Microbial signatures and continuum in endometrial cancer and benign patients

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Abstract

Endometrial cancer is a multifactorial disease with inflammatory, metabolic and potentially microbial cues involved in disease pathogenesis. Here we sampled different regions of the reproductive tract (vagina, cervix, endometrium, fallopian tubes and ovaries) of 61 patients and showed that the upper genital tract of a subset of women with and without endometrial cancer harbour microbiota quantitatively and compositionally distinguishable from background contaminants. A microbial continuum, defined by detection of common bacterial species along the genital tract, was noted in most women without cancer while the continuum was less cohesive in endometrial cancer patients. Vaginal microbiota were poorly correlated with rectal microbiota in the studied cohorts. Endometrial cancer was associated with reduced cervicovaginal and rectal bacterial load together with depletion of *Lactobacillus* species relative abundance, including *L. crispatus*, increased bacterial diversity and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* in the lower genital tract and endometrium. Treatment of benign and malignant endometrial organoids with *L. crispatus* conditioned media had minimal impact on cytokine and chemokine profiles. Our findings provide evidence that the upper female reproductive tract of some women contains detectable levels of bacteria, the composition of which is associated with endometrial cancer. Whether this is a cause or consequence of cancer pathophysiology remains to be elucidated.

Introduction

The incidence of endometrial cancer has increased by 55% since the early 1990s[1,2]. This is partly attributable to increased prevalence of obesity and diabetes[3–7]. Only a fraction (2–5%) of cases can be ascribed to inherited genetic predisposition[8], while epidemiological studies have also shown strong correlations with pelvic inflammatory disease (PID) caused by bacteria[9], suggesting a potential microbial link to endometrial carcinogenesis. Dysbiotic microenvironments have recently been associated with gynaecological precancer and cancer[10–15] and their role in endometrial oncogenesis warrants further investigation.

The sterile womb dogma has been recently challenged with the advent of sensitive molecular tools, including bacterial 16S rRNA gene profiling, which enables identification of microbes that were considered uncultivable[16]. Although recent studies report detection of metataxonomic signatures in 60–97% of non-malignant uteri[17–24], experimental design pitfalls have often failed to account for potential sources of contamination including niche-to-niche contamination caused by transcervical sample collection, or non-patient sources from airborne and “kitome” contaminants[25–27]. There is currently no consensus on a benign endometrial bacterial signature although commonly reported genera include *Lactobacillus, Prevotella*, *Gardnerella*, *Bifidobacterium, Staphylococcus* and *Streptococcus*[18–24, 28-31]. Intra-individual microbial correlations between the lower and upper genital tract, consistent with anatomical contingency, have recently been reported by Chen and colleagues[28]. Of the few studies to explore the endometrial oncobiome, an enrichment of seventeen taxa in the genital tract of endometrial cancer patients has been described, with *P. somerae* having the strongest association with disease[10,11].

In this prospective study, we aimed to characterise the bacterial load and composition of the female genital tract (FGT) in women with and without endometrial cancer. Where detectable above background contamination, we aimed to assess whether composition of the upper genital tract (endometrium, fallopian tubes, ovaries) was correlated with lower genital tract (vagina, cervix) composition. We next set out to compare the FGT and rectal microbiota in endometrial cancer patients in relation to benign controls. Finally, we attempted to investigate the impact of *L. crispatus*, which was depleted in women with endometrial cancer, on endometrial organoid proliferation and inflammation.

Methods

Study population and sample collection

We recruited women planned for laparoscopic or transabdominal hysterectomy for endometrial cancer or benign conditions (most commonly dysfunctional uterine bleeding and/or fibroids). Women were recruited irrespective of the surgical approach (transabdominal open or laparoscopic) or endometrial cancer histological type. We excluded women undergoing hysterectomy for other gynaecological malignancies or for pelvic inflammatory disease. Women who undertook vaginal douching or had taken antibiotics within the previous two weeks and women who had had sexual intercourse in the 48 hours prior to sampling were also excluded. All participants gave informed consent.

Microbiome swabs (VWR Swab Liquid Plastic Amies) were collected from seven distinct regions of the FGT and the rectum by rotating the swab five times against the sites of interest. FGT locations included the lower GT, i.e. lower two thirds of vagina, higher one third of vagina and cervical os and the higher GT, i.e. lower half of endometrium, fundal endometrium, fallopian tubes and ovaries (only for benign samples). Vaginal, cervical and rectal swabs were collected pre-operatively in the operating room, using an unlubricated, disposable, sterile plastic speculum (Medscope Intraspec) before antibiotic administration. In addition to this, a sterile cotton vaginal swab was collected and stored at -80^{o}C in 8% v/v DMSO for bacterial cryopreservation. The surgical specimen was placed in a sterile bag inside a histology pot and stored at 4^{o}C before being transferred to the histopathology lab on the same day. Under aseptic conditions, the fimbrial end of the fallopian tube and external surface of ovary were swabbed, while endometrial samples were collected after longitudinal dissection of uterus. Tissue samples from the cervix, endometrium and fallopian tube were also collected. All samples were stored at -80^{o}C within 30 minutes. For most cases, matched samples from the same patient along the genital tract were obtained. Two sets of technical controls were used in the pathology lab. A lysogeny broth (LB) agar plate was left open during sample collection and swabbed to account for airborne contaminants, while the packaged, non-sterile knife used for uterus dissection was also sampled.

DNA extraction and 16S rRNA gene sequencing

Bacterial DNA from swabs and tissue was extracted using the QiAmp Mini DNA kit. The V1–V2 hypervariable regions of 16S rRNA genes were amplified for sequencing using forward and reverse fusion primers. The forward primer consisted of an Illumina i5 adapter (5'-AATGATACGGCGACCACCGAGATCTACAC-3'), an 8-base-pair (bp) bar code, a primer pad (forward, 5'-TATGGTAATT-3') and the 28F primer (5'-GAGTTTGATCNTGGCTCAG-3'). The reverse fusion primer was constructed with an Illumina i7 adapter (5'-CAAGCAGAAGACGGCATACGAGAT-3'), an 8-bp bar code, a primer pad (reverse, 5'-AGTCAGTCAG-3') and the 388R primer (5'-TGCTGCCTCCCGTAGGAGT-3'). Sequencing was performed in the Digestion, Metabolism & Reproduction Department (St Mary's Hospital, London, UK) using an Illumina MiSeq platform (Illumina Inc).

16S rRNA gene sequence analysis

Sequence data was processed in Mothur using the MiSeq SOP Pipeline[32]. OTUs were defined using a cut-off value of 97% and result data analysed using Vegan package within the R statistical package for assessment of microbial composition and diversity (R Development Core Team 2008). OTUs were subsequently randomly sub-sampled to the lowest common read count for each site to avoid sequencing bias. This technique retained 96% of OTU counts and still provided coverage of greater than 95% for all samples. OTU taxonomies (from Phylum to Genus) were determined using the ribosomal database project (RDP) MultiClassifier script to generate the RDP taxonomy, whereas species level taxonomies of the OTUs were determined using the USEARCH algorithm (v.11)[33,34] combined with the cultured

representatives from the RDP and STIRRUPS databases. Alpha and beta indices were calculated from these datasets within Mothur and the Vegan package within the R environment.

Removal of contaminating sequence reads

As low bacterial biomass samples are prone to environmental and kit contamination, we collected four different sets of negative controls to account for air, equipment (knife) and kit (DNA extraction and 16S rRNA gene sequencing) sources of contamination. Taxonomic units were identified as likely contaminants using the Decontam R package[35] (v1.6.0) with a prevalence-based threshold of 0.5 where a p-value calculated using Chi-square/Fisher's Test below 0.5 was identified as a contaminant.

Determining genuine signatures and microbiota continuum

Following application of the Decontam R package to exclude likely contaminant OTUs, we additionally filtered our data by including only OTUs with at least 5 counts in 10% of samples and low variance (IQR) 5%. The remaining OTUs in both patient samples and controls were compared through hierarchical clustering analysis (HCA) at genera level to determine taxa enriched in low biomass samples versus contaminant controls.

We defined as a continuum the presence of bacterial species at a relative abundance of at least 0.5% in matched samples collected from the lower and upper genital tract. In benign patients this included the vagina, cervix, endometrium, fallopian tube and ovary. In endometrial cancer patients, samples included the vagina, cervix and endometrium. In both patient cohorts, the vagina and rectum were sampled. Venn diagrams were used to depict patterns of overlapping cooccurance of taxa among sites. For this, high vaginal and fundal endometrial samples were used.

Compositional comparison of microbiota in endometrial cancer versus benign controls

Analysis of statistical differences between microbiota of women with and without endometrial malignancy was performed using the Statistical Analysis of Metagenomic Profiles (STAMP) package (v.2.1.3)[36], ClustVis[37] and Marker-gene Data Profiling (MDP) module of MicrobiomeAnalyst[38]. Data were subjected to multivariate analysis using hierarchical clustering analysis by Ward clustering with a density threshold of 0.75 and were rarefied to minimum library size prior to analysis. The most commonly identified genera were included, while the remaining OTUs were classified as Others. Differences in mean α- diversity (Shannon index), which reflects richness and evenness within bacterial populations of each sample, was assessed using Mann-Whitney/Kruskal-Wallis statistical test as appropriate, while β- diversity was calculated using the Bray-Curtis index and compared using PERMANOVA.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis[39] was used to identify taxa significantly overrepresented in endometrial cancer patients when compared to benign controls at multiple taxonomic levels. This analysis was performed using taxonomic relative abundance, with per-sample normalisation and default settings for alpha values (0.05) for the factorial Kruskal–Wallis test among classes and pairwise Wilcoxon test between subclasses. A logarithmic LDA score > 2 was used to determine discriminative features.

Quantitative real-time PCR (qPCR) of the 16S rRNA gene

Quantitative real-time PCR was carried out for quantification of the 16S rRNA gene copy number to determine the bacterial load at various anatomical sites of benign and endometrial cancer patients. Real-time qPCR was performed with universal BactQUANT 16S rRNA gene primers (Forward primer: 5'-CCTACGGGAGGCAGCA, Reverse primer: 5'-GGACTACCGGGTATCTAATC) (Sigma) with the FAM labeled BactQUANT probe ((6FAM) 5'-CAGCAGCCGCGGTA-3'

(MGBNFQ)) on the Applied Biosciences StepOne machine (Thermo Fisher Scientific, Ashford, UK) with StepOne software version 2.3 (Life Technologies). Each 20 µl reaction included lyophilized genomic DNA from *Escherichia coli (E.coli)* Strain B (Sigma, Dorset, UK) serially diluted in diethylpyrocarbonate (DEPC) water to make a ten-fold standard curve and Platinum Supermix UDG (including ROX) (ThermoFisher Scientific). Sterile water was used as negative control. Cycling conditions were 50^0C for 2 minutes, 95^0C for 10 seconds, 95^0C for 15 seconds, 60^0C for 60 seconds, 95^0C for 15 seconds for 40 more times. Total DNA amount for each sample was calculated by multiplying 16S rRNA gene quantity in each loaded sample (5µL) by the total volume of extracted DNA (50µL).

Lactobacillus crispatus **culture**

L. crispatus isolated from the high vaginal swab of a benign patient and stored in 8% v/v DMSO at -80^{o}C or commercially sourced (*L. crispatus* strain ATCC 33820, DSMZ, Germany) was cultured in MRS broth (Sigma-Aldrich) overnight at 37°C to a density of ~ 10^8 CFU/ml. The culture supernatant was collected after 24 hours and neutralised with a 1M NaOH solution. *L. crispatus* isolated from a patient swab was identified using near full-length 16S rRNA gene sequencing and the USEARCH and BLASTn software.

Human endometrial glandular organoid cultures in L. crispatus**-conditioned media**

Pipelle endometrial biopsies were used to generate endometrial glandular organoids following the Cambridge protocol[40]. Briefly, endometrial glands were separated from stromal cells and following digestion with collagenase V/DNAse I, the pellet was re-suspended in Matrigel and 20µl droplets were deposited in pre-warmed 48-well plates. Organoids were grown in Advanced DMEM, supplemented with EGF, FGF10, HGF, Rspondin 1, Nicotinamide and Noggin at 37^{o}C and 5% CO2. Medium was changed every 2 days and organoids passaged at 1:2 ratio every 2–3 weeks. To explore the effect of *L. crispatus*- secreted biomolecules on endometrial organoid proliferation and inflammation, benign and malignant endometrial organoids were cultured in media conditioned with *L. crispatus* supernatant at increasing concentrations (10%, 20%, 30% v/v), with (benign organoids only) or without 1µg/ml LPS (*E. coli* O111:B4). Controls were grown in plain MRS broth- supplemented medium.

Organoid Viability Assay

To assess the effect of *L. crispatus* conditioned media on endometrial organoid proliferation, we performed the CellTiter-GLo® 3D cell viability assay, which quantifies intracellular ATP, according to manufacturer's protocol (Promega). Briefly, medium was removed following treatments and endometrial cells were lysed in 100 µl pre-warmed CellTiter-GLo 3D reagent. Samples were incubated for 30 min at room temperature and luminescence was recorded using a plate reader (PheraStar).

Confocal microscopy

To confirm that the LPS receptor, TLR4, is expressed in endometrial organoids, we fixed them in 10% v/v formaldehyde, permeabilized in blocking buffer plus 0.3% Triton-X and incubated with the anti-TLR4 primary antibody (Thermo Fisher Scientific) at 4°C overnight, followed by incubation with the fluorescent conjugated anti-rabbit Alexa Fluor secondary antibody (Thermo Fisher Scientific) for 2 h at room temperature. Both primary and secondary antibodies were diluted (1:100 primary, 1:200 secondary) in blocking buffer. Negative controls were prepared by omitting primary antibody. Organoids were imaged using the inverted Leica SP5 confocal system and software.

Magnetic Luminex Cytokine assay

To investigate if *L. crispatus*- conditioned medium influences cytokine and chemokine secretion by endometrial organoids, benign and malignant endometrial glandular organoids were stimulated with LPS (*E. coli* O111:B4) for 24h

or vehicle in the presence of *L. crispatus*- conditioned media at increasing concentrations (10%, 20%, 30% v/v). A total of 50 µl of undiluted supernatants were then collected and the concentrations of up to 12 different cytokines and chemokines were analyzed using a Magnetic Multiplex Cytokine Array (R&D systems) following the manufacturer's instructions. For endometrial cancer organoid supernatants the following analytes were measured: CCL4/MIP1beta, CCL5/Rantes, G-CSF, GM-CSF, IL-1rα, IL-1β/IL-1F2, IL-2, IL-6, IL-8/CXCL8, IFNγ, TNFα and VEGF. For benign organoid culture supernatants, the concentrations of IL-1β/IL-1F2, IL-6, IL-8/CXCL8, IL-10, IFNγ and TNFα were measured. All samples were assayed in duplicate. Total protein concentration of supernatant in each well was determined using a Bradford protein assay (Quick Start™ Bradford Protein Assay kit 2, Bio-rad). To correct for different cell number making up the organoids in each well, a correction factor was calculated by dividing the total protein concentration of each well (µg/mL) by the total protein concentration of one of the samples (separately for benign and malignant organoids), which was then multiplied by the cytokine/chemokine concentration (pg/mL) in each well.

Statistical Analyses

Other statistical analyses described in this study were performed using the statistical package GraphPad Prism v.8.0.1 (GraphPad Software Inc., California, USA). Viability and cytokine results were analysed by ANOVA Friedman test and two-tailed Mann-Whitney test. A *p*-value less than 0.05 was considered statistically significant.

Results

Patient demographics and characteristics

Sixty-one women undergoing hysterectomy were recruited; 37 had endometrial cancer (61%) and 24 were benign controls (39%). In total, 178 benign, 207 malignant and 51 technical control samples were sequenced and analysed. Patient and clinical characteristics are shown in Table 1. Key clinical characteristics of the two groups were largely comparable apart from age (endometrial cancer ≥ 65, 56.8%; benign 50–64, 58.3%, p = 0.0077). Most endometrial cancers were endometrioid tumours (30/37, 81%) and of stage I (31/37, 83.8%).

Bacterial Signatures above Background Contamination in Low Bacterial Biomass Sites

The presence of a microbial metataxonomic signature above background contamination in the endometrium, fallopian tubes and ovaries of benign patients and endometrium of endometrial cancer patients was performed in careful consideration of potential sources of contamination (Suppl Table 1). Sequencing read data was obtained from all samples however 5 vaginal, 10 cervical and 25 endometrial samples were excluded from analysis due to low read counts. Exploration of negative control samples identified a total of 1037/6818 (15%) OTUs in the dataset that were likely contaminants (Suppl Table 2). These were removed from further analyses. As expected, the effect of environmental and kit contaminant removal was more pronounced in low-biomass sites (Suppl Fig. 1).

We next estimated bacterial copy number at different locations within the FGT and rectum in both benign and endometrial cancer samples. Low biomasses were observed in the endometrium, fallopian tubes and ovaries that were comparable to negative controls. These values were one to four orders of magnitude lower than vagina, cervix and rectum samples (Fig. 1). Applying a cut-off above control counts at 700 bacterial copies based on sequencing reads, a microbial signature above background contamination was observed in 62% of benign endometrium, 50% of malignant endometrium, 85% of benign fallopian tube and 95% of benign ovary. We next compared bacterial composition at genus level between the low-biomass samples of the upper GT and controls. The compositional signature of benign endometrium, fallopian tube and ovary was comparable yet different from malignant endometrial samples and technical controls which displayed high overlap of taxa (Fig. 2).

Of note, comparison of ten paired swab-tissue samples (cervix, endometrium and fallopian tube) revealed compositional dissimilarity in most samples based on 16S rRNA gene amplicons (Suppl Fig. 2A). While α- and β-diversity was not significantly different between tissue and swab samples, this could probably be due to small sample size (Suppl Fig. 2B).

Most recruited patients underwent laparoscopic hysterectomy (49/61, 80.3%). We assessed whether practices adopted in laparoscopic hysterectomy (15/24, 62.5% of benign cases and 34/37, 91.9% of malignant cases) introduce intra-patient contamination in low bacterial biomass sites. To do this, we compared the microbial composition of fallopian tubes and ovaries against higher vaginal samples intra-individually to test for contamination during vaginal retrieval of specimen and lower/higher endometrium against cervical samples to identify contamination during uterine manipulation. Transabdominal (n = 13) and laparoscopic (n = 13) hysterectomies performed for either benign indications or endometrial malignancy were compared at species level to increase metataxonomic resolution (Suppl Fig. 3). In samples collected during transabdominal surgery, the microbiota of fallopian tubes and ovaries were either highly positively or negatively correlated with vaginal composition. In laparoscopic procedures, a spread of correlation was observable in 2/8 cases (25%). With regards to contamination of the lower endometrium by the adjacent cervix during instrumentation, correlation follows the same pattern in both surgical techniques. When comparing higher endometrium to matched cervical samples, a strong overlap of taxa was observed in around half of the patients in the transabdominal approach. In laparoscopy, a spread of correlation data is noted, suggestive of potential microbial transfer from the cervix into the fundal endometrium in one third (4/12, 33%) of patients.

Investigation of a Microbial Continuum in Benign and Endometrial Cancer Patients

We next compared microbial composition across FGT sites in 16 benign and 16 endometrial cancer patients. In 75% (12/16) of benign patients, the most abundant species of the lower GT were also detected from all sites of the upper GT (Fig. 3A). In two patients the microbial continuum was disrupted at the level of fallopian tube or ovary with microbial concordance of the rest of sites, while for the remaining two patients the lower GT microbial composition was dissociated from the upper. Conversely, in the endometrial cancer cohort, which was microbially more diverse, only 7.2% of bacterial species were shared by the lower GT and endometrium (Figs. 3B,C). Sub-analysis within the vagina (lower 2/3 vs higher 1/3) and endometrium (lower 2/3 vs fundal) revealed high intra-patient correlation ($R^2 \geq 0.7$) in 92% of benign and 86% endometrial cancer patients along the vagina and in only 62% of benign and 42% of malignant samples along the endometrium at genera level (Suppl Figs. 4A,B). The lower microbial correlation within the malignant endometrium compared to benign was statistically significant (Genus $p = 0.0007$; Species $p = 0.0003$) and probably driven by the low bacterial biomass and high microbial diversity of malignant endometrium as suggested by inspection of individual patient data.

The correlation of vaginal with rectal microbiota was sought in 16 women with benign pathology and 26 women with endometrial cancer (Suppl Fig. 5). We observed that rectal microbiota were more diverse than vaginal, and vaginal microbiota of endometrial cancer patients were more diverse than benign patients (median of bacterial species ≥ 0.5% relative abundance: benign vagina- 7.5, benign rectum- 32, malignant vagina- 19.5, malignant rectum- 33.5). The less diverse vaginal ecosystem of benign patients shared no microbiome with rectum in 44% (7/16) of cases, in 25% (4/16) of patients only one bacterial species was shared, whereas in 31% (5/16) of cases ≥ 2 bacterial species overlapped with a median of 4 OTUs. In endometrial cancer patients, 15% (4/26) of cases displayed no shared microbiome between vagina and rectum, 27% (7/26) shared one bacterial species, while 58% (15/26) had ≥ 2 bacterial species in common with a median of 5 OTUs. Taken together this evidence suggests that vaginal microbiota are poorly correlated with rectal microbiota in both benign and endometrial cancer patients.

The Genital Tract and Rectal Microbiota in Endometrial Cancer Patients and Benign Controls

We compared the microbial composition of 24 benign and 37 endometrial cancer patients at different anatomical sites (vagina, cervix, endometrium) and observed high diversity, *Lactobacillus* depletion and enrichment of *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus* in endometrial malignancy when compared to benign controls.

Hierarchical clustering analysis (HCA) at genera level revealed three distinct clusters of *Lactobacillus*- dominant (≥ 75% relative abundance), *Gardnerella/Lactobacillus*- dominant (≥ 50%/40%) and High diversity/Other at all sites examined with an additional *Streptococcus* (≥ 36%) cluster for vagina (Fig. 4A). High diversity/Other cluster included samples with higher Shannon α-diversity than other clusters, but also a few samples dominated by one or two bacteria forming small clusters (Suppl Fig. 6). We examined the α- and β-diversity at genera level of the microbial community composition in patients with and without endometrial cancer (Fig. 4B,C). We found that patients with endometrial cancer display higher α-diversity than patients without endometrial cancer (Vagina $p = 7.5763e-05$; Cervix $p = 0.006$; Endometrium $p = 0.006$). Similarly, β-diversity was significantly different between benign and endometrial cancer patients at all anatomical sites (Vagina $p < 0.001$; Cervix $p < 0.001$; Endometrium $p < 0.001$).

The rates and frequency of different clusters were compared between patients with and without endometrial cancer (Table 2). *Lactobacillus*- and *Gardnerella/Lactobacillus* dominant clusters were significantly higher among benign patients (Vagina 30% and 22.5%, $p = 0.000485$; Cervix 45.5% and 18.1%, $p = 0.003821$; Endometrium 41% and 20.6%, $p < 0.00001$ respectively), while High diversity/Other was the most prevalent cluster among endometrial cancer patients (Vagina 72.2%, $p = 0.000485$; Cervix 82.1%, $p = 0.003821$; Endometrium 87.8%, $p < 0.00001$), which was also accompanied by *Lactobacillus* depletion.

Linear discriminant analysis (LDA) effect size (LEfSe) modelling was also used to identify differences in microbiota composition of endometrial cancer patients versus benign controls (Figs. 5A,B). In benign patients, we observed an over-representation of *Lactobacillus* species (*L. crispatus*, *L. gasseri*, *L. iners* and *L. vaginalis*) at all sites examined (vagina, cervix, endometrium) and *Bifidobacterium breve* in vagina and cervix. Conversely, in endometrial cancer patients, an enrichment of several microbes was observed at all sites, including *Porphyromonas*, *Prevotella, Peptoniphilus* and *Anaerococcus*.

We next sought to determine differences in microbial composition based on histological type and grade of endometrial cancer. We included 30 endometrioid and 7 non-endometrioid endometrial tumours of different grades (Grade I: n = 12; Grade II: n = 11; Grade III: n = 14). PCA plots showed some divergence of microbial signals between endometrioid and non-endometrioid tumours only in the cervix, while the vagina and endometrium displayed extensive microbial overlap between the two histological types. Of note, microbial structure differed more between benign samples and non-endometrioid tumours in the vagina and cervix than benign and endometrioid endometrial cancer samples (Suppl Fig. 7A). With regards to grade, PCA plots revealed a degree of divergence between grade I and grade III cancers, while grade II tumours shared many microbial features with both grade I and grade III samples, implying an intermediate microbial identity between differentiated and poorly differentiated cancers. Intriguingly, benign samples displayed greater resemblance to grade I tumours in the vagina and cervix than high-grade tumours (Suppl Fig. 7B).

Given the high diversity observed in rectum, we compared the rectal microbial make-up of patients with and without endometrial cancer at phylum level. We noted that *Firmicutes* ($p = 0.002$), *Bacteroidetes* ($p = 0.002$), *Actinobacteria* ($p = 0.002$) and *Proteobacteria* ($p = 0.007$) were significantly depleted in endometrial cancer patients (Suppl Fig. 9A). On the other hand, rectal microbiota α- and β-diversity did not differ significantly between benign and endometrial cancer patients at genera level (α-diversity: $p = 0.455$; β-diversity: $p < 0.359$) (Suppl Fig. 9B).

Finally, we inquired about microbial load differences in the genital tract and rectum of benign and endometrial cancer patients. Quantitative real-time PCR targeting the 16S rRNA gene revealed lower actual abundances by one- two orders of magnitude in the vagina, cervix and rectum of patients with endometrial malignancy versus benign controls, whilst no significant differences were noted in the endometrium, where bacterial quantities were comparable to the bacterial biomass of controls (Fig. 1). Our findings indicate that endometrial cancer is marked by reduced bacterial colonisation of anatomical sites that normally display a high bacterial load.

L. crispatus **effect on Endometrial Organoid Proliferation and Inflammation**

We tested the hypothesis that *L. crispatus*, which dominates the vagina, cervix and endometrium of patients without endometrial cancer and is depleted in endometrial cancer patients, has an anti-inflammatory, anti-mitogenic effect on endometrial organoids. Organoids were derived from the endometrium of five benign and six endometrial cancer patients (Suppl Table 3).

Effect on proliferation

Firstly, the viability of endometrial organoids was assessed at increasing concentrations of *L. crispatus*- conditioned (LCC) media (10%, 20%, 30%, 50% v/v). We noted that 50% v/v LCC was lethal across all endometrial organoid lines within two days (data not shown), and therefore used 10%, 20% and 30% v/v concentrations for further experiments. In EC organoids, proliferation showed an inverse relationship with increasing LCC concentrations at 48h, irrespective of *L. crispatus* origin (*L. crispatus* vaginal isolate from a patient or commercial isolate), but this trend reached statistical significance only for the 30% v/v concentration (vaginal isolate: 0.0009; commercial isolate: 0.0003) (Fig. 6A). Benign endometrial organoids, on the other hand, demonstrated increased proliferation at 10% v/v LCC and decreased proliferation dynamics at 20% and 30% v/v concentrations, but relationships were not nominally significant (Fig. 6A).

Effect on inflammation

We next investigated the impact of LCC media on the constitutive and LPS- induced cytokine secretion by endometrial organoids and found that *L. crispatus* supernatant does not significantly affect cytokine secretion in benign and malignant endometrial organoids. Organoids were grown in expansion medium supplemented with 10%, 20% and 30% LCC medium and secreted cytokines were detected in culture supernatant after 48h. Basal cytokine secretion of IL-1β, IL-6, IL-8 and TNF-α by malignant organoids was higher than benign organoids but only IFN-γ reached statistical significance ($p = 0.0286$) (Suppl Fig. 10B). For EC organoids, the addition of *L. crispatus* supernatant at any concentration did not significantly alter the basal secretion of IL-6, IL-8, TNF-α, IFN-γ, IL-1ra, IL-2, G-CSF, GM-CSF, VEGF and chemokine CCL5/RANTES, while a significant increase was noted for IL-1β and CCL4/MIP1beta at certain LCC concentrations (Fig. 6C). When endometrial cancer organoids were treated with supernatant of commercially available *L. crispatus*, none of the cytokines/chemokines was significantly affected (Suppl Fig. 10C). Similarly, for benign endometrial organoids the secretion of IL-6, IL-8, IL-10, TNF-α, IFN-γ remained unaffected following LLC medium supplementation, whilst an increase of IL-1β and TNF-α and reduction of IL-10 production were observed at certain LCC concentrations (Fig. 6D). Finally, to mimic the chronic inflammatory signals predisposing to endometrial cancer, we simultaneously treated benign organoids, which express LPS receptor, TLR-4 (Toll-like receptor 4) (Fig. 6B), with *E. coli*-derived LPS (O111:B4) and increasing LCC media concentrations for 24h. No significant changes were noted in the inducible secretion of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ by benign organoids (Fig. 6E).

Discussion

In this study, we sought to determine the presence of a genuine microbial fingerprint in the upper and lower female genital tract of benign and endometrial cancer patients highlighting potential differences and trying to elucidate the putative role of the microbiota in endometrial cancer generation or suppression.

Contamination from patient and non-patient sources may obscure actual microbial signatures from low bacterial biomass sites. Our study integrated four different sets of technical controls for air and reagent/equipment contaminants during sample collection in the pathology laboratory, DNA extraction and 16S rRNA gene sequencing and their sequence reads (15% of total sequencing data) were removed to deduct background DNA contamination from further data analysis. Furthermore, we demonstrated potential intra-patient contamination in one quarter to one third of samples in laparoscopic procedures. A previous report described high bacterial similarity of endometrial samples between transcervical and transuterine collection methods, indicating low contamination[28].

We calculated actual microbial abundances using quantitative real time PCR and showed that endometrial, fallopian tube and ovarian microbiota are encountered in much lower quantities (2–4 $\log_{10}$) than vaginal and cervical microbiota and are similar to bacterial abundances of controls. Our findings are consistent with those of Mitchell and co-workers[18], who reported that mean bacterial quantities in the endometrium and upper endocervix are lower than vaginal levels by 2–4 $\log_{10}$ rRNA gene copies, which was further corroborated by the Chen study[28] showing that vaginal sites contained about four orders of magnitude more bacteria (10^{10}-10^{11}) than the endometrium and peritoneal fluid, still exhibiting much lower cycle threshold (Ct values) than negative controls. Another study confirmed high bacterial signals in vagina and comparable bacterial load in cervix and endometrium with most cervical and endometrial samples (60–72%) exceeding the bacterial copies of blank controls[17].

Overall, we observed a microbiota signature above background contamination in 62% of benign endometrium, 50% of malignant endometrium, 85% of benign fallopian tube and 95% of benign ovary based on sequencing reads. Winters and colleagues also reported a 60% (15/25) bacterial recovery above background DNA controls from the endometrium of women with benign pathology, mainly fibroids, undergoing transabdominal hysterectomy[17]. Mitchell and co-workers concluded that 95% (55/58) of non-cancer patients having total laparoscopic or laparoscopically-assisted vaginal hysterectomy without an intracervical manipulator had endometrial colonisation with at least one species[18], while Chen and colleagues enrolled 95 reproductive age women and isolated bacteria from the endometrium, fallopian tubes and peritoneal fluid during either laparotomy or laparoscopy[28]. With regards to bacterial composition, we identified a prominent microbial signature in benign endometrium, fallopian tube and ovary, while the microbiome of malignant endometrial samples and technical controls was largely shared. In the Mitchell and co-workers study, signature endometrial species were *L. iners* (45%), *Prevotella* spp. (33%) and *L. crispatus* (33%)[18], while in the Chen study *Lactobacillus, Vagococcus, Acinetobacter* and *Pseudomonas*, which were also identified in the fallopian tube[28]. Notably, our results are predominantly deduced from post-menopausal women > 50 years of age, while the aforementioned reports involved mainly premenopausal women < 50 years of age. The data suggests that a microbiota signature is detectable in the upper reproductive tract of a proportion of women regardless of age and menopausal status.

Investigation of a microbial continuum within the female genital tract revealed that in 75% of benign patients the most abundant species of the lower genital tract were also recoverable from all sites of the upper genital tract, lending credence to the theory of bacterial colonisation through ascension. Conversely, bacterial continuum was less cohesive in the endometrial cancer cohort. Similarly, intra-individual microbial correlations between the lower and upper genital tract consistent with anatomical contingency have been reported by Chen and colleagues[28], whereas Walsh and co-workers reported that the microbiome of lower genital tract is correlated with uterine microbiome in patients with

endometrial cancer[10]. In addition to this, associations of vaginal with rectal microbiota have been suggested based on reports of bacterial species overlap between the two sites and the presence of enteric oestrobolome, where the gut microbiome metabolises oestrogens and indirectly facilitates vaginal *Lactobacillus* growth[41–47]. We found that vaginal-rectal correlations were stronger in endometrial cancer patients compared to benign, still accounting for only 15% sharedness of microbiota.

We subsequently took cross-sectional snapshots of the FGT microbial structure in thirty-seven women with and twenty-four women without endometrial cancer. Our findings suggest that there is an unequivocal decrease of *Lactobacillus* abundance in the vagina, cervix and endometrium of endometrial cancer patients and an enrichment of *Anaerococcus*, *Porphyromonas*, *Prevotella, Fusobacterium, Bacteroides* and *Peptoniphilus*, which is in keeping with previous studies[10,11]. *Lactobacillus* reduction lends support to the more broadly described association between *Lactobacillus* depletion and adverse gynaecological and obstetric outcomes, including cervical cancer and precancerous lesions[13] as well as miscarriages[48], preterm labour[49] and *in vitro* fertilisation failure[24]. The biological significance of *Lactobacillus* communities includes pathogen antagonism by lactic acid, hydrogen peroxide and bacteriocin production but also immune and metabolic pathway as well as epigenetic regulation[50–59]. Anti-cancer effects of *Lactobacillus* species have been reported for *L. casei*, *L. plantarum*, *L. rhamnosus* and *L. acidophilus* through natural killer cell activation, dendritic cell maturation or probiotic-derived ferrichrome release[60–68] inducing cancer cell apoptosis *in vitro*[61,67,69] and increasing anticancer drug efficacy[70–71,82]. Continuous administration of *L. reuteri* strain to tumour-prone mice for several months reduces the frequency of intestinal pre-cancerous lesions[68]. Caution should be exercised, however, when analysing metataxonomic findings because routinely relative abundances are used, which conceal actual abundances. Therefore, the depletion in the relative abundance of a prevalent species will lead to an increase of the relative abundance of the least prevalent species. This could be misinterpreted as an enrichment of those taxa, even though their actual abundance has remained the same making the use of quantitative PCR imperative to confirm the enrichment of the taxa in question.

We sought to determine whether the disparities observed between benign and endometrial cancer patient microbiota can be attributed to malignancy, suggesting a cause-effect relationship or other confounders impacted on our findings. Ethnicity[70], menopausal status[10,71], obesity[72], use of sex hormones[71], smoking[73], sexual behaviour[74] and vaginal pH[11] have all been recognised as modulators of microbial composition in the female genital tract. Our group has previously demonstrated that obesity drives the vaginal microbiome towards a highly diverse, *Lactobacillus*-depleted eco-structure characterised by higher levels of *Dialister*, *Anaerococcus vaginalis* and *Prevotella timonensis*, and lower levels of *Lactobacillus crispatus* compared to non-obese women[72]. Another study highlighted the importance of menopausal status in FGT composition showing that postmenopausal women exhibit increased diversity and enrichment of *Anaerococcus*, *Peptoniphilus* and *Porphyromonas* species[10]. Interestingly, almost half (8/17) of the microbes enriched in postmenopausal women were also associated with endometrial cancer[10]. From a mechanistic standpoint, the interplay between estrogens and *Lactobacilli* is well-documented in the vaginal mucosa with oestrogens stimulating glycogen-dependent metabolism that increases colonisation with lactobacilli, which in turn metabolise glycogen to lactic acid maintaining an acidic vaginal environment[75]. In our study, no variable showed systematic bias with respect to the two comparison groups (benign vs endometrial cancer) apart from age; endometrial cancer patients were predominantly ≥ 65 years, while benign patients between 50–64 years of age. Importantly, postmenopausal women were equally distributed between the two cohorts.

To uncover a potential role of altered microbiota in endometrial carcinogenesis, we selected *L. crispatus* as a candidate health-promoting microbe, given that it is significantly depleted in endometrial cancer and substantial evidence suggests that commensal *Lactobacillus* species have anti-inflammatory and anti-cancer properties[54–69]. Endometrial

cancer is thought to arise in an environment of chronic, low-grade inflammation characterised by an increase of pre-diagnostic circulating levels of CRP, IL-6, TNFα, sTNFR1 and sTNFR2 and IL-1Ra (EPIC cohort)[76,77], which have also been linked to high BMI[78,79], highlighting the interrelationship of the obesity- inflammation- endometrial carcinogenesis axis. Following establishment of endometrial cancer, cytokines are secreted by cancer cells, the tumour microenvironment and infiltrating immune cells but their role in oncogenic processes remains ambiguous. We interrogated the effect of *L. crispatus* on endometrial cell proliferation and its intersection with inflammatory pathways and found that *L. crispatus*-conditioned medium reduced viability of endometrial cancer organoids, whilst had minimal effect on studied cytokines/chemokines. It is not clear whether LCC medium is actually cytotoxic or restricts proliferation and/or induces lethality of endometrial organoids due to dilutional effects.

A limitation of this study involves the use of women with benign pathology as controls instead of healthy individuals that may display differential microbial structure. Women undergoing risk-reducing surgery can provide an ethically acceptable source of healthy surgical specimens in future studies. Longitudinal studies monitoring the bacterial composition intra-individually over time to capture transition from a healthy state to precancerous conditions (complex atypical hyperplasia) and invasive endometrial carcinoma are required to provide useful information on microbiota dynamics and temporal shifts within each patient. Finally, viral and fungal metagenomic studies could be useful to complement and expand our knowledge of the human-microbial and microbial-microbial cell interactions in health and disease.

In conclusion, only a subset of women with and without endometrial cancer harbours a microbiome in the upper GT that is quantitatively and qualitatively distinct from background contamination. *Lactobacillus* depletion in the lower and upper GT is characteristic in endometrial malignancy but whether depletion precedes or follows the development of cancer is still unclear. *L. crispatus* does not significantly affect cytokine production in benign and malignant endometrial organoids but this observation does not preclude that *L. crispatus* or other bacterial species could influence metabolic or oestrogen signalling pathways locally or could have an impact on infiltrating immune cell cytokine secretion, which could subsequently exert an action on endometrium. Transitioning from reductionist cell models to more comprehensive, physiologically relevant cell systems that capture the cellular diversity of organs, including stromal, mesenchymal and immune cells, is mandatory to reliably assess the functional role of resident microbiota and devise ways of manipulation that could restore eubiosis.

Declarations

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Author Contributions

The study was conceived and designed by AS, DAM and MK. The samples and data were acquired and collated by AS, EW and MK. Data were analysed and interpreted by AS, DAM, and MK. The manuscript was drafted and revised critically for important intellectual content by all authors. All authors gave final approval of the version to be published and have contributed to the manuscript.

Declaration of competing interests

The authors declare no competing financial interests.

Ethics approval

National Research Ethics Service Committee London (Approval number 13/LO/0126 and 14/WS/1098)

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Table 1

Table 1 is available in the Supplementary Files section.

Table 2

Table 2. Genital tract microbial clusters in benign and endometrial cancer patients.

Genus	*Lactobacillus* dominant n/N (%)	*Streptococcus* dominant n/N (%)	*Gardnerella/Lactobacillus* dominant n/N (%)	High diversity & Other n/N (%)	p value
VAGINA					
Benign	12/40 (30)	5/40 (12.5)	9/40 (22.5)	14/40 (35)	0.000485
Endometrial cancer	4/54 (7.4)	8/54 (14.8)	3/54 (5.6)	39/54 (72.2)	
Total	16/94 (17)	13/94 (13.8)	12/94 (12.8)		
CERVIX					
Benign	10/22 (45.5)	-	4/22 (18.1)	8/22 (36.4)	0.003821
Endometrial cancer	3/28 (10.7)	-	2/28 (7.1)	23/28 (82.1)	
Total	13/50 (26)	-	6/50 (12)	31/50 (62)	
ENDOMETRIUM					
Benign	18/44 (41)	-	9/44 (20.6)	17/44 (38.6)	< 0.00001
Endometrial cancer	5/49 (10.2)	-	1/49 (2)	43/49 (87.8)	
Total	23/93 (24.7)	-	10/93 (10.8)	60/93 (64.5)	

p value was calculated by chi-squared test.

Figures

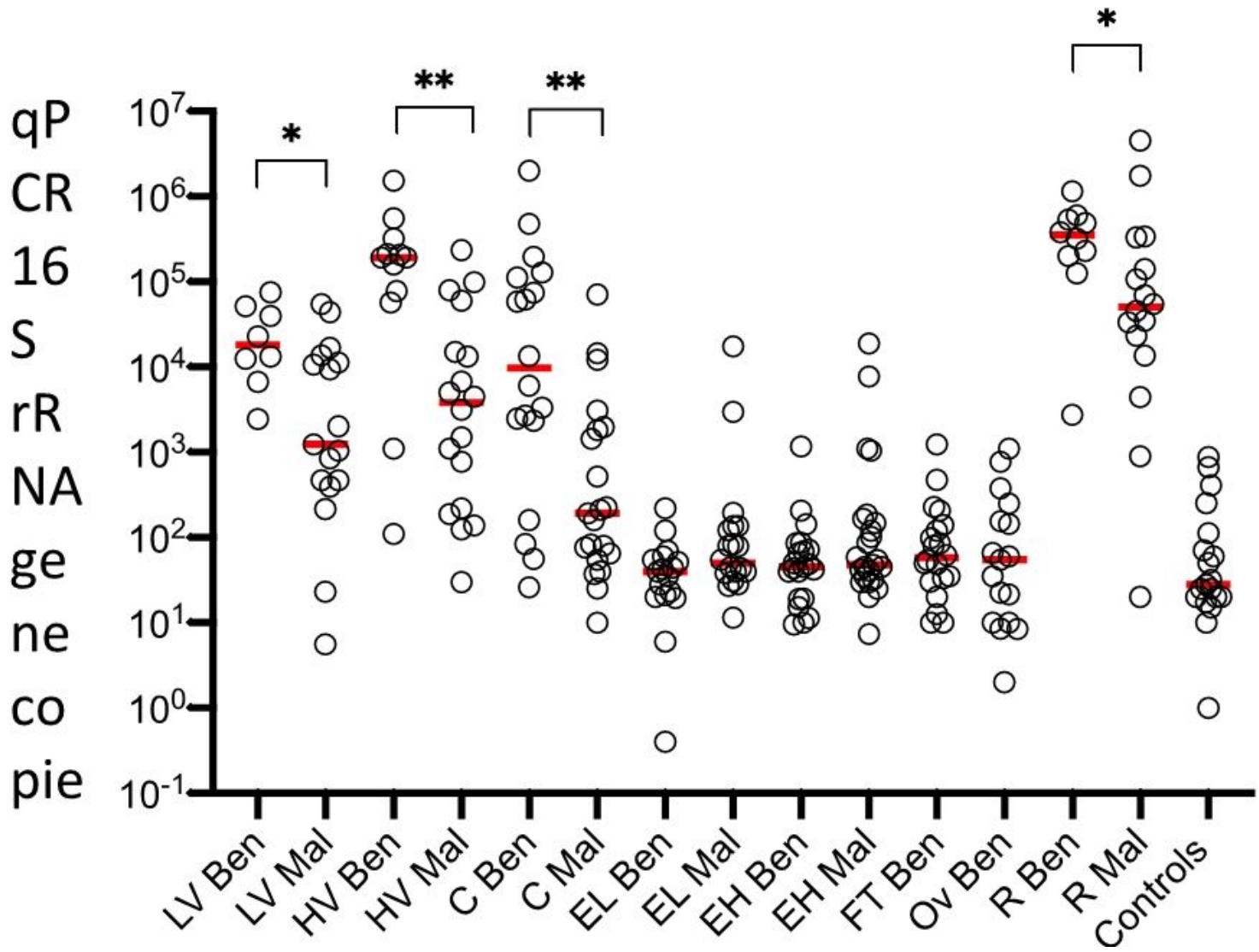

Figure 1

qPCR bacterial load at different locations in benign, endometrial cancer patients and controls. Red line represents median. *Ben: Benign, Mal: Malignant, LV: Lower Vagina, HV: Higher Vagina, C: Cervix, EL: Endometrium Lower, EH: Endometrium Higher, FT: Fallopian tube, Ov: Ovary, R: Rectum.* * p-value < 0.05, ** p-value < 0.005.

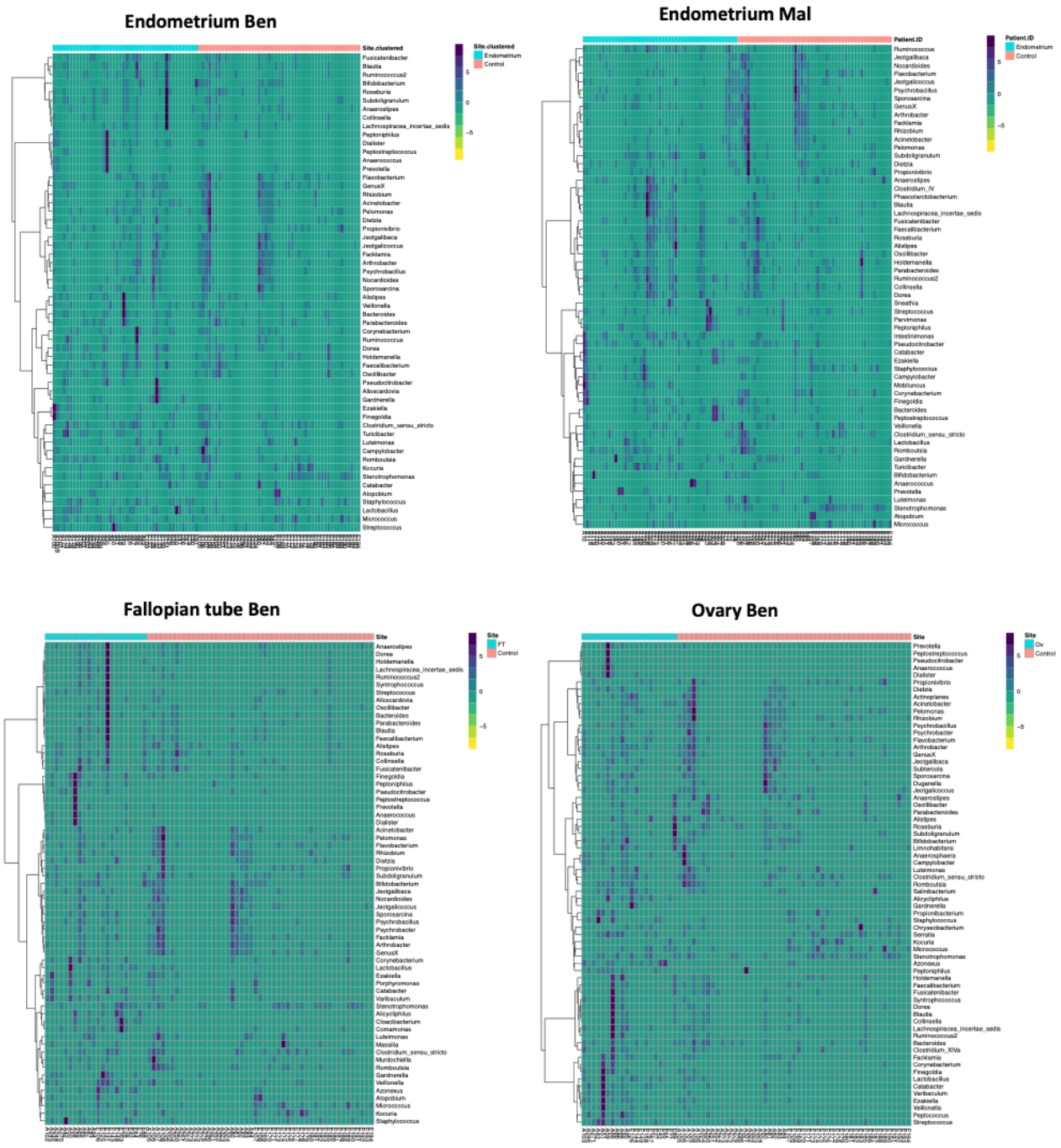

Figure 2

Prominent operational taxonomic units (OTUs) among low biomass patient samples and technical controls. *Ben: Benign, Mal: Malignant, FT: Fallopian tube, Ov: Ovary.*

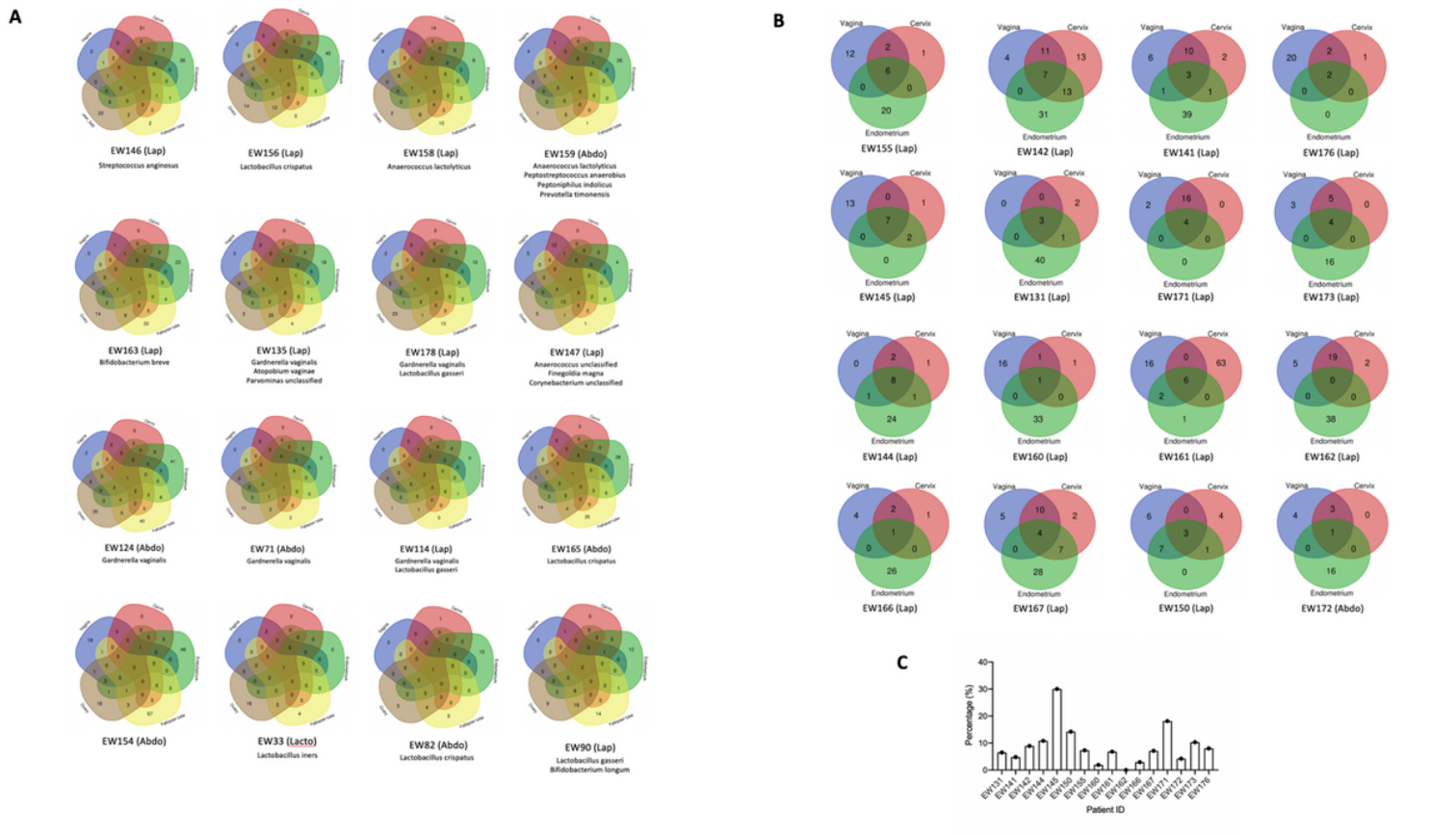

Figure 3

Microbial continuum along the female genital tract. Venn diagrams illustrating microbial species shared by **A.** all sites of the lower and upper genital tract (vagina, cervix, endometrium, fallopian tube, ovary) in 16 patients with benign pathology **B.**the vagina, cervix, and endometrium in 16 patients with endometrial cancer. Microbial species shared by all sites: **EW155***: Prevotella amnii, Atopobium vaginae, Gardnerella vaginalis, Anaerotruncus unclassified, Megasphaera unclassified, Sneathia unclassified;* **EW142***: Finegoldia magna, Prevotella timonensis, Peptoniphilus indolicus, Streptococcus mitis, Catabacter unclassified, Peptoniphilus harei, Campylobacter ureolyticus;* **EW141***: Finegoldia magna, Veillonalla parvula, Prevotella timonensis;* **EW145***: Sneathia unclassified, Anaerococcus unclassified, Streptococcus agalactiae, Anaerococcus obesiensis, Anaerotruncus unclassified, Peptoniphilus harei, Parvimonas unclassified;* **EW176***: Anaerococcus unclassified, Anaerococcus vaginalis;* **EW171***: Lactobacillus gasseri, Peptoniphilus indolicus, Prevotella bivia, Peptoniphilus harei;* **EW173**: *Fusobacterium gonidiaformans, Pseudocitrobacter anthropic, Peptostreptococcus anaerobius, Bacteriodes fragilis;* **EW131***: Lactobacillus iners, Gardnerella vaginalis, Lactobacillus crispatus;* **EW144***: Anaerococcus unclassified, Anaerococcus vaginalis, Varibaculum anthropi, Finegoldia magna, Peptoniphilus harei, Propionimicrobium lymphophilum, Streptococcus anginosus, Alloscardovia omnicolens;* **EW160***: Gardnerella vaginalis;* **EW161***: Peptococcus unclassified, Subbdoligranulum unclassified, Bacteroides vulgatus, Species X, Bacteroides coprocola, Faecalibacterium prausnitzii;* **EW166***: Bifidobacterium breve;* **EW167***: Species X, Corynebacterium pyruviciproducens, Corynebacterium faecal, Psychrobacillus insolitus;* **EW150***: Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum;* **EW172**: *Lactobacillus crispatus.* **C.** Proportion of overlapping bacterial species in all three sites of endometrial cancer patients. Only species with an at least 0.5% relative abundance were included. *Lap: Laparoscopic; Abdo: Abdominal.*

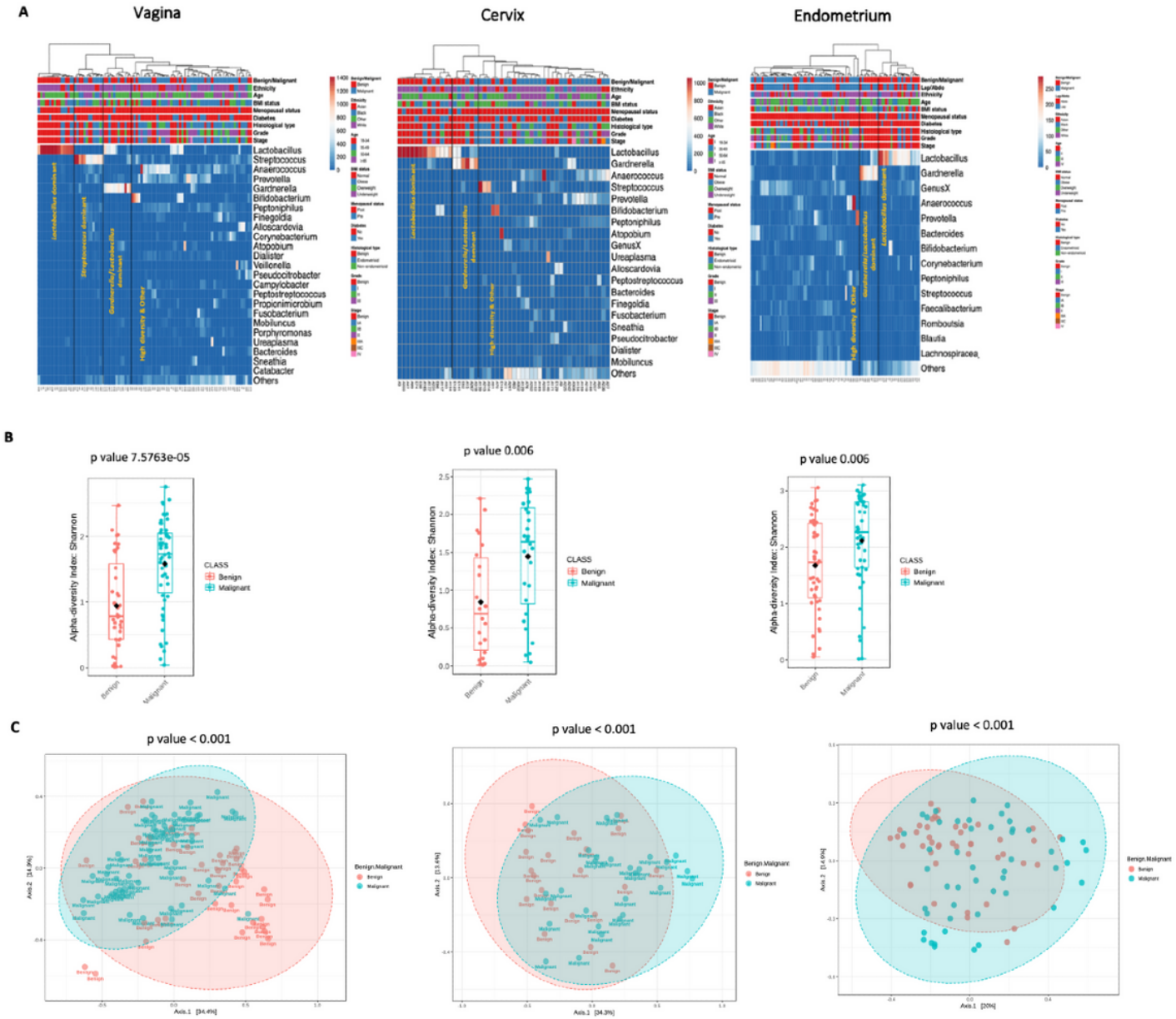

Figure 4

Vaginal, cervical and endometrial microbial composition at genera level according to presence of endometrial malignancy. A. Hierarchical clustering analysis per endometrial malignancy, ethnicity, age, BMI status, menopausal status, diabetes, histological type, grade and FIGO stage of disease using Ward linkage of the most commonly identified genera. Data were rarefied prior to analysis. **B.** Microbiome Shannon α- and **C.** β- diversity per anatomical site.

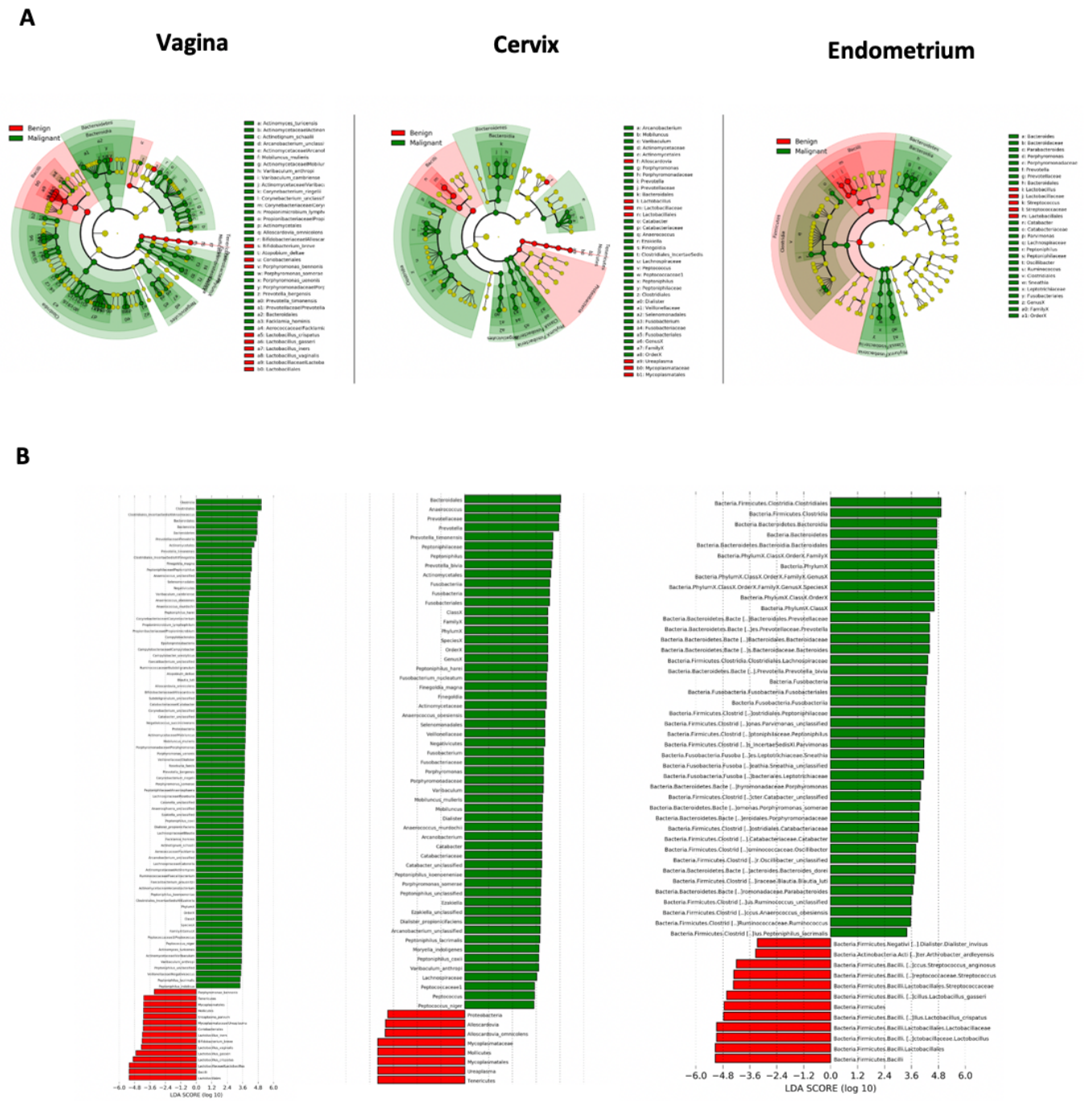

Figure 5

Significantly enriched taxa among patients with and without endometrial cancer in the upper and lower female genital tract. A. Cladograms representing taxa with different abundances between the comparison groups per site. **B.** Histograms of the LDA scores computed for features differentially abundant between endometrial cancer and benign patients. *LDA score: Linear discriminant analysis score.*

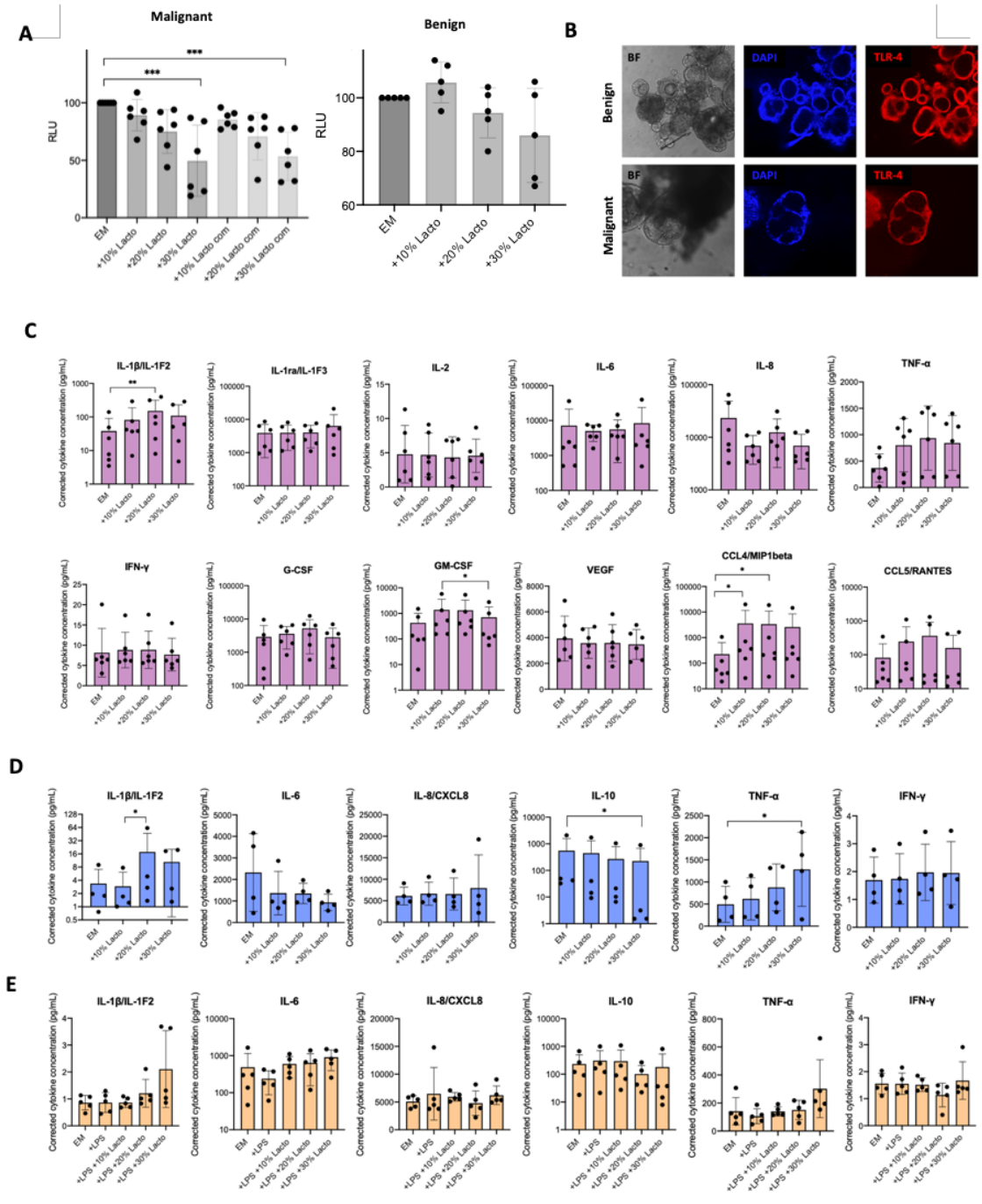

Figure 6

A. Benign and endometrial cancer organoid viability in response to increasing *L. crispatus*- conditioned media concentrations for 48h. **B.** Confocal microscopy in benign and malignant organoids stained for TLR-4 expression. **C.** Cytokine expression by endometrial cancer organoids in response to increasing *L. crispatus*- conditioned media concentrations for 48h. A significant increase of chemokine CCL4/MIP1beta was noted in response to 10% v/v LCC medium (p= 0.0105), of CCL4/MIP1beta (p= 0.0105) and IL-1β (p= 0.0048) in response to 20% v/v LCC medium and a significant decrease of GM-CSF was observed in the 30% vs 10% v/v concentration (p= 0.0437). **D.** Cytokine expression by benign endometrial organoids in response to increasing *L. crispatus*- conditioned media concentrations for 48h. IL-6, IL-8, IL-10, TNF-α and IFN-γsecretion remained unaffected following 10% and 20% v/v LLC medium supplementation. IL-1β increased significantly in 20% versus 10% v/v LCC media (p= 0.037), while 30% LCC significantly increased TNF-α (p= 0.037) and decreased IL-10 secretion (p= 0.0155). **E.** Cytokine expression by benign endometrial organoids in

response to LPS and increasing *L. crispatus*- conditioned media concentrations for 24h. Treatment of organoids with LPS failed to induce a significant inflammatory reaction, possibly due to low LPS concentration or organoids being refractory to the LPS type used. Lacto: *L. crispatus* isolated from patient vaginal swab, Lacto com: commercial *L. crispatus*. * p value< 0.05

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Supplementarymaterial.docx