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Decoding the Microbial Landscape of Endometrial Cancer: a case-control study

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Abstract

Background:

The human microbiome plays an emerging role in cancer biology, yet its contribution to endometrial cancer (EC) remains poorly defined. This study investigates the microbial composition of the vaginal, rectal, and endometrial sites in women with and without EC, aiming to uncover microbial signatures associated with the disease.

Results:

We performed shotgun metagenomic sequencing on vaginal, rectal, and endometrial samples from 25 patients with EC and 27 control women undergoing hysterectomy for benign conditions. Vaginal and rectal swabs were collected before surgery, while endometrial swabs were obtained post-hysterectomy using a sterile brushing technique to prevent cross-contamination. Vaginal microbiota in patients with EC showed significantly higher microbial diversity and distinct community composition compared to controls. These differences remained significant after adjusting for age and body mass index. Several bacterial species, including *Peptococcus niger, Anaerococcus murdochii, Mobiluncus, Porphyromonas,* and *Prevotella,* were more abundant in the vaginal microbiota of patients with cancer. In contrast, *Lactobacillus spp.* were more abundant in vaginal and rectal samples of control subjects.

Conclusions:

This work represents one of the few studies to comprehensively examine the relationship between the vaginal, rectal, and endometrial microbiomes in the context of EC, suggesting a potential role for

microbial imbalance in disease development. The findings underscore the importance of site-specific microbial analyses in gynecologic oncology and support further investigation into the microbiome as a possible biomarker for early detection and a target for preventive strategies.

Introduction

The microbiome of the female reproductive tract varies depending on the anatomical site [1] and a woman's age. Although there is a continuum between the upper and lower parts of the female reproductive tract, the composition and relative abundance of bacterial communities differ significantly [2]. The lower tract, particularly the vagina, is typically dominated by *Lactobacillus* species, which help maintain a low pH and protect against pathogen colonization. In contrast, the upper reproductive tract, including the uterus, fallopian tubes, and ovaries, harbors a more diverse microbial community [3,4]. Age and hormonal status also influence the microbial landscape. In premenopausal women, estrogen promotes glycogen accumulation in vaginal epithelial cells, supporting the growth of *Lactobacillus* spp. and the production of lactic acid. After menopause, decreased estrogen levels lead to a reduction in *Lactobacillus* dominance, an increase in vaginal pH, and a shift toward a more diverse and potentially pro-inflammatory microbial community. Several studies have highlighted that the menopausal reduction in *Lactobacilli* is associated with higher serum levels of follicle-stimulating hormone and lower estrogen levels [5]. Moreover, genital modifications such as vaginal atrophy during this period are accompanied by well-documented changes in the species composition of the vaginal microbiome [6–8], with a decreased proportions of *Lactobacilli* and lactic acid production, and an increased vaginal pH. Postmenopausal women undergoing hormone therapy show a restoration of Lactobacillus species, particularly *L. crispatus, L. iners,* and *L. gasseri*, emphasizing the strong hormonal influence on the vaginal microbial ecosystem.

The rectal and vaginal microbiota share several bacterial species, with the rectum potentially acting as a reservoir for both commensal and pathogenic vaginal colonization. Several studies have reported that *Lactobacilli* commonly found in the vagina can also be detected in the rectal microbiota. For example, in rectal swabs from fertile women, *Lactobacillus plantarum* was the most frequently identified species, followed by *L. vaginalis*, *L. crispatus, L. delbrueckii*, and *L. salivarius*. In postmenopausal women, *L. plantarum* remained the most often detected *Lactobacillus*, followed by *L. gasseri* and *L. ruminis*. The vaginal environment of fertile women with high or medium estradiol levels mainly comprised *L. crispatus, L. jensenii, L. reuteri,* and *L. vaginalis* [9]. Moreover, in a large cohort of 531 fertile women aged 14–35, 43% of those with vaginal *L. crispatus* also had rectal colonization of the same species, indicating a significant overlap between the rectal and vaginal

microbiota [10]. Notably, hormone levels and the abundance of vaginal bacteria did not always correlate with rectal microbial composition, and sex hormone levels were frequently unrelated to rectal microbiota. Compared to postmenopausal women, *L. crispatus* was more frequently identified in the vaginal microbiota of reproductive-age women, suggesting both age and hormonal status influence microbial distribution across these sites.

Variations in the microbiota of the female reproductive tract can be caused by several factors, including changes in endometrial pH, temperature, humidity, menstruation, and pregnancy [11–14]. Both exogenous and endogenous variables [15] can affect microbiota composition, as observed in tumor development [16–18], and, specifically, in endometrial cancer (EC) [19]. In developed nations, EC is the most common gynecological cancer and the fourth leading cause of cancer-related deaths among women [20]. Recognized oncological risk factors include pro-inflammatory conditions, metabolic syndrome, low parity, advanced age, ethnicity, hormonal dysregulation, and genetic predisposition [19,21,22]. In particular, body mass index (BMI) shows a strong association with type 1 endometrial cancer: the relative risk is 1.5 in overweight women (BMI of 25.0–29.9 kg/m²), 2.5 in obesity class I (30.0–34.9 kg/m²), 4.5 in obesity class II (35.0–39.9 kg/m²) and 7.1 in obesity class III (≥40 kg/m²) [21]. The mechanisms linking obesity to EC are not yet fully understood but probably involve elevated estrogen levels in postmenopausal women, hyperinsulinemia and a chronic pro-inflammatory state. The main source of excess estrogen is the aromatase-mediated conversion of androgens to estrogens by adipocytes; these estrogens, in turn, stimulate the proliferation of the endometrium, leading to hyperplasia and cancer development. In women with diabetes, the risk of developing EC is estimated to be 72% higher, mainly due to hyperinsulinemia, hyperglycemia and systemic inflammation levels. Hyperinsulinemia promotes carcinogenesis indirectly through the activity of Insulin-like Growth Factor 1 (IGF-1), which has strong mitogenic and anti-apoptotic effects [23]. Furthermore, hyperglycemia promotes tumor cell proliferation and metastasis formation. Diabetes is also associated with increased production of reactive oxygen species and consequent oxidative damage to DNA, which can lead to mutations in oncogenes and tumor suppressor genes [19,21,22].

In recent years, the potential role of microbiota in carcinogenesis has attracted increasing scientific attention [24]. Under physiological conditions, a balanced microbiota stimulates mucus production, antimicrobial peptide secretion and epithelial cell regeneration [4,25,26]. These protective effects can prevent toxins and pathogenic bacteria from entering the bloodstream, thereby reducing the risk of metabolic syndrome, cancer, obesity, and chronic inflammation [4,25–27]. When the physiological balance of the microbiota is disrupted, microbial communities lose stability and diversity, allowing opportunistic microorganisms to proliferate [28–30] and resulting in dysbiosis and inflammation.

Importantly, the initiation and persistence of a chronic inflammatory state, which plays a central role in carcinogenesis, may be closely linked to microbiota composition [31–33].

Microbiota imbalance also contributes to carcinogenesis through multiple mechanisms, such as genetic instability and the generation of a microenvironment conducive to tumor growth [34], both locally and systemically [35,36]. Interactions between the vaginal and intestinal microbiota, as well as metabolic, immunological, and hormonal imbalances of intestinal bacteria, can influence the female genital tract and contribute to the development of gynecologic cancers [25,37]. The elevated production of pro-inflammatory cytokines, such as Interleukin-17 (IL-17), Tumor Necrosis Factor (TNF)-α, and Interferon (IFN)-γ, and the activation of pattern recognition receptors, such as toll-like receptor 4 (TLR-4), facilitate this process [38].

Through the gut-vaginal microbiome axis, estrogen levels may influence the endometrium in gynecologic malignancies [39,40]. The intestinal microbiota may modulate circulating estrogen levels by secreting β-glucuronidase, an enzyme that deconjugates inactive estrogen metabolites and increases the levels of active circulating estrogens [39]. The microbiome may also promote conditions that facilitate carcinogenesis, such as insulin resistance and adipose tissue expansion [34,41]. By enhancing angiogenesis and disrupting epithelial or mucosal barriers, microbiota may further contribute to tumor development [42–44]. Pro-inflammatory molecules, including NOS2 (nitric oxide synthase), reactive nitrogen species (RNS), and other reactive oxygen species, are produced in greater quantities, altering normal microbiota composition.

Certain bacterial species can reduce apoptosis, promote cell invasion and migration, increase cell proliferation, and induce genomic instability, all of which favor carcinogenesis [45,46].

It is crucial to identify which species are present in the female genital tract and which changes are most closely linked to EC, since these many mechanisms may directly contribute to endometrial carcinogenesis [47]. Additionally, pathogenic alterations in the microbiota can affect the metabolism of carcinogenic compounds [46]. While inflammation is recognized as a key factor in EC development, the specific role of the genital microbiota remains unclear [48–51].

The aim of our study was to identify microbial signatures across the rectal, vaginal, and endometrial niches that distinguish EC cases from controls, leveraging shotgun metagenomic sequencing to achieve strain-level resolution. Previous studies addressing related questions have typically relied on 16S rRNA gene sequencing, which limits both taxonomic and functional resolution [52,53], or have focused exclusively on vaginal and/or rectal sites [52,54]. To ensure sample integrity and minimize contamination, we implemented a rigorous, site-specific sampling strategy, including sterile endometrial brushing performed immediately after hysterectomy. By comprehensively analyzing microbial diversity and composition across multiple niches within a well-characterized cohort, and

adjusting for key confounders such as age and BMI, we sought to identify site-specific microbial signatures associated with EC.

Methods

Patients and sample collection

This prospective, single-center, non-profit observational study was conducted at the Department of Obstetrics and Gynecology, AOU Maggiore della Carità, Novara, Italy. The study was approved by the local Ethics Committee (N. CE018/2023, 340CE) and carried out in accordance with the Declaration of Helsinki and current research regulations. The study population consisted of adult women undergoing hysterectomy for either EC (cases) or benign uterine conditions (controls). Participants were enrolled consecutively at hospital admission during routine clinical care, after providing written informed consent. Clinical and anamnestic were retrieved from standard medical records and entered into a secure, password-protected electronic database (REDCap, Electronic Data Capture). Each participant was assigned a progressive identification number following pseudonymization procedures to ensure data confidentiality.

Eligible participants were adult women who have not received antibiotics or probiotics within the previous two months and were diagnosed with either benign (controls) or malignant (cases) uterine pathology. Exclusion criteria included inability to provide informed consent and recent antibiotics or probiotics use. In the operating room, following induction of anesthesia, vaginal and rectal swabs were collected by a single trained operator to minimize inter-operator variability. Following hysterectomy, the uterus was immediately transported to the Pathological Department of our hospital. Before routine histopathological processing, the uterine cavity was surgically exposed, and an endometrial swab was collected directly from macroscopically tumorous (cases) or benign tissue (controls) using a sterile brush. This sampling procedure did not alter the tissue, that was processed according to standard diagnostic protocols.

Microbiota analyses on tumor samples

Microbiota analyses were performed on vaginal, rectal and endometrial e-NAT™ (Copan) swabs brushed on the corresponding anatomical sites. Vaginal and endometrial microbiota were extracted using the QIAamp® DNA Microbiome kit (QIAGEN), whereas rectal microbiota were extracted using the QIAamp PowerFecal Pro kit (QIAGEN), according to the manufacturer's instructions. Shotgun metagenomics sequencing was performed by Novogene (UK) Company Limited. Libraries were constructed with the NexteraXT DNA Library Preparation Kit (Illumina) and sequenced on the Illumina NovaSeq platform with 150-bp paired-end reads (target sequencing depth: 7 Gb/sample).

Taxonomic profiling with estimation of relative abundances at species-level was performed using MetaPhlAn version 4.1.0 with marker database version mpa_vJun23_CHOCOPhlAnSGB_202307 [55]. Pre-processing was performed to remove low-quality reads through Trimmomatic [56] and to remove host-contaminated reads by mapping the raw sequences against the T2T-CHM13v2.0 human genome. Downstream statistical analysis was performed through custom scripts written in the R environment. Figures were produced using a custom R script (ggplot-based) inspired by common visualization styles. A blank swab was also processed as negative control alongside biological samples. Taxa detected in this negative control were not found in any of our samples so did not impact downstream analyses.

Functional data in terms of pathway abundance profiles were generated with HUMAnN 4.0 with default parameters. The downstream statistical analysis was conducted by considering the same setting exploited for taxonomic profiles.

Statistical analyses

The sample size was determined based on the average number of participants examined in previous studies [57]. The Shannon index and estimated richness were used to compare α-diversity between cases and controls. Statistical significance was assessed using the Wilcoxon-Mann-Whitney test for unadjusted comparisons and linear models adjusted for age and BMI. β-diversity was analyzed through multidimensional scaling based on Bray-Curtis distance, and statistical significance was assessed by PERMANOVA (adonis2 function in R) with adjustment for age and BMI. To identify taxa that were significantly different between groups, we applied the Wilcoxon-Mann-Whitney test for unadjusted p-values and linear models for analyses adjusted for age and BMI ($p < 0.05$). We have also performed a sensitivity analysis in which we include menopausal status alongside age in the adjusted models. Multiple hypothesis testing was controlled using the false discovery rate (FDR), and taxa with q-values (FDR-adjusted p-values) < 0.1 were considered statistically significant. FDR threshold of $q < 0.1$ was selected a priori as an exploratory criterion to balance false discovery control with sensitivity in this relatively small, hypothesis-generating case-control study.

Results

Vaginal, rectal, and endometrial microbiota samples were collected from 25 patients with EC and 27 control subjects, resulting in a total of 156 samples. One rectal sample from a control subject (ID37) failed during library preparation prior to shotgun sequencing, so downstream analyses were performed on the final set of 155 metagenomes. We retained only samples with detectable microbial species (20 cases and 26 controls for vaginal samples, 25 cases and 26 controls for rectal samples, 6

cases and 9 controls for endometrial samples). In addition, to minimize noise from rare taxa and reduce false positives, we removed taxa with prevalence < 5% across samples. The distribution of non-human reads per site is reported in Supplementary Figure S1. No statistically significant differences were observed between cases and controls after host-read removal.

Clinical and demographic characteristics of the study population are summarized in Table 1 and reported in Table S1. The mean age was 67.1 ± 11.8 years in EC patients and 58.7 ± 10.7 in controls, while the mean BMI was 31.1 ± 8.2 and 25.5 ± 4.4 kg/m², respectively. Four EC patients had a previous diagnosis of other malignancies, including breast, thyroid, and renal cancers. The two groups were well matched for the remaining characteristics, with overlapping distributions for: menarche, parity, miscarriages, smoking history, and comorbidities (*i.e.* diabetes). However, age and BMI were significantly higher in cases compared to controls (p = 0.015 and p = 0.009, respectively), consistent with established risk factors for EC.

The cases were predominantly affected by type 1 (*i.e.*, endometrioid) EC (92%), including grade 1 (n=5, 20%), grade 2 (n=15, 60%), and grade 3 (n=3, 12%). The remaining 8% included other histological types. None of the cases were receiving hormone replacement therapy (HRT). Controls underwent surgery for benign gynecological conditions, primarily uterine leiomyomas (n=20, 74.1%) and urogenital prolapse (n=13, 48.1%), with smaller proportions having ovarian cysts (n=9, 33.3%) and ovarian endometriosis (n=1, 3.7%) (Table S1). None of the controls had a history of malignancy or were receiving HRT.

Table 1. Demographical and clinical characteristics of participants.

	Patients N=25	**Controls N=27**	**OR (95% CI)**	***p*-value**
Age Mean (SD)	**67.08 (11.75)**	**58.7 (10.67)**	**1.07 (1.01 - 1.13)**	**0.015**
BMI Mean in Kg/m^2 (SD)	**31.06 (8.18)**	**25.5 (4.42)**	**1.16 (1.04 - 1.30)**	**0.009**
Menarche Mean (SD)	12.14 (1.78)	12.3 (1.29)	0.93 (0.65 - 1.34)	0.711
Obesity categories Normal weight (%)	5 (20)	15 (55.56)	1	
Overweight (%)	4 (16)	7 (25.93)	1.71 (0.35 - 8.42)	0.507
Obesity (%):	**16 (64)**	**5 (18.52)**	**9.6 (2.31 - 39.94)**	**0.002**
class 1	*9 (36)*	*5 (18.52)*		
class 2	*4 (16)*	*0 (0)*		
class 3	*3 (12)*	*0 (0)*		
Smoking Never (%)	22 (88)	21 (80.77)	1	
Yes or only in the past (%)	3	5 (19.23)	0.57 (0.12 - 2.70)	0.481
Parity				

0 (%)	7 (28)	5 (18.52)	1	
>0 (%): *1* *2* *3*	18 (72) *8 (32)* *7 (28)* *3 (12)*	22 (81.48) *9 (33.33)* *12 (44.44)* *1 (3.70)*	0.58 (0.16 – 2.16)	0.420
Miscarriages 0 (%)	17 (73.91)	17 (62.96)	1	
>0 (%): *1* *2* *5*	6 (26.09) *5 (21.74)* *1 (4.35)* *0 (0)*	10 (37.04) *9 (33.33)* *0 (0)* *1 (3.7)*	0.67 (0.19 – 2.29)	0.519
Contraception No (%)	22 (91.67)	19 (73.08)	1	
Yes (%)	2 (8.33)	7 (26.92)	0.25 (0.05 - 1.33)	0.104
Hypertension No (%)	12 (48)	15 (55.56)	1	
Yes (%)	13 (52)	12 (44.44)	1.35 (0.45 - 4.03)	0.586
Diabetes No (%)	23 (92)	25 (92.59)	1	
Yes (%)	2 (8)	2 (7.41)	1.09 (0.14 - 8.36)	0.936
Family history of cancer No (%)	9 (37.5)	8 (30.77)	1	
Yes (%)	15 (62.5)	18 (69.23)	0.74 (0.23 - 2.39)	0.616
Menopause No (%)	3 (12)	8 (29.63)	1	
Yes (%)	22 (88)	19 (70.37)	3.09 (0.71 – 13.32)	0.131

OR: odds ratio; CI: confidence interval; BMI: body mass index

Microbiota analyses

Following quality filtering and host DNA decontamination of shotgun sequencing data, a mean of 64,874.81 (±47,245.999), 1,658,331 (±3,273,173.061) and 20,894,726 (±19,841,901.67) reads were obtained for endometrial, vaginal and rectal samples, respectively.

We analyzed both α-diversity, which indicates the richness and evenness of microbial taxa within a sample, and β-diversity, which quantifies the variation in bacterial community composition among samples [58]. The vaginal microbiota of EC patients showed significantly higher α-diversity (Shannon index) compared to controls ($p = 0.0071$). β-diversity also differed significantly between vaginal samples from cases and controls ($p = 0.001$) (Fig. 1). To account for age and BMI—two major clinical differences between cases and controls—we repeated the analyses using adjusted models. The differences in both α- and β-diversity for vaginal samples remained statistically significant after

age and BMI adjustment (corrected p = 0.0264 and p = 0.029, respectively), confirming the robustness of these findings (Fig. 1).

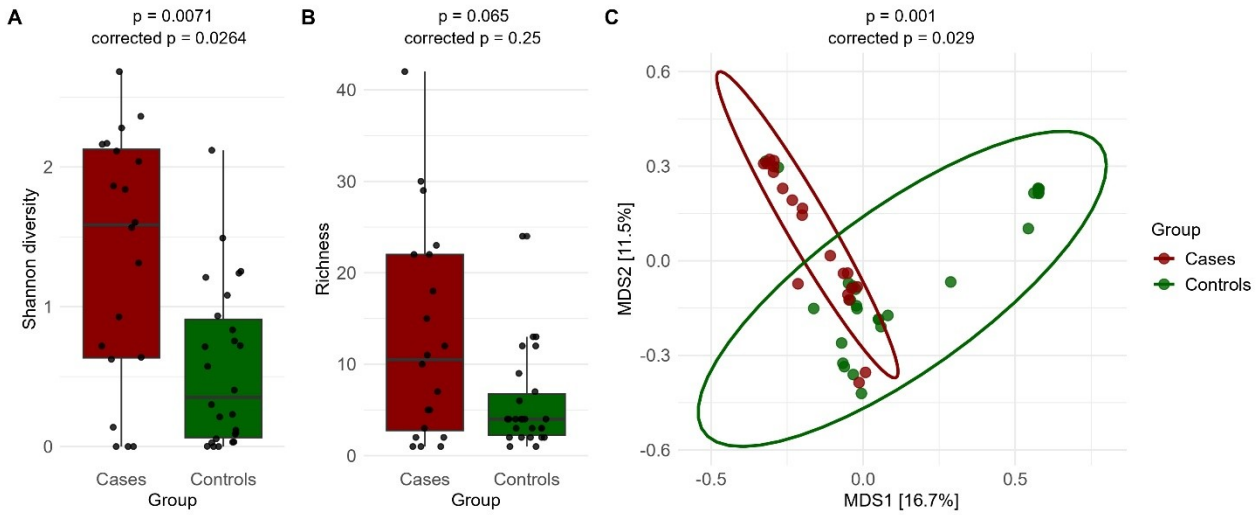

Figure 1. α-diversity and β-diversity analyses for vaginal samples. (A, B) Box plots showing the Shannon index (A) and the richness (B) to compare the α-diversity between cases (red) and controls (green). (C) Principal coordinates analysis (PCoA) of β-diversity shows the different microbial composition between the two groups. p: p-value; corrected p: p-value after correction for BMI and age.

No statistically significant differences in α-diversity or β-diversity were observed in the rectal (Fig. 2) or endometrial (Fig. 3) microbiota between groups, either before or after adjustment for age and BMI.

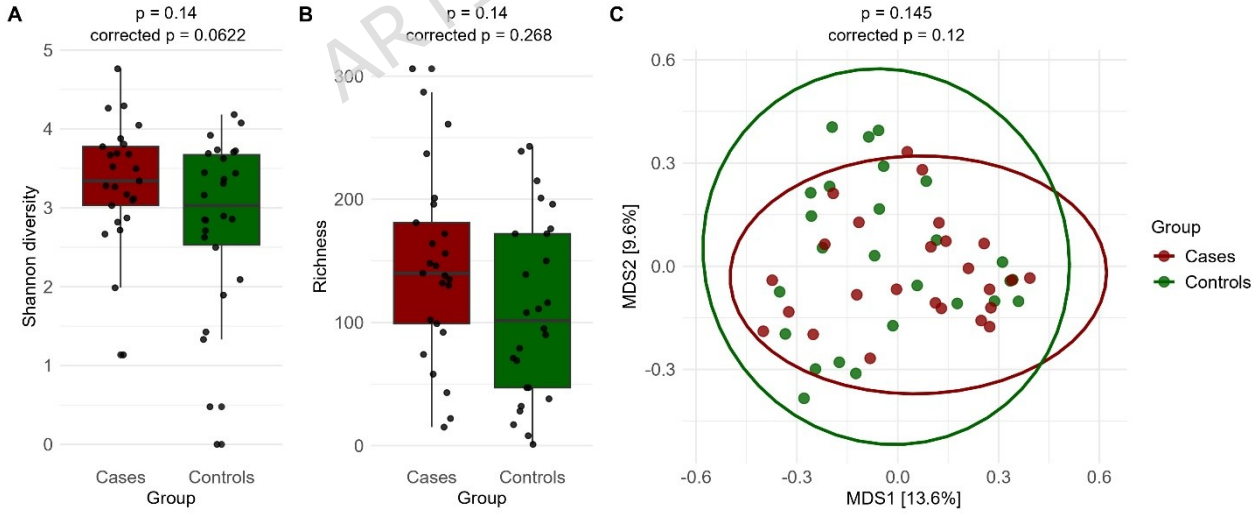

Figure 2. α-diversity and β-diversity analyses for rectal samples. (A, B) Box plots showing the Shannon index (A) and the richness (B) to compare the α-diversity between cases (red) and controls (green). (C) Principal coordinates analysis (PCoA) of β-diversity shows the different microbial composition between the two groups. p: p-value; corrected p: p-value after correction for BMI and age.

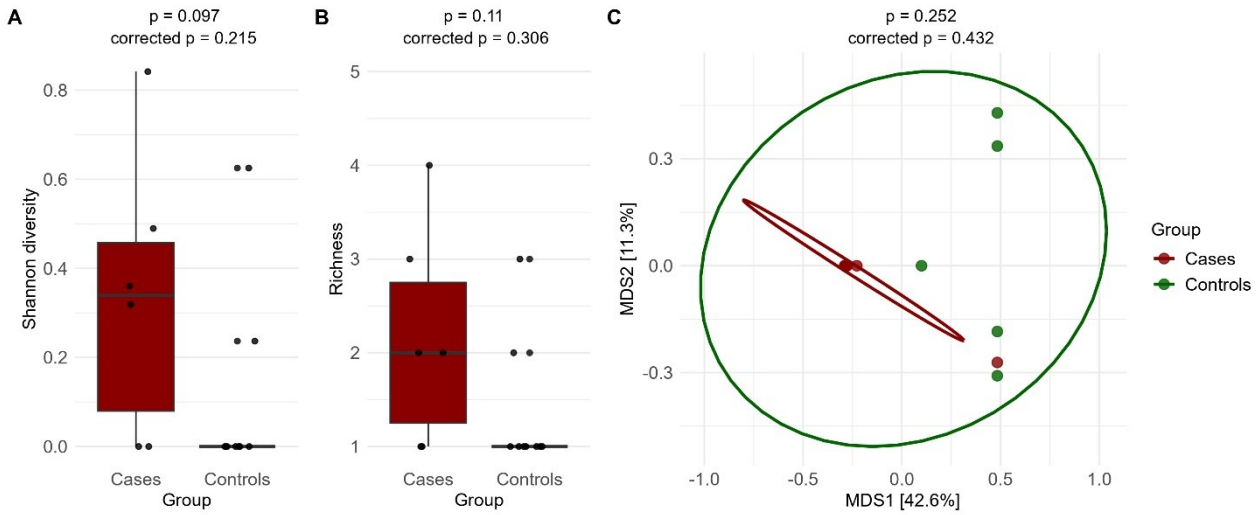

Figure 3. α-diversity and β-diversity analyses for endometrial samples. (A, B) Box plots showing the Shannon index (A) and the richness (B) to compare the α-diversity between cases (red) and controls (green). (C) Principal coordinates analysis (PCoA) of β-diversity shows the different microbial composition between the two groups. p: p-value; corrected p: p-value after correction for BMI and age.

We identified different bacterial signatures in cases *vs* controls. In particular, in vaginal samples, 33 taxa were differentially enriched in cases or controls (FDR-adjusted $q < 0.1$), including *Peptococcus niger, Anaerococcus murdochii, Mobiluncus SGB15488* (FDR-adjusted $q < 0.05$) and *Porphyromonas* (unadjusted $p < 0.05$), which were enriched in patients with EC, and *Lactobacillus iners* (FDR-adjusted $q < 0.05$), *Lactobacillus crispatus* and *Gardnerella* (unadjusted $p < 0.05$) which were enriched in the vaginal microbiota of controls (Fig. 4 and Table S2).

Most differentially enriched vaginal species showed no significant correlations with age or BMI (Fig. 4B, right). After correction for BMI and age, we found *Veillonella montpellierensis, Mobiluncus SGB15488, Alloscardovia omnicolens, Corynebacterium uberis, Prevotella bivia,* and *Gordonibacter sp. Marseille* enriched in vaginal samples of cases *vs* controls (unadjusted $p < 0.05$) (Table S2). To account for menopausal status, we included it alongside age in the adjusted models; the main vaginal community-level differences and key taxon-level associations remained consistent with the main analysis.

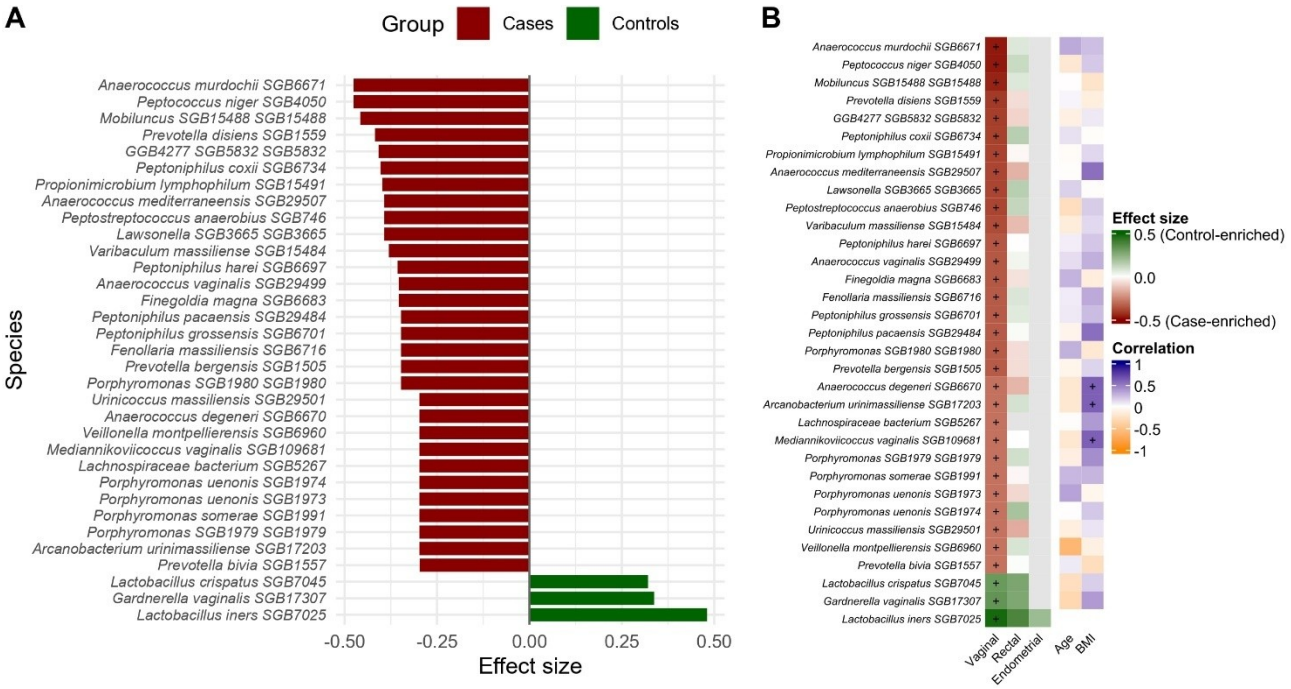

Figure 4. Bacterial signatures in vaginal samples. (A) Bacterial species differentially enriched in vaginal samples of EC cases (red) and controls (green). (B) Differentially enriched vaginal species in rectal and endometrial samples (left) and correlation with age and BMI (right). Intense purple indicates a strong positive correlation, and yellow a strong negative correlation, with age or BMI. +: FDR-adjusted $q < 0.1$.

In rectal samples, 46 taxa exhibited differential abundance between cases and controls based on unadjusted p-values. Among these, *Lactobacillus iners* and *Mobiluncus SGB15489* were enriched in controls and EC cases (unadjusted $p < 0.05$), respectively, consistent with the patterns observed in the vaginal microbiota (Table S2).

The low number of microbial reads after host decontamination of endometrial samples limited statistical power for differential abundance testing. No taxa reached statistical significance, though a trend toward increased *Lactobacillus iners* and *L. gasseri* in controls was observed (Table S2).

After correction for BMI and age, 20 species —including *Mobiluncus SGB15489*—were differentially enriched in the rectal samples of the two groups (unadjusted $p < 0.05$), while no statistically significant results were observed in endometrial samples (Table S2).

Functional pathway analysis was performed on the vaginal metagenomic data (Supplementary Figure S2 and Table S3). Enrichment of pathways related to amino acid fermentation and anaerobic metabolism, including L-lysine fermentation to acetate and butanoate, purine nucleobases degradation I (anaerobic), and 2-oxobutanoate degradation were observed in EC compared to control samples. Conversely, the chitin derivatives degradation pathway was enriched in controls. However, none of these differences remained significant after FDR correction.

After adjustment for age and BMI, the following pathways were enriched in controls at an unadjusted p-value < 0.05: superpathway of fatty acids biosynthesis (E. coli); D-galactarate degradation I, and

superpathway of D-glucarate and D-galactarate degradation (Table S3). No pathway reached statistical significance following FDR adjustment.

Discussion

Our study explores the relationship between the vaginal, rectal, and endometrial microbiomes in the context of EC, compared with control patients who underwent hysterectomy for non-cancer-related reasons. To ensure high-quality, contamination-free samples, we collected vaginal and rectal swabs prior to hysterectomy, and obtained endometrial samples using a sterile brushing technique after uterine removal, thereby avoiding contamination from adjacent anatomical sites. This careful sampling strategy allowed us to generate accurate microbial profiles and identify potential associations between site-specific microbiota and EC. In this study, we analyzed the vaginal, rectal, and endometrial microbiota of 52 individuals (25 patients with EC and 27 controls).

Vaginal microbiota of EC patients exhibited significantly higher α-diversity compared to controls, consistent with previous reports [53,57]. β-diversity also differed significantly between the two groups, suggesting distinct microbial community structures. Conversely, rectal microbiota did not differ significantly in either α- or β-diversity between cases and controls, in line with previous observations [53]. Importantly, differences in specific taxa may occur even in the absence of significant global diversity differences, as diversity metrics reflect overall community structure; therefore, taxon-level findings in rectal and endometrial samples should be considered exploratory.

We show that vaginal microbiota of EC cases is associated with a depletion of *Lactobacillus iners.* Notably, *L. iners* was more abundant in controls *vs* cases also in rectal and endometrial samples, although these differences did not reach statistical significance after multiple testing correction. Moreover, *L. crispatus* was found depleted in EC cases both in vaginal and rectal samples. Importantly, our data confirms the notion that *Lactobacilli* are more prevalent in benign gynecological conditions [57,59]. *Gardnerella* was found enriched in controls *vs* patients and its abundance may distinguish between EC and cervix cancer, because it is enriched in the vaginal microbiota of patients with cervix cancer, but not in those with EC [60]. Nevertheless, comparisons across different gynecologic malignancies should be made cautiously. We found an enrichment of *Porphyromonas, Peptococcus niger, Anaerococcus murdochii, Mobiluncus SGB15488, Peptoniphilus spp.* and *Lachnospiraceae spp.* associated with vaginal samples of EC cases *vs* controls (FDR-adjusted $q < 0.1$). The enrichment of *Porphyromonas* species in the vaginal microbiota of patients with EC is well known [53]. Interestingly, these species are enriched also in fecal microbiota both in endometrial and cervix cancer [61]. Among *Mobiluncus* species, *M. curtisii* has previously been reported to be enriched in EC patients *vs* healthy controls [57]. Additionally, *Peptococcus*

niger has been identified as enriched in the vaginal microbiota of EC *vs* hyperplasia patients [62]. Our finding of *Anaerococcus murdochii* enrichment associated with vaginal samples of EC patients aligns with previous reports of *Anaerococcus* genus enrichment in vagina, cervix and endometrium microbiota of EC patients *vs* benign conditions (dysfunctional uterine bleeding and/or fibroids) [53]. Moreover, in our study, three *Prevotella* species—*P. disiens*, *P. bergensis*, and *P. bivia*—were enriched in vaginal samples from EC patients compared to controls, in accordance with previous reports [53,54]. *Prevotella* spp. are well-documented contributors to genital tract infections and have been associated with bacterial vaginosis [63]. We also observed an enrichment of *Peptostreptococcus anaerobius* associated with vaginal samples from EC patients compared to controls, supporting previous findings of its role in gynecologic cancers. Elevated levels of *P. anaerobius* have also been reported in cervicovaginal fluid of cervical cancer patients, where macrophages activated by this bacterium promote tumor migration and angiogenesis [64]. However, comparisons between different diseases should be interpreted cautiously. Likewise, in EC, *P. anaerobius* is more abundant in the endometrium, cervix, and posterior fornix compared to benign or healthy samples, and is implicated in facilitating immune evasion [65]. In agreement with previous reports, we also found an enrichment of *Lachnospiraceae* spp. [52] and *Peptoniphilus* spp. [52,53] associated with EC samples.

Notably, we identified *Finegoldia magna* as enriched in cases compared to controls. Interestingly, our group previously reported an enrichment of this species in the mucosa-associated microbiota of obese versus normal-weight patients with colorectal polyps [66]. Previously, this species was found in the vulvar microbiota of obese women [67].

Enrichment of *Anaerococcus degeneri, Arcanobacterium urinimassiliense*, and *Mediannikoviicoccus vaginalis* in EC patients showed a strong positive correlation with BMI, suggesting that their increased abundance is associated with obesity rather than EC itself. To our knowledge, these species have not previously been reported in literature in association with obesity. These findings support the interpretation that their presence likely reflects metabolic confounding rather than a direct link to EC.

After adjusting for age and BMI, the differences in α- and β-diversity in vaginal samples remained statistically significant. Although FDR-adjusted p-values did not reach significance in taxonomic analyses, *Mobiluncus* remained differentially enriched in both vaginal and rectal samples after BMI adjustment, supporting the robustness of this finding. Additional taxa distinguishing cases from controls emerged in the adjusted analysis. In particular, *Veillonella montpellierensis* and *Alloscardovia omnicolens* were more abundant in EC patients. These species have previously been reported to be higher in the vaginal microbiota of patients with endometriosis/adenomyosis (EM/AM) compared with patients with chronic pelvic pain without EM/AM and women without chronic pelvic

pain [68]. Moreover, *Veillonella* was previously found enriched in low-grade endometrioid carcinoma compared to other EC subtypes [52] while *Prevotella bivia* was reported to be more abundant in high-grade *vs* low-grade EC [54]. The previously mentioned *F. magna* did not reach statistical significance in vaginal samples from EC cases compared to controls after adjusting for age and BMI, suggesting that its enrichment is more closely associated with obesity than with EC. This is further supported by its previously observed enrichment in obese patients with colorectal polyps [66].

Notably, the enrichment of anaerobic taxa observed in our EC cohort overlaps with microbial patterns reported across other gynecologic malignancies. Recent large-scale meta-analyses of the cervical cancer microbiome [69] have identified increased abundance of anaerobic genera including *Peptococcus*, *Anaerococcus*, *Porphyromonas*, and *Prevotella*, alongside depletion of *Lactobacillus* spp., as recurrent features of cervical cancer–associated vaginal microbiota. The concordance between these findings and our results suggests that shifts toward anaerobe-dominated microbial communities may be commonly observed in association with gynecologic malignancies, potentially reflecting common tumor-associated microenvironmental changes rather than disease-specific effects alone, although cross-disease comparisons should be interpreted cautiously.

Moreover, recent work by Muraoka et al. [70] identified *Fusobacterium* as a key microbial driver of endometriosis through fibroblast activation and TGF-β signaling. In our study, only one control subject was affected by endometriosis, localized to the ovary, making it unlikely that endometriosis-associated microbial signatures influenced our results.

Functional pathway analysis did not show statistically significant differences between EC cases and controls after multiple testing correction, but highlighted trends that may provide useful exploratory insights. The chitin derivatives degradation pathway was enriched in the vaginal microbiota of controls, compared to EC cases. Chitin is a structural polysaccharide found in fungal cell walls, and its degradation products, such as chitosan and N-acetylglucosamine (GlcNAc), have been reported to modulate immune and inflammatory responses [71,72]. The increased abundance of this pathway in controls may indicate enhanced microbial capacity to limit fungal overgrowth, and could be consistent with a metabolically balanced vaginal ecosystem.

In contrast, the enrichment of anaerobic and fermentative pathways in EC patients may reflect ecological adaptation of the vaginal microbiota to a tumor-associated microenvironment characterized by altered nutrient availability and inflammation. For example, the enrichment of L-lysine fermentation pathway suggests increased amino acid fermentation activity, consistent with a *Lactobacillus*-depleted microbiota in which anaerobic bacteria utilize amino acids as fermentative substrates [73,74].

After adjustment for age and BMI, control samples showed enrichment of pathways involved in fatty acid biosynthesis and carbohydrate catabolism, including D-galactarate and D-glucarate degradation. These pathways are associated with bacterial anabolic activity and may reflect a functionally balanced vaginal microbiota.

Strengths and limitations

This study has several notable strengths. First, the comprehensive sampling of vaginal, rectal, and endometrial microbiota within the same individuals provides a unique, multi-site perspective on microbial alterations associated with EC. Moreover, we adopted a sampling approach designed to maximize accuracy and minimize contamination through direct endometrial brushing performed on the uterine tissue. Unlike most studies, our protocol involved collecting endometrial samples after surgical exposure of the uterine cavity and greatly reduced potential sampling bias. Furthermore, we deliberately excluded patients who had recently undergone therapy with antibiotics or probiotics to avoid possible iatrogenic interference, thereby increasing the objectivity of our data.

Second, the use of shotgun metagenomic sequencing enabled high-resolution taxonomic profiling, surpassing the capabilities of 16S rRNA gene sequencing and allowing species-level identification of microbial signatures relevant to disease status.

Third, we adjusted for key clinical confounders, including age and BMI, which strengthened the robustness of the observed associations between microbiota composition and EC. This adjustment is essential given the high prevalence of obesity that characterizes EC patients.

Finally, the identification of consistent microbial signatures across sample types—particularly the depletion of *Lactobacillus iners* and the enrichment of *Mobiluncus* species—supports the biological relevance of our findings and provides a solid foundation for future mechanistic and translational research.

However, this study also has limitations. First, the sample size was relatively small (n = 52), which may have limited the statistical power to detect subtle microbial differences in relative abundances, especially in endometrial samples where microbial load was low—although comparable to that reported in previous studies on this topic [57]. The low microbial read counts following host DNA decontamination in endometrial tissues particularly constrained our ability to perform robust differential abundance testing in that compartment; accordingly, findings related to the endometrial microbiome should be considered exploratory. Absolute quantification and/or spike-in approaches would be valuable in future work to validate changes in microbial load.

Second, the cross-sectional study design precludes causal inference regarding the role of microbiota in the development or progression of EC. Longitudinal studies are needed to assess whether specific microbial shifts precede tumor development or arise as a consequence of the disease.

Third, while shotgun metagenomics offers detailed taxonomic resolution, the use of relative abundance data without absolute quantification limits conclusions about actual bacterial load.

Fourth, the patients were older than the controls, although adjusting for age did not alter the results. Moreover, most women in both groups were postmenopausal and were not receiving hormonal therapy. Thus, the higher abundance of *Lactobacilli* in controls cannot be explained by younger age or premenopausal status, nor by hormonal treatment. Since cases had a higher BMI compared to controls, the estrogen produced by adipose tissue should not be responsible for the change in *Lactobacilli*.

Finally, validation in independent cohorts and integration with host transcriptomic, metabolomic, or immune profiling data would also help elucidate potential mechanistic links between microbial alterations and endometrial carcinogenesis.

Conclusions

This work adds valuable knowledge to a rapidly evolving field, offering novel insights that could inform both basic research and translational applications in women's health. For the first time, we used metagenomics analyses to characterize the vaginal, rectal and endometrial microbiota in a large cohort of patients with EC *vs* controls. The identification of distinct microbial signatures associated with EC may open new avenues for early detection, risk stratification, and even preventive interventions based on microbiota modulation. The potential clinical implications of these findings extend to screening, diagnostic and prognostic applications. Future studies should aim to determine whether specific microbial taxa are predictive of EC risk or reflect disease-associated changes, confer protection or increase susceptibility to EC, whether certain microbial profiles correlate with more advanced disease stages, and whether the routine use of probiotics could play a protective role for EC.

Author Contributions

Conceptualization, methodology, validation C.I.A., M.L.V., I.D., A.A., D.S., V.R.; formal analysis, visualization E.P., D.F.; investigation, data curation C.I.A., M.L.V., E.P., G.S., A.L., R.B.; writing — original draft preparation C.I.A., M.L.V., I.D., A.A., D.S.; writing—review and editing C.I.A., M.L.V., E.P., R.B., I.D., A.A., D.S., V.R.; visualization, supervision, project administration C.I.A., M.L.V., I.D., A.A., D.S., V.R.; funding acquisition: V.R., I.D. All authors have read and agreed to the published version of the manuscript.

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Ethics declarations

Ethics approval and consent to participate

This study was carried out according to the Declaration of Helsinki and approved by the Ethics Committee of AOU Maggiore della Carità, Novara, Italy (Protocol number N. CE018/2023, 340CE, in date 21/03/2023). Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable. The manuscript does not contain identifying personal or clinical details that could compromise the anonymity of participants.

Competing interests

The authors declare that they have no conflicts of interest.

Supplementary Material

Table S1. Clinical features of each case and each control.

Table S2. Bacterial taxa differently enriched in EC cases and controls in endometrial, rectal and vaginal samples (uncorrected and corrected for age and BMI).

Table S3. Functional pathway abundance analysis in vaginal samples. Differential abundance of predicted metabolic pathways between cases and controls based on vaginal microbiota profiles (Sheet1, uncorrected and Sheet 2, corrected for age and BMI).

Figure S1. Percentage of non-human reads remaining after host-read removal in cases vs. controls for each body site.

Figure S2. α-diversity and β-diversity analyses for vaginal samples based on functional pathway abundance profiles.

Availability of data and materials

The raw data for the rectal, vaginal, and endometrial are available in NCBI-SRA under the BioProject PRJNA1347038.

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