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MINI REVIEW

Analysing endometrial microbiome: methodological considerations and recommendations for good practice

Nerea M. Molina[1,2], Alberto Sola-Leyva[1,2], Thor Haahr[3], Lusine Aghajanova[4], Piotr Laudanski[5], Jose Antonio Castilla[2,6,7], and Signe Altmäe[1,2,8,*]

[1]Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Granada 18071, Spain [2]Instituto de Investigación Biosanitaria ibs.GRANADA, Granada 18014, Spain [3]The Fertility Clinic, Skive Regional Hospital, Skive 7800, Denmark [4]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Stanford School of Medicine, Sunnyvale, CA 94087, USA [5]Department of Obstetrics and Gynecology, Medical University of Warsaw, Warsaw 02-015, Poland [6]Unidad de Reproducción, UGC de Obstetricia y Ginecología, Hospital Universitario Virgen de las Nieves, Granada 18012, Spain [7]CEIFER Biobanco—NextClinics, Granada 18004, Spain [8]Competence Centre on Health Technologies, Tartu 50410, Estonia

*Correspondence address. Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Fuente Nueva S/N, 18071 Granada, Spain. Tel: +34 958 241 383; E-mail: signealtmae@ugr.es

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ABSTRACT: There is growing evidence that the upper female genital tract is not sterile, harbouring its own microbial communities. However, the significance and the potential effect of endometrial microorganisms on reproductive functions remain to be fully elucidated. Analysing the endometrial microbiome, the microbes and their genetic material present in the endometrium, is an emerging area of study. The initial studies suggest it is associated with poor reproductive outcomes and with different gynaecological pathologies. Nevertheless, studying a low-biomass microbial niche as is endometrium, the challenge is to conduct well-designed and well-controlled experiments in order to avoid and adjust for the risk of contamination, especially from the lower genital tract. Herein, we aim to highlight methodological considerations and propose good practice recommendations for future endometrial microbiome studies.

Key words: endometrium / metagenome / microbiome / microbiota / uterus

Introduction

Few, if any, tissues in our body are totally sterile, with most having a coevolved microbiota, i.e. community of microorganisms that inhabits a defined site. Indeed, recent studies have sequenced hyper-variable regions of the bacterial 16S rRNA gene and have identified a unique endometrial microbiome, i.e. microorganisms and their genomes (Baker *et al.*, 2018; Benner *et al.*, 2018; Koedooder *et al.*, 2019). The uterine (endometrial) microbiome is considered as a low microbial biomass site since the estimation of the uterine bacterial load is 100–10 000 times lower than that of the vagina (Baker *et al.*, 2018).

Endometrial microbiome composition has been associated with various gynaecological diseases such as endometriosis, chronic endometritis, dysfunctional endometrial bleeding, endometrial cancer or hyperplasia, and poorer outcomes in assisted reproduction (Molina *et al.*, 2020) (see Table I for all studies). Especially, the potential implications for human reproduction have sparked research in a previously overlooked infectious cause of infertility. However, there is in fact only one study to date that has detected a statistically significant difference in microbiome profiles between successful and unsuccessful reproductive outcomes (Moreno *et al.*, 2016). Other studies have not detected any significant associations between endometrial microbiome and reproductive outcomes (Franasiak *et al.*, 2016; Verstraelen *et al.*, 2016; Kyono *et al.*, 2018, 2019; Liu *et al.*, 2018; Wee *et al.*, 2018; Hashimoto and Kyono, 2019; Carosso *et al.*, 2020; Riganelli *et al.*, 2020). The study by Moreno *et al.* analysed 35 infertile women undergoing IVF and detected *Lactobacillus* dominance in the uterus. Interestingly, non-*Lactobacillus*-dominated microbiome was associated with decreased implantation, pregnancy and live birth rates among infertile women undergoing IVF (Moreno *et al.*, 2016). In most of the studies, the endometrial sample was obtained transcervically, which is prone to the bacterial 'contamination' from the lower genital tract. Notably, studies that obtained endometrial biopsies from hysterectomy (Winters *et al.*, 2019), laparoscopy (Chen *et al.*, 2017) and/or during caesarean section (Leoni *et al.*, 2019; Younge *et al.*, 2019) (lowering the contamination risk from the vagina and cervix) conclude that

Table I Microbiome studies in human endometrium.

Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/ quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Mitchell *et al.* (2015)	Hysterectomy patients (N = 58); avg 43 ± 7 y; Caucasian, African American, Hispanic	Endometrial swab from excised uterus (−80°C)	NR	MoBio Bacteraemia DNA Isolation Kit; qPCR for 12 vaginal bacterial species and broad-range 16S rRNA gene qPCR	*Lactobacillus iners*, *Prevotella* spp., *L. crispatus*	NR	95% of women had endometrial colonisation with bacteria Quantity of bacteria in uterus was lower than that in the vagina Detection of bacteria in the uterus was not associated with inflammatory immune response
Franasiak *et al.* (2016)	IVF patients (N = 33); avg 35.9 y; Caucasian, Asian, African American, Hispanic	Transfer catheter (snap-frozen and stored at −20°C after cell lysis)	Negative controls from reagents; positive control: *Escherichia coli*	Cell lysis and DNA purification (Agencourt AMPure® XP Reagent); Ion PGM™ sequencing (V2-4-8, V3-6, 7-9)	*Flavobacterium*, *Lactobacillus*	NR	No significant differences in endometrial microbiome between successful and unsuccessful IVF
Verstraelen *et al.* (2016)	RIF (N = 11); RPL (N = 7); RIF+RPL (N = 1); avg 32 y; Caucasian	Tao Brush™ Endometrial Sampler (-80°C)	Disinfection of the cervical surface and external os	Mechanical cell lysis and DNA purification; Illumina MiSeq® (V1-2)	*Bacteroides xylanisolvens*, *B. thetaiotaomicrom*, *B. fragiles*, *B. vulgatus*, *B. ovatus*, *Pelomonas*, *Betaproteoacteria*, *Escherichia/Shigella*, *Chitinophagaceae*	Supplementary material	The uterine core microbiome composed of three main bacterial phyla: *Proteobacteria*, *Firmicutes* and *Bacteroidetes*
Fang *et al.* (2016)	EP (N = 10); 34.4 ± 2.4y; EP/CE (N = 10); avg 35.2 ± 1.8y; Control (N = 10); 30.9 ± 1.6y; Chinese	Uterine swab (−80°C)	Vaginal and cervical canal disinfection	TIANamp Swab DNA Kit; Illumina MiSeq® (V4)	*Lactobacillus*, *Enterobacter*, *Pseudomonas*, *Euryarchaeota* (Archaea)	PRJEB9626 deposited in ENA	Bacterial populations in uterus greatly differed from vaginal microbiome Low levels of Archaea were detected in all samples Women with EP and EP/CE showed different endometrial microbiome composition compared to controls
Khan *et al.* (2016)	Endometriosis (N = 32); 21–47 y; Control (N = 32); 21–54 y; Japanese	Seed swab (−80°C)	NR	Ultra Clean PowerSoil® DNA Isolation Kit; Illumina MiSeq® (custom primers)	*Lactobacillacae*, *Streptococcaceae*, *Staphylococaceae*, *Enterobacteriaceae*, *Moraxellaceae*	NR	*Lactobacillaceae* were decreased and *Streptococcaceae* and *Moraxellaceae* increased in women with endometriosis GnRH treatment influenced bacterial proportions in uterus Slight increase in the microbial colonisation during the menstrual phase

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Table I Continued

Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Walther-António *et al.* (2016)	Endometrial cancer (N = 17); avg 64 y; Endometrial hyperplasia (N = 4); avg 54 y; Benign condition (N = 10); avg 44.5 y; Caucasian	Endometrial swab, uterine scrapes and uterine biopsies from excised uterus at hysterectomy (−80°C)	Negative controls: air culture during sample collection and controls of DNA extraction and microbiome enrichment processes	MoBio PowerSoil Kit; Illumina MiSeq® (V3-5)	*Shigella, Barnesiella, Staphylococcus, Blautia, Parabacteroides*	SRP064295 deposited in SRA repository	Significant subject-specific correlations in endometrial microbiome distribution Existence of structural microbiome shift in the cancer and hyperplasia cases Higher diversity in cancer cohort Detection of *A. vaginae* and *Porphyromonas* sp. 99% matching *P. somerae* as predictor of endometrial cancer Significant effects of collection type in profiling the microbiome
Moreno *et al.* (2016)	IVF (N = 35); 25–40 y; Control (N = 35); 18–35 y; Spanish	Endometrial fluid with Wallace Classic transfer catheter (snap-frozen, −80°C)	Cervix cleaning with a cotton swab and mucus aspirated	Extra MagNA Pure compact nucleic acid isolation Kit I; Pyrosequencing on Roche 454 (V3-5)	*Lactobacillus, Gardnerella, Bifidobacterium, Streptococcus, Prevotella*	SRP078557 deposited in SRA repository	Different bacterial communities detected between uterus and vagina Endometrial microbiome was highly stable throughout menstrual cycle Non-*Lactobacillus* dominance was associated with significant decrease in implantation, pregnancy, ongoing pregnancy and live birth rates in IVF
Miles *et al.* (2017)	Hysterectomy patients (N = 8); 41–57 y; North American	Endometrial swab from excised uterus (−80°C)	Positive and negative controls (not specified)	Pyrosequencing on Roche 454 (V1-3)	*Lactobacillus, Acinetobacter, Blautia, Corynebacterium, Staphylococcus*	NR	Bacteria identified in 95% of endometrial samples *Lactobacillus* was highly abundant Microbial communities were highly related across the uterine and cervical samples and patients
Tao *et al.* (2017)	IVF (N = 70); avg 36.2 y; Caucasian, Asian, African, American, Hispanic	Wallace Classic transfer catheter (NR)	Negative control: blank extraction control; Positive controls: different concentrations of mock communities	Genomic DNA isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Corynebacterium* spp., *Streptococcus, Staphylococcus* spp., *Bifidobacterium* spp.	NR	*Lactobacillus* was detected in all patients Provide method to study ultra-low amount of bacteria

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Cregger *et al.* (2017)	Endometriosis (N = 6); premenopausal; Control (N = 3); North American	Uterine wash (−80°C)	NR	Power Soil® DNA Isolation Kit; Illumina MiSeq® (V3-5)	*Lactobacillus* sp., *Barnesiella*, *Flavobacterium*, *Pseudomonas*	PRJNA387551 deposited in SRA repository	Uterine and cervical bacterial communities were significantly different Uterine microbiome was not significantly different across endometriosis stages
Chen *et al.* (2017)	Various conditions not known to involve infection (N = 95); 22–48 y; Asian	Nylon flocked swabs at laparoscopy or laparotomy (snap-frozen, -80°C)	Negative controls: some reagents (PBS, physiological saline and ultrapure water), diluent negative controls, dry sterile swabs rubbed on patient's preoperative skin area and surgeon's gloves	Chloroform: isoamyl alcohol extraction; Ion Torrent PGM™ (V4-5); conventional culturing methods and qPCR to detect 4 *Lactobacillus* spp.	*Pseudomonas*, *Acinetobacter*, *Vagococcus*, *Sphingobium*, *Comamonadaceae*, *Lactobacillus*	PRJEB16013 and PRJEB21098 deposited in ENA	Distribution of bacteria in the endometrial samples taken through the cervical os showed high similarity to that taken during surgery Upper reproductive tract showed higher bacterial diversity and lower bacterial biomass than vaginal microbiome Differences in the uterine microbiome were identified between menstrual phases Live bacteria detected in the upper reproductive tract
Wee *et al.* (2018)	Infertile (N = 6); avg 37.6 y; Control (N = 5); avg 42.6 y; Australian	Endometrial curettage at hysteroscopy (−80°C in RNALater)	Vagina treated with betadine antiseptic solution; Negative controls for lysis, extraction and PCR	DNeasy Blood and Tissue Kit; Illumina MiSeq® (V1-3); qPCR to detect *Ureaplasma* spp.	*Lactobacillus*, *Bifidobacterium*	PRJEB18626 deposited in SRA repository	Endometrial microbiome differed from that in the lower reproductive tract, having generally lower relative abundance
Pelzer *et al.* (2018)	Menorrhagia or dysmenorrhoea (N = 57); 14–52 y; Control virgo intacta (N = 3); Australian	Endometrial curettage collected at hysteroscopy or laparoscopy (NR)	Perineum, vagina and ectocervix were disinfected with povidone iodine	QIAmp Mini DNA extraction Kit; Pyrosequencing on Roche 454 (V5, V8)	*Lactobacillus*, *Gardnerella vaginalis*, *Veilonella*, *Prevotella*, *Sneathia*	NR	Endocervix and endometrium showed different microbiome Endometrial microbial communities were different between women with menorrhagia and dysmenorrhoea Microbial communities were significantly different between menstrual phases

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Table I Continued

Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Kyono *et al.* (2018)	IVF (N = 79); avg 37 ± 4.2 y; Infertile non-IVF (N = 23); avg 33.2 ± 3.6y; Control (N = 7); avg 36.6 ± 6.6y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucous from cervix; Negative controls: blank control of clean water; Positive control: ZymoBIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood and Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Gardnerella*, *Streptococcus*, *Atopobium*, *Bifidobacterium*, *Sneathia*, *Prevotella*, *Staphylococcus*	NR	Endometrial microbiome of healthy women was highly stable throughout menstrual cycle Considerable percentage of non-*Lactobacillus* dominated microbiome was found in the endometrium of infertile women Contaminants: *Acinetobacter*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*
Moreno *et al.* (2018)	CE undergoing IVF (N = 13); 21–53 y	Novak curette connected to a 20-ml syringe (frozen)	Negative controls to detect any contamination from reagents; Positive control: *E. coli* DNA	QIAamp Cador Pathogen Mini Kit; Ion Torrent S5 XL (V2-4-8, V3-6, 7-9); qPCR to detect the nine most common bacteria responsible for causing CE	*Lactobacillus*, *Streptococcus*, *Gardnerella*, *Bifidobacterium*, *Magasphaera*, *Parvimonas*, *Prevotella*, *Propionibacterium*, *Veillonella*	NR	16S rRNA gene sequencing and qPCR were effective techniques to discriminate between positive and negative cases of CE
Li *et al.* (2018)*	Surgery for non-infectious condition (N = 3); avg 28.3 y; Asian	Endometrial swab at laparoscopy or laparotomy (snap-frozen, −80°C)	Negative controls: some reagents (PBS, physiological saline and ultrapure water), diluent negative controls, dry sterile swabs rubbed on patient's preoperative skin area and surgeon's gloves	Chloroform: isoamyl alcohol extraction; BGISEQ-500	*Pseudomonadaceae*, *Propionibacteriaceae*, *Streptococcaceae*, *Moraxellaceae*	PRJEB24147 deposited in the European Bioinformatics Institute database	Presence of intra-individual continuum of microorganisms Endometrial microbiome analysis using shotgun metagenomics requires greater depth and more samples to meet the full coverage scale in sequencing
Liu *et al.* (2018)	RPL (N = 25); avg 35.2 y; Chinese	Endometrial fluid collected using double-lumen ET catheter and endometrial tissue with Pipelle (−80°C)	Removal of excessive vaginal secretions; Negative controls: collection medium blank and swabs exposed to the air of the clinic	QIAmp DNA Mini Kit plus Triton-X, mutanolysin and proteinase K; Illumina MiSeq® (V4)	*Lactobacillus*, *Stenotrophomas*, *Gardnerella*, *Bifidobacterium*, *Atopobium*, *Prevotella*, *Megasphaera*, *Staphylococcus*, *Escherichia*	NR	Microbial composition in the endometrial fluid did not fully reflect that of the endometrial tissue

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Kyono *et al.* (2019)	IVF (N = 92); avg 37 ± 4.1 y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucus from cervix	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Atopobium, Bifidobacterium, Gardnerella, Megasphaera, Sneathia, Prevotella, Staphylococcus, Streptococcus*	NR	Microbial composition gradually changed along the upper reproductive tract Recovering *Lactobacillus* dominance might benefit implantation in IVF Percentage of endometrial *Lactobacillus* >80% might be favourable for embryo implantation
Kitaya *et al.* (2019)	RIF (N = 28); avg 38.7 ± 3.2 y; Control undergoing IVF (N = 18); avg 37.6 ± 4.2 y; Asian	Endometrial fluid collected using MedGyn Pipette IV (stabilising medium)	Perineum, vagina and cervix were cleansed with benzalkonium chloride solution; mucus was removed; Negative control: clean water; Positive control: ZymoBIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood and Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus* spp., *Gardnerella*, *Streptococcus*, *Prevotella*	NR	Bacterial species in endometrial fluid and vaginal samples were similar within the same individual, however, fluid had higher diversity and broader bacterial species Significant variation in community composition between the RIF and controls Contaminants: *Acidovorax*, *Acinetobacter*, *Chryseobacterium*, *Citrobacter*, *Elizabethkingia*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Leptothrix*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Yersinia*
Winters *et al.* (2019)	Hysterectomy patients (N = 25); avg 45 y; Italian	Endometrial swab from excised uterus (−80°C)	Negative controls from reagents	QIAGEN DNeasy PowerLyzer PowerSoil Kit; Illumina MiSeq® (V4); qPCR to detect *L. iners* and *L. crispatus* and V1-2 16S rRNA gene	*Acinetobacter*, *Pseudomonas*, *Cloacibacterium*, *Comamonadaceae*	PRJNA543861 deposited in SRA repository	Bacterial profiles of the endometrium differed from those of the oral cavity, rectum, vagina and background DNA controls, but not of the cervix Endometrial microbiome was not dominated by *Lactobacillus* Forty per cent of patients did not present detectable microbiome over contamination Contaminants: *Veillonella*, *Escherichia*, *Streptococcus*, *Acinetobacter*

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Liu *et al.* (2019)	Infertile CE (N = 12); avg 35 y; Control infertile without CE (N = 118); avg 36 y; Chinese	Endometrial fluid collected using double-lumen ET catheter (−80°C)	Removal of excessive vaginal secretions; Negative controls: collection medium blank and swabs exposed to the air of the clinic; Positive control: microbial mock community HM-277D	QIAmp DNA Mini Kit plus Triton-X, mutanolysin and proteinase K; Illumina MiSeq® (V4)	*Lactobacillus*, *Dialister*, *Bifidobacterium*, *Prevotella*, *Gardnerella*, *Anaerococcus*	PRJNA530321 deposited in SRA repository	CE associated with a significantly higher abundance of certain bacterial taxa in uterus Median relative abundance of *Lactobacillus* in CE group was lower than in non-CE group
Younge *et al.* (2019)	Caesarean delivery (N = 10); North American	Endometrial swab from the lining of the anterior uterine wall at caesarean section (−80°C)	Negative controls: no DNA template for each extraction kit and PCR	Bead-beating and commercial extraction kits; Illumina MiSeq® (V4)	*Escherichia*, *Acinetobacter*, *Lactobacillus*, *Bacillus*,	PRJNA557826 available at NCBI BioProject	Endometrial microbiome did not differ from that of the vagina
Garcia-Grau *et al.* (2019)	Infertile (N = 1); 37 y; Spanish	Endometrial fluid collected using a Wallace transfer catheter (−80°C in RNALater)	Cervix cleaning with a cotton swab and mucus aspirated	QIAamp DNA Blood Mini Kit; Ion Torrent S5 XL system (V2-4-8 and V3-6, 7-9); NextSeq 500 system; qPCR targeting clade-specific genes for *Gardnerella*	*Gardnerella vaginalis*, *Lactobacillus*, *Pseudoalteromonas*, *Bifidobacterium*, *Rhodanobacter*	PRJNA545633 deposited in SRA repository	Persistent *G. vaginalis* colonisation associated with reproductive failure (based on both 16S rRNA gene seq and metagenome seq)
Hashimoto and Kyono (2019)	IVF (N = 99); avg 35.3 ± 3 y; Asian	Endometrial fluid collected by IUI catheter (stabilising medium)	Removal of the mucus from cervix; Negative control: clean water; Positive control: Zymo-BIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood & Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Gardnerella*, *Atopobium*, *Streptococcus*, *Lactobacillus*	NR	Pregnancy rate in IVF was comparable between *Lactobacillus* dominated microbiome and non-*Lactobacillus* dominated microbiome Species-level resolution might be required for identifying the true pathogenic bacteria and avoiding over-intervention against non-*Lactobacillus* dominated microbiome Contaminants: *Acinetobacter*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Leoni *et al.* (2019)	Caesarean delivery (N = 19); avg 32.3 ± 7.6y; Caucasian	Endometrial biopsy at caesarean section (−80°C)	Negative controls: fake extraction procedure and clean water	Fast DNA Spin Kit for Soil; Illumina MiSeq® (V5-6)	*Cutibacterium* (formerly *Propionibacterium*), *Escherichia*, *Staphylococcus*, *Acinetobacter*, *Streptococcus*, *Corynebacterium*	PRJNA557586 deposited in SRA repository	Bacterial composition could differ between different sites of the endometrium *Lactobacillus* can be found in the endometrium, but without being a part of the core microbiome
Walsh *et al.* (2019)	Endometrial cancer (N = 66); avg 61.8 ± 10.3y; Hyperplasia (N = 7); avg 55 ± 3.3 y; Benign condition (N = 75); avg 49.9 ± 10.5 y; Caucasian, Native American, Filipino, Chinese	Endometrial swab, uterine scrapes and uterine biopsies at hysterectomy (−80°C)	Negative controls: during sample collection and controls of PCR amplification; Positive control	MoBio PowerSoil Kit; Illumina MiSeq® (V3-5)	*Shigella, Barnesiella, Staphylococcus, Blautia, Parabacteroides*	PRJNA481576 deposited in SRA repository	Each factor (menopause, BMI, vaginal pH) independently alters the endometrial microbiome *Porphyromas somerae* presence was the most predictive microbial marker of endometrial cancer
Moreno *et al.* (2020)	IVF (N = 1); 28 y; Spanish	Endometrial fluid collected using a double-lumen ET catheter (−80°C in RNALater)	Cervix cleaning with a cotton swab and mucus aspirated; Negative controls: blank controls from reagents; Positive control: *E. coli* DNA	QIAamp DNA Blood Mini Kit; Ion Torrent S5 XL system (V2-4-8 and V3-6, 7-9); NextSeq 500 system	*Lactobacillus*, *Enterobacteriaceae*, *Streptococcus*, *Pseudomonas*, *Staphylococcus*	PRJNA514966 deposited in SRA repository	Taxonomic and functional differences in endometrial microbiome between spontaneous abortion and successful pregnancy in the same patient
Hernandes *et al.* (2020)	Endometriosis (N = 10); 18–50 y; Control (N = 11); Brazilian	Endometrial curettage (snap-frozen, −80°C)	Negative controls: reagents from extraction procedure and PCR steps	DNeasy Power Soil Kit; Illumina MiSeq® (V3-4)	*Lactobacillus*, *Gardnerella*, *Streptococcus*, *Prevotella*	PRJNA546137 available at NCBI BioProject	Endometrium samples showed lower amount of relative reads and higher diversity than vaginal samples Endometriotic lesions demonstrated the highest microorganism diversity

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Wei *et al.* (2020)	Endometriosis (N = 26); avg 31.47 y; Control with conditions not known to involve infection (N = 11); Asian	Endometial fluid collected using Huales Medical sampler at laparoscopy (dry-ice, −80°C)	Negative controls: saline	QIAamp DNA Mini Kit; Ion Torrent PGM™ (V4-5)	*Lactobacillus*, *Pseudomonas*, *Acinetobacter*, *Vagococcus*	Available from the corresponding author on reasonable request	Existence of distinct community composition in the lower compared to the upper genital tract Alteration in microbial community composition associated with endometriosis
Riganelli *et al.* (2020)	Infertile (N = 24); avg 37 y; Italian	Endometrial biopsy collected using Pipelle covered by IUI catheter (−80°C in Allprotect Tissue reagent)	External genitals and cervical canal disinfection; Negative controls: blank controls from reagents; Positive control: ZymoBIOMICS Microbial Community DNA Standard D6305	DNeasy Blood and Tissue Kit; Illumina MiSeq® (V4-5)	*Kocuria dechangensis*, *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia*, *Agrobacterium tumefaciens*, *Delftia tsuruhatensis*, *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Aerosakkonema fusiforme*, *Bacteroides ovatus*	PRJNA603234 deposited in SRA repository	Significant difference between vaginal and endometrial microbiome, with higher number of species and biodiversity in endometrium *Lactobacillus* was exclusively detected in the group that displayed unsuccessful IVF *Kocuria dechangensis* was significantly predominant in endometrium among women who did not achieve pregnancy in IVF
Kadogami *et al.* (2020)	RIF (N = 392); avg 38.6 y; Asian	Endometrial fluid collected using Pipette IV (stabilising medium)	Removal of the mucous from cervix; Negative controls: UltraPure™ Nase/RNase-Free Distilled Water; Positive control: ZymoBIOMICS Microbial Community Standard	Agencourt Genfind v2 Blood and Serum DNA Isolation Kit; Illumina MiSeq® (V4)	*Lactobacillus*, *Bifidobacterium*, *Gardnerella*, *Atopobium*, *Streptococcus*, *Prevotella*	NR	*Lactobacillus*/*Bifidobacterium* presence associated with the stage of follicular development Increasing *Lactobacillus* colonisation during the luteal phase Combination of a vaginal probiotic suppository and antibiotics may represent an effective treatment for endometrial health Contaminants: *Acidovorax*, *Acinetobacter*, *Chryseobacterium*, *Citrobacter*, *Elizabethkingia*, *Escherichia*, *Flavobacterium*, *Janthinobacterium*, *Leptothrix*, *Methylobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Yersinia*

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Study	Sample (N); age (y); ethnicity/race	Sampling technique (storage)	Controlling contamination	DNA extraction and sequencing platform (hypervariable regions); validation/quantification	Top identified taxa	Raw data availability	Main findings (including identified contaminants)
Carosso *et al.* (2020)	IVF (N = 15); avg 35.1 y; Caucasian	Guardia® Access catheter (−80°C)	Negative controls: blank controls	Cell lysis and DNA purification (Agencourt AMPure® XP Reagent); Illumina MiSeq® (V3-4-6)	*Lactobacillus*, *Gardnerella*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Atopobium*, *Delftia*, *Pelomonas*, *Veillonella*, *Escherichia coli*/*Shigella*	PRJNA634237 available at NCBI BioProject	Biodiversity was greater in the endometrial microbiome than in the vagina Biodiversity was significantly greater after COS when compared with pre-COS in the same patients Contaminants: *Sphingomonas*, *Arthrobacter*, *Renibacterium*
Chen *et al.* (2020)	Control group (infertility, leiomyoma, ovarian borderline tumour or teratoma) (N = 2); avg 36.07 y; Asian	Vacuum suck tube (−80°C)	Cervical canal was sterilised with iodine	CTAB/SDS method; Illumina HiSeq 2500® (V3-4)	*Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Atopobium*	http://atm.amegroups.com/article/view/54025/38885 (available from June 2021)	The outcome of the PCR from uterine samples was barely satisfactory No valid evidence of uterine sterility
Lu *et al.* (2020)	Endometrial cancer (N = 25); 18–75 y; Benign condition (N = 25); Asian	Endometrial biopsy at hysterectomy (−80°C)	Negative control: blank buffer control	SDS method; Illumina HiSeq® (V3-4)	*Rhodococcus*, *Phyllobacterium*, *Sphingomonas*, *Bacteroides*, *Bifidobacterium*	Available from the corresponding author on reasonable request	Significant differences in alpha and beta diversities between cancer and benign condition groups Increased abundance of *Micrococcus* in endometrial cancer group

The systematic literature search was performed up to 5 January 2021 using PubMed, Web of Science and Cochrane databases, and was registered with the International Prospective Register of Systematic Reviews (PROSPERO registration number: CRD42020161218). Keywords 'endometrium', 'endometrial fluid', 'uterus', 'uterine fluid' were paired with terms 'microbiome', 'microbiota', 'microbes', 'microorganisms'. A total of 15 292 studies were identified and following critical selection 119 manuscripts of endometrial microbiome studies were selected. Next, after careful abstract and full-text evaluation, 34 manuscripts remained eligible.

Avg, average; CE, chronic endometritis; COS, controlled ovarian stimulation; ENA, European Nucleotide Archive; EP, endometrial polyps; ET, embryo transfer; NR, non reported; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss; spp., species; SRA, Sequence Read Archive.

*The first metagenomics study performed in human endometrium.

Lactobacillus does not dominate the uterine cavity, and bacteria such as *Pseudomonas*, *Acinetobacter*, *Vagococcus* and *Sphinogobium* constitute a notable fraction of the endometrial microbiome, contradicting the findings of *Lactobacillus*-dominance in the uterus in studies using transcervical sampling method (Moreno *et al.*, 2016; Kyono *et al.*, 2018; Hashimoto and Kyono, 2019). Even more, 40% of the endometrial samples collected from abdominal hysterectomy did not present any detectable uterine microbiome above the negative controls (Winters *et al.*, 2019), which adds to the ongoing debate whether there is in fact a unique endometrial microbiome in all women, and whether the detected bacterial sequences refer to tourists, residents, invaders or contamination.

Difficulty in establishing the endometrial core microbiome is further hindered by the fact that the original works performed so far are barely comparable (see Table I), with different study protocols and several other limitations, leaving us far from drawing any conclusions on the composition and role of the microbial communities in the endometrium in health and disease. It is important to be meticulous in designing, analysing and interpreting studies of the endometrial microbiome, as many factors starting from patient selection, sampling methods and handling, laboratory experiments, statistical analyses and other confounding factors can lead to potential bias and hamper study validity, reliability and generalisability (Molina *et al.*, 2020). There is an urgent need for standardised methodologies and data processing of the obtained results in the fast-growing field of endometrial microbiome in order to improve comparability of studies and facilitate meta-analyses. With this review, we give an overview of the methodology that is currently used to study the endometrial microbiome and highlight factors that can influence studies in niches with low microbial abundance, as is the endometrium. Furthermore, we aim to provide recommendations for conducting well-designed, clinically relevant studies with detailed metadata in order to adequately analyse and explore the clinical implications of reproductive tract exposure to microorganisms and to ensure that quality research in endometrial microbiome studies is undertaken.

Considerations on study design

Designing an experiment that generates meaningful data is the first important step. Differences in microbial community structure, composition and genetics or function between separate cohorts (case-control or cross-sectional studies) or over time (longitudinal studies) can be studied in the context of endometrial microbiome. Prospective studies should aim to collect the samples at identical time points as well as sequential samples from the same individual at different time points (Knight *et al.*, 2018). For instance in the gut, microbial community instability rather than the specific taxa present at a single time point has been considered as a predictor of a disease (Knight *et al.*, 2018).

Adequate sample size is another important point. Majority of the endometrial microbiome studies performed to date are underpowered, including on average 30–60 participants (Table I). Statistical power and effect size analyses are a challenge, and proper methods such as Dirichlet Multinomial, PERMANOVA or random forest analyses should be applied in order to determine technical variability and to obtain true biological results (Knight *et al.*, 2018; Qian *et al.*, 2020).

The next important aspect to consider is the reproducibility of the studies. Similar microbiome studies can produce different results, and without detailed documentation of the study population, sample type, collection method, data processing and analysis workflow, i.e. the creation of metadata, it is difficult to reproduce the data (Jurburg *et al.*, 2020). The recorded metadata would ensure that as much variability as possible is accounted for, and it should be made publicly available together with the raw data. However, metadata deposited in repositories (e.g. sequence read archive) are not standardised, creating difficulty for sample reanalysis (Kasmanas *et al.*, 2021). We also recommend the researchers to follow the Genomic Standards Consortium minimum information standards for marker genes (MIMARKS) and metagenome (i.e. sequencing of microbiome) analyses (MIMS) (Yilmaz *et al.*, 2011; Quiñones *et al.*, 2020), or other initiatives such as BioProject and BioSample project, which outline the minimum necessary information about a metagenomic sample (Kasmanas *et al.*, 2021).

Considerations on study population

Accurate selection of patients and controls is the next crucial step in planning a quality research project, where it is important to consider endometrial microbiome dynamics and factors that could influence the uterine environment (see review for factors by (Molina *et al.*, 2020)). It is still debatable whether endometrial microbiome changes throughout the menstrual cycle or not (Altmäe, 2018). Some studies have detected that endometrial microbiome is highly stable throughout the menstrual cycle (Khan *et al.*, 2016; Moreno *et al.*, 2016; Kyono *et al.*, 2018), while other groups have found significant differences in the composition of uterine microbes between proliferative, secretory and menstrual phases (Chen *et al.*, 2017; Pelzer *et al.*, 2018; Kadogami *et al.*, 2020; Sola-Leyva et al., 2021). Clearly, more investigation into identification of the uterine 'baseline' microbial continuum along the menstrual cycle is warranted.

The selection of proper control/patient groups depends on the specific research question; nevertheless, a detailed metadata information is required for all participants in order to have comparable study groups and to control for possible confounding factors. Furthermore, nullipara, para and multipara women should be distinguished, as it has been shown that pregnancy and childbirth can influence microbial composition of the female reproductive tract (Koedooder *et al.*, 2019).

Considerations on endometrial sampling and storage

The biggest concern regarding endometrial sampling for microbiome analysis is the extremely high risk of contamination from lower genital tract. Sampling during invasive surgical procedures, such as hysterectomy and laparoscopy, avoids contamination with microbes from the vagina and/or cervix, however, these samples are usually obtained from women with an existing medical condition and from peri- and

postmenopausal women, thus the results are not necessarily applicable to healthy reproductive age women.

In addition to the conventional endometrial sampling devices such as Pipelle and swabs, a few uterine sampling methods have been adapted for microbiome studies in order to minimise the risk of contamination: double-sheathed embryo transfer catheter (Franasiak et al., 2016), intrauterine insemination catheter (Kyono et al., 2018) and a transcervical sheathed brush device (Verstraelen et al., 2016). Like with every step in a study protocol, effort should be made to collect samples in a standardised manner throughout the study for minimising unintentional variability. A recent report compared microbial colonisation in endometrial fluid and endometrial tissue samples that were collected simultaneously and concluded that the microbiome composition in the fluid does not fully reflect that in the tissue (Liu et al., 2018).

Once collected, storage and transport of the samples under bacteriostatic conditions are critical, as sample handling and treatment can influence the composition of a microbial community (Karstens et al., 2018; Fricker et al., 2019). A quality microbiome study should also record sampling and storing steps in metadata collection. Direct freezing of the collected samples at –80°C is considered as the reference method in microbiome studies (Koedooder et al., 2019). A comparative study on gut showed that there were no considerable differences between 16S rRNA gene sequencing outcomes from fresh samples, samples frozen at –80°C, or samples that were snap-frozen on dry ice and then at −80°C (Fouhy et al., 2015). Of note, snap-freezing reduces ice crystal formation in the sample compared with direct freezing at −80°C, thus retaining a better cell integrity (Fouhy et al., 2015).

In a clinical setting, however, –80°C freezer is often not available and different storage buffers/media could serve as alternatives that can stabilise nucleic acids at 4°C or even at room temperature for days (Karstens et al., 2018). Certain concentrated buffered solutions such as RNAlater, PSP stabilisation buffer, Allprotect Tissue Reagent or medium that stabilises microbes and maintains RNA and DNA integrity of bacteria and viruses (e.g. stabilisers by COPAN Diagnositics, DNA Genotek, Norgen Biotek and others) are available in market. Microbial community analysis from samples stored in RNAlater, however, has shown the least similar bacterial communities compared to fresh samples and the samples stored at −80°C (Pollock et al., 2018). Another study compared the use of six storage solutions (Norgen, OMNI DNA Genotek, RNAlater, CURNA, HEMA and Shield) and found that samples collected in Norgen and OMNI showed the least shift in community composition relative to −80°C standards compared with other storage media (Chen et al., 2019).

In short, different endometrial microbiome studies using different sampling and storage methods (Table I) are hardly comparable and the field is yearning for meticulously designed large cohort studies specifically adapted to the low microbial biomass niche.

Detection of microbiome

Technical variation among experimental methods ranging from nucleic acid extraction to sequencing is high (Fricker et al., 2019). Commercial kits for microbial nucleic acid isolation show different efficacy in lysing specific microbes, which can impact the observed microbial patterns (Pollock et al., 2018). Different methods for detecting microbial communities including marker gene (prokaryotic 16S rRNA and fungal internal transcribed spacer, ITS), metagenome and metatranscriptome sequencing (see Fig. 1 for illustration of detection techniques) can also give rise to diverse results (Knight et al., 2018). All these detection methods have their strengths and limitations (see Table II), and the choice of method would depend on the study question, design and budget.

Marker gene analysis

The most commonly used marker gene is 16S rRNA gene (Fig. 1). Despite 16S rRNA gene being specific to bacteria and archaea, additional marker genes for eukaryotic microorganisms, like fungi, exist 18S rRNA gene, the 28S rRNA gene and the ITS (Fricker et al., 2019).

Marker gene amplification and sequencing is well-tested, fast and cost-effective for attaining a low-resolution information of microbial communities. This method is especially suitable for samples contaminated by host DNA (Knight et al., 2018) such as tissues and low microbial biomass samples as is the endometrium. However, different methodological considerations should be considered when opting for this detection method (Table II). In the endometrial studies, the most frequently used 16S rRNA hyper-variable region is V4 (Table I). A study comparing V1-2 and V3-4 hyper-variable regions when analysing vaginal microbiome concluded that V3-4 identifies more taxa and displays higher diversity than the V1-2 region, since V1-2 region failed to identify some important species harbouring the female lower genital tract (Graspeuntner et al., 2018).

Despite several limitations in marker gene analysis (Table II), this approach is the most commonly used and preferred for the low microbial biomass microbiome studies today (Knight et al., 2018; Liu et al., 2020). In fact, all endometrial microbiome studies conducted so far are based on the marker gene analysis, except for the preliminary analysis of the five endometrial samples where the whole metagenome analysis approach has been applied (Li et al., 2018; Garcia-Grau et al., 2019; Moreno et al., 2020) (Table I).

Whole metagenome analysis

This approach yields more detailed genomic information and taxonomic resolution than the marker gene sequencing method, capturing all microbial genomes present in the sample, including viral and eukaryotic DNA (Liu et al., 2020; Qian et al., 2020) (Fig. 1; strengths and limitations in Table II). As the metagenomics field matures, the current limitations (especially the annotation steps) will continue to improve.

The preliminary results in metagenome analysis have identified different taxonomies of bacteria, archaea, fungi and viruses within the endometrial samples (Li et al., 2018); however, greater depth of sequencing, better reference database and more sample material are required in order to meet the full coverage scale. Undoubtedly, assessing microorganisms other than bacteria in endometrial/uterine health and disease is an important future research area.

Metatranscriptome analysis

Unlike marker gene and metagenomic sequencing, where DNA sequences in a sample are analysed regardless of the cell viability or activity, metatranscriptomics uses RNA sequencing to profile transcripts of microorganisms to provide information of gene expression

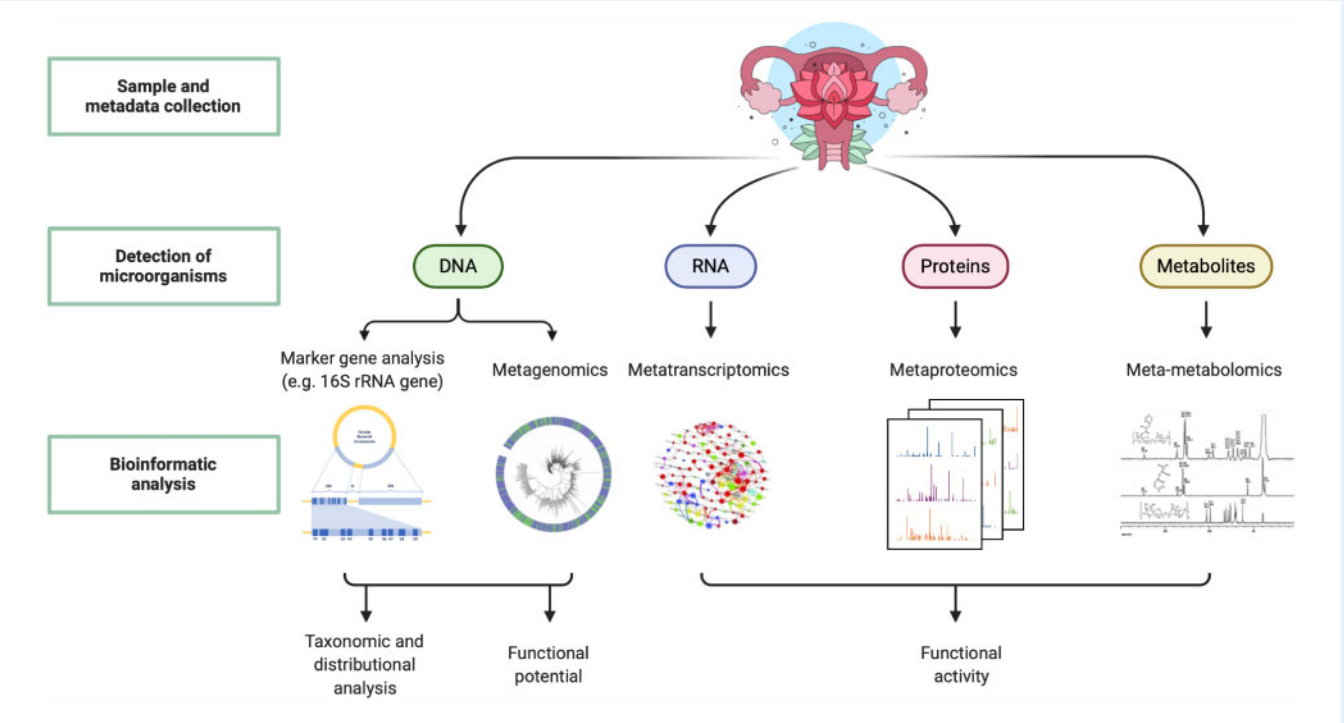

Figure 1. Different techniques for studying human microbiome: marker gene analysis, whole genome sequencing, metatranscriptomic sequencing, metaproteome and meta-metabolome analyses. Marker gene analysis targets a specific sequence of a gene in order to identify microbial phylogenies in a sample. The most commonly used marker gene is 16S rRNA gene. The 16S and 23S rRNA genes are highlighted together with the ITS region. V1–9 marks the hyper-variable regions, with the conserved regions between them. Whole metagenome sequencing analysis (metagenomics) consists of sequencing of all genes and genomes in a microbial community, which does not depend on amplifying and sequencing specific taxonomically informative genes. Metagenome represents the genetic/functional potential of the microbes, not the actual activity of the community. To analyse the functional activity (metatranscriptomics), the isolation of messenger RNA (mRNA) is necessary. It is also possible to characterise the function of microbial communities through the analysis of proteins expressed by microorganisms (metaproteomics), which offers better knowledge of the intricate host–microbiome interactions. Generally, metatranscriptome and/or metaproteome studies are complemented by identification and quantification of chemical compounds present in the sample (meta-metabolomics) in order to correlate gene expression to the metabolite profiles.

and the functional activity of the microbiome (Knight *et al.*, 2018). One of the biggest challenges for metatranscriptome analysis is the identification and removal of the host ribosomal sequences (>95% of extracted bacterial RNA is non-coding rRNA and thus not informative for active expression) in order to enrich the microbiome transcripts (see Table II for pros and cons).

Recently, the first endometrial metatranscriptomic analysis revealed that endometria of healthy women harbour more than 5000 functionally active microorganisms (Sola-Leyva et al., 2021). This study also demonstrated that several bacteria, viruses and archaea are differentially regulated along the menstrual cycle and these cyclical changes could be associated with metabolic activity in the host-microbiota crosstalk during receptive phase endometrium (Sola-Leyva et al., 2021).

Computational considerations

Microbiome data analysis methods are quickly advancing, and computational considerations and challenges are evolving alongside them. Several platforms and algorithms exist, with a major issue being their ability to guarantee proper alignment and fidelity of the data while successfully subtracting contamination and background noise (Callahan *et al.*, 2017). The recommendations regarding differential abundance testing using amplicon sequence variants (ASVs) over operational taxonomic units (OTUs) have advanced especially fast (Knight *et al.*, 2018). ASV methods deduce the biological sequences in the sample before amplification and sequencing errors, and distinguish sequence variants differing by as little as one nucleotide (Callahan *et al.*, 2017). These new ASV methods are expected to replace OTUs as the unit of analysis.

Many pipelines are available today to process and analyse 16S rRNA gene sequencing data, where QIIME, QIIME 2, UPARSE, USEARCH, VSEARCH and Mothur are the most commonly used (Liu *et al.*, 2020). An additional factor that could influence the resolution of the analysis is database selection for taxon identification (Knight *et al.*, 2018). Different curated databases of 16S rRNA gene sequences exist, such as Greengenes, SILVA, NCBI and Ribosomal Database Project (RDP) classifier (Karstens *et al.*, 2018). The last three contain taxonomic information for the domains of Bacteria, Archaea and Eukarya,

Table II High-throughput techniques to analyse the endometrial microbiome.

Technique	Function	Advantages	Limitations
Marker gene analysis (e.g. 16S rRNA gene sequencing)	Amplification and sequencing of the 16S rRNA gene—a specific hyper-variable region or a set of contiguous hyper-variable regions	Relatively rapid, simple and affordable technique Classifies microorganisms to taxonomic classification Correlates well with genomic content Applicable to many sample types and study designs Adaptable to low-biomass and highly host-contaminated samples (e.g. endometrial tissue) Requires lower quantity of DNA input (≈ 50 ng)	Requires a priori knowledge of microbial community Limited to explore microbial diversity Taxonomical resolution typically limited to genera level Some bacteria contain several copies of 16S rRNA Indistinguishable species (some species have <0.5% sequence divergence) Limited identification of variant strains (does not contemplate horizontal gene transfer) Different hyper-variable regions yield different results, making comparisons between studies challenging Technical bias where some species are amplified more than others Limited functional analyses No information about bacteria viability Chimeric errors DNA sequencing errors Contaminant errors
Whole metagenome (shotgun) analysis	Breaks the full DNA content into small constant fragments that can be sequenced and aligned to a reference catalogue	Captures the full genetic information (bacteria, archaea, viruses, eukaryotic microorganisms), including that of unknown species Does not require knowledge of microbial community No PCR-related biases Allows direct quantification of microbial gene abundance and microbial genome reconstruction Predicts the potential function of the microbiota based on their genomes Enables a more specific taxonomic resolution at the species level Identification of strains Can estimate *in situ* growth rates for target organisms	More expensive and laborious Requires complex bioinformatics and biostatistics pipelines Assembly artefacts Requires higher quality and higher amounts of DNA (> 1 μg) Requires removal of host and ribosomal sequences Just gives information about the potential functionality Huge amounts of data produced (specific information is diluted) Lacking universal reference databases Difficult quantification of a particular organism in a sample Viruses and plasmids are not typically well annotated by default pipelines No information about bacteria viability
Metatranscriptome analysis (meta-RNAseq)	Sequencing of the transcribed RNA in a microbial community after undergoing samples to microbial rRNA and host nucleic acid removal	Directly evaluates microbial functional activity Informs about the viability/dormancy of microorganisms Represents a link between the metagenome and community phenotype Quantifies gene expression level, detecting genes that are differentially expressed/regulated Captures dynamic intra-individual variations	Expensive and laborious technique Difficult to maintain the resemblance of the *in situ* expression levels Abundance of the microorganisms and their gene expression levels can exhibit variation in transcript levels Methods used for single-organism transcriptomics cannot be applied Host RNA and microbial rRNA must be removed (ribodepletion, mRNA enrichment) Requires creating a complex and proper reference library Requires paired DNA sequencing to decouple transcription rates from bacterial abundance changes

(continued)

Table II Continued

Technique	Function	Advantages	Limitations
			Different strains, or even different species, can maintain a similar functional profile Data are biased towards organisms with high transcription rates
Metaproteome analysis	Shears the peptides into small fragments that undergo a mass spectrometry and the resulting amino acid sequence is compared to a reference databases	Informs about taxonomic distribution Characterises all gene translation products (sequential variants resulting from splicing processes) Provides insights on post translational modification (identification of alterations in structure) Informs about the viability/dormancy of microorganisms Analyses interactions between species present in a community Informs about protein stability and localisation Identifies habitat-specific functions covered by the community	Time-consuming methodology High sensitivity to sample impurities Subject to inefficient chemical labelling leading to compromised biological coverage Does not provide information on protein abundance Requires an environment-specific protein database Requires high amount of protein biomass from samples
Meta-metabolome analysis	Examines metabolites through liquid/gas chromatography, mass spectrometry and nuclear magnetic resonance and metabolomic data are compared to spectral databases	Analyses of numerous metabolites in a given sample High predictive capacity for phenotype Resolution of microbial metabolic products/signalling molecules	High cost Rather emerging (currently not possible to translate all the data produced into a meaningful biological context) Databases may contain low-quality reference mass spectrometry spectra Enormous chemical diversity hinders molecular identification Rapid turnover of metabolites Difficult to determine whether a metabolite was produced by the host or by the microbiome

rRNA, ribosomal RNA; mRNA, messenger RNA.

while Greengenes is dedicated to Bacteria and Archaea (Balvočiūtė and Huson, 2017). SILVA is the most updated and largest database, allowing identification at the species level in a good number of taxa (Balvočiūtė and Huson, 2017), and is commonly used for 16S rRNA gene studies. However, one should consider using the NCBI database in studies that perform both targeted 16S rRNA gene sequencing and shotgun sequencing (Balvočiūtė and Huson, 2017).

For further reading about bioinformatics tools and how they can be applied for targeted sequencing, metagenomic and metatranscriptomic analyses please see previous comprehensive reviews (Knight *et al.*, 2018; Liu *et al.*, 2020; Qian *et al.*, 2020). In the last years, several initiatives have arisen to harmonise metadata from multiple human metagenomic studies in a single standardised database in order to readily filter the data for samples of interest, including standardised attributes describing host characteristics, sampling site and/or condition information. Specifically, HumanMetagenomeDB aims to explore possibilities of human metagenomes from different body sites (e.g. gut, skin, vagina, however, not including uterus) in a user-friendly interface, leading to accurate meta-analyses (Kasmanas *et al.*, 2021). Furthermore, Vaginal Microbiome Consortium is creating a specific database for vaginal samples (http://vmc.vcu.edu/vahmp) and similar activities are strongly encouraged for endometrial samples.

Combating contamination

It is clear that low-biomass microbial niches such as uterus are sensitive to contamination and data misinterpretation. Two fundamental types of contamination can arise in microbiome studies, contaminant DNA and cross-contamination (Eisenhofer *et al.*, 2019). To start with, the contaminant DNA can originate from the technique of endometrial sampling, but also from laboratory environment, plastic consumables, researchers and reagents (Eisenhofer *et al.*, 2019). Many groups have demonstrated that laboratory stocks used in processing and analysing DNA are contaminated with bacterial DNA—jointly termed as 'kitome' (unsurprising as many enzymes/compounds in them are derived from bacteria) (Kim *et al.*, 2017). The kitome varies between kits, and can even be different between different lots of the same kit

Table III List of contaminant genera most commonly identified in human microbiome studies.

Phylum	List of contaminant genera
Proteobacteria	Alpha-proteobacteria: *Afipia*,[a] *Aquabacterium*,[b] *Asticcacaulis*,[b] *Aurantimonas*,[a] *Beijerinckia*,[b] *Bosea*,[b] *Bradyrhizobium*,[a] *Brevundimonas*,[a] *Candidatus*,[a] *Caulobacter*,[a] *Chelatococcus*,[a] *Craurococcus*,[b] *Devosia*,[a,b] *Gluconobacter*,[a] *Hoeflea*,[b] *Mesorhizobium*,[a] *Methylopila*,[a] *Methylobacterium*,[a,b] *Novosphingobium*,[a,b] *Ochrobactrum*,[a] *Pannonibacter*,[a] *Paracoccus*,[b] *Pedomicrobium*,[b] *Phenylobacterium*,[a] *Phyllobacterium*,[a] *Pseudochrobactrum*,[a] *Rhizobium*,[b] *Rhizomicrobium*,[a] *Roseomonas*,[a] *Rubellimicrobium*,[a] *Ruegeria*,[a] *Sphingobium*,[a,b] *Sphingomonas*,[a,b] *Sphingopyxis*,[b] *Telmatospirillum*[a] Beta-proteobacteria: *Achromobacter*,[a] *Acidovorax*,[a] *Alicycliphilus*,[a] *Azoarcus*,[b] *Azospira*,[b] *Burkholderia*,[a] *Comamonas*,[a,b] *Cupriavidus*,[a,b] *Curvibacter*,[a] *Delftia*,[b] *Duganella*,[b] *Herbaspirillum*,[a] *Janthinobacterium*,[a] *Kingella*,[a] *Leptothrix*,[a] *Limnobacter*,[b] *Limnohabitans*,[a,b] *Massilia*,[a] *Methylophilus*,[b] *Methyloversatilis*,[b] *Neisseria*,[a] *Oxalobacter*,[a] *Parasutterella*,[a] *Pelomonas*,[a] *Polaromonas*,[b] *Ralstonia*,[a,b] *Roseateles*,[a] *Schlegelella*,[a] *Sulfuritalea*,[a] *Tepidimonas*,[a] *Undibacterium*,[b] *Variovorax*,[a] *Vogesella*[a] Gamma-proteobacteria: *Acinetobacter*,[a,b] *Alcanivorax*,[a] *Cardiobacterium*,[a] *Citrobacter*,[a] *Dokdonella*,[a] *Dyella*,[a] *Enhydrobacter*,[a] *Enterobacter*,[a] *Escherichia*,[a] *Haemophilus*,[a] *Halomonas*,[a] *Klebsiella*,[a] *Lysobacter*,[a] *Nevskia*,[b] *Pseudomonas*,[a,b] *Pseudoxanthomonas*,[b] *Psychrobacter*,[b] *Serratia*,[a] *Shewanella*,[a] *Stenotrophomonas*,[a] *Xanthomonas*,[b] *Yersinia*[a]
Actinobacteria	*Actinomyces*,[a] *Aeromicrobium*,[b] *Agrococcus*,[a] *Arthrobacter*,[a] *Atopobium*,[a] *Beutenbergia*,[b] *Bifidobacterium*,[a] *Blastococcus*,[a] *Brevibacterium*,[a] *Collinsella*,[a] *Corynebacterium*,[a] *Curtobacterium*,[b] *Dermacoccus*,[a] *Dietzia*,[a] *Eggerthella*,[a] *Geodermatophilus*,[b] *Gordonia*,[a] *Janibacter*,[b] *Kocuria*,[b] *Microbacterium*,[a] *Micrococcus*,[a,b] *Microlunatus*,[a] *Patulibacter*,[b] *Pilimelia*,[a] *Propionibacterium*,[a] *Pseudoclavibacter*,[a] *Renibacterium*,[a] *Rhodococcus*,[a] *Rothia*,[a] *Slackia*,[a] *Tsukamurella*,[b] *Zimmermannella*[a]
Firmicutes	*Abiotrophia*,[a] *Alicyclobacillus*,[a] *Anaerococcus*,[a] *Anaerotruncus*,[a] *Bacillus*,[a] *Blautia*,[a] *Brevibacillus*,[a] *Brochothrix*,[b] *Catenibacterium*,[a] *Christensenella*,[a] *Clostridium*,[a] *Coprococcus*,[a] *Dialister*,[a] *Dorea*,[a] *Enterococcus*,[a] *Erysipelatoclostridium*,[a] *Eubacterium*,[a] *Exiguobacterium*,[a] *Facklamia*,[a] *Faecalibacterium*,[a] *Fastidiosipila*,[a] *Finegoldia*,[a] *Flavonifractor*,[a] *Gemella*,[a] *Geobacillus*,[a] *Granulicatella*,[a] *Halocella*,[a] *Intestinibacter*,[a] *Johnsonella*,[a] *Lachnoanaerobaculum*,[a] *Lachnoclostridium*,[a] *Lachnospira*,[a] *Lactobacillus*,[a] *Lactococcus*,[a] *Leuconostoc*,[a] *Megasphaera*,[a] *Moryella*,[a] *Oscillospira*,[a] *Paenibacillus*,[a] *Papillibacter*,[a] *Parvimonas*,[a] *Peptococcus*,[a] *Peptoniphilus*,[a] *Pseudobutyrivibrio*,[a] *Pseudoflavonifractor*,[a] *Quinella*,[a] *Roseburia*,[a] *Ruminiclostridium*,[a] *Ruminococcus*,[a] *Sanguibacter*,[a] *Selenomonas*,[a] *Solobacterium*,[a] *Staphylococcus*,[a] *Streptococcus*,[a] *Thermicanus*,[a] *Trichococcus*,[a] *Tumebacillus*,[a] *Turicibacter*,[a] *Tyzzerella*,[a] *Veillonella*[a]
Bacteroidetes	*Alistipes*,[a] *Bacteroides*,[a] *Capnocytophaga*,[a] *Chryseobacterium*,[a] *Cloacibacterium*,[a] *Dyadobacter*,[b] *Elizabethkingia*,[a] *Filimonas*,[a] *Flavobacterium*,[a] *Fluviicola*,[a] *Hydrotalea*,[a] *Hymenobacter*,[a] *Niastella*,[b] *Olivibacter*,[b] *Parabacteroides*,[a] *Pedobacter*,[a] *Porphyromonas*,[a] *Prevotella*,[a] *Sediminibacterium*,[a] *Sphingobacterium*,[a] *Wautersiella*,[a] *Xylanibacter*[a]
Deinococcus-Thermus	*Deinococcus*,[a] *Meiothermus*[a]
Fusobacteria	*Fusobacterium*,[a] *Leptotrichia*[a]
Spirochaetes	*Leptospira*[a]
Acidobacteria	Predominantly unclassified *Acidobacteria* Gp2 organisms[a]

The listed genera were detected in sequenced negative controls, consisting of DNA extraction blanks from different DNA extraction kits, and no-template PCR amplification controls from PCR kits (Salter *et al.*, 2014; Glassing *et al.*, 2016; Kyono *et al.*, 2018; Eisenhofer *et al.*, 2019; Hashimoto and Kyono, 2019; Kitaya *et al.*, 2019; Weyrich *et al.*, 2019; Winters *et al.*, 2019; Carosso *et al.*, 2020; Kadogami *et al.*, 2020).
Source of contamination: [a]Extraction blank control; [b]No-template amplification control.

(Salter *et al.*, 2014; Glassing *et al.*, 2016). Thus, it is strongly recommended to process all samples side by side using the same batches of reagents. In fact, over 100 common contaminant taxa have been detected in DNA extraction blank controls and no-template controls across many studies (Table III). Cross-contamination is another challenge during microbiome sample processing and can originate from other samples and sequencing runs (Eisenhofer *et al.*, 2019). It has been demonstrated that lower levels of microbial DNA within low-biomass samples enable contaminant DNA and cross-contamination to outcompete and dominate the biological signal within the sample (Salter *et al.*, 2014; Glassing *et al.*, 2016).

Regardless of the study approach, appropriate controls are vital and mandatory for microbiome studies. Low-biomass environments, such as the endometrium, require controls that have gone through the entire sampling process in order to fully detect contaminants and to distinguish the low-abundance microorganisms that truly originate from the sampling site (Salter *et al.*, 2014; Benner *et al.*, 2018; Qian *et al.*, 2020). Three types of negative controls are recommended to adequately detect contaminants: (i) sampling blank control, (ii) DNA extraction blank control and (iii) no-template amplification control (Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019). As for the positive controls, two types are recommended to use for determining the limit of detection and to evaluate the effect of cross-contamination during the sample processing: (i) DNA extraction positive control and (ii) positive amplification control (Eisenhofer *et al.*, 2019). A single known microbial isolate or a mock community (known mixture of microbial species or a synthetic non-biological DNA) could serve as positive controls (Kim *et al.*, 2017; Karstens *et al.*, 2018; Pollock *et al.*, 2018).

The contaminant taxa must be addressed in the final analysis and interpretation of the results. As biomass decreases, the influence of contaminating sequences becomes more evident (Weyrich *et al.*, 2019; O'Callaghan *et al.*, 2020). Three different strategies for assessing the impact of contamination in the microbiome datasets are available: (i) comparison of controls to biological samples, (ii) subtracting contaminants from biological samples (e.g. Decontam, microDecon) and (iii)

Table IV The current good practice recommendations for performing endometrial microbiome studies.

Study design	• Set study hypothesis • Define study type (e.g. case-control, longitudinal)
Study population	• Calculate sample size and power (Dirichlet Multinomial, PERMANOVA, random forest analyses) • Specify participant inclusion/exclusion criteria • Select and define control group • Define confounding factors (e.g. age, medical history, medication, ethnicity, BMI, diet, sexual habits, pregnancy) • Create metadata (detailed information of participants, sample collection and storage, microbiome detection and analysis)
Endometrial sampling	• Record cycle day for sampling as microbiome can change throughout the cycle • Using adapted sampling methods (e.g. double-sheathed catheter, sheathed brush device)—currently, the best method as reduces cervical/vaginal bacterial contamination risk and minimally invasive • Using surgical procedures (hysterectomy, laparoscopy) avoids transcervical/vaginal bacterial contamination, but limitation with obtaining samples from healthy controls • Using transcervical sampling (e.g. Pipelle)—the most commonly used technique, but high bacterial contamination risk from cervix/vagina • Include negative controls (e.g. from gloves, unused surgical tools, swab of air)—allows adequate monitoring of contaminants throughout sample collection and handling
Sample storage	• Snap-freezing and direct freezing of samples at –80°C are the reference methods (alternative is to use storage media that stabilises nucleic acids at higher temperatures) • Avoid repeated freezing-thawing • Record sampling and storage protocols in metadata
Detection of microbiome	• Adequate cell lysis and host DNA elimination are crucial in extracting microbial DNA—one of the recommended kits today for low microbial biomass site is QIAamp DNA Microbiome Kit (Bjerre *et al.*, 2019; Heravi *et al.*, 2020) • Include positive controls (e.g. bacterial mock community) • Include negative controls (e.g. blank sample) • Report in detail DNA extraction method and controls • Assess microbial DNA quality and quantity (e.g. spectrophotometry) • 16S rRNA marker gene analysis is the recommended method for low microbial biomass site analysis as is endometrium • Sequencing 16S rRNA gene hyper-variable regions 3-4 is the most commonly used and has been shown to be more sensitive over V1-2 • Utilise the same protocols throughout the study
Microbiome data analysis	• Process and analyse sequencing data with updated methods (e.g. QIIME2, Mothur) • Use amplicon sequence variants (ASV) over operational taxonomic units (OTU) • SILVA is the most updated and largest database for alignment—commonly used for 16S rRNA gene studies • Remove contaminant sequences *in silico* (e.g. Decontam, microDecon, Sourcetracker) • Predict microbial functional activity (PICRUSt, Tax4Fun, BugBase) • Report in detail the analysis steps
Data validation	• Quantify detected bacteria (e.g. flow-cytometry, qPCR, spiking of exogenous bacteria into crude samples) • Validate in separate cohort
Data presentation	• Deposit raw data and metadata to public database (e.g. SRA repository, European Nucleotide Archive) • Characterise and report contaminant microbes • Publish in detail the study protocols • Address limitations/strengths of the study

using predictive modelling for detecting contaminants (e.g. Sourcetraker) (see Eisenhofer *et al.* (2019) for further reading).

To sum up, contamination has been and will be one of the biggest combats in analysing endometrial microbes and it is clearly challenging to control for all possible contaminants. Nevertheless, we should aim transparency in presenting study results, provide raw data together with metadata (also for negative/positive controls) and address the study limitations adequately.

Concluding remarks and future perspectives

Our understanding of the endometrial microbiome is still in its infancy and myriads of questions remain to be addressed. Whether the microbiome detected in the endometrium is merely the result of contamination and an artefact of the study design, and/or whether the microbes in the endometrium are tourists, invaders or residents need to be

established by large and well-designed, well-controlled and well-conducted studies.

Based on the currently available studies' designs, no conclusive information on microbial detection and distribution of a 'core uterine microbiome' is available. Before the presence of a microbiome can be attributed to a disease risk and pathogenesis, and before any treatment options for bacterial 'dysbiosis' are offered for patients (Haahr et al., 2020), acquisition and development of the 'normal' microbiome must be well established (Chu et al., 2019). To that point, the role of the endometrial microbiome and/or microbiota is not yet established, and the nature of the relationship between the endometrial microbiome and the function of the female reproductive tract remains an open issue. Furthermore, a sole presence of a microbial DNA sequence does not equate to the presence of a live microorganism, and the question of the viability of microbes detected by the next-generation sequencing (NGS) requires further research (Benner et al., 2018; Altmäe et al., 2019). DNA sequences can represent microbial breakdown products (e.g. DNA from dead microbes) or background DNA contamination (Kim et al., 2017), thus detection of a nucleotide sequence of a bacteria is not the same as the identification of a living microorganism; DNA can be used to characterise a microbiome but not to establish its existence (Salter et al., 2014; Glassing et al., 2016; Kim et al., 2017).

Although the functional activity of a given community could be predicted through analysing the microbial gene content (e.g. PICRUSt), future analyses of the expressed proteins within a microbial community (i.e. metaproteome) and characterisation of metabolites generated by microbes (i.e. meta-metabolome) (see Fig. 1 for methods) would further unravel the complexity of microbial functions in the endometrium.

As the endometrial microbiome field continues to rapidly expand, there is a great need for clear, concise and well-tested protocols starting with study planning and sample collection up to bioinformatic analyses. Three minimal standard requirements that researchers should follow for human microbiome studies have been proposed (Greathouse et al., 2019): (i) describe in detail nucleic acid extraction methods employed in the study so that all extraction procedures can be reproduced; (ii) include and describe negative and positive controls, and report contamination in blank samples; and (iii) follow the same study protocol along entire project. It is unlikely that the entire field will agree to the exact protocols and workflows, but scientists, reviewers and journals should aim to improve our current protocols and reporting criteria to ensure reliability and consistency between studies. With this review, we hope to provide the much-needed guidance, recommendations and best practices for designing and conducting endometrial microbiome studies and to draw attention to the weak points that could be improved or avoided in order to ensure that quality microbiome research in endometrial microbiome field is undertaken (see Table IV for the specific recommendations). This methodological review provides an initial framework to help to establish community guidelines and maximise the potential of this emerging and highly interdisciplinary field of research.

Data availability

The data underlying this article are available in the article, including Figure and Tables.

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Authors' roles

N.M.M. and S.A. conceived and drafted the original version of the article; A.S-L. and T.H. participated in the conception of the article; L.A., P.L. and J.A.C critically revised the article and approved the final draft.

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Conflict of interest

T.H. reports honoraria for lectures from Ferring, IBSA, Besins and Merck and a grant from Osel. T.H. is listed as an inventor in an international patent application (PCT/UK2018/040882). S.A. has received honoraria for lectures from Merck. The funder had no role in this study. No potential conflicts of interest were reported by the remaining authors.

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