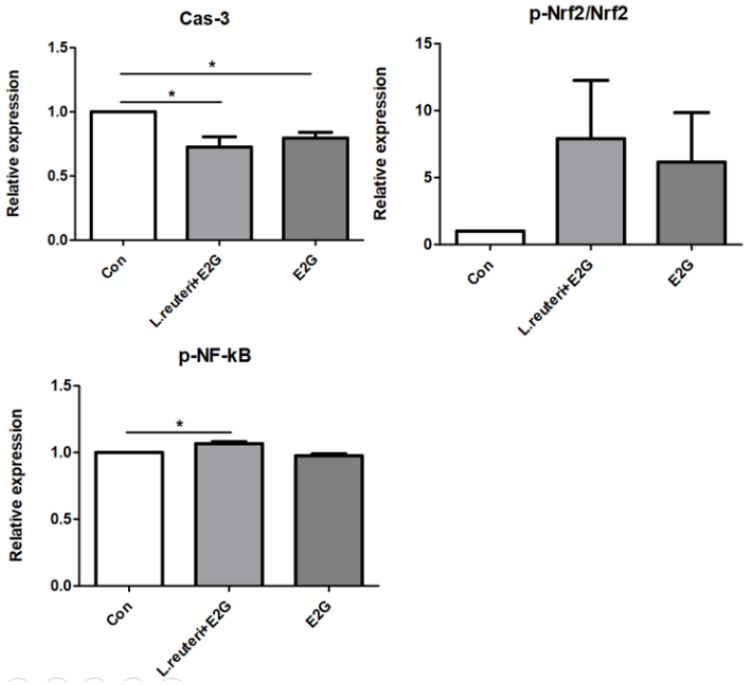


Figure 7. Protein expression of endometriosis-associated genes in EM cells co-cultured with *Lactobacillus reuteri* and estradiol-17-glucuronide(E2G). (N=5, MOI=1, E2G: 10mM, p(*)<0.05, p(**)<0.01, p(***)<0.001)

3.9. Expression of estrogen and progesterone receptors in endometrial cells co-cultured with *Lactobacillus reuteri* and estradiol-17-glucuronide

When EM cells were co-cultured with *L. reuteri* and E2G for 24 hours, changes in the expression of ER and PR were observed through western blot analysis (Figure 8). The ER-α/ER-β ratio was significantly reduced during the 24-hour co-culture with *L. reuteri* and this reduction was similarly pronounced with the addition of E2G. The most substantial decrease in expression was observed in EM cells cultured with E2G alone, without *L. reuteri.*

When EM cells were co-cultured with *L. reuteri* or when E2G was added to the culture medium, the expression of both PR-α and PR-β decreased. Notably, the expression of PR-α and PR-β was significantly reduced only when E2G was included in the culture medium with *L. reuteri.*

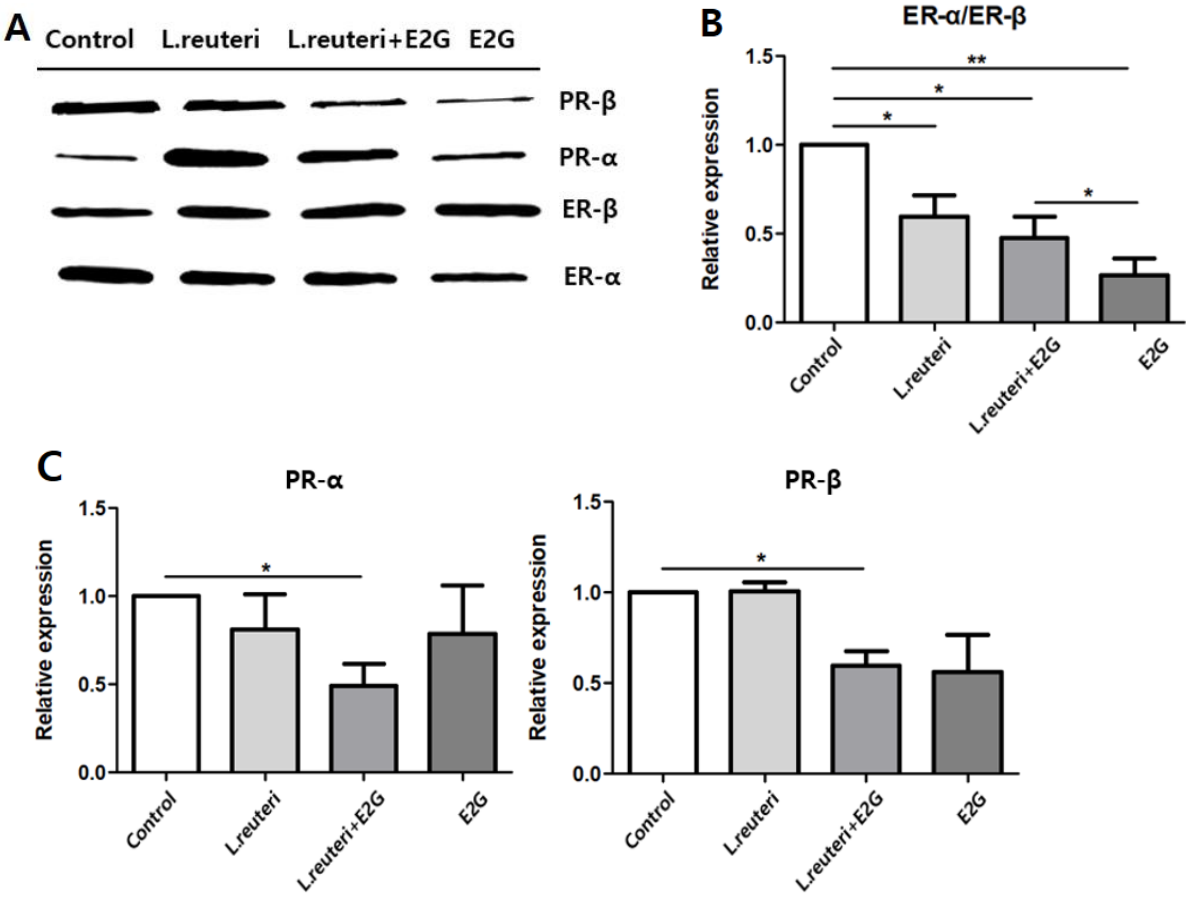


Figure 8. Expression of estrogen and progesterone receptors in EM cells co-cultured with *Lactobacillus reuteri* and estradiol-17-glucuronide(E2G) for 24 hours. (N=5, MOI=1, E2G: 10mM, p(*)<0.05, p(**)<0.01)

3.10. Production of β-glucuronidase and estradiol in co-culture of endometrial cells and *Lactobacillus reuteri*

When EM cells were co-cultured with *L. reuteri* and E2G for 24 hours, production of β-glucuronidase and estradiol was measured using ELISA and LC-MS/MS system, respectively. A significant increase in β-glucuronidase levels in the culture medium was observed only when EM cells were co-cultured with *L. reuteri* and E2G, compared to the control (Figure 9).
In contrast, no significant changes were observed when comparing EM cells cultured with E2G alone to those co-cultured with both E2G and *L. reuteri*. Despite the increased β-glucuronidase levels, the estradiol/E2G ratio did not indicate enhanced conversion of E2G to estradiol in the co-culture (Table 5).

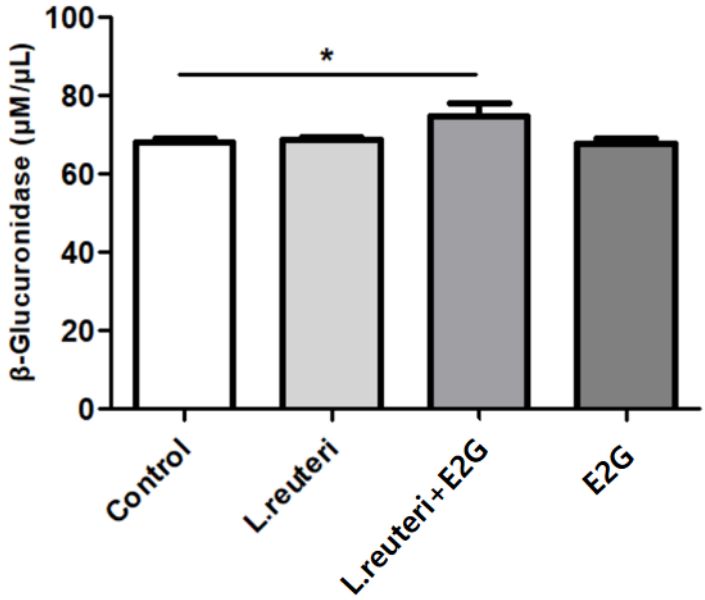


Figure 9. β-Glucuronidase levels measured by ELISA in four different groups after 24-hour co-culture: EM cells alone (control), EM cells with *Lactobacillus reuteri*, EM cells with *Lactobacillus reuteri* and estradiol-17-glucuronide (E2G), and EM cells with E2G (N=5, MOI=1, E2G: 10mM, p(*)<0.05)

Table 5. Estradiol-17-glucuronide (E2G) and estradiol levels measured by LC-MS/MS in four different groups after 24-hour co-culture. EM cells alone (control), EM cells with *Lactobacillus reuteri* (*L. reuteri*), EM cells with *L. reuteri* and E2G, and EM cells with E2G.

	E2G	**Estradiol**	**Estradiol/ E2G**
Control	-	-	-
L. reuteri	-	-	-
L. reuteri+ E2G	0.36 (0.02)	3.32 (2.21)	9.11(5.75)
E2G	0.35 (0.08)	2.97 (2.06)	8.68 (5.63)
P-value	0.009	0.656	0.837

4. DISCUSSION

In this study, microbiome composition in samples obtained from the vagina, endometrium, and PF was compared between women with EMS and control women using 16S rRNA gene sequencing. Although no overall differences in α or β diversity were observed between the two groups, specific bacterial taxa demonstrating significant differences were identified at each anatomical site. Among these, *L. reuteri* was the only species that exhibited significant differences in both the vagina and endometrium, with a relatively greater disparity between the two groups compared to other species.

To evaluate the potential protective effect of *L. reuteri* on EMS, *L. reuteri* was co-cultured with EM cells at a MOI of 1 for 24 hours. This methodology aimed to replicate the distinct microenvironment associated with retrograde menstruation, which is a critical factor implicated in the pathogenesis of EMS. When EM cells were co-cultured with *L. reuteri* at a multiplicity of infection of 1, no significant changes were observed in the expression of EMS-related proteins after 24 hours of co-culture, appeared to have a neutral effect. Furthermore, to simulate the follicular phase, EM cells were co-cultured with *L. reuteri,* and E2G was added to the culture medium. This resulted in a further reduction in BAX/Bcl-2 expression and a significant increase in p-NF-κB expression. Therefore, to investigate how *L. reuteri* modifies the effects of estrogen, hormone receptor expression was analyzed alongside measurements of β-glucuronidase and estrogen metabolites. While expression of ERs was not significantly affected by the presence of *L. reuteri*, PR-α and -β expression showed notable reductions in EM cells co-cultured with *L. reuteri* and E2G. ELISA results revealed a significant increase in β-glucuronidase levels after 24 hours of co-culture. However, estradiol levels and the estradiol/E2G ratio, measured via LC-MS/MS, remained unchanged, indicating that the conversion of E2G to estradiol was not facilitated.

Not only the NGS results of this study, but also previous research has consistently shown that women with endometriosis have lower levels of *Lactobacillus*, including *L. reuteri*, compared to control groups[24]. Similar findings have been observed in cases of infertility and other gynecological disorders, suggesting that *Lactobacillus* plays a crucial role in reproductive health of women[25,26].

However, the *in vitro* findings of this study revealed a significant reduction in BAX/Bcl-2 expression in EM cells co-cultured with *L. reuteri* and supplemented with E2G. This indicates a potential shift towards anti-apoptotic condition under estrogenic influence, which could inadvertently contribute to the development of EMS by promoting the survival of affected cells. This raises questions about the role of *L. reuteri*, traditionally considered a beneficial bacterium, in the

EM environment.

One possible explanation is that the mere presence of *L. reuteri* may not be sufficient to elicit a protective or modulatory response within the context of EMS-related pathways. The transient nature of the initial changes in BAX/Bcl-2 and p-NF-κB suggests an acute cellular response that does not persist with prolonged exposure. Another possible explanation is that the usual protective effects of *L. reuteri* might be mitigated or altered in the presence of other microbial species naturally occurring in the endometrium. Unlike the vaginal microbiome, where *Lactobacillus* constitute the majority, the EM microbiome is more diverse, with *Lactobacillus* comprising a smaller fraction, approximately 30%[27]. This diversity may play a crucial role in balancing the effects of individual microbial species, potentially offsetting any adverse outcomes associated with *Lactobacillus* alone. Furthermore, the reduced abundance of *Lactobacillus* in the endometrium compared to the vagina might reflect a necessary adaptation to prevent excessive anti-apoptotic signaling, which could otherwise facilitate pathological conditions such as EMS.

Since *Lactobacillus* secretes β-glucuronidase, it has the potential to function as part of the estrobolome[7,28]. Estradiol produced by the ovaries is metabolized in the liver into conjugated estrogens with lower potency. β-glucuronidase, secreted by various bacteria, can de-conjugate these conjugated estrogens back into estradiol, creating an estrogenic environment that may contribute to the development of estrogen-dependent diseases, such as EMS[7,28].

Normally, *Lactobacillus* in the female reproductive system does not have direct contact with blood. However, during menstruation, the EM lining sheds, exposing blood vessels. This allows *Lactobacillus* to interact with conjugated estrogens present in the blood. To investigate the dynamics of this interaction, particularly between estrogen metabolites and the microbiome, we devised an experimental study. By adding E2G, the most common conjugated estrogen, to a co-culture of EM cells and *L. reuteri*, we aimed to simulate these conditions and observe any potential interactions between them. This setup allowed us to investigate how the microbiome might influence estrogen metabolism in a scenario mimicking menstrual exposure.

The expression changes of BAX/Bcl-2 and p-NF-κB in EM cells observed in this study suggest that *L. reuteri* and E2G may exhibit a synergistic effect. This prompted an investigation into how *L. reuteri* modulates the action of estrogen. We first assessed the expression of estrogen receptors, however, the ER-α/ER-β ratio was not significantly affected by the presence of *L. reuteri*. In contrast, the expression of progesterone receptor-α and -β exhibited significant reductions in EM cells co-

cultured with *L. reuteri* and E2G, suggesting that *L. reuteri* may influence progesterone resistance.

This experiment did not provide evidence that *L. reuteri* directly participates in estrogen metabolism. When analyzing β-glucuronidase, which plays a crucial role in estrogen metabolism, using ELISA, a significant increase was observed only in the group that included E2G with *L. reuteri*. However, estradiol levels and the estradiol/E2G ratio in the group with E2G and *L. reuteri* showed no significant differences compared to the group where only E2G was added during the culture of EM cells. This indicates that the conversion of E2G to estradiol may not have been facilitated, possibly due to factors such as temperature affecting de-conjugation or a low β-glucuronidase activity from *L. reuteri.*

Recently, Wei et al. reported higher β-glucuronidase expression in bowel lesions and uterosacral ligament lesions obtained from women with EMS compared to normal endometrium[29]. In the same study, it was demonstrated that β-glucuronidase promotes EMS development by causing macrophage dysfunction *in vitro* and *in vivo*. Although an increase in the conversion of E2G to estradiol during the co-culture process was not confirmed, the findings of Wei et al. suggest that it cannot be discounted that β-glucuronidase may directly influence the expression changes of BAX/Bcl-2 and p-NF-κB.

It is important to emphasize that, while these results offer insights into the molecular interactions among EM cells, *L. reuteri*, and estrogen metabolites, the experimental setup cannot entirely replicate the complexities of menstrual blood, which encompasses a diverse array of microbial communities. This research underscores the importance of considering hormonal and microbial influences in EMS. Future studies should explore the interactions of multiple bacterial species and varying hormonal conditions to better represent *in vivo* environments. Understanding these dynamics could lead to novel therapeutic approaches for managing EMS, potentially leveraging the protective effects of beneficial bacteria within the reproductive tract.

5. CONCLUSION

In this study, 16S rRNA gene sequencing was used to identify bacterial taxa with differing distributions between women with EMS and controls across vaginal, EM, and PF samples. Among these, *L. reuteri* was the only species that exhibited significant differences in both the vagina and endometrium, with a relatively greater disparity between the two groups compared to other species.

When *L. reuteri* was co-cultured with EM cells, it had a neutral effect. However, when E2G

was added to the culture medium, there was a significant reduction in BAX/Bcl-2 expression and a significant increase in p-NF-κB expression. Additionally, EM cells co-cultured with *L. reuteri* and E2G showed a significant increase in β-glucuronidase levels, though the study did not confirm whether β-glucuronidase was directly involved in estrogen metabolism under these conditions.

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Abstract in Korean

자궁내막증 환자에서의 미생물군유전체 조성과 *Lactobacillus reuteri* 가 자궁내막에 미치는 영향에 대한 연구

배경:

자궁내막증은 가임기 여성의 약 10%에서 발병하는 만성 염증성 질환이다. 최근 미생물군유전체가 자궁내막증의 발병과 진행에 영향을 줄 수 있다는 연구 결과들이 보고되고 있다. 본 연구는 자궁내막증 여성과 대조군의 생식 기관- 질, 자궁내막, 복막액-에서의 미생물군유전체 조성 차이를 분석하고, *Lactobacillus reuteri (L. reuteri)*가 자궁내막에 미치는 영향을 시험관내 공동 배양 실험을 통해 평가하고자 한다.

방법:

연구에 포함된 총 41명의 환자들의 질, 자궁내막, 복막액에서 샘플들을 채취하여, V3 및 V4 영역을 표적으로 하는 16SrRNA유전자 서열분석을 사용하여 미생물군유전체 조성을 분석하였다. 또한, 자궁내막세포를 *L. reuteri*와 공동 배양하면서 western blot을 시행하여 발현 차이를 보이는 단백질들을 확인하였고, 배양액에 에스트라디올 글루쿠로니드 (estradiol-17-glucuronide, E2G)를 추가하여 같은 과정을 반복하였다. *L. reuteri*에 의한 에스트로젠 대사를 확인하기 위해, ELISA를 통해 β-glucuronidase 농도를 측정하였고, LC-MS/MS를 통해 에스트라디올, E2G, 에스트라디올/E2G 비율을 평가하였다.

결과:

자궁내막증 여성과 대조군의 전체적인 미생물군유전체 조성을 질, 자궁내막, 복막액에서 비교하였을 때, 알파 및 베타 다양성 지표는 두 군 간에 유의미한 차이가 없었다. 그러나, 미생물 운영 분류 단위 (operational taxonomic unit)의 상대 풍부도와 LEfSe 분석을 시행하였을 때, 두 군 간 유의미한 차이를 보이는 세균들이 존재하였으며, 질, 자궁내막, 복막액에서 두 군 간 유의미한 차이를 보이는 세균들의

분류군을 속 (genus), 종(species) 단계에서 확인할 수 있었다. 여러 세균들 중, *L. reuteri*는 질과 자궁내막에서 모두 유의미한 차이를 보인 유일한 종이며, 다른 종들에 비해 두 군 간의 차이가 상대적으로 컸다. 월경 주기에 따라 미생물군유전체 조성이 변하는지 분석하였는데, 자궁내막과 질에서는 월경주기에 따라 속 또는 종 단계에서 조성의 변화가 없었으나, 복막액에서는 총 60개의 속과 76개의 종이 난포기와 황체기 동안 통계적으로 유의미한 풍부도의 차이를 나타냈다.

*L. reuteri*와 감염 배율(multiplicity of infection, MOI) 1에서 공동 배양된 자궁내막 세포가 *L. reuteri*에 의해 직접적으로 나타내는 반응을 western blot을 이용하여 확인하였을 때, 자궁내막증 관련 단백질들은 24시간의 공동 배양 후 유의미한 변화가 나타나지 않았다. 그러나 E2G를 배양액에 추가했을 때, *L. reuteri*와 E2G를 모두 포함한 공동 배양에서 BAX/Bcl-2 발현이 유의미하게 감소하였고, p-NF-κB 발현은 유의미하게 증가하였다. 또한, 에스트로젠 수용체 발현은 *L. reuteri*의 존재에 의해 뚜렷한 영향을 받지 않았지만, 프로게스테론 수용체 α 및 β 발현은 *L. reuteri*와 E2G를 공동 배양한 자궁내막 세포에서 현저하게 감소했다. *L. reuteri*에 의한 에스트로젠 대사를 확인하기 위해, 24시간 공동배양 후 ELISA를 통해 확인한 β-glucuronidase 수치가 유의미하게 증가했으나, LC-MS/MS를 통해 확인한 에스트라디올/E2G 비율은 변하지 않아 E2G가 에스트라디올 로 변환되는 과정이 촉진되지 않았음이 나타났다.

결론:

본 연구는 자궁내막증 여성과 대조군의 질, 자궁내막, 복막액에서 차이를 보이는 미생물군유전체 조성을 규명하였으며, *L. reuteri*는 유익균으로 알려져 있으나 세포실험에서는 오히려 자궁내막세포의 세포사멸을 억제하는 반응을 유도하였다. 향후 연구에는 다양한 박테리아 종과 호르몬 조건의 상호 작용을 고려함으로써 생체 내 환경을 더 잘 반영하는 실험 조건이 수립되어야 할 것이다.

핵심되는 말 : 자궁내막증, 미생물군, 미생물군유전체, *Lactobacillus reuteri,* 16S rRNA유전자 서열분석, 에스트로젠/대사, β-글루쿠론산분해효소, 에스트라디올, 공동 배양 기법.