

CAN AUTOCHTHONOUS ENDOMETRIAL MICROBIOTA BE DETECTED BY TRANSCERVICAL SAMPLING?

Zornikov DL¹✉, Simarzina VM¹, Islamidi DK^{1,2}, Belykh NS², Kornilov DO¹, Abakumova EI², Khayutin LV², Plotko EE², Voroshilina ES^{1,2}¹ Ural State Medical University, Yekaterinburg, Russia² Medical Center "Garmonia", Yekaterinburg, Russia

Transcervical endometrial sampling is widely used to study the uterine microbiota, but the risk of cervical contamination raises doubts about whether detected microorganisms genuinely originate from the endometrium. This study aimed to determine whether autochthonous endometrial microbiota can be detected in transcervically collected samples by quantitative PCR after accounting for cervical contamination. Paired cervical and endometrial samples were collected from 185 reproductive-age women using an Endobrush catheter for endometrial sampling. Microbiota composition was assessed by real-time PCR targeting 27 microbial groups. An endometrial signal was considered true if its quantity exceeded that in the paired cervical sample, adjusted for transfer thresholds of 30% (expected, based on prior *in vitro* data) and 100% (conservative). Before correction, microbial signals were detected in endometrial samples from 144 (77.8%) women. After correction, positive signals remained in 44.3% (30% threshold) and 37.3% (100% threshold) of women. Most *Lactobacillus* spp. positives fell into an uninterpretable gray zone (47.6% and 55.1% at the 30% and 100% thresholds, respectively). In contrast, opportunistic microorganisms largely remained true positives after correction. Autochthonous endometrial microbiota was detected in approximately 37% of women, typically comprising 1–3 predominantly non-lactobacillus taxa. Detection of opportunistic microorganisms in the endometrium therefore likely reflects their genuine presence, whereas *Lactobacillus* detection is, in most cases, indistinguishable from cervical contamination.

Keywords: endometrial microbiota, autochthonous microbiota, cervical contamination, transcervical sampling, quantitative PCR.

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Compliance with ethical standards: the study was approved by the Ethics Committee of Ural State Medical University (Protocol No. 1 dated January 24, 2020; Protocol No. 4 dated May 26, 2023). Informed consent was obtained from all study participants.

✉ **Correspondence should be addressed:** Danila L. Zornikov
Klyuchevskaya, 17, Yekaterinburg, 620109, Russia; zornikovdl@gmail.com

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МОЖНО ЛИ ОБНАРУЖИТЬ АУТОХТОННУЮ МИКРОБИОТУ ЭНДОМЕТРИИ ПРИ ТРАНСЦЕРВИКАЛЬНОМ ВЗЯТИИ ПРОБ?

Д. Л. Зорников¹✉, В. М. Симарзина¹, Д. К. Исламиди^{1,2}, Н. С. Белых¹, Д. О. Корнилов¹, Е. И. Абакумова², Л. В. Хаютина², Е. Э. Плотнок², Е. С. Ворошилина^{1,2}¹ Уральский государственный медицинский университет, Екатеринбург, Россия² Медицинский центр «Гармония», Екатеринбург, Россия

Трансцервикальный сбор образцов эндометрия традиционно используют для изучения микробиоты полости матки, однако неизбежный риск контаминации цервикальной микробиотой ставит под сомнение происхождение обнаруживаемых микроорганизмов. Цель исследования — определить, можно ли обнаружить аутохтонную микробиоту эндометрия в трансцервикально собранных образцах методом количественной ПЦР с учетом цервикальной контаминации. В одномоментное методологическое исследование включены 185 женщин репродуктивного возраста, у которых проводили забор парных цервикальных и эндометриальных образцов (для эндометриальных образцов использовали зонд Endobrush, Пайпель С2). Микробиоту оценивали методом ПЦР в реальном времени на 27 групп микроорганизмов. Сигнал в эндометрии считали истинным, если его количество превышало таковое в цервикальном образце с учетом порогов переноса 30% (ожидаемый) и 100% (консервативный). Микробные сигналы в эндометрии до поправки обнаружены у 144 (77,8%) пациенток; после поправки положительные сигналы сохранялись у 44,3% (порог 30%) и 37,3% (порог 100%) женщин. Большинство положительных проб на *Lactobacillus* spp. попадали в неинтерпретируемую «серую зону» (47,6 и 55,1% от всех пациенток при порогах 30 и 100% соответственно). Напротив, условно-патогенные микроорганизмы в большинстве случаев оставались истинно положительными после поправки. Аутохтонная микробиота эндометрия обнаруживалась у 37,3% женщин и была представлена 1–3 таксонами, преимущественно не лактобациллами. Таким образом, обнаружение условно-патогенных микроорганизмов в эндометрии можно интерпретировать как маркер их реального присутствия, тогда как лактобациллы в большинстве случаев неотличимы от цервикальной контаминации.

Ключевые слова: микробиота эндометрия, аутохтонная микробиота, цервикальная контаминация, трансцервикальный забор, количественная ПЦР

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✉ **Для корреспонденции:** Данила Леонидович Зорников
ул. Ключевская, д. 17, г. Екатеринбург, 620109, Россия; zornikovdl@yandex.ru

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In 2015, culture-based methods and quantitative PCR (qPCR) revealed a distinct microbial community within the uterine cavity using tissue specimens obtained during hysterectomies [1]. By utilizing post-hysterectomy tissue, that study effectively eliminated the risk of vaginal and cervical contamination. A subsequent study, which also evaluated hysterectomy specimens alongside rigorous negative controls, provided a definitive answer to a foundational question: does the uterine cavity harbor a detectable microbiota when assessed by molecular methods? [2]. The resulting data prompted a paradigm shift away from the traditional assumption of a sterile uterine cavity. Today, it is generally accepted that the endometrium harbors its own low-biomass microbial community, making the study of the endometrial microbiota a priority within human microbiome research [3, 4].

Clinical studies, however, cannot rely on hysterectomy specimens. Consequently, nearly all published literature evaluating the endometrial microbiota and its association with reproductive outcomes relies on transcervical sampling—most commonly performed using devices such as Pipelle or Endobrush (Tao Brush) [5–13]. To collect a sample, these instruments must traverse the heavily colonized cervical canal.

Studies employing transcervical sampling have reported associations between the endometrial microbiota and early pregnancy loss [14], chronic endometritis [15], endometrial polyps [5, 16, 17], endometrial hyperplasia [12], and endometriosis [18]. Yet, a critical question remains unanswered: do these associations reflect genuine alterations within the endometrial cavity, or do they largely capture cervical microbiota carried upward during sampling? This uncertainty stems from an unavoidable technical limitation: any instrument traversing the cervical canal risks contaminating the endometrial specimen with cervical microorganisms. This concern is particularly acute given that the baseline bacterial load in the uterine cavity is often substantially lower than that of the cervix [19]. Consequently, even minor contamination could mask a true endometrial signal or, conversely, be misinterpreted as evidence of an indigenous uterine microbial community.

Most published studies attempt to mitigate this risk by pre-treating the vagina and cervix with saline or antiseptics, and by using vaginal or cervical samples as contamination controls [5–10, 12, 13, 15–17, 20, 21]. While these measures reduce contamination risks, they do not eliminate them, nor do they permit reliable quantification of the carryover.

In a recent *in vitro* study using a physical model of the cervical canal, we quantified the extent of microbial carryover associated with Pipelle and Endobrush devices [22]. We demonstrated that both instruments transfer a substantial amount of microbiota from cervical mucus into model endometrial samples, yielding a median carryover of 81.6% for Pipelle and 29.8% for Endobrush. These findings cast serious doubt on the true origin of the microbiota detected in clinical, transcervically collected specimens. However, because *in vitro* models cannot fully replicate the physiological complexity of a clinical setting, the actual extent of contamination in patient samples remains unknown.

Therefore, the objective of the present study was to determine whether a true, autochthonous endometrial microbial signal — previously demonstrated in hysterectomy specimens [1, 2] — can be detected behind the inevitable “curtain” of cervical contamination introduced during routine clinical sampling. In this study, we classify an endometrial signal as autochthonous (true endometrial) only if the concentration of bacterial DNA in the endometrial sample exceeds the level that could be explained by cervical carryover alone.

Aim: To determine whether an autochthonous endometrial microbiota can be detected in transcervically collected samples by quantitative PCR after adjusting for cervical contamination.

METHODS

Patients

The study included 185 reproductive-age women (median 32.9, Q₁–Q₃ [29.9–37] years). All patients were examined at the Garmonia Medical Center (Yekaterinburg, Russia) between January 2020 and August 2023 for either a history of reproductive dysfunction or suspected endometrial pathology.

Inclusion criteria were: reproductive age (18–45 years), non-pregnant status, preserved menstrual cycle, clinical or ultrasound signs of endometrial pathology or infertility, feasibility of simultaneous collection of paired clinical samples (cervical and endometrial), and written informed consent.

Exclusion criteria at enrollment were: use of hormonal or intrauterine contraception at the time of examination or within the preceding six months, malignancy of any site, acute inflammatory diseases of the lower reproductive tract or pelvic organs at the time of examination, antibiotic therapy within four weeks prior to enrollment, and refusal to participate.

Post-enrollment exclusion criterion was: detection of sexually transmitted infections caused by obligate pathogens — *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium* (formerly *Mycoplasma genitalium*), *Trichomonas vaginalis* — as determined by the Androflor assay (DNA-Technology, Russia).

Microbiota composition in cervical and endometrial samples was assessed by real-time PCR targeting 27 microbial groups.

Sample Collection

All procedures were performed on days 8–12 of the menstrual cycle. To minimize contamination risk, samples were collected in ascending order (vagina → cervical canal → uterine cavity).

Vaginal sample

A vaginal sample was collected from the posterior-lateral fornix before antiseptic treatment using a sterile standard urogenital swab. The biomaterial was transferred to a tube containing sterile saline (0.9% NaCl). Vaginal samples were not analyzed in the present study.

Vaginal preparation

Following vaginal sampling, the vagina was irrigated with 150–200 mL of 0.05% chlorhexidine bigluconate using a low-frequency ultrasonic cavitation device (AUZKh-100; Fotek, Russia) according to the manufacturer's protocol [23]: exposure time, 1–2 min; power setting, 6–8 units. This procedure served as prophylaxis against infectious-inflammatory complications prior to intrauterine sampling [24].

Cervical sample

After vaginal preparation, a cervical mucus sample was collected using a new sterile standard urogenital swab inserted into the cervical canal to a depth of 1–1.5 cm. The biomaterial was transferred to a tube containing sterile saline.

Endometrial sample

Endometrial sampling was performed using the Endobrush Standard for Endometrial Cytology device (Laboratoire C.C.D., France). The device is equipped with a protective sheath that shields the internal brush from contact with the cervical mucosa: the brush is deployed only after the device enters the uterine cavity and is retracted into the sheath before withdrawal. The cervix was visualized with a speculum and recleaned with a swab soaked in 0.05% chlorhexidine. The device was then introduced into the uterine cavity transcervically, avoiding contact with the vaginal walls. Once inside the uterine cavity, the protective sheath was retracted to expose the brush, and 3–5 rotational movements were performed to collect the sample. The brush was then retracted into the sheath and the device was withdrawn. The outer surface of the sheath was thoroughly wiped with a sterile swab soaked in 96% ethanol to remove residual cervical mucus and prevent sample contamination. Finally, the brush was extended and the biomaterial was transferred to a tube containing sterile saline.

DNA Extraction

DNA was extracted using the Proba-NK-PLUS kit (DNA-Technology, Russia) according to the manufacturer's instructions. Endometrial samples underwent a preliminary deproteinization step: sample tubes were centrifuged at 13,000 rpm for 10 min on a MiniSpin centrifuge (Eppendorf, Germany), the supernatant was discarded, and the pellet was resuspended in 100 μ L of lysis solution from the Proba-NK-PLUS kit and vortexed to homogeneity. A 50 μ L aliquot of the resulting homogenate was transferred to a clean tube containing a mixture of 25 μ L lysis solution, 5 μ L proteinase K (20 mg/mL; VWR Life Science, USA), and 120 μ L sterile saline. After mixing, samples were incubated at 60 °C for 30 min, then at 95 °C for 10 min. Following incubation, tubes were centrifuged at 13,000 rpm for 60 s. A 100 μ L volume of the supernatant was used for DNA extraction according to the Proba-NK-PLUS kit protocol. The deproteinization step for endometrial samples was necessitated by their high viscosity due to abundant protein and mucus, which could reduce DNA extraction efficiency. Cervical samples did not require this step owing to their different consistency. Negative controls (sterile saline) were included in each DNA extraction and PCR run to rule out reagent contamination.

Microbiota Analysis

Each sample was analyzed by real-time PCR for 27 microbial groups: *Lactobacillus* spp., *Lactobacillus crispatus*, *Lactobacillus acidophilus*, *Lactobacillus iners*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus vaginalis*, *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Gardnerella vaginalis*, *Megasphaera* spp., *Veillonella* spp., *Dialister* spp. (MVD group), *Sneathia* spp., *Leptotrichia* spp., *Fusobacterium* spp. (SLF group), *Atopobium cluster*, *Bacteroides* spp., *Porphyromonas* spp., *Prevotella* spp. (BPP group), *Anaerococcus* spp., *Peptostreptococcus* spp., *Parvimonas* spp. (PP group), *Eubacterium* spp., *Haemophilus* spp., *Pseudomonas aeruginosa* / *Ralstonia* spp., *Burkholderia* spp. (PRB group), *Enterobacteriaceae* / *Enterococcus* spp., *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Metamycoplasma hominis*, and *Candida* spp.

PCR was performed using the Androflor kit and a kit for species-level identification of vaginal lactobacilli (both

manufactured by DNA-Technology, Russia) on DT-Prime and DT-96 thermal cyclers with the accompanying software and amplification protocols (DNA-Technology, Russia). According to the kit instructions, samples were considered positive for *Ureaplasma urealyticum*, *Ureaplasma parvum*, and *Metamycoplasma hominis* at any non-zero quantity. For all other microbial groups, the detection threshold was 10³ genome equivalents per sample (GE/sample).

Statistical Analysis

Data processing and visualization were performed in R version 4.6.0 (R Foundation for Statistical Computing; Vienna, Austria). Central tendency is reported as median with first and third quartiles (Q₁ and Q₃). Differences in detection frequencies were assessed using two-tailed Fisher's exact test; differences in quantitative variables were assessed using the Mann–Whitney U test. All differences were considered statistically significant at $p < 0.05$.

RESULTS

Clinical Characteristics of the Patients

At the time of examination, patients presented with menstrual cycle abnormalities, ultrasound signs of endometrial pathology, or were undergoing evaluation for infertility. These conditions served as the indications for the workup, which included intrauterine sampling for microbiota analysis. The majority of patients had a history of obstetric-gynecological pathology, while 17 (9.2%) women had an uneventful history (Table 1).

Comparison of the Microbiota in Cervical and Endometrial Samples

Microbial signals were detected in endometrial samples from 144 (77.8%) patients. For most microbial groups, the detection frequency in cervical samples was 1.3–3 times higher than in endometrial samples (Fig. 1). The groups detected significantly more frequently in cervical samples included *Lactobacillus* spp. (88.6% vs. 68.1%), *L. crispatus* (39.5% vs. 15.7%), *L. iners* (41.6% vs. 28.1%), *L. jensenii* (31.9% vs. 10.8%), *L. gasseri* (16.8% vs. 8.1%), *L. johnsonii* (3.2% vs. 0%), *L. vaginalis* (23.8% vs. 2.7%), *Anaerococcus* spp. (10.8% vs. 2.2%), *Eubacterium* spp. (31.9% vs. 20%), *Enterobacteriaceae/Enterococcus* spp. (13% vs. 5.9%), *U. parvum* (6.5% vs. 0%).

Statistically significant differences in microbial quantity between cervical and endometrial samples were found for 13 microbial groups: *Lactobacillus* spp., *L. crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*, *G. vaginalis*, *Anaerococcus* spp., PP group, *Eubacterium* spp., *Enterobacteriaceae/Enterococcus* spp., *U. parvum* (Fig. 1). *Lactobacillus* spp. exhibited the highest quantities at both anatomical sites, with 10^{4.9} (10^{3.8}–10^{6.1}) GE/sample in cervical samples versus 10^{3.5} (0–10^{4.2}) GE/sample in endometrial samples ($p < 0.001$). Median quantities for all other microbial groups were zero at both sites; the significant differences were driven entirely by higher concentrations in the upper quartiles of the cervical sample distributions.

Endometrial Signals After Correction for Potential Contamination

For each positive signal in an endometrial sample, we calculated the ratio of the microorganism's quantity in the endometrium to

Table 1. Clinical characteristics of the patients

Parameter	Value
Age, years	32.9 (29.9–37.0)
Body mass index, kg/m ²	22.1 (20.2–24.4)
Obstetric and gynecological history	
Age at menarche, years	13 (12–14)
Menstrual cycle length, days	29 (28–30)
Duration of menses, days	5 (5–7)
Total pregnancies	1 (0–2)
Parity (deliveries)	78 (42.4%)
History of abortion	44 (23.8%)
History of pregnancy loss	40 (21.6%)
Gynecological pathology*	
Infertility	77 (41.6%)
Endometriosis	32 (17.3%)
Uterine fibroids	17 (9.2%)
Endometrial hyperplasia	13 (7.0%)
Pelvic inflammatory disease	10 (5.4%)
HPV carrier	7 (3.8%)
Hyperprolactinemia	7 (3.8%)
Recurrent pregnancy loss	6 (3.2%)
Heavy menstrual bleeding	6 (3.2%)
Chronic cervicitis	6 (3.2%)
Cervical dysplasia	4 (2.2%)
Endometrial polyp	4 (2.2%)
Polycystic ovary syndrome	2 (1.1%)
Teratoma	1 (0.5%)
Chronic endometritis	1 (0.5%)
No gynecological pathology	17 (9.2%)

Note: * — the sum of obstetric-gynecologic diagnoses exceeds 100% because several patients had more than one diagnosis.

its quantity in the paired cervical sample from the same patient. A signal was considered truly endometrial if this ratio exceeded the established cervical carryover threshold. Two carryover thresholds were applied: 30% (the expected threshold, based on our prior *in vitro* experiment [22]) and 100% (a conservative threshold, requiring the microbial load in the endometrium to exceed that in the cervix). When the target microorganism was absent from the cervical sample, any positive endometrial signal was considered to exceed the carryover threshold, as it could not be explained by cervical contamination.

After applying these thresholds, microorganisms were detected in 82 (44.3%) samples at the 30% threshold and 69 (37.3%) samples at the 100% threshold (Fig. 2). The majority of positive samples contained no more than three microbial groups. Specifically, at the 30% threshold, one, two, and three groups were detected in 31 (16.8%), 13 (7%), 15 (8.1%) cases, respectively; at the 100% threshold, these counts were 28 (15.1%), 12 (6.5%), 12 (6.5%) cases. In a few exceptional samples, up to 11 microbial groups were present at the 30% threshold and up to 10 groups at the 100% threshold.

We next evaluated the detection frequency of each microbial group in endometrial samples after correcting for potential cervical contamination. Based on the results, each sample was assigned to one of three categories: negative (no signal detected), “gray zone” (signal detected but not exceeding the established carryover threshold), or true endometrial signal (exceeding the threshold, thereby ruling out cervical contamination as the sole source) (Fig. 3):

$$x = \frac{\text{Quantity of target microorganism}_{(\text{endometrium})}}{\text{Quantity of target microorganism}_{(\text{cervix})} \times \text{Carryover threshold}}$$

where $x > 1$ — indicates a true signal and $x \leq 1$ indicates the gray zone. Carryover thresholds were set at 0.3 (30% carryover) and 1.0 (100% carryover).

At the 30% carryover threshold, *Lactobacillus* spp. fell into the gray zone in 88 (47.6% of all patients), as did *L. crispatus* in 18 (9.7%), *L. iners* in 38 (20.5%), *L. jensenii* in 12 (6.5%), and *L. vaginalis* in 1 (0.5%). Among opportunistic microorganisms (OMs), the highest proportions of gray-zone results were observed for *G. vaginalis* and *Eubacterium* spp.—16 (8.6%) and 7 (3.8%), respectively. For the remaining OMs, gray-zone results accounted for 0–1.6% of samples.

At the 100% carryover threshold, *Lactobacillus* spp. fell into the gray zone in 102 (55.1% of all patients), as did *L. crispatus* in 19 (10.3%), *L. iners* in 42 (22.7%), *L. jensenii* in 17 (9.2%), and *L. vaginalis* in 1 (0.5%). Among OMs, the highest proportions of gray-zone results were again observed for *G. vaginalis* and *Eubacterium* spp.—18 (9.7%) and 13 (7%), respectively. For the remaining OMs, gray-zone results accounted for 0–3.2% of samples.

After correction, the detection frequency of the most prevalent group, *Lactobacillus* spp., dropped from 126 (68.1%) pre-correction to 38 (20.5%) at the 30% threshold and 24 (13.0%) at the 100% threshold. Following correction, individual *Lactobacillus* species and OMs were detected in 0.5–16.2% of samples at the 30%

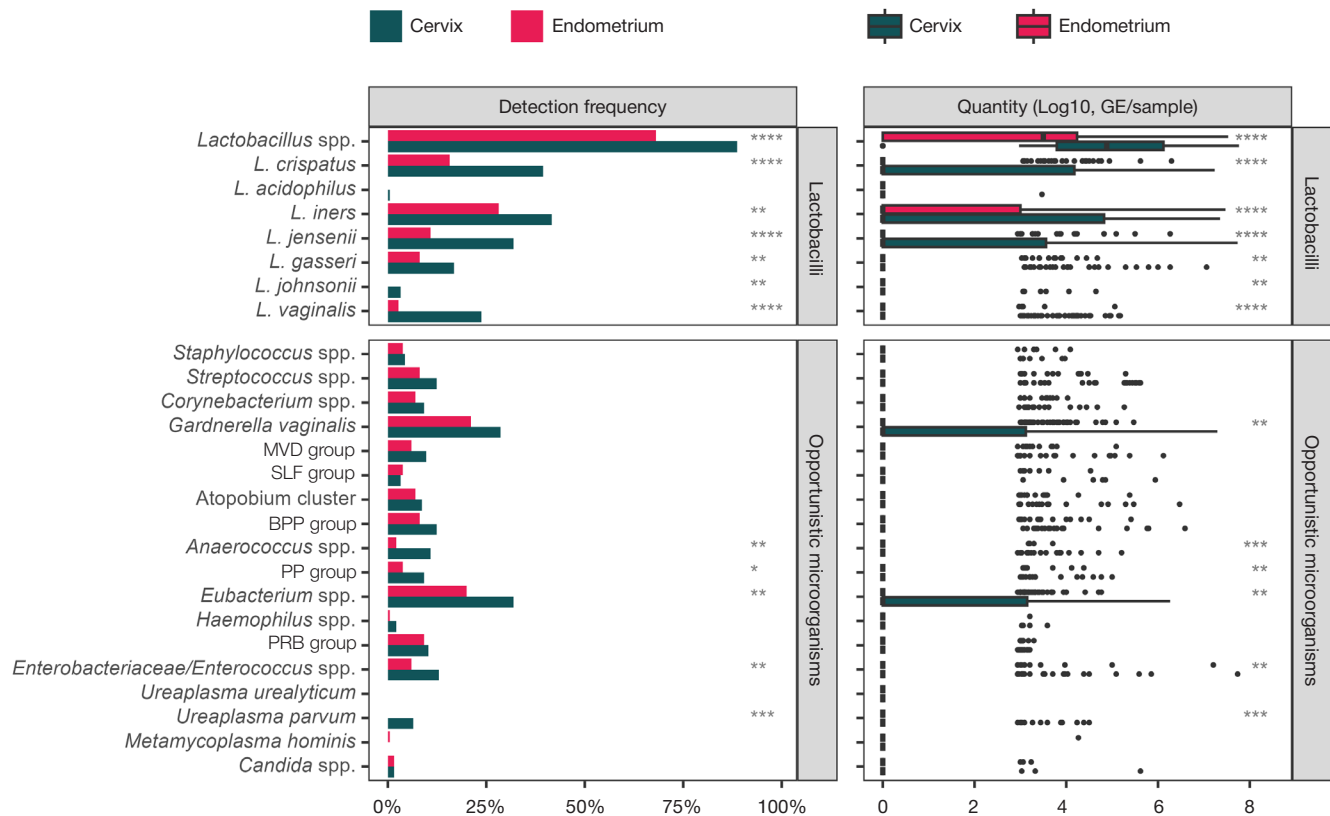


Fig. 1. Microbiota of cervical and endometrial samples. Detection frequency (left panel) and quantity (right panel) of target microbial groups. MVD — *Megasphaera* spp. / *Veillonella* spp. / *Dialister* spp.; SLF — *Sneathia* spp. / *Leptotrichia* spp. / *Fusobacterium* spp.; BPP — *Bacteroides* spp. / *Porphyromonas* spp. / *Prevotella* spp.; PP — *Peptostreptococcus* spp. / *Parvimonas* spp.; PRB — *Pseudomonas aeruginosa* / *Ralstonia* spp. / *Burkholderia* spp. Significance levels: * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$, **** — $p < 0.0001$

threshold and 0.5–13% at the 100% threshold; signals for *L. acidophilus*, *L. johnsonii*, *U. urealyticum*, and *U. parvum* were completely absent from endometrial samples after correction, regardless of the threshold applied (Table 2).

DISCUSSION

Endometrial samples from the patient cohort yielded 22 of the 27 target microbial groups (Fig. 1). *Lactobacillus* spp. were

detected in 68.1% of samples at a median quantity of $10^{3.5}$ GE/sample, whereas all other groups were identified in fewer than one-third of patients. This is consistent with the established understanding of the endometrial microbiota as a low-diversity, low-biomass community [1, 2, 21]. Notably, detection frequencies for all 22 groups were higher in cervical samples than in endometrial samples. *Lactobacillus* spp., for instance, were detected in 88.6% of cervical samples at a median quantity of $10^{4.9}$ GE/sample ($p < 0.0001$ for both frequency

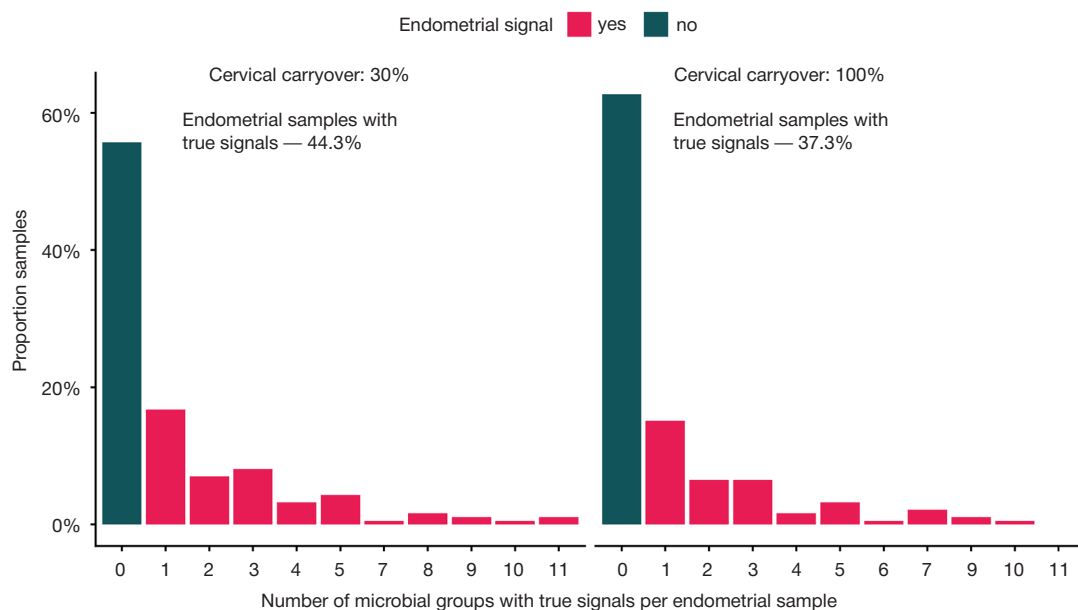


Fig. 2. Samples with endometrial microbiota after applying a 30% (left panel) and 100% (right panel) cervical DNA carryover threshold. A sample was considered to harbor endometrial microbiota if at least one target microbial group exceeded the established carryover threshold

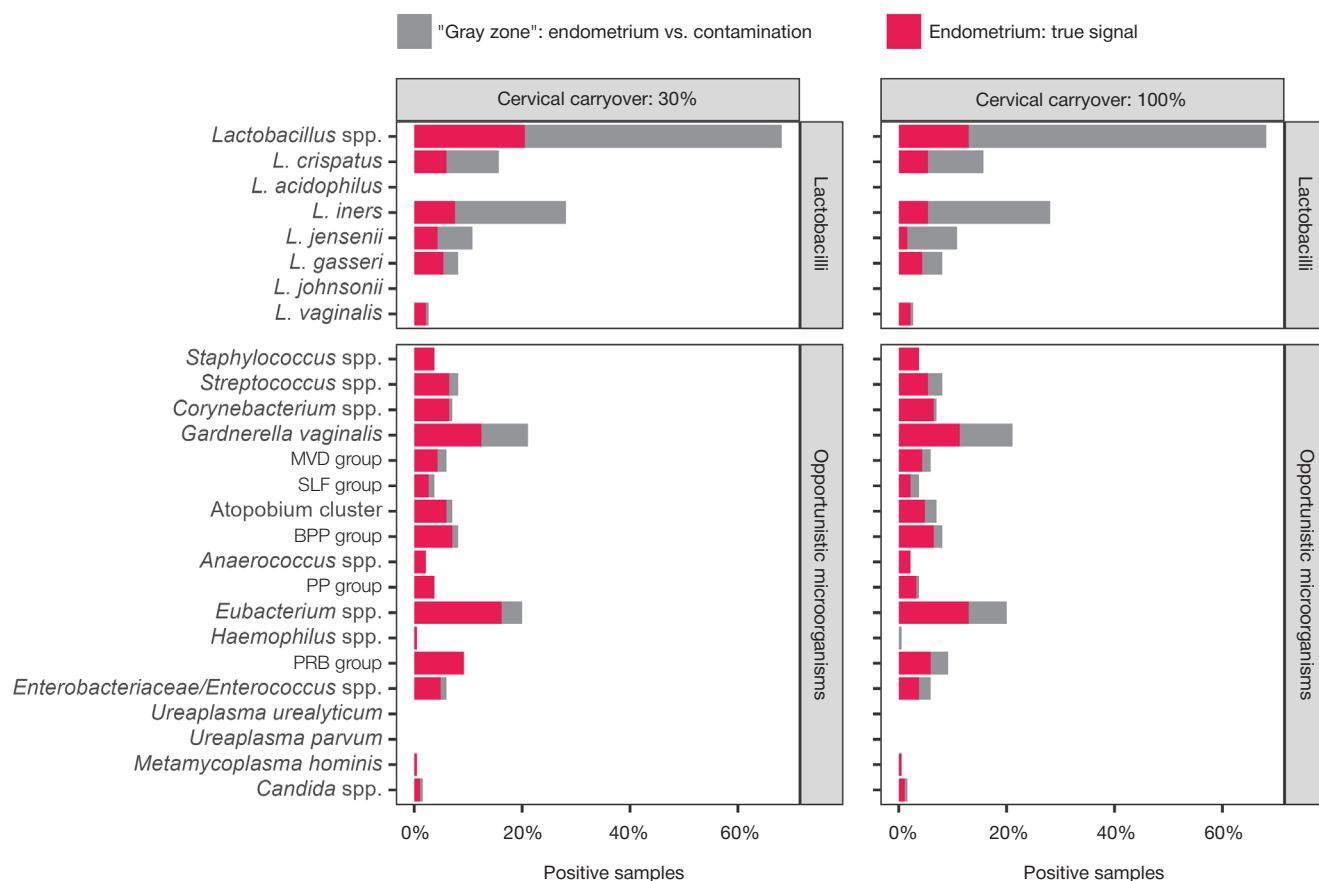


Fig. 3. Detection frequency of target microbial groups in endometrial samples after correction for 30% (left panel) and 100% (right panel) cervical DNA carryover. MVD — *Megasphaera* spp. / *Veillonella* spp. / *Dialister* spp.; SLF — *Sneathia* spp. / *Leptotrichia* spp. / *Fusobacterium* spp.; BPP — *Bacteroides* spp. / *Porphyromonas* spp. / *Prevotella* spp.; PP — *Peptostreptococcus* spp. / *Parvimonas* spp.; PRB — *Pseudomonas aeruginosa* / *Ralstonia* spp. / *Burkholderia* spp.

and quantity). This observation, combined with our earlier *in vitro* finding that the Endobrush device transfers approximately 30% of bacterial DNA from cervical mucus into the endometrial sample [22], strongly suggests that a substantial proportion of positive endometrial signals are attributable to cervical contamination.

To distinguish true endometrial signals from cervical carryover, we compared the quantity of DNA for each microorganism in the endometrial sample to that in the paired cervical sample from the same patient. We applied two carryover thresholds: 30% (the expected threshold based on our *in vitro* experiment [22]) and 100% (a conservative threshold requiring the quantity in the endometrium to exceed that in the cervix, thereby ruling out contamination entirely). At both thresholds, more than half of the endometrial samples lacked positive signals above the cutoff (Fig. 2). The presence of microorganisms in these samples could be fully explained by cervical carryover, meaning they could not be considered truly positive. Among the validated positive samples (44.3% at the 30% threshold and 37.3% at the 100% threshold), the majority contained no more than three microbial groups. Thus, after contamination correction, nearly half of the 144 (77.8%) initially positive endometrial samples were reclassified as non-interpretable, and the remaining positive samples harbored only a few taxa.

We next evaluated the results for each target microbial group after applying the contamination correction, assigning each sample to one of three categories: negative (no signal detected), gray zone (signal detected but not exceeding the established carryover threshold), or true endometrial signal (exceeding the threshold, thereby ruling out cervical

contamination as the sole source). For *Lactobacillus* spp. and individual lactobacillus species, the majority of initially positive samples — ranging from two-thirds to three-quarters — fell into the gray zone (Fig. 3). Before correction, *Lactobacillus* spp. were detected in 126 (68.1%) of patients; after applying the 30% threshold, this dropped to 38 (20.5%), and after the 100% threshold, to 24 (13.0%). Similar patterns were observed for the three predominant lactobacillus species: *L. crispatus*, *L. iners*, and *L. jensenii*. For OMs, the proportion of samples falling into the gray zone was lower than for lactobacilli: 8.6% and 9.7% for *G. vaginalis*, and 3.8% and 7.0% for *Eubacterium* spp. at the 30% and 100% thresholds, respectively. For the remaining OMs, gray-zone results accounted for 0–1.6% and 0–3.2% of samples. Thus, among OMs-positive samples, the majority remained true positives after correction, although their overall detection frequency did not exceed 16.2% at the 30% threshold and 13% at the 100% threshold.

Our findings align with the cautious view advanced earlier [2] that the predominance of lactobacilli reported in transcervically collected endometrial samples [5, 18, 25–27] may reflect cervical or vaginal contamination. This interpretation is further supported by historical culture-based studies demonstrating higher rates of bacterial isolation from transcervically obtained endometrial samples compared with transabdominal sampling [28]. In the present study, we directly quantified the contribution of cervical contamination during transcervical sampling: using paired cervical samples and a threshold-based carryover model demonstrated that cervical contamination can account for most positive signals in endometrial samples. The observation that *Lactobacillus* spp. exhibited the highest proportion of gray-zone results supports the hypothesis that *Lactobacillus*-

Table 2. Detection frequency of microorganisms in endometrial samples after correction for potential contamination. Microorganisms are ranked by detection frequency at the 30% threshold

Microbial group	Frequency at the 30% threshold	Frequency at the 100% threshold
<i>Lactobacillus</i> spp.	38 (20,5%)	24 (13%)
<i>Eubacterium</i> spp.	30 (16,2%)	24 (13%)
<i>Gardnerella vaginalis</i>	23 (12,4%)	21 (11,4%)
PRB group	17 (9,2%)	11 (5,9%)
<i>L. iners</i>	14 (7,6%)	10 (5,4%)
BPP group	13 (7%)	12 (6,5%)
<i>Streptococcus</i> spp.	12 (6,5%)	12 (6,5%)
<i>Corynebacterium</i> spp.	12 (6,5%)	10 (5,4%)
<i>L. crispatus</i>	11 (5,9%)	10 (5,4%)
<i>L. gasseri</i>	11 (5,9%)	9 (4,9%)
<i>Atopobium cluster</i>	10 (5,4%)	8 (4,3%)
<i>Enterobacteriaceae/Enterococcus</i> spp.	9 (4,9%)	7 (3,8%)
<i>L. jensenii</i>	8 (4,3%)	3 (1,6%)
MVD group	8 (4,3%)	8 (4,3%)
PP group	7 (3,8%)	6 (3,2%)
<i>Staphylococcus</i> spp.	7 (3,8%)	7 (3,8%)
SLF group	5 (2,7%)	4 (2,2%)
<i>L. vaginalis</i>	4 (2,2%)	4 (2,2%)
<i>Anaerococcus</i> spp.	4 (2,2%)	4 (2,2%)
<i>Candida</i> spp.	2 (1,1%)	2 (1,1%)
<i>Haemophilus</i> spp.	1 (0,5%)	0 (0%)
<i>Metamycoplasma hominis</i>	1 (0,5%)	1 (0,5%)
<i>L. acidophilus</i>	0 (0%)	0 (0%)
<i>L. johnsonii</i>	0 (0%)	0 (0%)
<i>Ureaplasma parvum</i>	0 (0%)	0 (0%)
<i>Ureaplasma urealyticum</i>	0 (0%)	0 (0%)

Note: PRB — *Pseudomonas aeruginosa* / *Ralstonia* spp. / *Burkholderia* spp. BPP — *Bacteroides* spp. / *Porphyromonas* spp. / *Prevotella* spp.; MVD — *Megasphaera* spp. / *Veillonella* spp. / *Dialister* spp.; PP — *Peptostreptococcus* spp. / *Parvimonas* spp.; SLF — *Sneathia* spp. / *Leptotrichia* spp. / *Fusobacterium* spp.

dominance in endometrial samples may be an artifact of the sampling procedure [2]. Moreover, after applying the contamination correction, *G. vaginalis* and *Eubacterium* spp. emerged as the most frequently detected microorganisms in endometrial samples alongside *Lactobacillus* spp., surpassing all individual lactobacillus species (Table 2).

Detection of a target microorganism in the endometrium at quantities exceeding the established cervical carryover threshold does not constitute absolute proof of its endometrial origin. However, it likely reflects a genuine presence in the uterine cavity, as such a signal cannot be explained by cervical contamination within our model framework. In this study, 37.3% of patients retained positive microbial signals in endometrial samples even at the conservative 100% threshold. This finding cannot be fully explained by cervical contamination, pointing to the presence of an autochthonous endometrial microbiota. In most cases, however, this microbiota was represented by only one to three non-lactobacillus taxa, and the detection frequency of even the most prevalent group did not exceed 13%.

The fact that lactobacilli predominantly fell into the gray zone does not necessarily imply their absence from the endometrium. It is plausible that lactobacilli were present in some samples but at quantities too low to be reliably distinguished from cervical carryover. The anatomical proximity of the cervix and uterine cavity may lead to genuine overlap in the microbial communities of these two sites. Notably, even when post-hysterectomy

specimens and 16S rRNA gene sequencing were used, no significant differences were found between the cervical and endometrial microbiota [2].

Crucially, the majority of OM s detected in endometrial samples remained true positives even after applying the conservative 100% contamination threshold (Fig. 3). While the biological mechanisms underlying this pattern require further investigation, the finding has immediate clinical relevance. Unlike lactobacilli, which largely fell into the gray zone, the detection of OM s in an endometrial sample likely reflects genuine presence in the uterine cavity rather than a sampling artifact. This is highly meaningful in a clinical context, as OM s are traditionally associated with endometrial pathology and represent the primary targets of microbiological investigation in intrauterine samples.

A key strength of this study is its paired-sample design, in which cervical and endometrial specimens were collected from the same patient during a single procedure. This approach allowed each patient's individual cervical microbial load to serve as the reference for contamination assessment rather than relying on averaged cohort values, thereby eliminating the confounding effect of inter-individual clinical variability. The use of two distinct carryover thresholds — 30% (expected, based on our *in vitro* model [22]) and 100% (conservative, completely ruling out contamination) — enabled an assessment of the robustness of our findings across threshold choice. The use

of quantitative PCR targeting 27 microbial groups provided high analytical sensitivity and allowed assessment of both the frequency and absolute quantity of each target group, offering a distinct advantage over studies relying solely on 16S rRNA gene sequencing.

A practical implication of our findings is the need to reconsider current approaches to investigating the endometrial microbiota. The use of sheathed sampling devices such as the Endobrush, even when combined with antiseptic preparation, does not reliably distinguish endometrial from cervical microorganisms. In the short term, it may be more appropriate to study the combined cervico-endometrial microbiota by pooling the cervical and endometrial samples into a single specimen obtained during transcervical sampling. This approach, at a minimum, does not create the illusion of selective endometrial sampling. In the longer term, methods that effectively minimize cervical contamination must be developed.

Limitations of the study

This study has several limitations. First, our contamination model assumed that a single cervical sample adequately reflects the microbial load along the entire length of the cervical canal; however, regional variability in microbial concentrations may exist, potentially leading to under- or overestimation of the carryover level. Second, the DNA extraction protocol differed between cervical and endometrial samples: endometrial samples underwent a preliminary deproteinization step, which could have influenced DNA extraction efficiency and the ratio of microbial quantities between the two biotopes. Third, the PCR panels were limited to 27 target microbial groups; unlike 16S rRNA gene sequencing, this approach does not capture the full taxonomic composition of the microbiota and cannot detect microorganisms not included in the panels. Fourth,

quantitative PCR cannot distinguish viable microorganisms from extracellular DNA; therefore, some of the detected signals may have originated from non-viable bacteria or free DNA that reached the uterine cavity from the lower reproductive tract, a consideration that should be taken into account when interpreting results clinically. Fifth, vaginal samples were collected but not analyzed in the present study. Although the risk of endometrial contamination by vaginal microbiota is considered low when the device is introduced under visual guidance, the absence of these data limits our ability to assess the full continuum of microbial communities along the lower reproductive tract. Finally, this was a single-center study, and its findings require independent replication.

CONCLUSIONS

This study demonstrates that transcervical endometrial sampling using the Endobrush device does not reliably distinguish endometrial from cervical microbiota in the majority of patients. True endometrial signals were detected by quantitative PCR in approximately 40% of women and were typically limited to one to three taxa. The majority of endometrial samples positive for lactobacilli (approximately two-thirds of those initially positive for *Lactobacillus* spp.) fell into the gray zone and could reflect either genuine endometrial presence or cervical contamination. In contrast, detection of OMIs in endometrial samples can be interpreted as a marker of their genuine presence, as their quantities exceeded the established cervical carryover thresholds in most cases. These findings underscore the need for either the development of novel endometrial sampling methods that effectively eliminate cervical contamination or the interpretation of results from such specimens as representing a combined cervico-endometrial microbiota.

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