International Doctoral Thesis / Tesis Doctoral Internacional

Microbial landscape in female reproductive health: unravelling its composition and host-microbe interactions

Atlas microbiano en la salud reproductiva femenina: descubriendo su composición y las interacciones huésped-microorganismo

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The bittersweet side of appreciating life's most precious moments is the unbearable awareness that those moments are passing.

– Mark Parents –

"No hay más." "Ya no hay más." ¡Se acabaron los ratones! El retrato del hombre de la barba, frente a mí, que lo vio todo y que libró al pueblo ibero de su inferioridad nativa ante la ciencia, escrutador e inmóvil, presidiendo la falta de cobayas. Su sonrisa comprensiva y liberadora de la inferioridad explica, comprende, la falta de créditos. Pueblo pobre, pueblo pobre. ¿Quién podrá aspirar otra vez al galardón nórdico, a la sonrisa del rey alto, a la dignificación, al buen pasar del sabio que en la península seca espera que fructifiquen los cerebros y los ríos?

– Luis Martín Santos –
Tiempo de Silencio. 1961.

A mis padres, por allanar previamente cada paso que doy

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LIST OF ABBREVIATIONS

AE-PCOS: Androgen Excess Society

AMPs: antimicrobial peptides

ANOVA: analysis of variance

BMI: body mass index

CE: chronic endometritis

Cis: confidence intervals

CSS: cumulative sum scale

DEGs: differentially expressed genes

EBERs: herpesvirus EBV-encoded non-coding small RNAs

ES-C: early-secretory controls

FC: fold change

FDA: Food and Drug Administration

FDR: false discovery rate

FMT: faecal microbiota transfer

GEO: gene expresión omnibus

GSEA: gene set enrichment analysis

GTEx: Genotype-Tissue Expression

HIV: human immunodeficiency virus

HMP: human microbiome project

iHMP: integrative human microbiome project

IQR: interquartile range

ITS: internal transcribed spacer

IVF: *in vitro* fertilization

MetaHIT: Metagenomics of the Human Intestinal Tract

MS-C: mid-secretory controls

MS-RIF: mis-secretory RIF

NGS: next generation sequencing

NIH: National Institutes of Health

PCA: principal component analysis

PCOS: polycystic ovary syndrome

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PROSPERO: International Prospective Register of Systematic Reviews

PRRs: pattern recognition receptors

RIF: recurrent implantation failure

rRNA: ribosomal RNA

SD: standard deviation

SMD: standardised mean difference

STORMS: Strengthening The Organization and Reporting of Microbiome Studies

T2D: type 2 diabetes

TLRs: Toll-like receptors

UMT: uterine microbiota transfer

VMT: vaginal microbiota transfer

ABSTRACT

The human body is colonised by more microbes than its own cells, and as more knowledge about the human microbiome is obtained, the clearer its significance in human physiology becomes. Most bacterial communities in humans coexist synergistically with their host. However, an imbalance in this relationship can lead to a disease. In the context of human reproductive health, it is well known that the female reproductive tract, specifically the vaginal milieu, possesses a highly active microbiota dominated by *Lactobacillus*. Despite the evidence, the complete influence of the microbes of adjacent sites and its association with female fertility remains still unclear. Recent studies have shown that the endometrium (i.e., the inner layer of the uterus) has its own microbial profile. Furthermore, it has been shown that microorganisms of the upper reproductive tract not only influence the functions of the uterus but may also play an important role in the embryo implantation and gynaecological complications such as recurrent implantation failure (RIF).

This Doctoral Thesis studies the relationship between the microbiome and the female reproductive health. The objectives of this Doctoral Thesis were: 1) to summarise the existing knowledge of the endometrial microbiome studies, the current treatments offered in the clinical setting and the future possibilities for modifying the uterine microbial composition; 2) to highlight the methodological considerations in meta-transcriptome analyses when applying either the poly(A) enrichment or random hexamer primer protocols for RNA sequencing (meta-transcriptomic analysis); 3) to detect the entire cartography of functionally active microorganisms in the endometrium from healthy women and whether there are changes in its composition throughout the menstrual cycle; 4) to determine functionally active microorganisms in the receptive phase endometria in women

with RIF *vs.* healthy control women; and 5) to summarise and meta-analyse the current knowledge of the composition and diversity of the microbiome in polycystic ovary syndrome (PCOS).

Five studies were carried out to address the objectives, with the main findings being: 1) uterus harbours its own microbial composition that is dysregulated in different gynaecological conditions like infertility, endometriosis, chronic endometritis, endometrial polyps, dysfunctional menstrual bleeding, and endometrial cancer. Nevertheless, the core/consensus endometrial microbiome has not been established. Modulation of the endometrial microbiome by antibiotics, pro- and prebiotics is a promising field with high clinical relevance, but it is too early to offer these treatment options for patients today (**Study I**). 2) Many microorganisms are able to generate poly(A) tails in the process of transcription (similar to the host), while several microbes may lack poly(A) tails, therefore the wide application of microbial RNA sequence analysis (meta-RNA-seq) must be supported by a well-prepared protocol for a comprehensive understanding of the entire microbial atlas (**Study II**). 3) The analysis of the functionally active endometrial microbiota shows that >5000 microorganisms (bacteria, fungi, viruses, and archaea) are present in the endometrium of healthy women and changes in composition and function along the menstrual cycle are detected. Microbes have possible metabolic activity in the host-microbiota crosstalk in receptive phase endometrium related to prostanoid biosynthesis pathway and L-tryptophan metabolism. Our study confirms the presence of active microbes in the human endometrium with implications in receptive phase endometrial functions, meaning that microbial dysfunction could impair the metabolic pathways important for endometrial receptivity (**Study III**). 4) Women suffering RIF have significantly different functionally active microbial profile,

where retinol biosynthesis and serotonin degradation metabolic pathways in the host-microbe interactions were dysregulated when compared to healthy controls. Our study confirms the presence of the core microbiota in the human endometrium in health and that in women with implantation failures the microbial composition demonstrates less richness which could impair the metabolic pathways important for endometrial functions (**Study IV**). 5) The relation between the microbial composition and the aetiology of PCOS is an active field of research. Most of the studies performed in the field focus on gut microbiome analysis, nevertheless the studies are barely comparable and findings inconsistent. Our meta-analysis gathers 17 studies with microbiome data of 1868 women (737 women with PCOS and 631 controls) were meta-analysed, with a special focus on alpha diversity indexes and demonstrates that women with PCOS possess lower richness in the gut microbial composition when compared to control women. These findings support the potential importance of microbiome in PCOS development with possible future biomarker/treatment options (**Study V**).

RESUMEN

El cuerpo humano está colonizado por más microorganismos que por células propias, y a medida que se adquiere más conocimiento sobre el microbioma humano, más evidente resulta su significativo efecto en la fisiología humana. La mayoría de las comunidades bacterianas presentes en humanos coexisten de manera sinérgica con su hospedador. Sin embargo, un desequilibrio en esta relación puede contribuir al desarrollo de enfermedades. En el contexto de la fertilidad humana, se ha demostrado que el tracto reproductor femenino, especialmente el medio vaginal, posee un microbioma altamente activo, formado fundamentalmente por *Lactobacillus*. Por el contrario, la influencia del microbioma de sitios adyacentes y su relación con la fertilidad femenina está por conocer. Estudios recientes han demostrado que el endometrio (capa interna del útero) tiene su propio perfil microbiano. Además, se ha demostrado que los microorganismos del tracto reproductor superior no solo influyen en las funciones del útero, sino que además podrían desempeñar un papel importante en la implantación embrionaria y en ciertas complicaciones ginecológicas, entre las que se incluye el fallo recurrente de implantación.

La presente Tesis Doctoral estudia la relación del microbioma con la salud femenina. Así, los objetivos de esta Tesis Doctoral fueron: 1) resumir el conocimiento actual acerca del microbioma endometrial, los tratamientos actuales ofrecidos en el ámbito clínico y las posibilidades futuras de modificar la composición microbiana uterina; 2) poner de manifiesto las consideraciones metodológicas en los análisis del meta-transcriptoma cuando se aplican los protocolos de enriquecimiento de poli(A) o cebadores hexámeros aleatorios para la secuenciación del ARN (análisis meta-transcriptómico) 3) detectar toda la composición microbiana funcionalmente activa en el endometrio de mujeres

sanas y si hay cambios a lo largo del ciclo menstrual; 4) determinar los microorganismos funcionalmente activos en el endometrio durante la fase receptiva en mujeres con fallo de implantación recurrente frente a mujeres control; y 5) resumir y metaanalizar los conocimientos actuales sobre la composición y la diversidad del microbioma en el síndrome de ovario poliquístico.

Para el abordaje de los objetivos planteados se realizaron cincos estudios, obteniéndose como principales hallazgos: 1) el útero alberga su propia composición microbiana, diferente en determinadas condiciones ginecológicas como la infertilidad, la endometriosis, la endometritis crónica, los pólipos endometriales, la hemorragia menstrual disfuncional y el cáncer de endometrio. Sin embargo, no se ha establecido el núcleo/consenso del microbioma endometrial. Por tanto, la modulación del microbioma endometrial mediante antibióticos y pro y prebióticos es un campo prometedor con gran relevancia clínica, pero es demasiado pronto para ofrecer esta opción de tratamiento a las pacientes (**Estudio I**). 2) Muchos microorganismos son capaces de generar colas de poli(A) en el proceso de transcripción (de forma similar al huésped), mientras que varios microbios pueden carecer de colas de poli(A), por lo que la aplicación del análisis de la secuencia del ARN microbiano (meta-ARN-seq) debe estar respaldada por un protocolo bien preparado para una comprensión integral de todo el atlas microbiano (**Estudio II**). 3) El análisis de la microbiota endometrial funcionalmente activa muestra que >5000 microorganismos (bacterias, hongos, virus y arqueas) habitan en el endometrio de mujeres sanas y se detectan cambios en su composición y función a lo largo del ciclo menstrual. Los microorganismos tienen una posible actividad metabólica en la interacción huésped-microbiota en el endometrio en fase receptiva relacionada con la ruta de biosíntesis de

prostanoides y el metabolismo del L-triptófano. Nuestro estudio confirma la presencia de microbios activos en el endometrio humano con implicaciones en las funciones endometriales de la fase receptiva al embrión, lo que significa que la disfunción microbiana podría perjudicar a rutas metabólicas importantes para el establecimiento de la receptividad endometrial (**Estudio III**). 4) Las mujeres que padecen de fallo recurrente de implantación tienen un perfil de microbiota funcionalmente activo significativamente diferente, donde las rutas metabólicas de biosíntesis de retinol y degradación de serotonina en las interacciones huésped-microbio estaban alteradas en comparación con las controles fértiles. Nuestro estudio confirma la microbiota en el endometrio humano sano y que en las mujeres con fallos de implantación la composición de microorganismos demuestra una menor riqueza que podría perjudicar rutas metabólicas importantes para las funciones endometriales (**Estudio IV**). 5) La relación entre el microbioma y la etiología del síndrome de ovario poliquístico es un campo de investigación muy activo. La mayoría de los estudios realizados en este ámbito se centran en el análisis del microbioma intestinal, pero los estudios son poco comparables y los resultados son inconsistentes. Nuestro metaanálisis reúne 17 estudios y en total 1368 mujeres (737 con síndrome de ovario poliquísticos y 631 controles) y demuestra que las mujeres con este síndrome metabólico poseen una menor riqueza en la composición microbiana intestinal en comparación con las mujeres de control. Estos hallazgos apoyan la importancia potencial del microbioma en el desarrollo del síndrome de ovario poliquístico con posibles opciones futuras de biomarcadores/tratamiento (**Estudio V**).

1. General introduction

1.1. The human microbiome

Microbes in and on the human body account for 1-3% of our total weight and comprise slightly more cells than our own body (Power *et al.*, 2017). Specifically, $3.8x10^{13}$ is the estimation for the number of bacterial cells across the whole body whereas it is estimated that there are $3.0x10^{13}$ human cells (Sender *et al.*, 2016). This data supports the coined term for the microbiota as our "last organ" (Baquero and Nombela, 2012). The microorganisms inhabiting the human body are principally bacteria but also viruses (i.e., virome), fungi, archaea, and bacteriophages (Perez-Muñoz *et al.*, 2017). It is becoming increasingly evident that microorganisms play an important role in our health and well-being, via producing bioactive molecules both necessary for and harmful to other microbes, and for interacting with our cells to regulate and influence our metabolism, physiology, and immune functions that ultimately shape our health and resistance to a disease (Figure 1) (Young, 2017).

Over the past two decades, a remarkable development in microbiome research is noted due to the methodological advances that have allowed more robust, consistent, and complete experimental designs for the identification of previously unknown microorganisms. With the advances in the field, new terms like microbiota, microbiome, and metagenome -among others- have been created. Microbiota is the total community of microorganisms (bacteria, archaea, microeukaryotes, and viruses) that occupies a defined site or habitat (Cho and Blaser, 2012). The microbiome, however, often used as a synonym for microbiota, defines the genomes of the microbes (Berg *et al.*, 2020).

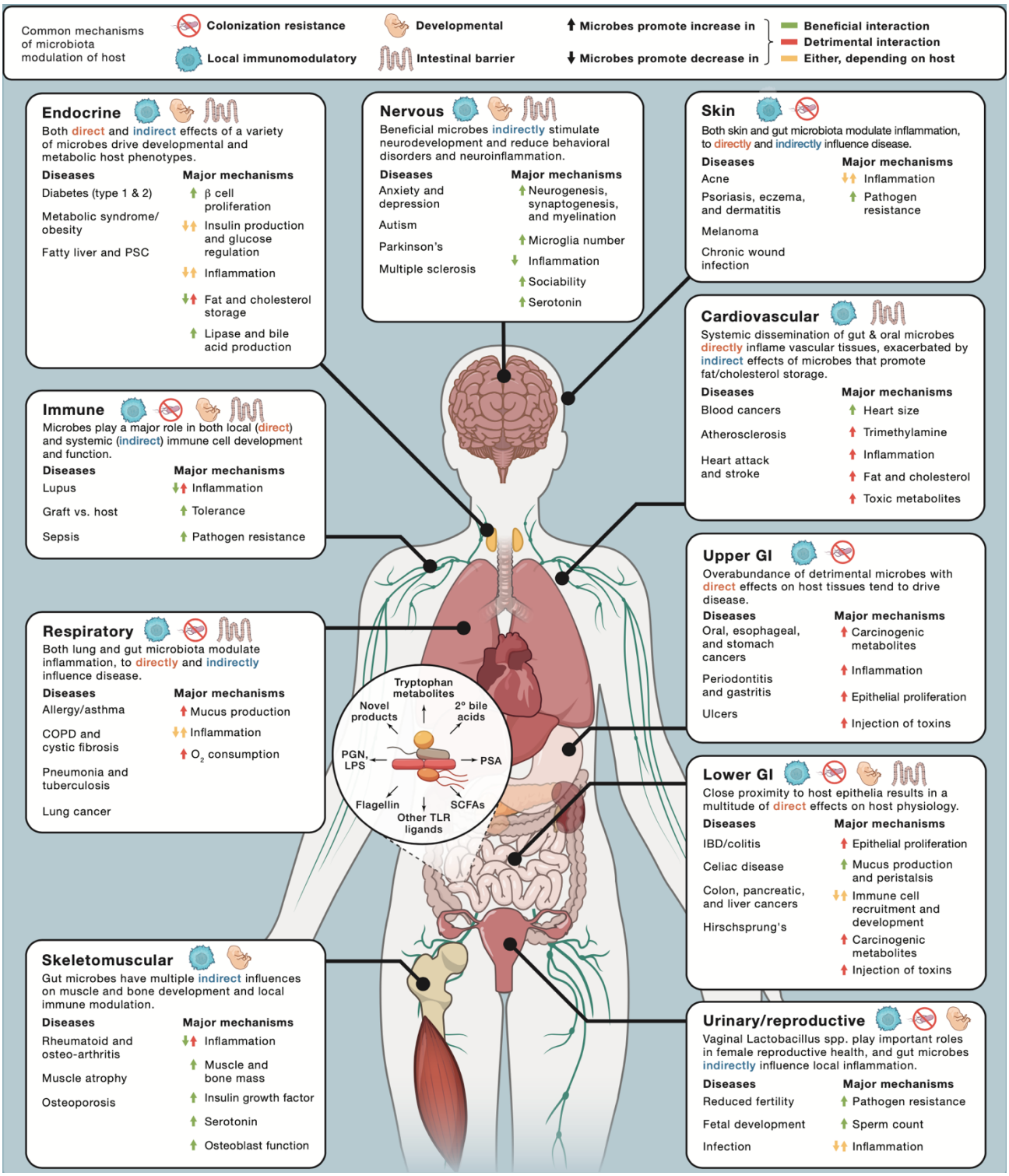

Figure 1. Microbiome effects in human physiology. Representation of the major organ systems and the implication of microbes in maintenance of the homeostasis according to germ-free animal models (Hill and Round, 2021). *This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5293110281193).*

1.2. The human microbiome composition

The Human Microbiome Project (HMP), launched in 2007, aimed to increase the knowledge about microbial communities involved in human physiology and pathology. This project was divided into two phases (Figure 2). The first phase aimed to determine the microbial composition shared among healthy individuals to establish the core microbiome of multiple body sites. The second phase, released in 2019, comprised studies of dynamic modifications in the microbiome and host under different conditions: pregnancy and preterm birth; inflammatory bowel diseases; and stressors that affect individuals with prediabetes, raising more new questions than it had answered about the inter-individual differences in microbial composition and its dynamics (Huttenhower *et al.*, 2012; The Integrative HMP (iHMP) Research Network Consortium, 2014; Lloyd-Price *et al.*, 2017; Proctor *et al.*, 2019). The results from the HMP pave the way for understanding and unravelling the microbes and host-microbe interactions in the urogenital tract in both eubiosis and dysbiosis (i.e., balanced or imbalanced commensal species, respectively, mainly regarding *Firmicutes* and *Bacteroidetes* phyla).

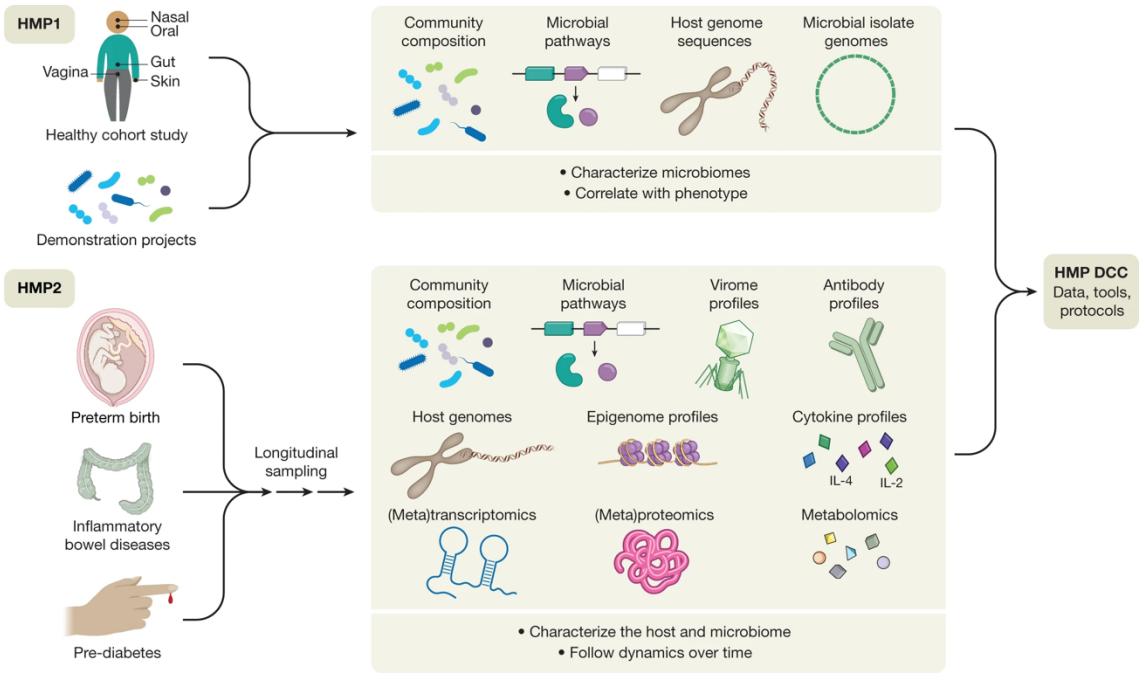

Figure 2. The Human Microbiome Project (HMP) is distributed in two different phases (HMP1 and HMP2). The first part (HMP1) focussed on the description of microbial communities in different body sites (oral and nasal cavities, vagina, gut, and skin) in healthy adults. The HMP2 studied both host and microbial communities in three longitudinal cohort studies in pregnancy and preterm birth (vaginal microbiomes of pregnant women), inflammatory bowel diseases (gut microbiome), and prediabetes (gut and nasal microbiomes) using multi-omic analyses at multiple time-points (Proctor *et al.*, 2019). *This figure is reproduced under a Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.*

The study design of the HMP1 cohort represented by 300 United States individuals consisted of the sampling of representing five main body parts: the oral cavity and oropharynx: saliva; buccal mucosa, gums, palate, tonsils, throat, tongue, gingival dental plaque, gut, vagina, and skin (Huttenhower *et al.*, 2012). This large-scale study revealed that every single part of the human body has its microbial signature, dynamics, and interaction with human tissue (Grice and Segre, 2012; Huttenhower *et al.*, 2012). Likewise, it was demonstrated that interpersonal variation was significantly higher than intrapersonal variability (Grice and Segre, 2012). A summarised overview of the microbial composition along the human body is shown in Figure 3.

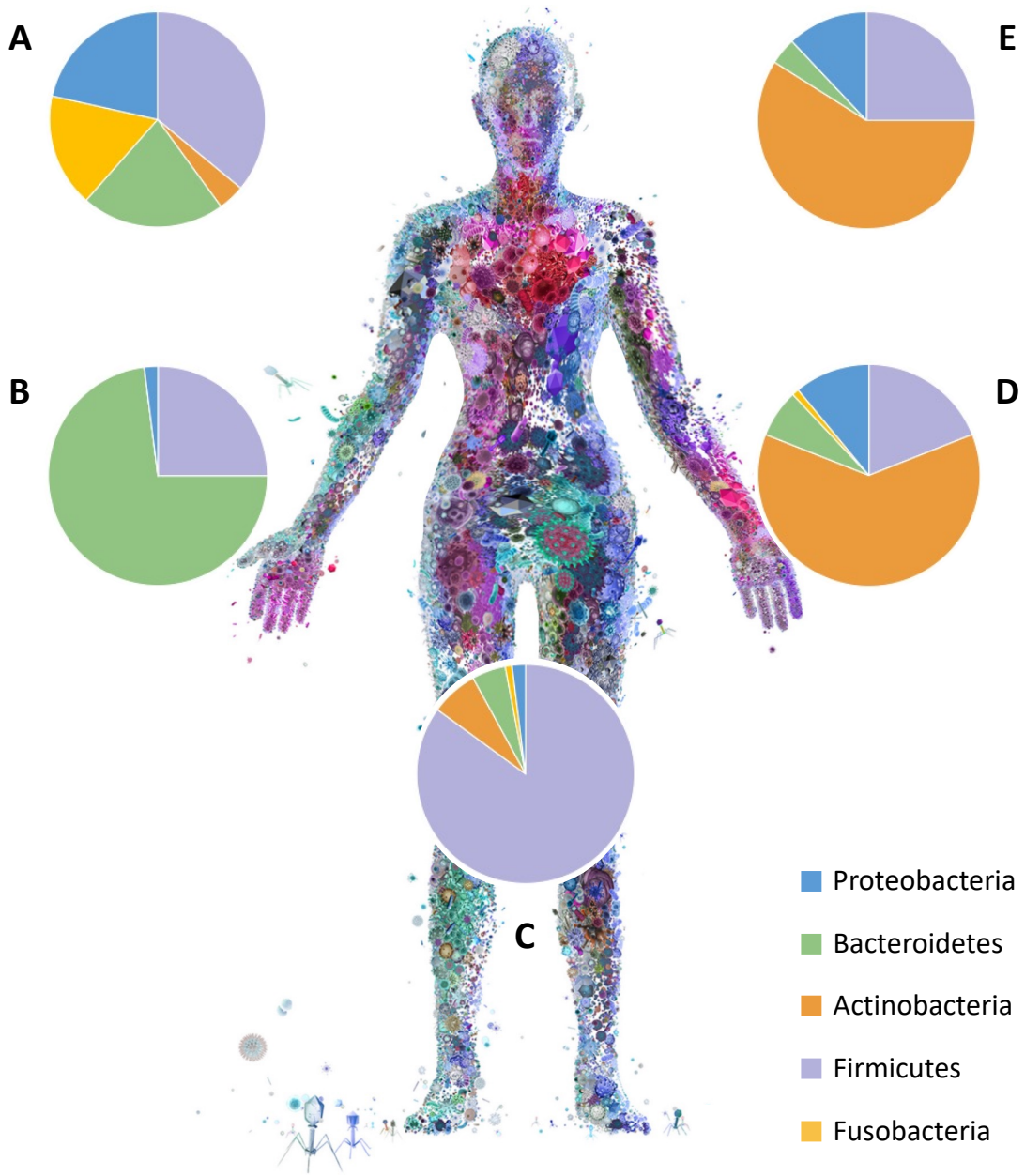

Figure 3. Phylum-level classification of bacteria residing in diverse body sites. Data resulting from the NIH Human Microbiome Project (HMP). Different niches of the body were sampled and analysed distinctly: A. oral samples; B. stool samples; C. vaginal samples; D. skin specimens; E. nares specimens.

During the lifespan the microbial dynamics, the microbiome diversity and composition changes. In neonates, there is a gradual development of the microbiome characterised by low diversity and instability (Cho and Blaser, 2012). The composition of microbes is strongly influenced by the mode of delivery (i.e., caesarean section or vaginal delivery) as well as by the type of feeding in the early

stages of life (Cho and Blaser, 2012). During childhood and adolescence, the microbial dynamics stabilises, although strongly influenced by hormonal, nutritional, and metabolic changes (Uhr *et al.*, 2019). In adulthood, microbial diversity increases significantly and remains stable, with rare fluctuations generally associated with a pathology. In old age, microbial diversity decreases gaining more similarity among individuals (Uhr *et al.*, 2019). Generally, the higher the microbial diversity the better for a healthy state (Mosca *et al.*, 2016).

1.3. The gut microbiome

Most commensal microbes inhabit the colon, making it the most studied ecosystem within the human body. Certainly, the MetaHIT (Metagenomics of the Human Intestinal Tract) Consortium was launched in 2008 by the European Commission including 13 academic and business partners from different countries to produce a catalogue of the microbial genes from the human intestinal tract and to determine relations among the microbial genes and human illnesses (Ehrlich, 2011). The integration of the results from HMP and MetaHit has recognised 2172 different bacterial species that belong to 12 different phyla in human beings: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, which represent up to 94% of gut bacteria, and *Spirochaetes*, *Tenericutes*, *Fusobacteria*, *Chlamydiota*, *Synergistetes*, *Verrucomicrobia*, *Lentisphaerae*, and *Deinococcus-Thermus* as rare species (Li *et al.*, 2014; Hugon *et al.*, 2015). Also, regarding the gut microbiome, bacteria, archaea, fungi, and viruses promptly diversify after birth and stabilise in adulthood (Coyte *et al.*, 2015). Several studies have investigated the relationship between the gut microbiome and metabolic disorders like obesity, dyslipidemia, type 2 diabetes (T2D) and insulin resistance, low-grade inflammation, and polycystic ovary syndrome (PCOS), among others (Dabke *et*

al., 2019). In this context, the gut microbiota modulation has prompted a promising treatment option for several diseases via modulating/improving human gut microbes; nevertheless, the long-term effects on health need to be well established (Sanna *et al.*, 2022).

1.4. Microbiome of the female reproductive tract

The lowest part of the female reproductive tract (i.e., vagina) is mainly dominated by *Lactobacillaceae,* but also *Bifidobacteriaceae*, *Prevotellaceae,* and *Veillonellaceae* form part of the vaginal microbiome (Figure 4) (Koedooder *et al.*, 2019a; France *et al.*, 2022). The vaginal microbiome produces lactic acid to maintain vaginal pH of 3.5 to 4.5 helping to avoid pathogens infection. Alterations in the vaginal microbiome play a role in common conditions such as bacterial vaginosis, sexually transmitted diseases, urinary infections, and preterm birth (Goldenberg *et al.*, 2000; Ma *et al.*, 2012; Hyman *et al.*, 2014). Also, dysbiosis of vaginal microbiome has been widely associated with pathologies and adverse assisted reproductive techniques outcomes (Schoenmakersa *et al.*, 2020). Specifically, low *Lactobacillus* abundance in the vagina has been proven as a predictor for unfavourable *in vitro* fertilisation (IVF) outcomes (Koedooder *et al.*, 2019b).

Despite the anatomical proximity of the cervical canal to the vagina, cervix composes different microbial composition, being the transition zone between the lower and the upper reproductive tract (Figure 4) (Chen *et al.*, 2017). Cervical microbiome is mainly composed of *Lactobacillus*-related species but more diversity with bacterial families like *Clostridiaceae*, *Enterobacteriaceae*, *Staphylococcaceae,* and *Streptococcaceae* has been detected (Moreno *et al.*, 2016; Chen *et al.*, 2017; Koedooder *et al.*, 2019a).

Despite the traditional vision of the endometrium as a sterile cavity, it harbours its own microbial composition that differs from that of the vagina, conforming a low biomass body site (Chen *et al.*, 2017). Because endometrium has 100-10000 times less microbial biomass than vagina, important methodological considerations should be taken into account for the establishment of the endometrial core microbiome (Molina *et al.*, 2021). The modulation of the endometrial microbiome seems to be promising clinical application for several gynaecological conditions like endometriosis, endometritis, endometrial cancer, and IVF outcomes (Toson *et al.*, 2022). Indeed, supplementations with oral and vaginal probiotics and antibiotics modulated effectively endometrial microbiome composition (Kadogami *et al.*, 2020). Despite the advances in the field, the core endometrial microbiome in health and disease needs to be established and the different microbial modulation protocols need to be thoroughly tested.

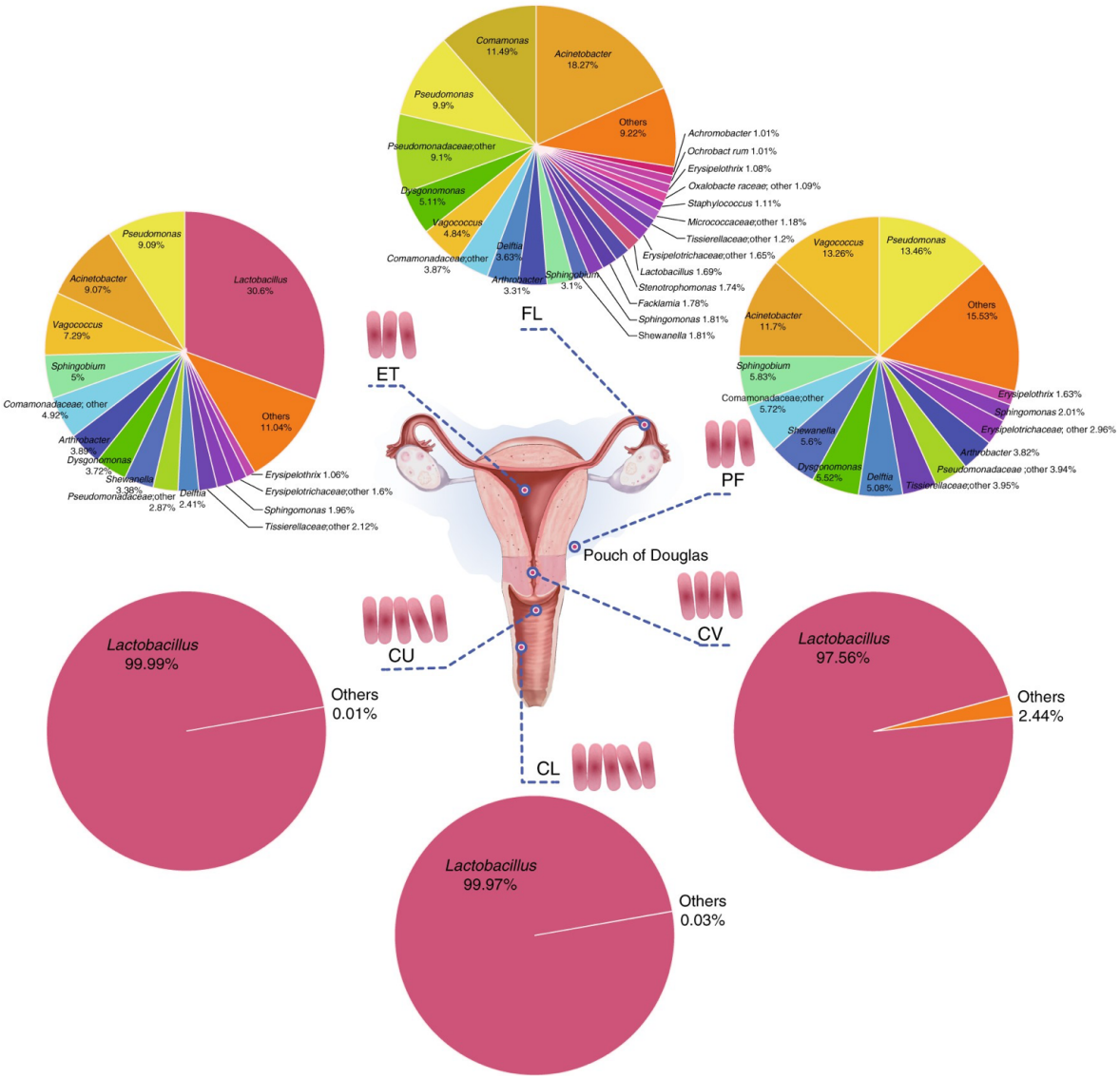

Figure 4. Microbiome of the female reproductive tract. CL – lower third of the vagina; CU – posterior fornix; CV – cervical mucus drawn from cervix; ET – endometrium; FL – fallopian tubes; PF – peritoneal fluid from the pouch of Douglas (Chen *et al.*, 2017). *This figure is reproduced under a Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.*

1.5. Endometrial microbiome in implantation failure (RIF)

RIF is a common and burdening diagnosis in infertility treatment. Depending on the clinic and country, RIF definition is based on unsuccessful IVF cycles (2 to 6), the number of embryos transferred (3 to 10) or a combination of both factors (Tan *et al.*, 2005). Notwithstanding scientific advances, the origin of RIF remains unclear and the treatment options ambiguous. Recent studies are suggesting that

endometrial microbial composition plays a role in successful embryo implantation (Benner *et al.*, 2018; Molina *et al.*, 2021; Toson *et al.*, 2022) and therefore the microbial dysbiosis could lead to implantation failure in RIF (Fu *et al.*, 2020; Patel *et al.*, 2022). Endometrial microbiome studies in women with RIF have demonstrated enrichment of bacterial taxa such as *Gardnerella* (Kitaya *et al.*, 2019; Ichiyama *et al.*, 2021), *Burkholderia* (Kitaya *et al.*, 2019; Ichiyama *et al.*, 2021), *Atopobium* (Ichiyama *et al.*, 2021), *Delftia* (Ichiyama *et al.*, 2021), *Prevotella* (Diaz-Martínez *et al.*, 2021; Ichiyama *et al.*, 2021) and *Sneathia* (Diaz-Martínez *et al.*, 2021) when compared to controls, nevertheless there is no consensus on the endometrial microbiome in health and in RIF. The endometrial microbial diversity analyses of RIF patients reveal that the endometrial microbiome potentially regulates endometrial immune cell proliferation and differentiation via microbial metabolites, which may alter the process of embryo implantation (Chen *et al.*, 2021). The exact microbial composition and its role in RIF need to be studied further.

1.6. Microbiome in PCOS

PCOS is one of the most common gynaecological disorders affecting up to 20% of the reproductive-aged women world-wide (Skiba et al., 2018). PCOS diagnosis is commonly based on at least 2 out of the 3 following features from the National Institutes of Health (NIH) (Zawadski and Dunaif, 1992), Rotterdam ("Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS)," 2004) and Androgen Excess Society (AE-PCOS) (Azziz *et al.*, 2009) criteria: 1) hyperandrogenism; 2) oligoanovulation; and 3) polycystic ovarian morphology. Despite its high prevalence, the aetiology of PCOS remains largely unknown (Dokras *et al.*, 2017).

Recent studies suggest that microbial composition could be involved in the development of PCOS (Hill and Round, 2021). Microbes can influence the microenvironment and thereby the host by the production of several metabolites like bile acids, ceramides, short-chain fatty acids, branched-chain amino acids, and trimethylamine N-oxide. These metabolites could interfere in the synthesis of vitamins like folic acid and B12; metabolism of bile acids, neurotransmitters, and hormones; altering intestinal permeability that could contribute to pathologic phenotypes; detoxification mechanisms; defence against potential pathogens; and modulation of immune system and metabolism (Chen and Pang, 2021). Changes in specific bacterial taxa and biodiversity of microbiome have been studied along the body sites (e.g., oral cavity, reproductive tract, blood, and gut) indicating that microbes may have a potential role in the development and progression of PCOS (Giampaolino *et al.*, 2021). However, there is no consensus about the microbial composition, its dysbiosis, and concrete influence of the microbes related to PCOS.

1.7. Challenges in microbiome studies

Since the human microbiome could be influenced by multiple factors from methodological aspects to physiological/pathological conditions, the big challenge in the field is to integrate and unify different microbiome studies (Knight *et al.*, 2018). Several efforts have been made to implement methodological considerations and guidelines to conduct replicable and reliable microbiome studies that could be applied to reproductive sciences (Knight *et al.*, 2018; Mirzayi *et al.*, 2021; Molina *et al.*, 2021).

Before modifying the microbiome for diagnostical, prognostic, and treatment purposes, it is essential to understand the factors that can influence microbial

composition and dynamics. Wide range of factors like age, ethnicity, geographic localisation, diet, body mass index (BMI), diseases, therapies (i.e., antibiotic, antifungal, antiviral treatments), administration of pre- and probiotics, stress, physical activity, smoking, and alcohol consumption among others are important modulators of the microbiota (Hall *et al.*, 2017; Altmäe *et al.*, 2019; Daliri *et al.*, 2021). Among these, diet stands out as one of the most important modulators of microbial composition (Shanahan *et al.*, 2021) especially for the gut microbiota, as metabolites present in diet can be metabolised by microbes into end products like short-chain fatty acids, such as acetate, butyrate, and propionate produced as a result of protein and carbohydrates degradation, having physiological impact on human health (Blaut, 2002). Altogether, this makes diet an important factor to be considered in microbiome studies and studies analysing microbial composition, especially in the gut.

Another important factor in microbiome studies is contamination, especially when analysing low microbial biomass sites such as uterus/endometrium (Molina *et al.*, 2021). A big part of the microbiome studies performed in the field of human reproduction has not considered proper negative and positive controls, making thereby the interpretation and comparability between studies difficult (Molina *et al.*, 2021). The addition of negative and positive controls, as well as the use of *in silico* decontamination approaches like Decontam and microDecon R packages, could be used to generate meaningful results by avoiding/reducing microbial contamination (Molina *et al.*, 2021). Further, a new term has been coined to define well-to-well contamination during sequencing plate preparation, i.e., "splashome" (Olomu *et al.*, 2020). To avoid this source of contamination, it has been proposed that sequencing of samples high microbial biomass should be done separately from those of low biomass to avoid cross-

contamination (Olomu *et al.*, 2020). Furthermore, the term "kitome" has been proposed to refer to the contamination of bacterial genomic material in molecular biology reagents, DNA purification kits, PCR master mixes, and other laboratory stocks used for processing and analysing nucleic acids, meaning that most of them are 'contaminated' with bacterial DNA (Salter *et al.*, 2014; Glassing *et al.*, 2016; Zulian *et al.*, 2016; Kim *et al.*, 2017).

For all that, careful study design and protocol are required to avoid inconsistent results and diverse interpretations. An international effort has been made to overcome these difficulties when microbiome analyses are performed (Santiago *et al.*, 2014; O'Donnell *et al.*, 2016; Costea *et al.*, 2017) and the consensus proposes that at least 3 critical aspects are required in conducting microbiome studies (Greathouse *et al.*, 2019; Mirzayi *et al.*, 2021): (1) to report a detailed extraction protocol, (2) to use the same protocol along all the study, and (3) detailed explanation of inclusion and description of positive and negatives controls to denoise contaminants are required to ensure the reproducibility of the studies (Leigh Greathouse *et al.*, 2019). Also, a STORMS checklist (Strengthening The Organization and Reporting of Microbiome Studies) has arisen to provide direction for concise and comprehensive reporting of microbiome studies that will enable manuscript preparation, peer review, selection of publications, and comparative analysis of published data (Figure 5) (Mirzayi *et al.*, 2021).

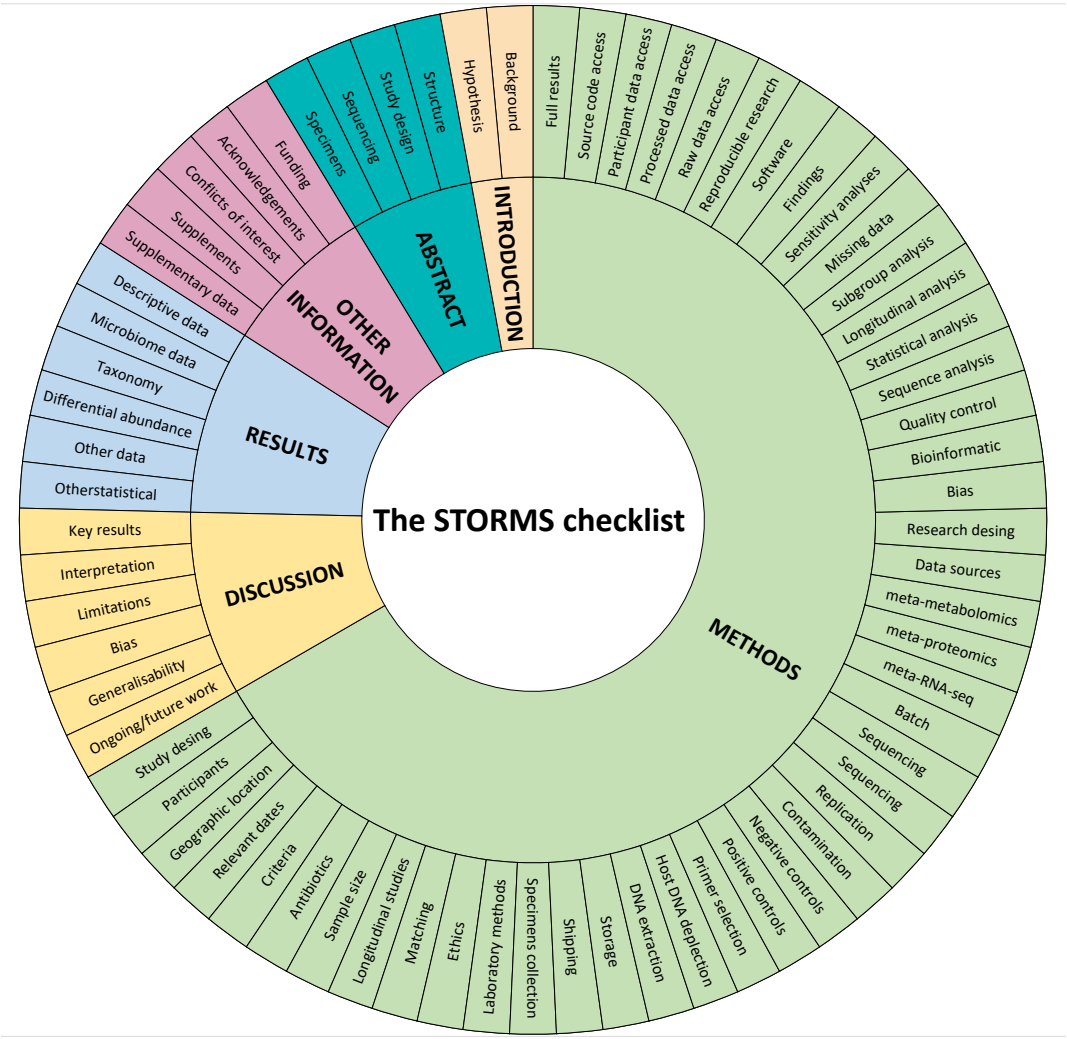

Figure 5. Strengthening The Organization and Reporting of Microbiome Studies (STORMS) is composed of a checklist structured into six sections. Figure adapted from (Mirzayi *et al.*, 2021).

1.8. Analysing microbial composition

As less than 1% of microbes can grow and form colonies on agar plates, analysis of the genomes of the microorganisms (i.e, microbiome) overcomes two limitations of the traditional culture-based microbe characteristics: non-culturability and genomic diversity (Giudice, 2016), opening up a new research field. Different methodological approaches for analysing microbial composition are available. A summary of the methodologies together with its strengths and limitations are highlighted in Figure 6.

1.8.1. Marker gene sequencing

Amplicon sequencing or ribosomal RNA (rRNA) gene sequencing is used to establish the relative abundance of microbes by detection of microbial genes. Bacteria and archaea communities are assayed by sequencing the conserved and hypervariable (V1-V9) regions of the 16S rRNA gene (Tao *et al.*, 2017). Fungal communities are detected by sequencing 18S and 28S rRNA (Nilsson *et al.*, 2016) and the internal transcribed spacer (ITS) region (Figure 6) (Schoch *et al.*, 2012). Notwithstanding the extensive use, effectiveness, and low cost of gene marker sequencing methods, some limitations have been addressed like underestimations of microbial diversity and abundance (Poretsky *et al.*, 2014; Callahan *et al.*, 2021). Despite these limitations, the marker gene analysis is the most used approach and is preferred for the low microbial biomass microbiome studies today (Knight *et al.*, 2018; Liu *et al.*, 2021).

1.8.2. Metagenome sequencing

This technique is based on the sequencing of DNA from all microbial genomes within the community, providing high coverage of taxonomic composition comprising bacterial, viral, and eukaryotic DNA (species and strain level detection) (Figure 6). Additionally, this methodology can provide information about the potential function of the microbial communities. Nevertheless, this method is relatively expensive, and biases that are introduced by library construction, assembly, and reference databases for microbial annotation are easy to occur (Quince *et al.*, 2017). As the metagenomics field matures, the current limitations (especially the annotation part) continue to

improve and metagenomic approach is gradually taking over 16S rRNA gene sequencing technique.

1.8.3. Meta-transcriptome analysis

Meta-transcriptome, also named meta-RNA-seq, is the total collection of the microbial gene transcripts in a community. The meta-transcriptome analysis provides insight into the functional activity of a microbial community (Figure 6); however, sequencing microbial RNA can be biased by the more active microorganisms (Knight *et al.*, 2018). Also, the high risk of contamination with host RNA could be an obstacle. Further, sequencing the RNA of the whole ecosystem compared to typical transcriptomic analysis is extremely challenging for several reasons: 1) it can be difficult to coordinate the collection and processing of samples to maintain the resemblance of their *in situ* expression levels (Macklaim and Gloor, 2018); 2) it can be challenging to identify and create a proper reference library to map all the generated sequenced reads for all the organisms in the sample (Jiang *et al.*, 2016); 3) methods used for single-organism comparative transcriptomics cannot capture the variance between changing expression and gene content/organism abundance (Macklaim *et al.*, 2013); and 4) the ecosystem can contain different bacterial strains, or even species, that have a similar functional profile (Macklaim *et al.*, 2013). Regardless of these limitations, the existence of functionally active microbes could be tested by RNA-based method, providing information about the regulation and expression profiles of microbes (i.e., archaea, bacteria, viruses, and fungi) reaching beyond the marker gene and/or metagenome analyses where only DNA sequences are analysed.

1.8.4. Meta-proteome analysis

The functional activity of the microbial communities could be represented also directly by microbiome/host proteins, adding complementary information to the meta-transcriptomics analyses. Meta-proteomics has been defined as the analysis of the whole protein composition of microbes providing information about their activity and metabolism (Mills *et al.*, 2019). The critical steps for an efficient meta-proteomic analysis include sample collection and preservation, protein extraction and isolation method, liquid chromatography-mass spectrometry analysis, and database searching (Xiao *et al.*, 2017). One of the bigger challenges in the meta-proteomics is to distinguish between the host and the microbe protein species.

1.8.5. Meta-metabolome analysis

Meta-transcriptome and/or meta-proteome studies are often completed with the meta-metabolome analyses to associate gene expression with metabolites profile (Hassa *et al.*, 2018). Meta-metabolome is the identification and quantification of the global small microbial molecules or metabolites present in a sample (Figure 6) (Sandhu *et al.*, 2019; Weckx *et al.*, 2019). One of the biggest challenges for meta-metabolomic studies, likewise in meta-proteome analysis, is to differentiate between the host- and microbiome-source metabolites and to link them directly with a concrete microbial taxon (Nicholson *et al.*, 2012). To overcome that, protein stable-isotope probing could link specific metabolites to phylogenetic information by monitoring mass spectrometers (Jehmlich *et al.*, 2010).

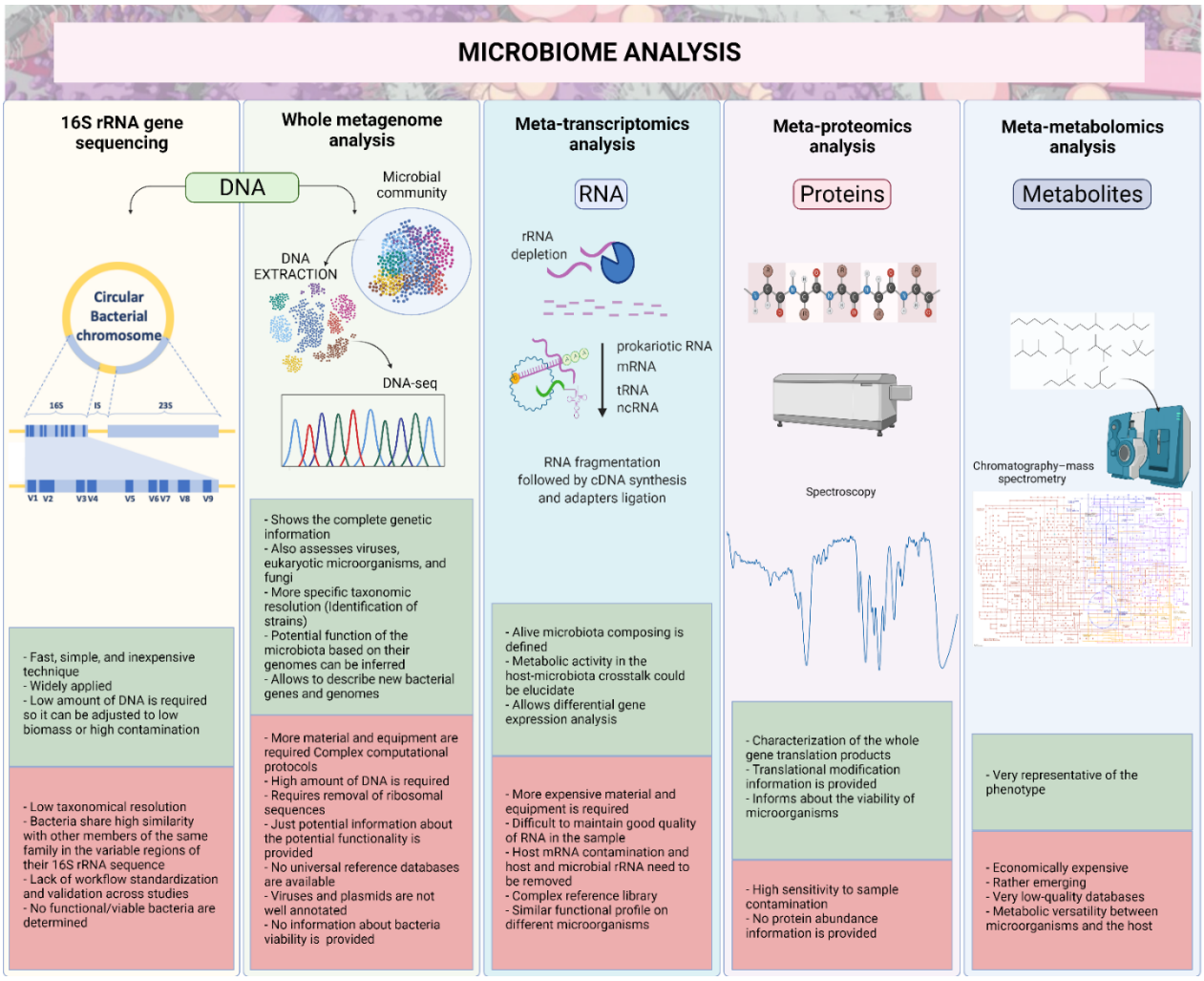

Figure 6. Methodologies to study human microbiota: marker gene sequencing, whole-genome sequencing, meta-transcriptomic sequencing, meta-proteome, and meta-metabolome analyses. Each methodology's strengths (green) and limitations (red) are highlighted. Created with BioRender.

1.8.6. Integrating multi-omics data

The study of the human microbial communities is a fast-expanding and maturing research field. The use of high-throughput techniques like next-generation sequencing linked to mass spectrometry-based metabolomics could provide a comprehensive version of microbial function and composition as well as knowledge of the microbiome-human crosstalk. Novel studies are integrating metagenome, meta-transcriptome, and meta-proteome/meta-metabolome (Figure 7) providing holistic and mechanistic information about the microbes and

their genomic material, proteins, and metabolites in the human body, and their genomic material, proteins, and metabolites (Daliri *et al.*, 2021). Specifically, the combination of metagenomics and meta-transcriptomics reveals interesting results like while same species-level microbial composition was found in monozygotic twins, there were notable differences in genetic information and transcriptional activities of their gut microbes, indicating that similar microbial composition could have different roles (Turnbaugh *et al.*, 2010). Also, by combining metagenomics and meta-proteomics, some studies have revealed that the gut microbial dysbiosis and increased microbial antigenic cell wall proteins associates with Crohn's disease (Erickson *et al.*, 2012)

Despite the valuable opportunities that multi-omics analysis offers for understanding microbial dynamics, its application is still limited today due its novelty and methodological limitations. More and more analysis methods are being developed, but the robust nature of the data and the processing requirements are complex. Future research will contribute to furthering the application of multi-omics networks to elucidate unresolved questions in the microbiota field (Jiang *et al.*, 2019).

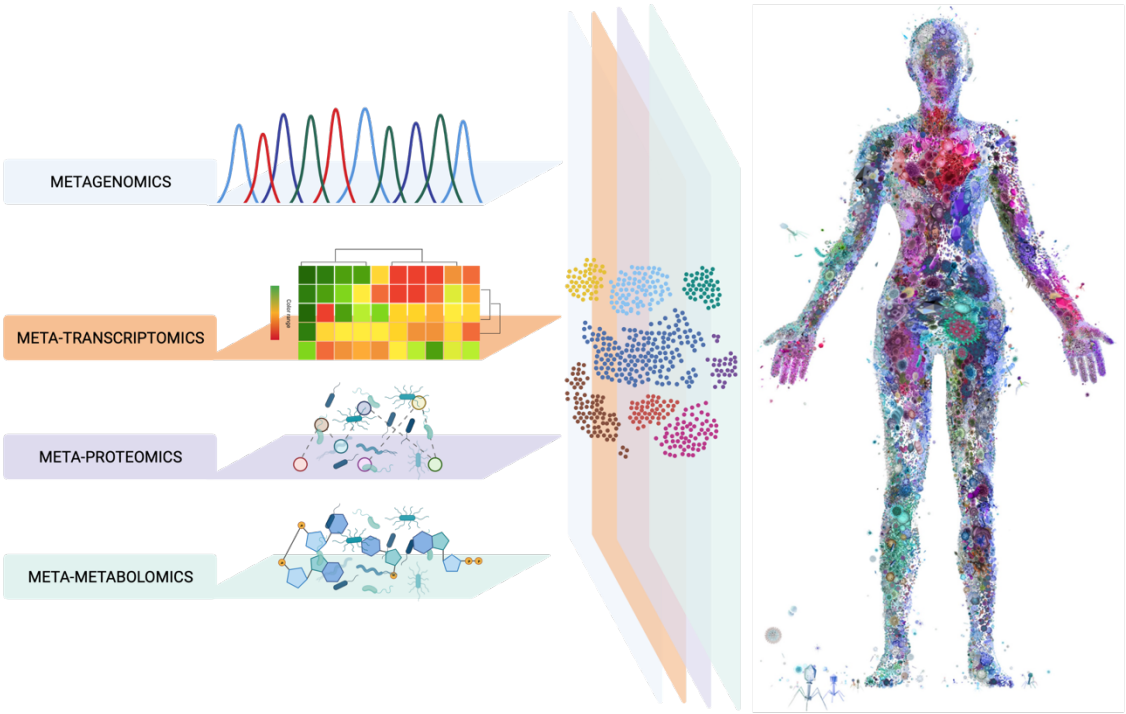

Figure 7. Multi-omics integration including metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics with the main goal to understand the dynamics of microbial communities and host-microbiome interactions. Created with BioRender.

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2. Aims

The general aim of this Doctoral Thesis was to provide current knowledge of the microbiome studies in female reproductive health and to identify the functionally active microbes in the uterus together with their potential role in host-microbe interactions. These aims are addressed in five studies:

1. To summarise the existing knowledge of endometrial microbiome studies, the current treatments offered in the clinical setting and the future possibilities for modifying the uterine microbial composition.

2. To highlight the methodological considerations in meta-transcriptome analyses when applying either the poly(A) enrichment or random hexamer primer protocols for RNA sequencing (meta-transcriptomic analysis).

3. To detect the entire cartography of functionally active microorganisms in the endometrium from healthy women and whether there are changes throughout the menstrual cycle.

4. To determine functionally active microorganisms in the receptive phase endometria in women with RIF *vs.* healthy control women.

5. To summarise and meta-analyse the current knowledge of the composition and diversity of the microbiome in PCOS.

3. Materials and Methods

This section recapitulates the methodology applied along this Doctoral Thesis. In the respective studies can be found a detailed description of the methodologies. An overview of the methods is presented in Table 1.

In **study I**, a comprehensive revision of the literature was performed to gather information about the microbial composition of human uterus in health and disease, as well as the current treatments and future possibilities for microbial composition modification in the clinical setting.

In **Study II**, an opinion letter for a study by (Kumata *et al.*, 2020) "A tissue level atlas of the healthy human virome" in BMC Biology 2020;18(1):55; regarding methodological consideration in meta-transcriptome analysis was prepared.

Study III and IV aimed to investigate the endometrial meta-transcriptome together with the host transcriptome profiles in healthy women at different menstrual phases (**Study III**) and women with RIF (**Study IV**) and to predict possible host-microbe interactions.

In **Study V,** we systematically searched all publications involving microbiome analysis studies in women with PCOS to perform a meta-analysis assessing the microbial diversity differences in PCOS *vs.* control women.

Table 1. Methodological overview of the studies included in the Doctoral Thesis

Study	Design	Participants characteristics	Sampling and study material	Main outcome	Methodological approach analyses
Study I	Comprehensive review	Microbiome studies in human endometrium in health and disease	Endometrium	Endometrial microbiome in different gynaecological conditions and potential dysbiosis treatments	NA
Study II	Opinion letter	NA	NA	Methodological consideration for meta-transcriptomics	NA
Study III	Cross-sectional study	7 healthy women (24-31 years)	Endometrial samples from mid-secretory menstrual phase (LH+ 7-9) and from the proliferative phase (cycle days 6–8) using a suction curette	Transcriptome and meta-transcriptome analyses Microbial and human functional enrichment and interactions	RNA sequences converted into FASTQ files were processed using miARma-Seq Cutadapt was used to remove the adapter sequences. Non-human sequences resulting from the alignment with HISAT2 against human genome GRCh38 were aligned with Kraken2 to download bacterial, archaea, viral, and fungal libraries. Krona software was used to visualise the taxonomical results Differential abundance tests based on the zero-inflated Gaussian model integrated in metagenomeSeq were used for identifying taxon changes between the study groups. HUMAnN2 was used to identify the abundance of protein-coding sequences for microbial functional enrichment. Host transcriptome profile was analysed with GSEA with the metabolic pathways contained in MetaCyc

					To investigate the possible role of the microorganisms identified in the endometrium, we compared the metabolic pathways obtained from the host transcriptome and microbial meta-transcriptome analyses, since both were run ultimately using MetaCyc One-way analysis of variance (ANOVA) was used to determine normalised metabolic pathways abundances values between groups
Study VI	Case-control study	22 fertile women (30.2 ± 3.3 years) and 21 RIF patients (35.1 ± 3.9 years)	Endometrial samples early-secretory menstrual phase (LH+ 1-3) and mid-secretory (LH+ 7-9) using pipelle catheter	Transcriptome and meta-transcriptome analyses Microbial and human functional enrichment and interactions	RNA sequences converted into FASTQ files were processed using miARma-Seq Cutadapt was used to remove the adapter sequences. Non-human sequences resulting from the alignment with HISAT2 against human genome GRCh38 were aligned with Kraken2 to download bacterial, archaea, viral and fungal libraries. Krona software was used to visualise the taxonomical results Differential abundance tests based on the zero-inflated Gaussian model integrated in metagenomeSeq wew used for identifying taxon changes between the study groups HUMAnN3 was used to identify the abundance of protein-coding sequences for microbial functional enrichment. Host transcriptome profile was analysed with GSEA with the metabolic pathways contained in MetaCyc. Also functional

					enrichment was carried out with Gene Ontology (GO) To investigate the possible role of the microorganisms identified in the endometrium, we compared the metabolic pathways obtained from the host transcriptome and microbial meta-transcriptome analyses, since both were run ultimately using MetaCyc One-way analysis of variance (ANOVA) was used to determine normalised metabolic pathways abundances values between groups
Study V	Systematic review and meta-analysis	Women with and without PCOS	Saliva, vagina, blood, and faecal samples	Microbial composition and alpha, beta diversities	Statistical heterogeneity across studies was assessed using the I^2 value, considering 25%, 50%, and 75% as low, moderate, and high heterogeneity, respectively To evaluate the internal quality and the possible bias in the study design of the works, the Joanna Briggs Institute Critical Appraisal Tool for Systematic Reviews was used. Low risk of bias was considered when the study achieved at least 75% of the items listed The effect size was calculated as standardized mean difference (SMD) based on Cohen's and 95% confidence intervals

4. Results and discussion

STUDY I: New Opportunities for Endometrial Health by Modifying Uterine Microbial Composition: Present or Future?

ABSTRACT

Current knowledge suggests that the uterus harbours its own microbiota, where the microbes could influence the uterine functions in health and disease; however, the core uterine microbial composition and the host-microbial relationships remain to be fully elucidated. Different studies are indicating, based on next-generation sequencing techniques, that microbial dysbiosis could be associated with several gynaecological disorders, such as endometriosis, chronic endometritis, dysfunctional menstrual bleeding, endometrial cancer, and infertility. Treatments using antibiotics and probiotics and/or prebiotics for endometrial microbial dysbiosis are being applied. Nevertheless, there is no unified protocol for assessing the endometrial dysbiosis and no optimal treatment protocol for the established dysbiosis. With this review we outline the microbes (mostly bacteria) identified in the endometrial microbiome studies, the current treatments offered for bacterial dysbiosis in the clinical setting, and the future possibilities such as pro- and prebiotics and microbial transplants for modifying uterine microbial composition.

INTRODUCTION

For long it was assumed that the uterus is a sterile organ, with microbial colonisation present only in infection or in a pathological process (Evans *et al.*, 2016). The 'sterile womb' hypothesis has been challenged by recent studies using next-generation sequencing (NGS) where unique uterine microbial composition has been detected. As less than 1% of microbes can grow and form colonies on agar plates (Wade, 2002), analysis of the genomes of the microorganisms, i.e., microbiome, overcomes two common limitations of the traditional culture-based

microbe characteristics: nonculturability and genomic diversity (Giudice, 2016), opening a new research field in reproductive medicine.

The initial studies of endometrial microbiome suggest its association with reproductive outcomes in assisted reproduction (Franasiak *et al.*, 2016; Moreno *et al.*, 2016; Verstraelen *et al.*, 2016; Kyono *et al.*, 2018, 2019; Liu *et al.*, 2018; Wee *et al.*, 2018; Garcia-Grau *et al.*, 2019; Hashimoto and Kyono, 2019; Kitaya *et al.*, 2019) and with different gynaecological pathologies such as chronic endometritis (CE) (Fang *et al.*, 2016; Moreno *et al.*, 2018; Liu *et al.*, 2019), endometriosis (Khan *et al.*, 2016; Chen *et al.*, 2017; Cregger *et al.*, 2017; Li *et al.*, 2018; Wee *et al.*, 2018; Hernandes *et al.*, 2020), dysfunctional endometrial bleeding (Pelzer *et al.*, 2018), endometrial polyps (Fang *et al.*, 2016), and endometrial cancer or hyperplasia (Walther-Antonio *et al.*, 2016; Walsh *et al.*, 2019; Winters *et al.*, 2019). Nevertheless, causality has been difficult to prove because the reproductive tract represents a polymicrobial niche (O'Callaghan *et al.*, 2020), and it is not clear whether dysbiosis within the uterus is a cause or a consequence of a pathology.

Furthermore, the uterine microbial transmission is not clearly established, whereas different routes have been proposed including ascension of bacteria through the cervix, retrograde spread through fallopian tubes, haematogenous spread of oral and/or gut bacteria, through gynaecological procedures (e.g., assisted reproductive technology-related procedures; insertion/removal of the intrauterine devices), sexual habits, and/or with sperm (Mändar *et al.*, 2015; Altmae, 2018; Altmäe *et al.*, 2018; Baker *et al.*, 2018; Altmae *et al.*, 2019). Ascension of radioactively labelled macrospheres from vagina to the uterus by a 'uterine peristaltic pump' activity has been shown (Kunz *et al.*, 1997; Zervomanolakis *et al.*, 2007), highlighting the most probable way for bacterial route into the uterus. Indeed, different studies are suggesting that microbial composition is highly

influenced by the vaginal microbes (Mitchell *et al.*, 2015; Moreno *et al.*, 2016; Kyono *et al.*, 2018; Vornhagen *et al.*, 2018; Romero *et al.*, 2019).

Evidence for existence of certain microbiota (i.e., microbial community) in the uterus of healthy women is accumulating (Egbase *et al.*, 1996; Moore *et al.*, 2000; Salim *et al.*, 2002; Benner *et al.*, 2018; Moreno *et al.*, 2018; Smolnikova, 2019), but the confirmation of living microbes in the uterus is controversial and, up to now, no consensus on the core uterine microbial composition can be deduced. Uterine microbiome studies are challenged by many factors starting from the hormonal and physiological changes within the menstrual cycle, difficulty in obtaining uterine samples without contaminating the sample with vaginal/cervical bacteria, up to a high contamination risk also during samples processing and sequencing (see Figure 1 for factors). Uterine sample requires invasive sampling methods, and even when avoiding biopsying via cervix using surgical or explorative procedures, women undergoing these procedures usually present existing medical conditions and often are peri- and postmenopausal, which could contribute to the compositional shifts in the microbiome (Peric *et al.*, 2019). Furthermore, the NGS-based studies conducted so far are focussing on detecting microbial DNA sequences. While this approach provides knowledge of possible taxa present and describes the microbiome, a mere presence of a DNA sequence does not equate with the presence of a live bacteria. Nevertheless, there are studies showing that short bacterial DNA fragments and microbial components can induce a physiological inflammatory response in the host (Schindler *et al.*, 2004; Potgieter *et al.*, 2015; Kell and Kenny, 2016). Furthermore, other NGS methods are being applied in order to detect active microorganisms, such as meta-transcriptomics which analyses microbial RNA transcripts, identifying thereby potential functionally active microorganisms (Bashiardes *et al.*, 2016;

Knight *et al.*, 2018). Clearly more knowledge about the microbial composition, dynamics and function within the uterus in health and disease is warranted in order to better understand the microbial homeostasis and dysbiosis in endometrial functions.

In this review, we summarise the bacteria identified in endometrial microbiome studies in human endometrium, the current treatments offered for bacterial dysbiosis in clinical setting, and the future possibilities for modifying uterine microbial composition.

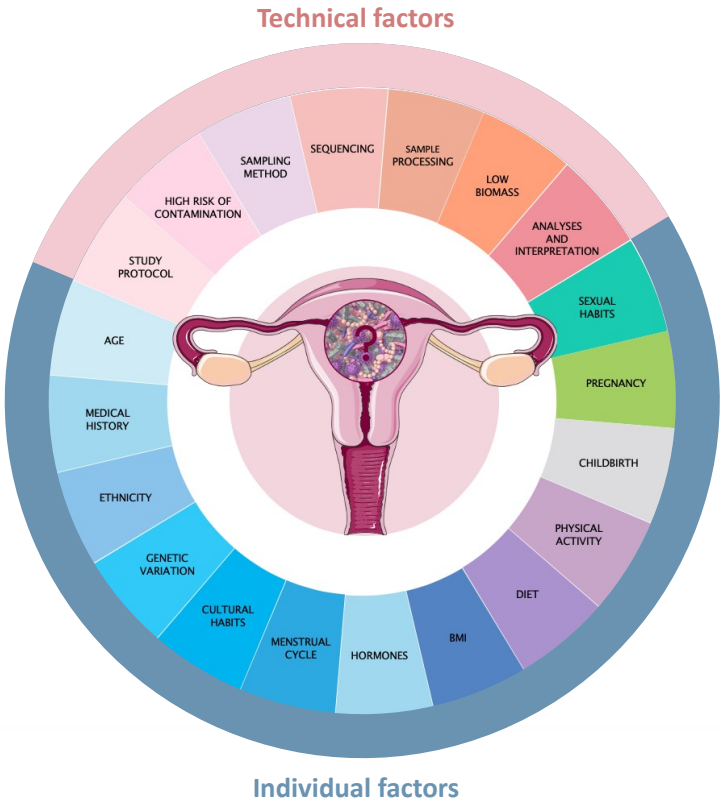

Figure 1. Dynamics and factors that could influence the endometrial microbiome and its analysis, including technical factors in the experimental design and individual/participant/group factors that could fluctuate the microbial composition in the uterus. Age: microbiome changes along lifespan (Takagi *et al.*, 2019), and the diversity and composition decline in the elderly (Hopkins *et al.*, 2001; Salazar *et al.*, 2014). Medical history: genital and extragenital diseases (Khan *et al.*, 2016; Walther-Antonio *et al.*, 2016; Mändar *et al.*, 2017; Pelzer *et al.*, 2018), including history of sexually transmitted infections can influence microbiome analysis. Ethnicity and different geographic regions: the ethnic origin of individuals seems to be an important factor to consider in microbiome research (Zhou *et al.*, 2007, 2010; Consortium *et al.*, 2014; Dunlop *et al.*, 2019), as African

American and Hispanic women show a trend to be more colonised with a non-Lactobacillus species in the upper genital tract than Caucasian women (Mitchell *et al.*, 2015). Genetic variation: host genetics can affect microbiome composition and maintenance (Genc and Onderdonk, 2011; Antonio Garcia-Velasco *et al.*, 2017). Cultural habits: these habits have shown to lead to microbial variations (Rayanakorn *et al.*, 2019). Menstrual cycle and hormones: fluctuations in circulating oestrogen and progesterone levels might influence endometrial microbiome (Wilson *et al.*, 2007; Khan *et al.*, 2016; Antonio Garcia-Velasco *et al.*, 2017; Bracewell-Milnes *et al.*, 2018). BMI: body mass index, especially obesity has been associated with altered intestinal microbiome composition (Dominianni *et al.*, 2015; Postler and Ghosh, 2017). Diet: it has been shown that the gut microbiome not just metabolizes ingested food but is itself shaped by the mode and type of food consumption (Claesson *et al.*, 2012; Javurek *et al.*, 2017). Physical activity: exercising has shown to impact microbial composition (Lynch and Pedersen, 2016; Deschasaux *et al.*, 2018). Childbirth and pregnancy (Huang *et al.*, 2014; DiGiulio *et al.*, 2015; Fox and Eichelberger, 2015; Selma-Royo *et al.*, 2019). Sexual habits: the concept of seminovaginal microbiome, i.e., the partners share their microbial communities, has gained support (Mändar *et al.*, 2015). Spermicidal agents could disrupt endometrial microbiota (McGroarty *et al.*, 1992); also sexual debut and activity can influence genital microbiome (Vodstrcil *et al.*, 2017; Mändar *et al.*, 2018). Study protocol: different protocols (collection technique, sample storage, extraction method, choice of primers) can lead to different results (Gill *et al.*, 2016; Hallmaier-Wacker *et al.*, 2018; Lim *et al.*, 2018; Macklaim and Gloor, 2018; Bjerre *et al.*, 2019; Fricker *et al.*, 2019; Greathouse *et al.*, 2019). High risk of contamination: laboratory and reagent contamination can critically impact sequence-based microbiome analyses (Goodrich *et al.*, 2014; Salter *et al.*, 2014; Glassing *et al.*, 2016; Kim *et al.*, 2017); negative controls consisting in blanks are recommended (van der Horst *et al.*, 2013; Laurence *et al.*, 2014; Salter *et al.*, 2014; Glassing *et al.*, 2016). Sampling method: an important point to consider consists in avoiding the surface of the vagina and the walls of cervical canal when taking the endometrial sample (Williams *et al.*, 2008; Du *et al.*, 2016). Sequencing: different sequencing platforms and sequencing filtering and processing can result in different outcomes (Aron-Wisnewsky and Clément, 2016; Clooney *et al.*, 2016; Knight *et al.*, 2018). Sample processing: amplicon sequencing has shown to cause great variance (Clooney *et al.*, 2016; Hallmaier-Wacker *et al.*, 2018). Low biomass: high risk of misinterpretation of the results (Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019; O'Callaghan *et al.*, 2020). Analyses and interpretation: statistical analyses and interpretation can induce bias (Goodrich *et al.*, 2014; Clooney *et al.*, 2016; D'Amore *et al.*, 2016).

POSSIBLE FUNCTION OF MICROBES IN THE UTERUS

It is becoming increasingly evident that microorganisms play an important role in our health and well-being through the production of bioactive molecules shaping a healthy microbiota, which, in turn, interacts with our own cells to regulate and influence our metabolism, physiology, and immune functions that ultimately shape our health and resistance to a disease (Cerdo *et al.*, 2016; Power

et al., 2017; Marques *et al.*, 2018). The exact role and relationship between microbes and the female reproductive tract remain to be established.

Endometrium seems to be an immunologically suited niche for microbiota with its possible function in modulating inflammatory and immune responses (Baker *et al.*, 2018; Benner *et al.*, 2018; Agostinis *et al.*, 2019) (Figure 2). The mucosal layers of the female reproductive tract constitute a part of the mucosal immune system, which exhibits a broad repertoire of immune responses (Nguyen *et al.*, 2014). Further support for the possible immune responses in the uterus is providing by the fact that the genital epithelial cells express a wide range of pattern recognition receptors (PRRs) that promote their ability to recognise and differentially respond to various pathogens (Nguyen *et al.*, 2014). The PRRs found in the female reproductive tract include Toll-like receptors (TLRs) and NOD-like receptors, which have important roles in protecting against pathogenic invasion, in tissue adaptation and ultimately in successful reproduction (Nguyen *et al.*, 2014; Benner *et al.*, 2018). Also pathophysiological effects of uterine microbes on the endometrial epithelium have been proposed: 1) the genomic stability of uterine epithelia could be impacted via modulation of transcription factors and other genomic and epigenetic alterations; 2) the integrity of the epithelial barrier could be impaired; and 3) the microbial-secreted metabolites and the inflammation triggered by TLR activation could lead to suppression and/or overgrowth of specific bacteria (Baker *et al.*, 2018). The potential molecular functions of the endometrial microbes have been linked to cell metabolism, motility, genetic information, immune system, and signalling processes in the NGS studies (Chen *et al.*, 2017; Garcia-Grau *et al.*, 2019). Regardless of all these hypotheses, future studies are needed to identify the basal uterine microbiota, its diversity and its functions and interactions with the endometrium.

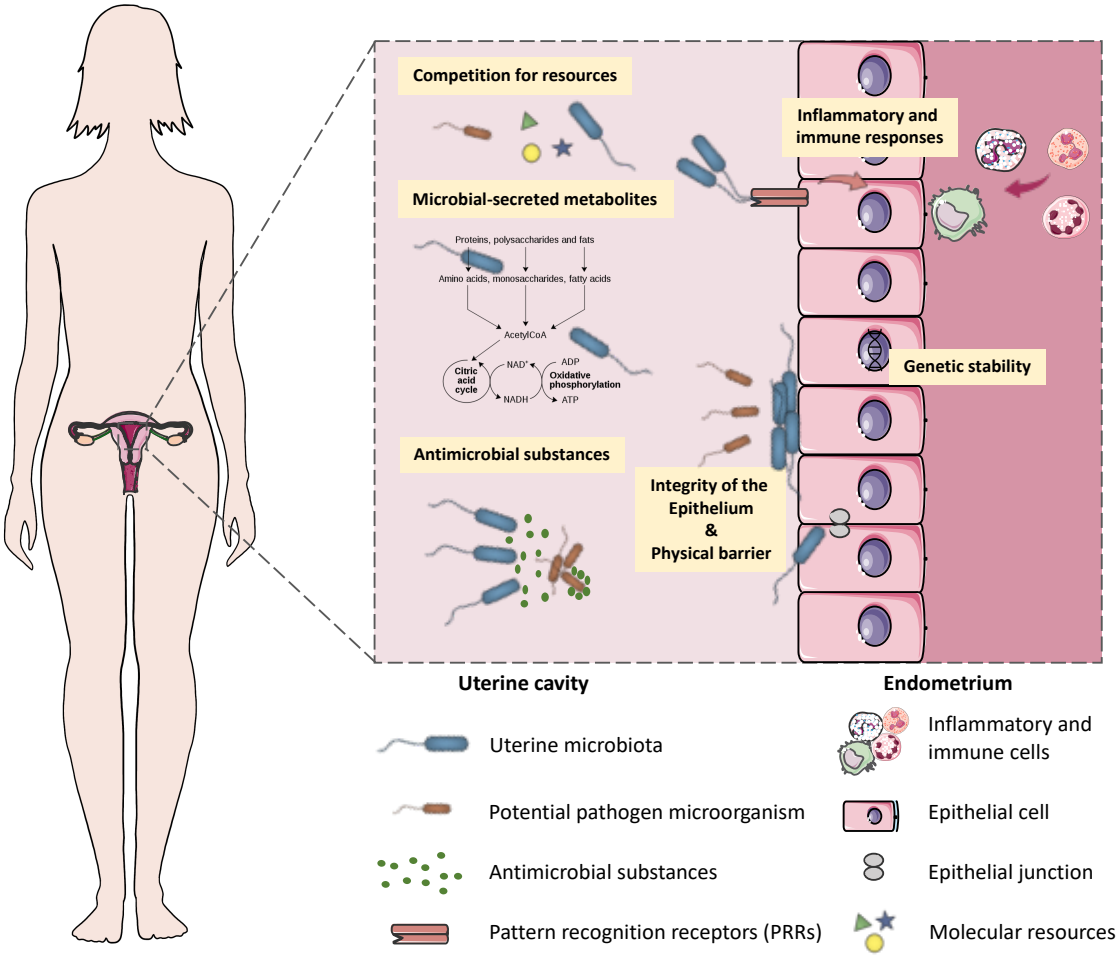

Figure 2. Endometrial-microbiota interplay in the uterus. Uterine microbes could impact the genomic stability of uterine epithelia through modulation of transcription factors and other genomic and epigenetic alterations; also, microbial-secreted metabolites may support the growth of specific species and suppress the growth of other bacteria; and the consumption of a limited resource can starve the pathogenic invaders. In short, the endometrium is an immunologically suited niche for microbes: the endometrial immune system needs to be well adapted to withstand the continuous threat caused by microbial colonisation of the large endometrial mucosal surface, separated from host tissue by only a single layer of epithelial cells. Thus, tissue invasion of microbes must be limited in order to prevent potentially harmful inflammation or imbalance in the symbiotic relationship. Endometrial microbial homeostasis is probably regulated in three different ways: 1) a single layer of columnar epithelial cells forming a strong barrier through tight junctions anatomically limiting exposure of resident bacteria to the systemic immune system; 2) immune mediators such as infection-controlling molecules (antimicrobial peptides, AMPs) that are present in the endometrial mucosal surface and the endometrial fluid (Wira *et al.*, 2014) and could restrict direct contact between epithelia and microbes; and 3) a rapid detection (epithelial cells express pattern recognition receptors (PRRs) that recognise and act to pathogens) and killing of bacteria upon a barrier breach by the endometrial lymphocytes that are present thromughout all stages of the menstrual cycle (Givan *et al.*, 1997; Vallve-Juanico *et al.*, 2019).

UTERINE/ENDOMETRIAL MICROBIAL COMPOSITION IN HEALTH AND DISEASE

Several studies have demonstrated that asymptomatic women harbour commensal microbial communities in their uterus (Fang *et al.*, 2016; Moreno *et al.*, 2016; Kyono *et al.*, 2018; Pelzer *et al.*, 2018; Leoni *et al.*, 2019), and that the uterine microbiome seems to be altered in women who suffer gynaecological pathologies such CE (Fang *et al.*, 2016; Moreno *et al.*, 2018; Liu *et al.*, 2019), endometriosis (Khan *et al.*, 2016; Chen *et al.*, 2017; Hernandes *et al.*, 2020), dysfunctional endometrial bleeding (Pelzer *et al.*, 2018), endometrial polyps (Fang *et al.*, 2016), endometrial cancer or hyperplasia (Fang *et al.*, 2016; Walther-Antonio *et al.*, 2016; Walsh *et al.*, 2019; Winters *et al.*, 2019), and infertility (Franasiak *et al.*, 2016; Moreno *et al.*, 2016; Kyono *et al.*, 2018; Wee *et al.*, 2018) (Table 1). All these studies support the evidence that the uterine microbial composition is clinically relevant and requires further investigation.

Healthy Women

The uterine microbiome of 'healthy' women has been mostly investigated as a control group in studies of infertile women or those with a gynaecological pathology (Fang *et al.*, 2016; Khan *et al.*, 2016; Moreno *et al.*, 2016, 2018; Walther-Antonio *et al.*, 2016; Cregger *et al.*, 2017; Kyono *et al.*, 2018; Pelzer *et al.*, 2018; Wee *et al.*, 2018; Kitaya *et al.*, 2019; Liu *et al.*, 2019; Walsh *et al.*, 2019; Hernandes *et al.*, 2020) (Table 1). Nevertheless, these data suggest that commensal microorganisms can inhabit the upper reproductive tract of healthy women. Regardless of the growing body of literature, the core uterine microbial composition remains an open issue. There are studies concluding that the endometrium has a resident microbiome dominated by *Lactobacillus* species being similar to that of the vagina

(Fang *et al.*, 2016; Moreno *et al.*, 2016; Kyono *et al.*, 2018), while other studies from surgical procedures (where vaginal bacterial contamination is minimised) demonstrate that *Lactobacillus* is rare and that *Acinetobacter*, *Pseudomonas* and *Comamonadaceae* dominate (Chen *et al.*, 2017; Winters *et al.*, 2019; Younge *et al.*, 2019). Also, a previous study analysing virgo intacta women identified the obligate anaerobes *Jonquetella* and *Fusobacterium* together with *Preovotella* as the predominant taxa in endometrium (Pelzer *et al.*, 2018). Furthermore, as 40% of the endometrial samples obtained by abdominal hysterectomy did not present any detectable microbes above the negative controls (Winters *et al.*, 2019), this raises further doubts whether there is a unique uterine microbiome in all women, whether these detected bacteria are temporarily or permanently in the uterus, or whether we are dealing with contamination.

Table 1. Predominant taxa in endometrial microbiome for different gynaecological disorders revealed by next-generation sequencing studies.

Gynaecological Condition	**Predominant Taxa**
Healthy	*Acinetobacter*, *Bacillus*, *Barnesiella*, *Bifidobacterium*, *Blautia*, *Corynebacterium*, *Desulfosporosinus*, *Enterobacter*, *Escherichia*, *Fusobacterium*, *Gardnerella*, *Jonquetella*, *Lactobacillus*, *Parabacteroides*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Ralstonia*, *Shigella*, *Staphylococcus*, *Streptococcus*
Infertility	*Atopobium*, *Bacteroides*, *Betaproteobacteria*, *Bifidobacterium*, *Burkholderia*, *Chitinophagaceae*, *Corynebacterium*, *Escherichia/Shigella*, *Flavobacterium*, *Gardnerella*, *Lactobacillus*, *Megasphaera*, *Pelomonas*, *Prevotella*, *Pseudoalteromonas*, *Rhodanobacter*, *Sneathia*, *Staphylococcus*, *Streptococcus*
Endometriosis	*Acinetobacter*, *Barnesiella*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacterium*, *Gardnerella*, *Lactobacillus*, *Moraxellaceae*, *Prevotella*, *Pseudomonas*, *Sphingobium*, *Staphylococaceae*, *Streptococcaceae*, *Vagococcus*
Chronic endometritis	*Alteromonas*, *Anaerococcus*, *Atopobium*, *Bifidobacterium*, *Dialister*, *Gardnerella*, *Lactobacillus*, *Magasphaera*, *Parvimonas*, *Prevotella*, *Propionibacterium*, *Streptococcus*, *Veillonella*
Endometrial polyps	*Alteromonas*, *Bifidobacterium*, *Euryarchaeota* (Archaea), *Gardnerella*, *Lactobacillus*, *Streptococcus*
Dysfunctional menstrual bleeding	*Gardnerella*, *Lactobacillus*, *Prevotella*, *Sneathia*, *Veilonella*
Endometrial cancer	*Acinetobacter*, *Anaerostipes*, *Anaerotruncus*, *Arthrospira*, *Atopobium*, *Bacteroides*, *Cloacibacterium*, *Comamonadaceae*,

	Dialister, *Escherichia*, *Peptoniphilus*, *Porphyromonas*, *Pseudomonas*, *Ruminococcus*, *Treponema*

Infertility

Several publications support the theory that alterations in the endometrial microbiome may also impact the reproductive potential of infertile patients and perhaps, correcting microbial dysbiosis would lead to improve success (Franasiak and Scott, 2015). There are numerous studies trying to associate endometrial microbiome with assisted reproduction outcomes, but just one has found a statistically significant difference in microbiome composition with success in infertility treatment outcomes (Moreno *et al.*, 2016). In that study, *Lactobacillus* dominance (>90% of all bacteria) correlated positively with embryo implantation, pregnancy and live birth rates among infertile women undergoing *in vitro* fertilisation (IVF) (Moreno *et al.*, 2016). Other studies have not detected any significant associations between endometrial microbiome and the treatment 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; Kitaya *et al.*, 2019).

Endometriosis

The pathophysiology of endometriosis is still unclear (Vargas *et al.*, 2020). The 'Bacterial contamination' hypothesis in endometriosis has been proposed (Khan *et al.*, 2010), where the inflammatory mediator lipopolysaccharide could be the initial trigger and bacterial contamination its source in the intrauterine environment serving as the primary cause in the growth regulation of endometriosis (Khan *et al.*, 2018). Also, considering the altered inflammatory condition in this disease, it is probable that the microbial pathogens activate the immune response by binding to host receptors. Indeed, complex bidirectional

interaction between the microbiome and endometriosis is gaining evidence (Leonardi *et al.*, 2020). Nevertheless, studies on endometrial microbiome and endometriosis have yielded contradicting results: *Streptococcaceae*, *Moraxellaceae*, *Staphylococaceae* and *Enterobacteriaceae* families were significantly increased while *Lactobacillus* species decreased in samples obtained from women with endometriosis (Khan *et al.*, 2016), and uterine wash samples from women both with and without endometriosis detected *Lactobacillus* together with *Barnesiella*, *Flavobacterium* and *Pseudomonas* as the most predominant genera (Cregger *et al.*, 2017). Another study investigating women with endometriosis identified the presence of *Pseudomonas*, *Acinetobacter*, *Vagococcus* and *Sphingobium* in uterus and revealed that uterine microbiota composition is significantly different in infertile women due to endometriosis (Chen *et al.*, 2017). A recently published study analysing endometriotic lesions found that the microbial diversity of lesions was higher compared to eutopic endometrium, where *Lactobacillus*, *Enterococcus*, *Gardnerella*, *Pseudomonas*, *Alishewanella*, *Ureaplasma* and *Aerococcus* prevailed (Hernandes *et al.*, 2020).

Chronic Endometritis

CE is a medical condition implicated in 12 – 46% cases of infertile patients (Takebayashi *et al.*, 2014). Indeed, different studies have demonstrated that CE is favourably prevalent in patients affected by infertility, especially in case of repeated IVF failures (Cicinelli *et al.*, 2018). In CE, the persistent inflammation of the endometrial mucosa is caused by the presence of bacterial pathogens in the uterine cavity (Cicinelli *et al.*, 2008). The most common responsible bacteria for CE are *Enterococcus faecalis*, *Enterobacteriaceae*, *Streptococcus* spp., *Staphylococcus* spp., *Gardnerella vaginalis*, *Mycoplasma* spp. and other pathogens associated with

sexually transmitted infections, such as *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (Moreno *et al.*, 2018).

Endometrial Polyps

Endometrial polyps, a common gynaecologic disease featured as a localized overgrowth of mucosa, has been correlated with CE, and continuous stimulation of biological inflammatory factors are believed to contribute to the disease (Al-Jefout *et al.*, 2009; El-Hamarneh *et al.*, 2013). A previous study found increased *Lactobacillus*, *Bifidobacterium*, *Gardnerella*, *Streptococcus*, *Alteromonas* and *Euryarchaeota* (archaea) and decreased *Pseudomonas* and *Enterobacteriaceae* among endometrial samples from women with endometrial polyps when compared to non-diseased women (Fang *et al.*, 2016) (Table 1).

Dysfunctional Menstrual Bleeding

Distinct microbial communities are believed to have a role in gynaecological pathologies such as menorrhagia and dysmenorrhea (Pelzer *et al.*, 2018). The study identified *Lactobacillus* dominance together with *Gardnerella vaginalis*, *Veillonella* spp., *Prevotela* spp., and *Sneathia* spp. in the endometrial samples (Table 1), and also detected menstrual cycle dependent changes within the endometrial microbiome during the proliferative and secretory phases of the cycle (Pelzer *et al.*, 2018).

Endometrial Cancer

Prevailing hypotheses for endometrial cancer aetiology are focussed on obesity and hormones, and do not consider the potential role of endometrial microbiota (Walsh *et al.*, 2019). However, studies in other fields, such as cervical

cancer, prostate cancer and gastrointestinal tumours, have demonstrated that microorganisms play important roles in cancer causation and development (Scree *et al.*, 2007; Wroblewski *et al.*, 2010; Mira-Pascual *et al.*, 2015; Audirac-Chalifour *et al.*, 2016; Altmae *et al.*, 2019). In fact, 15% of tumours are estimated to be related to different infectious agents (Shahanavaj *et al.*, 2015). The first study analysing endometrial microbiome in patients with endometrial cancer revealed a compositional microbiome shift in the cancer cases, distinguishable from the benign cases (Walther-Antonio *et al.*, 2016). The authors suggested that the presence of *Atopobium vaginae* and *Porphyromonas somerae* is associated with endometrial cancer (Walther-Antonio *et al.*, 2016). Recently, the same group investigated endometrial cancer risks and they confirmed *Porphyromas somerae* presence as the most predictive microbial marker of endometrial cancer (Walsh *et al.*, 2019). Meanwhile, another group studied women undergoing hysterectomy for endometrial tumours and detected *Acinetobacter*, *Pseudomonas*, *Cloacibacterium*, *Comamonadaceae*, and *Escherichia* as predominant taxa in the diseased endometrium (Winters *et al.*, 2019).

CURRENT TREATMENTS IN CLINICAL PRACTICE

In the clinical setting, there is a high interest and demand to detect and to improve endometrial dysfunctions in order to treat uterine dysbiosis and to enhance infertility treatment outcomes. However, there is no unified protocol for assessing the endometrial microbial composition, neither for the treatment of uterine dysbiosis.

Pioneering studies based on NGS approaches have already developed commercially available tests for assessing endometrial microbiome: EMMA test by iGenomix® (https://www.igenomix.com/genetic-solutions/emma-clinics/)

and Endometrial Microbiome Test by Varinos Inc. (https://www.varinos.com/english). EMMA test is based on the previous study findings where *Lactobacillus* was dominating in the uterus, and that *Lactobacillus* dominance correlated with reduced miscarriage and implantation failures and thus improved pregnancy rates in women undergoing IVF (Moreno *et al.*, 2016). EMMA test classifies endometrial samples into *Lactobacillus* dominant and non-*Lactobacillus* dominant profiles. Once the sample is classified as non-*Lactobacillus* dominant, adequate treatment including antibiotic, probiotics and prebiotics could be applied. In the same line, Varinos test is supporting the *Lactobacillus* dominance in the uterus and categorises endometrial microbiome as *Lactobacillus* dominant and non-*Lactobacillus* dominant (Kyono *et al.*, 2019). Then, choices for intervention are suggested such as uterine lavage for microbial eradication, eradication treatment with antibiotics and/or taking probiotics and prebiotics for improving the microbiota. Undoubtedly useful, these tests are based on mostly observational studies and include limited number of patients, resulting in rather limited evidence for testing and subsequent clinical decision-making in a clinical setting (Haahr *et al.*, 2020). Clearly more research in the field is required starting with establishment of the core uterine microbiome before any treatment recommendations for 'dysbiosis' are offered for patients.

Antibiotics

The use of antibiotics is widely applied in gynaecology and obstetrics (Pereira *et al.*, 2016). The antibiotics use ranges from diagnostic techniques (e.g., hysterosalpingography, sonography, hysteroscopy, and laparoscopy) to embryo transfer and up to diseases such as endometriosis and endometritis (Pereira *et al.*,

2016). It is clear that the use of antibiotics produces fluctuation in microorganism communities within the female reproductive tract.

In the infertility clinical setting, a broad-spectrum antibiotic therapy (i.e., amoxicillin or levofloxacin) was recently used to modulate the non-*Lactobacillus* dominant endometrial environment into *Lactobacillus* dominant, followed by the combination of antibiotics, prebiotics and/or probiotics, and 53% (N=17) of patients achieved *Lactobacillus* dominant state in the uterus while showing a higher but not statistically significant pregnancy rates compared to the non-*Lactobacillus* dominant group (Kyono *et al.*, 2019). These results are found to be encouraging, and this treatment approach is gaining popularity in the clinics.

CE is the gynaecological condition that is believed to benefit the most from the microbial modulation with antibiotics, as the bacterial infection in this condition is known (Kitaya *et al.*, 2018). Several studies have shown that the use of antibiotics in CE has improved the reproductive outcomes. CE patients treated with antibiotics before embryo implantation had significantly better reproductive outcomes compared to those not treated with antibiotics (Cicinelli *et al.*, 2014), suggesting that the negative impact of CE on reproductive outcomes may be in part attributable to the presence of uterine bacteria. In line, McQueen et al. demonstrated that antibiotics treatment among the recurrent pregnancy loss patients with CE improved live birth rate from 7% to 56% the after treatment (McQueen *et al.*, 2014). In a retrospective study conducted by Cicinell et al. the pregnancy and live birth rates were significantly higher in cured CE women treated with antibiotics when compared with the persistent CE group and even with the non-CE group (Cicinelli *et al.*, 2018). Further, Kitaya et al. reported that women with recurrent implantation failure (RIF) treated with antibiotics for CE had higher live birth rates compared with RIF patients with no CE (Kitaya *et al.*,

2017). Also, the study conducted by Zhang et al. demonstrated that patients diagnosed with RIF and CE when treated with intrauterine antibiotic infusion therapy had significantly higher implantation and pregnancy rates when compared with RIF women without CE and with persistent CE groups (Zhang *et al.*, 2019). Further, intrauterine infusion of antibiotics after poor oral antibiotic therapy outcomes has also been applied in order to restore the physiological condition of the uterus in CE, and the three patients assessed achieved pregnancy after the treatment (Sfakianoudis *et al.*, 2018).

Women with endometriosis is another group of patients that could benefit from the uterine microbial modulation by antibiotics, as the hypothesis of 'bacterial contamination' in endometriosis has been proposed (Khan *et al.*, 2010). Among patients who suffer endometriosis, the prophylactic antibiotic therapy has been applied prior to oocyte retrieval with the aim to reduce infection; with the treatment the infection risk reached 0% (Weinreb *et al.*, 2010). In fact, prophylactic administration of antibiotics prior to oocyte retrieval in patients suffering from severe endometriosis is today commonly applied due to the risk of infection (Pereira *et al.*, 2016). In animal models, mice with endometriosis were treated with broad-spectrum antibiotics and it resulted in a reduction of endometriotic lesions, supporting the possible role of microbes in endometriosis progression (Chadchan *et al.*, 2019).

Undoubtedly effective in modulating uterine microbiome, best administration regimens and their effects on endometrial microbiome should be tested further in order to understand the impact on health and to avoid unnecessary antibiotic use.

Probiotics

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit in the host', and capturing the essence of probiotics, as microbial, viable and beneficial to the health (Hill *et al.*, 2014). Probiotic bacteria produce bioactive molecules that could act on the body and promote good health. These formulations might be a safe and effective alternative to antibiotics in restoring the imbalance of the uterine microbiota as seen in female urogenital disorders (Khalesi *et al.*, 2019; Trush *et al.*, 2020). In the clinical setting, there is a high interest and demand to improve aberrant endometrial microbial environment in order to overcome dysbiosis and to enhance infertility treatment outcomes.

Probiotic interventions have been extensively investigated in relation to systemic inflammation (Pelzer *et al.*, 2017). The first probiotics were composed predominantly of members of *Lactobacillus* and *Bifidobacterium*, but they showed a lack of precision when targeting a specific biological function (Kashyap *et al.*, 2017). In the male reproductive field, the use of probiotics and synbiotics (formulations including probiotics and prebiotics) has been related to improved semen quality (Maretti and Cavallini, 2017; Valcarce *et al.*, 2017), and in females the protective role of probiotics in relation to vaginal infections has been observed (Selma-Royo *et al.*, 2019; Lopez-Moreno and Aguilera, 2020). A recent study aimed to select potential probiotic strains from vaginal samples to improve the health of the female reproductive tract and selected the *Lactobacillus rhamnosus* BPL005 strain for its capacity to reduce pH and produce short chain fatty acids, protective effects against pathogen colonisation and lactate production (Chenoll *et al.*, 2019).

Women suffering from endometriosis are shown to benefit from the administration of oral *Lactobacillus* by reduction in pain (Itoh *et al.*, 2011b;

Khodaverdi *et al.*, 2019). In the mouse model, the beneficial effect of *Lactobacillus* on endometriosis by increasing interleukin-12 levels and the activity of natural killer cells has been suggested, and the administration of probiotics resulted in a reduction of endometriotic lesions (Somigliana *et al.*, 1999; Itoh *et al.*, 2011a; Uchida and Kobayashi, 2013).

Female genital microbiota modulation could also be used to fight or protect against infection. A recent research has demonstrated how probiotic Lactobacilli (*Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1) can improve endometrial epithelial cells barrier function in response to human immunodeficiency virus-1 (HIV-1) (Dizzell *et al.*, 2019). It was also detected that bacterial strains could modulate the immune profile suggesting that the microbiota of the female reproductive tract is an important factor in the acquisition of resistance to the virus (Dizzell *et al.*, 2019).

The field of probiotics in modulating uterine microenvironment is very promising, nevertheless we have to bear in mind that probiotics are not medical drugs and they often lack detailed product information, and only few probiotics have been investigated thoroughly in clinical trials (https://www.igenomix.com/genetic-solutions/emma-clinics/).

Prebiotics

Prebiotics target microbes living in/on human with the result of improving health, providing non-viable substrates that serve as nutrients for beneficial microorganisms harboured by the host (Gibson *et al.*, 2017). In the attempt to modify endometrial microbiome lactoferrin a prebiotic agent with favourable prebiotic activity has been administered orally during and after treatment with antibiotics among women undergoing infertility treatment (Kyono *et al.*, 2019).

Among non-*Lactobacillus* dominant patients treated with lactoferrin for three months after the antibiotics therapy, 67% (6/9) of them reached *Lactobacillus* dominance in the endometrium (Kyono *et al.*, 2019). Additionally, lactoferrin administration has demonstrated effective result against bacterial vaginosis, leading to pregnancy and full-term birth in women with a previous medical history of preterm birth (Otsuki and Imai, 2017).

Prebiotics use in human reproductive field is in its infancy and it is for future studies to unravel its usefulness in modifying microbial niches in female reproductive tract.

Microbial Transplants

Faecal microbiota transfer (FMT) is increasingly being used for various indications, however clear evidence for the efficacy of FMT currently exists only for recurrent *Clostridioides difficile* infection (Stallmach *et al.*, 2020). Over 100 clinical trials using FMT for different conditions are currently ongoing (ClinicalTrials.gov). The problem arises from the fact that FMT is not only being used in clinical trials, but also applied on individual patients with methods that are not publicly documented (Stallmach *et al.*, 2020). In this scenario, the required screening of FMT donors is not always performed in a standardised way, which can cause different side effects and complications among patients. In fact, Food and Drug Administration (FDA, USA) has communicated a warning regarding the risk of severe bacterial infection after FMT (Stallmach *et al.*, 2020). The hypothesis for the uterine bacterial transmission route originating from the gut exists (Donnet-Hughes *et al.*, 2010; Baker *et al.*, 2018), thus FTM has been proposed as a promising (future) tool for treatment female reproductive tract diseases (Quaranta *et al.*, 2019). It was been shown on broad-spectrum antibiotics-

treated endometriosis mice that the FMT from mice with endometriosis resumed the growth of endometriotic lesions suggesting that the gut microbiota could promote endometriosis progression (Chadchan *et al.*, 2019). In human, differences in gut microbial composition between healthy women and women with endometriosis (Ata *et al.*, 2019) and PCOS have been reported (Lindheim *et al.*, 2017; Liu *et al.*, 2017; Insenser *et al.*, 2018; Torres *et al.*, 2018; Jobira *et al.*, 2020; Zhou *et al.*, 2020).

A new area of microbial transplants is arising – vaginal microbiota transplants (VMT), which is opening new frontiers for reproductive health (DeLong *et al.*, 2019). VMT involves the transfer of cervicovaginal fluid from a 'healthy' donor to a patient who aims to restore the most beneficial microenvironment. A pioneering study has tested the use of VMT from healthy donors as therapeutic alternative for patients suffering from symptomatic, intractable, and recurrent vaginosis, and reported positive treatment outcomes (Lev-Sagie *et al.*, 2019). Since the uterine colonisation of microorganisms by vaginal-cervical ascension is known (Vornhagen *et al.*, 2018; Romero *et al.*, 2019), VMT could open up a future way for managing endometrial dysbiosis. And what about uterine microbiota transfer (UMT)? To conclude, microbial transplants are highly promising ways for modifying uterine microbiota, nevertheless thorough research and testing in randomised, placebo-controlled trials is warranted.

CHALLENGES IN DEVELOPING TARGETED MODULATORS FOR UTERINE MICROBIOTA

The uterus represents an ideal organ for drug administration, possessing advantages such as the possibility of bypass first-pass metabolism, high permeability for low molecular weight drugs, considerable surface area for

absorption, and rich blood supply (Leyva-Gomez *et al.*, 2019). However, the effectiveness of the site would depend on intrinsic factors that include pH, temperature, uterine fluid composition, viscosity, enzymatic metabolism, clearance, and others, together with the hormonal fluctuations throughout the menstrual cycle. For instance, uterine pH has been shown to change along the menstrual cycle, oscillating between 6.4 and 7 (Feo, 1955) and this could modify the drug-release system since the vast majority of drugs possess an ionisable group (mostly weak bases) (Manallack, 2007). Other biophysical parameters such as oxygen tension (pO_2) and temperature are also factors with probable influence on the modulators. pO_2 is shown to have cyclical variation and minute-to-minute oscillations within human uterus (Ng *et al.*, 2018). Also, the temperature variation is cyclical by day and month, increasing in the luteal phase and is influenced by hormones, density of uterine vascular beds and effectiveness of local heat exchange (Ng *et al.*, 2018). It is clear that many factors could influence the drug-release system inside the uterus, and a detailed study of these properties are required in order to develop effective targeted microbiota modulators with the exact dose required, necessary time and right place.

Regardless of the different intrinsic factors, probably one of the most important challenges that arise in modulating the endometrial microbiome is the fact that it is a low microbial biomass niche (Winters *et al.*, 2019; Selway *et al.*, 2020). It is clear that analyses of low-biomass microbial sites are sensitive to contamination (especially from lower genital tract) and data misinterpretation. Thus, researchers face hurdles when describing the baseline microbial communities in endometrium and require well-designed and well-controlled experiments in order to avoid and adjust for the risk of contamination (Weyrich *et al.*, 2019; O'Callaghan *et al.*, 2020). This makes especially necessary to set up

standardised detection and description methods for analysing the uterine microbial composition, as well as strategies to re-establish/maintain these microbial populations. The challenge of assessing the true microbial composition in endometrial eu- and dysbiosis, the adequate dosing and evaluating these effects remains for future studies.

CONCLUSIONS AND FUTURE DIRECTIONS

The conventional approach to target bacterial dysbiosis has been and continues to be the use of antibiotics, which have been shown to be both essential and effective for treating infections usually resulting from pathogen proliferation. Nevertheless, the antibiotic drugs lead to unintended off-target effects on microbial community structure which frequently causes adverse effects, making them less appealing as precise therapy to target microbiome (Kashyap *et al.*, 2017). In many cases, its administration aggravates the underlying dysbiosis in long term and may promote resistance (Chenoll *et al.*, 2019). For instance, prescription of antibiotics in a prophylactical way has not resulted in improved pregnancy outcome not even in the cases of high risk of preterm birth on the presence of pathogens (Tita *et al.*, 2007).

New therapies, such as pro- and prebiotic administration, and microbiota transplants are gaining popularity for improving and maintaining the optimal composition of the microbiota (Figure 3). These approaches attempt to modulate the microbial communities in a way that is beneficial to human health. However, several important questions related to these new clinical strategies remain unresolved, such as indication for prescription, comparative efficacy of monostrain and multistrain probiotics, choice of excipients, and methods of administration and delivery (Trush *et al.*, 2020). Furthermore, procedures to

develop reliable and reproducible microbiome-based therapeutic approaches represent a challenge (Kashyap *et al.*, 2017). Currently, the pharmacological mechanisms of probiotics and prebiotics' action are poorly understood, providing not enough evidence to support the use of probiotics for medicinal purposes (Pelzer *et al.*, 2017). Of those commercially available probiotics, it is especially difficult to prove their clinical efficacy as they are based on studies on small sample size, and the heterogeneity in strains of bacteria used, duration of treatment and the lifestyle of patients, which can also influence the effects of probiotic supplementation (Selway *et al.*, 2020).

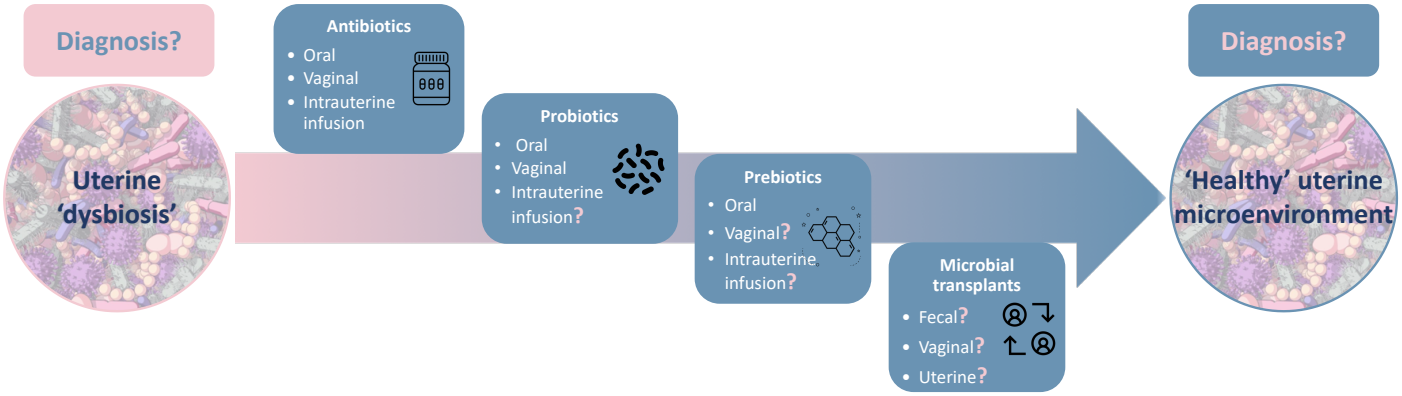

Figure 3. Current and future strategies for modifying uterine microbial composition. Intrauterine drug delivery represents an attractive alternative to achieve local and systemic effects due to the high contact surface exposed, the mucoadhesion of the epithelium, and the high absorption of drugs into the bloodstream. Several strategies for modifying endometrial microbial composition are being applied, nevertheless the core microbial composition is not established. The standard protocols for detecting uterine microbes and treatment protocols of dysbiosis are yet to be established.

Regarding the human uterus, there are a number of studies where the effect of probiotic supplementation on the endometrial microbiota has been studied, although mainly in combination with antibiotic treatment (Itoh *et al.*, 2011b, 2011a; Chenoll *et al.*, 2019; Khodaverdi *et al.*, 2019; Kyono *et al.*, 2019). The use of alternative modulators for uterine microbes is a highly demanded and relevant

area of investigation with direct clinical application. Nevertheless, before any treatment strategies could be offered the core uterine microbial composition needs to be established. In fact, there is an active debate ongoing whether uterus harbours a unique microbiota or not (Winters *et al.*, 2019). If microbiome is certainly present in the uterine environment in the absence of pathologic infection, current data support that it is of low abundance (Łaniewski *et al.*, 2020; O'Callaghan *et al.*, 2020), and several technical challenges in studies of low-biomass samples exist and make difficult to distinguish microorganisms that are truly present in small quantities from those arisen from contamination (Eisenhofer *et al.*, 2019; Weyrich *et al.*, 2019; O'Callaghan *et al.*, 2020). Thus, the appropriate uterine microenvironment for non-pathological conditions has yet to be established and applying methods for targeted modification of microbial communities is premature.

It is clear that in the case of infection caused by a pathogen, antibiotic treatment is required. However, in the case of prophylactics or suggested dysbiosis based on the molecular detecting methods (endometrial microbiome tests), today is too early to intervene and offer treatment recommendations for patients. In fact, no clinical recommendations are today available for diagnosis of 'abnormal/unfavourable uterine microbiota' (Haahr *et al.*, 2020). To sum up, modulation of uterine microbiota for restoring and maintaining microbial composition is a promising field of research and application with high clinical relevance, but we are not there yet and hopefully soon this promising future will be present.

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STUDY II: Omission of non-poly(A) viral transcripts from the tissue level atlas of the healthy human virome

ABSTRACT

A recent paper in BMC Biology entitled "A tissue level atlas of the healthy human virome" by Kumata et al. describes a meta-transcriptomic analysis of RNA-sequencing datasets from the Genotype-Tissue Expression (GTEx) Project. Using a workflow that maps the GTEx sequences to the human genome, then screens unmapped sequences to detect viral transcripts, the authors present a quantitative analysis of the presence of different viruses in the non-diseased tissues of over 500 individuals and assess the impact of these viruses on host gene expression. Here we draw attention to an issue not acknowledged in this study. Namely, by relying solely on GTEx datasets, which are enriched for transcripts with poly(A) tails, the analysis will have missed non-poly(A) viral transcripts, rendering this tissue level atlas of the virome incomplete.

A COMMENTARY ON KUMATA ET AL. (BMC BIOL 18:55, 2020)

Viruses are obligate parasites and require a living cell to complete their life cycles. Like mRNAs in the eukaryotic host cell, RNAs of many DNA and RNA viruses generate polyadenylated transcripts (i.e., transcripts containing 3′ poly(A) tails) that are synthesized post-transcriptionally (Carter *et al.*, 2013), and in some RNA viruses also by direct transcription from poly(U) sequence on the stretched template strand (Cross *et al.*, 2019; Geng *et al.*, 2019). The viral poly(A) tails are important for regulating RNA stability and translation initiation, mimicking roles of the stable poly(A) tails in eukaryotic mRNA (Barr and Fearns, 2010).

Many viruses, however, generate transcripts without poly(A) tails, a feature that has been maintained over evolution, especially in positive-strand RNA viruses as for instance are dengue virus, West Nile virus, Japanese encephalitis

virus, yellow fever virus, Zika virus, bovine viral diarrhea virus, and hepatitis C virus in the *Flaviviridae* family (Barr and Fearns, 2010; Gomila *et al.*, 2011; He *et al.*, 2015). Other important examples of non-poly(A) viral RNA transcripts are adenovirus-encoded non-coding RNA viral-associated RNAs and herpesvirus EBV-encoded non-coding small RNAs (EBERs) (the gold standard clinic markers for detection of EBV latent infection in specimens) (Yin *et al.*, 2020). Viral-encoded non-poly(A) RNAs have an important role in different physiological conditions and illnesses, including viral life cycle and function, and host cell immune evasion and transformation (Tycowski *et al.*, 2015).

Next-generation sequencing offers high sensitivity, specificity, and reproducibility in detection of low levels of transcripts thereby serving as a sensitive and reliable tool to qualify and quantify viruses at DNA and RNA levels (Noell and Kolls, 2019). Nevertheless, depending on the exact sequencing protocol of choice, the non-polyadenylated viral RNA sequences could be detected or discarded (Figure 1). The recent BMC Biology article by Kumata et al. presented the first tissue level atlas of the human virome by analyzing the RNA-seq data from the GTEx database (Kumata *et al.*, 2020). GTEx uses oligo (dT) primers for obtaining poly(A)-enriched fraction in the initial RNA purification step, meaning that only the RNA transcripts with poly(A) tail will be enriched and sequenced (The GTEx Project, https://www.gtexportal.org/home/documentationPage#staticTextDataProduction, Accessed 20 July 2020). We believe that Kumata et al. study has overlooked this important aspect, and although the first comprehensive investigation of the human virome in somatic tissues was presented, an important part of the human virome was not detected. A recently published study comparing poly(A)-enriched RNA-seq and non-poly(A)-selected RNA-seq in the lung virome

analysis from the same samples supports our concern, as in this study it was demonstrated clearly that poly(A)-enriched RNA-seq failed to detect several viruses (Yin *et al.*, 2020). Furthermore, Kumata et al. conclude that mainly DNA viruses shape the healthy human virome as most of the detected viruses in their study were DNA viruses, although they acknowledge the possibility that the detection sensitivity of RNA viruses could have been lower (Kumata *et al.*, 2020). Indeed, especially RNA viruses lack poly(A) tail (Gomila *et al.*, 2011; He *et al.*, 2015), which could be one solid explanation why RNA viruses were under-detected and DNA viruses predominated in the study by Kumata et al.

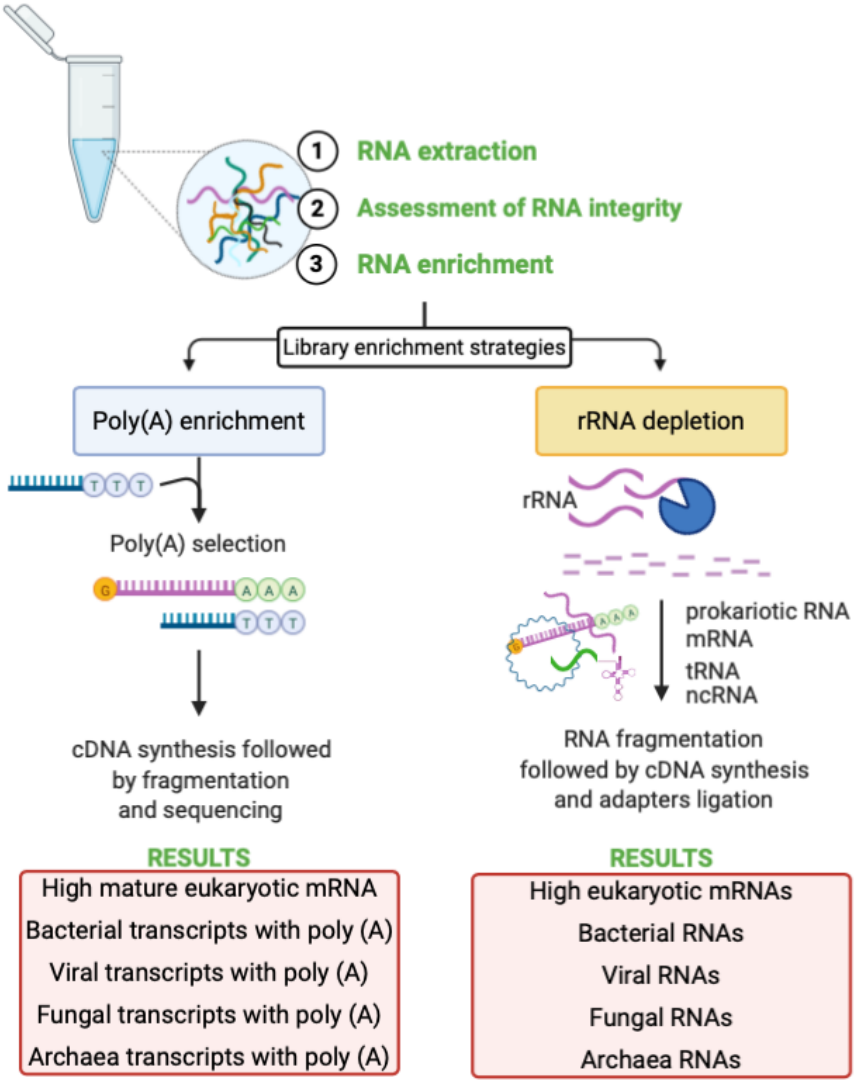

Figure 1. Simplified illustration of the two main protocols for analyzing RNA-seq. Created with BioRender.

Before other researchers are motivated to apply their meta-transcriptomic study approach (Kumata *et al.*, 2020) to other datasets with the aim of revealing the impact of viral infections on human health, we would like to highlight that the choice of sequencing protocol is crucial in obtaining and interpreting the study findings. In short, the recently presented tissue level atlas of the healthy human virome should be acknowledged as a partial tissue level atlas, and the comprehensive investigation should be completed with meta-transcriptome analysis of data generated using the total RNA extraction method in order to achieve a more complete view of the human virome.

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STUDY III: Mapping the entire functionally active endometrial microbiota

ABSTRACT

STUDY QUESTION

Does endometrium harbour functionally active microorganisms and whether the microbial composition differs between proliferative and mid-secretory phases?

SUMMARY ANSWER

Endometrium harbours functionally alive microorganisms including bacteria, viruses, archaea, and fungi whose composition and metabolic functions change along the menstrual cycle.

WHAT IS KNOWN ALREADY

Resident microbes in the endometrium have been detected, where microbial dysfunction has been associated with reproductive health and disease. Nevertheless, the core microorganismal composition in healthy endometrium is not determined and whether the identified bacterial DNA sequences refer to alive/functionally active microbes is not clear. Furthermore, whether there are cyclical changes in the microbial composition remains an open issue.

STUDY DESIGN, SIZE, DURATION

RNA sequencing (RNA-seq) data from 14 endometrial paired samples from healthy women, 7 samples from the mid-secretory phase and 7 samples from the consecutive proliferative phase were analysed for the microbial RNA sequences.

PARTICIPANTS/MATERIALS, SETTING, METHODS

The raw RNA-seq data were converted into FASTQ format using SRA Toolkit. The unmapped reads to human sequences were aligned to the reference database Kraken2 and visualised with Krona software. Menstrual phase taxonomic differences were performed by R package metagenomeSeq. The functional analysis of endometrial microbiota was obtained with HUMANn2 and the comparison between menstrual phases was conducted by one-way ANOVA.

Human RNA-seq analysis was performed using miARma-Seq and the functional enrichment analysis was carried out using gene set enrichment analysis (GSEA; HumanCyc). The integration of metabolic pathways between host and microbes was investigated. The developed method of active microbiota mapping was validated in independent sample set.

MAIN RESULTS AND THE ROLE OF CHANCE

With the novel meta-transcriptomic approach, we mapped the entire alive microbiota composing of >5300 microorganisms within the endometrium of healthy women. Microbes such as bacteria, fungi, viruses, and archaea were identified. The validation of three independent endometrial samples from different ethnicity confirmed the findings. Significant differences in the microbial abundances in the mid-secretory *vs*. proliferative phases were detected with possible metabolic activity in the host-microbiota crosstalk in receptive phase endometrium, specifically in the prostanoid biosynthesis pathway and L-tryptophan metabolism.

LARGE SCALE DATA

The raw RNA-seq data used in the current study are available at GEO GSE86491 and at BioProject PRJNA379542.

LIMITATIONS, REASONS FOR CAUTION

These pioneering results should be confirmed in a bigger sample size.

WIDER IMPLICATIONS OF THE FINDINGS

Our study confirms the presence of active microbes, bacteria, fungi, viruses, and archaea in the healthy human endometrium with implications in receptive phase endometrial functions, meaning that microbial dysfunction could impair the metabolic pathways important for endometrial receptivity. The results of this study contribute to the better understanding of endometrial microbiota

composition in healthy women and its possible role in endometrial functions. In addition, our novel methodological pipeline for analysing alive microbes with transcriptional and metabolic activities could serve to inspire new analysis approaches in reproductive medicine.

INTRODUCTION

The uterus has traditionally been considered sterile, while new studies using molecular techniques are suggesting that the endometrium harbours its resident microbiota, i.e., microorganismal communities (Evans *et al.*, 2016; Benner *et al.*, 2018; Koedooder *et al.*, 2019). The recent studies analysing endometrial microbiome, the genomes of the microbes, are indicating that microbial dysbiosis could be associated with different gynaecological disorders and with the treatment success in assisted reproductive techniques (Moreno *et al.*, 2016; Molina *et al.*, 2020). Nevertheless, the significance and the potential function of the microorganisms in the endometrium remains to be fully established.

Further, in comparison to the lower reproductive tract, the endometrium is considered a low microbial biomass site, hosting 10000 times fewer bacteria than vagina (Chen *et al.*, 2017; Winters *et al.*, 2019; O'Callaghan *et al.*, 2020). Due to the methodological complications in detecting low microbial biomass (Eisenhofer *et al.*, 2019), specifically high contamination risk with microbes from the lower reproductive tract while obtaining uterine sample (Koedooder *et al.*, 2019; Molina *et al.*, 2021) and the invasive nature of endometrial sampling, the core microbial composition in the healthy endometrium remains to be determined.

The difficulty in identifying the core/baseline endometrial microbiome is further complicated by a number of factors that can influence the microbial composition (Altmäe *et al.*, 2019), including cyclic menstrual phases in women.

Whether endometrial microbiome changes throughout the menstrual cycle have been explored in several studies (Khan *et al.*, 2016; Moreno *et al.*, 2016; Chen *et al.*, 2017; Cregger *et al.*, 2017; Pelzer *et al.*, 2018b; Kyono *et al.*, 2019), resulting in contradicting findings and with no clear consensus (Altmäe, 2018; Rosenberg and Zilber-Rosenberg, 2020). The cyclic influence on vaginal microbial composition is known (Garcia-Velasco *et al.*, 2017; Power *et al.*, 2017; Bracewell-Milnes *et al.*, 2018), also in the fallopian tubes microbiome differences between the proliferative and secretory phases have been detected (Pelzer *et al.*, 2018a). Further, gonadotrophin-releasing hormone agonist use resulted in a shift of intrauterine bacterial colonization (Khan *et al.*, 2016), supporting further the possibility of cyclical microbiome changes within the uterus. Nevertheless, a fundamental question remains as to whether endometrial microbiota fluctuates along the menstrual cycle or is stable.

All the next-generation sequencing (NGS)-based microbiome studies conducted so far in the endometrium have analysed the microbial DNA sequences (16S rRNA gene or metagenome analyses). While this approach provides knowledge of the possible taxa present, the sole presence of a microbial DNA sequence does not equate to the presence of an alive microorganism. DNA sequences could originate from microbial breakdown (e.g., DNA from dead microorganisms (Kliman, 2014)) as DNA molecules may persist for decades (Glassing *et al.*, 2016) or from background DNA contamination (Kim *et al.*, 2017; de Goffau *et al.*, 2018; Marsh *et al.*, 2018). Therefore, 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; de Goffau *et al.*, 2018; Willyard, 2018). Hence, whether the endometrial microbiome is functionally active or merely presents DNA sequences remains an open issue and needs further investigation.

We set out to detect the entire cartography of functionally active microorganisms in the endometrium from healthy women by applying meta-transcriptome, also named as meta-RNA sequencing (meta-RNA-seq) analysis. Meta-transcriptomics uses RNA-seq to profile transcripts of all microorganisms, thus identifying the alive microbes and the active functional output of the microbes, providing a snapshot of functional exploration of microbial community in situ (Macklaim and Gloor, 2018; Liu *et al.*, 2021). We also assessed whether the active microbial composition differs between the proliferative and mid-secretory phases, and investigated possible functions of the microorganisms in endometrial receptivity.

MATERIALS AND METHODS

Study material

A total of 14 paired endometrial samples from 7 healthy donors undergoing normal general physical and gynaecological examination were analysed. The women were 24–31 years old, with regular menstrual cycles, no hormonal or other treatment for at least 3 months, and no history of any chronic diseases (Sigurgeirsson *et al.*, 2017). The raw RNA-seq data were obtained from the Gene Expression Omnibus (GEO) repository, accession number GSE86491. As previously described (Sigurgeirsson *et al.*, 2017), the endometrial biopsies were collected from the mid-secretory phase (days LH+ 7-9 based on urinary ovulation test) and from the consecutive proliferative phase (cycle days 6–8) using a suction curette. Total RNA was extracted using miRNeasy Mini Kit, followed by RiboZero kit (Qiagen, Venlo, Netherlands) processing for removing rRNA, and the library was prepared with TruSeq Stranded Total RNA that used random hexamer primers for the first-stranded cDNA synthesis (Illumina, San Diego, CA,

USA). Sequencing was performed on the Illumina HiSeq2500 that generated 125 base pair pair-end reads with an average of 76.8 million reads per sample (Sigurgeirsson *et al.*, 2017).

Meta-transcriptomics: microbial transcriptome analysis

In order to analyse the microorganismal RNA sequences, the raw RNA-seq SRA files were converted into FASTQ format using the SRA Toolkit (Sequence Read Archive Submissions Staff, 2011). These paired FASTQ files were processed using miARma-Seq (Andres-Leon *et al.*, 2016), a pipeline that contains all the programs necessary for a RNA-seq study. This software is capable of performing a quality study, removing adapter sequences, aligning against a reference genome, obtaining the raw counts and doing a paired statistical study to obtain those differentially expressed genes in endometrium between the proliferative and mid-secretory phases. In detail, in the quality check step, miARma-seq uses FastQC (Andrews, 2010), in this way, the accumulation of rRNA and adapter sequences typical of the Truseq kit was observed. To remove the adapter sequences, Cutadapt (Martin, 2011) was applied, additionally filtering out all sequences with a quality score below 20. After this pre-processing, remaining reads were aligned using HISAT2 (Kim *et al.*, 2015) against the human genome GRCh38 from Gencode v26. In order to filter rRNA contamination, SortmeRNA (Kopylova *et al.*, 2012) was used in the aligned files. The parameters of this aligner were set so that the sequences that aligned to the reference genome were saved in a SAM format file, and those sequences that did not align to the reference genome (i.e., non-human, microbial sequences) (parameter –un-con-gz) were saved in paired FASTQ files. Next, these non-human sequences were aligned to a reference database with Kraken2 (Wood *et al.*, 2019). As a reference database,

we used the Kraken-build utility to download bacterial, archaea, viral, and fungal libraries including National Centre for Biotechnology Information (NCBI) taxonomic information as well as complete genome sequences from RefSeq. All the 14 samples were analysed independently, as well as in two meta-samples representing proliferative or mid-secretory phase groups. To visualise the set of taxa identified by Kraken2 in each of the sample sets analysed, we used the Krona software (Ondov *et al.*, 2011). Once all samples were processed and all reads were taxonomically classified, the report by Kraken was further processed to analyse all species found. Consequently, to study the phase specific differences in microorganismal communities, the species identified in the 14 samples together with the number of reads assigned directly to this taxon were processed using the metagenomeSeq R package (Paulson *et al.*, 2013). First, rare species (those that were not detected in any sample but included by the software, or in other words, those whose sum of reads was equal to 0) were removed. Next, to account for differences in the number of reads in each of the samples, the number of sequences was normalised using the Cumulative Sum Scale (CSS) method (Paulson *et al.*, 2013). This algorithm processes raw counts by dividing them by the cumulative sum of counts to a percentile that captures the relatively invariant count distribution in the data set. This method was applied as it appears to be more sensitive than ratio-based normalisation or random sampling in the taxon abundance measurement process (Paulson *et al.*, 2013). Subsequently, normalised data were subject to differential abundance tests based on the zero-inflated Gaussian model integrated in metagenomeSeq for identifying taxon changes between the two menstrual phases.

Validation of the functional microbiota mapping method

In order to validate our protocol in an independent endometrial sample set, a literature search was performed in PubMed, where the search criteria were set: endometrial samples from control women, and the use of ribodepletion and random hexamers for cDNA synthesis in the RNA-seq protocol. The meta-transcriptome analysis protocol for identifying functionally active microorganisms used with our experimental sample set was identically followed with the validation set.

Functional enrichment analysis of metabolic pathways among endometrial microorganisms

The functional enrichment analysis of the identified microorganisms was performed with HUMAnN2 (Franzosa *et al.*, 2018). HUMAnN2 identifies the abundance of each evolutionarily related protein-coding sequences, and clusters them into family if they perform similar function. As this software uses MetaCyc, the link between protein functions and pathways was directly obtained and stored in a pathway abundance file. The data was thereafter normalised using the HUMAnN2_renorm_table script and the results from each sample were joined using the HUMAnN2_join_tables utility. Each of the resulting pathways was studied using a one-way ANOVA, comparing the normalised abundance values between both menstrual phases. The abundance of a given pathway was considered statistically different between both stages at p-value <0.05.

Host transcriptome analysis: search for metabolic pathways

The SAM files (pre-processing explained above) where the sequences aligned to human reference genome were used for host transcriptome analysis. The calculation of gene expression values by miARma-Seq was performed using

featureCounts (Liao *et al.*, 2014) against the corresponding Gencode v26 annotation file. The raw counts were analysed using edgeR (Nikolayeva and Robinson, 2014) performing a paired study (each patient provided a sample from proliferative and mid-secretory phases). A gene was considered differentially expressed between both menstrual phases if the false discovery rate (FDR) value was below 0.05 and the log2-fold change $\geq |1.5|$. Next, the functional enrichment analysis was carried out using GSEA (gene set enrichment analysis), where the GSEAPreranked test (Subramanian *et al.*, 2005) was performed against the human metabolic pathways contained in MetaCyc, a database for all domains of metabolism (Caspi *et al.*, 2020). The HumanCyc pathway genome database constructed by the Pathway Tools utility from MetaCyc to obtain all human pathways was used. Thereafter, the obtained pathyway.col file was converted into a GMT GSEA compatible file, containing a total of 367 different metabolic pathways (Subramanian *et al.*, 2005). Once the GSEAPreranked method was executed using selected genes over the HumanCyc database, the results highlighted which pathways were statically enriched among differentially expressed genes.

RESULTS

Mapping the active microorganisms in the endometrium

Meta-transcriptome analysis enabled us to identify the RNA transcripts of all existent microorganisms in the endometrium, providing information of gene expression and thus the functional activity of these microbes. Out of all the detected RNA sequences, ~15% of the sequences were identified as non-human and aligned for microorganisms in the endometrial samples (Figure 1).

Interactive microbial atlas for each sample and the menstrual phase groups are available at http://bioinfo.ipb.csic.es/Metatranscriptome/.

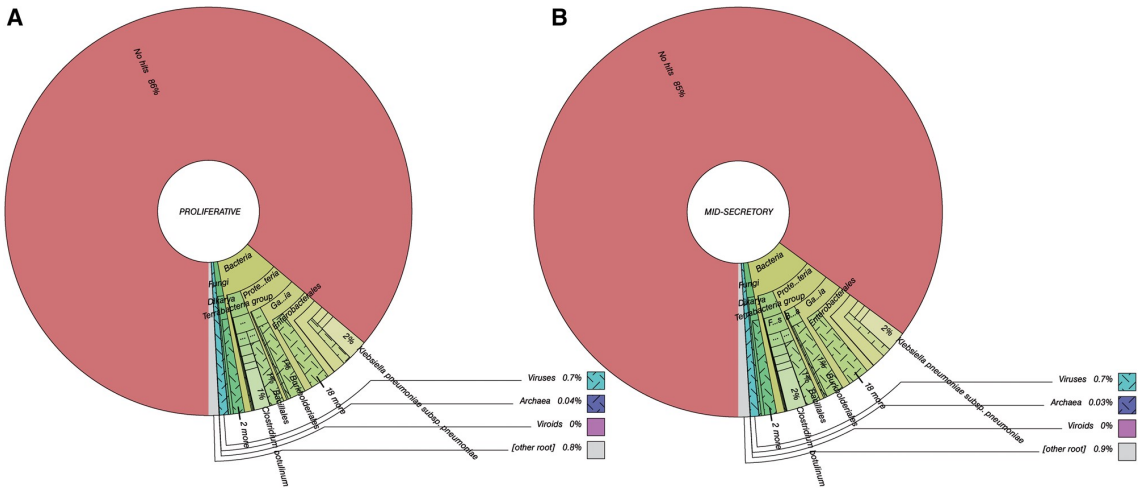

Figure 1. Krona plots of microorganisms identified in the proliferative (A) and mid-secretory phase (B) endometria. The results are illustrated in an interactive zooming and multi-layered microbial map obtained by Krona (html format), showing the mean taxonomic distribution and relative abundance of taxa. The interactive maps are available at http://bioinfo.ipb.csic.es/Metatranscriptome/.

In total, 5326 transcriptionally active microorganisms were identified in the endometrial samples, where 85% belonged to different bacteria, 10% to fungi, 5% to viruses and 0.3% to archaea (Supplementary Table SI). Microorganisms with relative abundance >1% among the samples are illustrated in Figure 2. The most abundant microorganisms in the endometria were *Klebsiella pneumoniae* subsp. *Pneumoniae*, *Clostridium botulinum*, *Pasteurella multocida* subsp. *Multocida* and *Hydrogenophaga* sp. NH-16, while the majority of the identified microbes had relative abundance below 1%, highlighting endometrium as the site of low microbial biomass. Comparison of our study findings with previously detected bacteria in the endometria from control women using 16S rRNA gene sequencing is summarised in Supplementary Table SII.

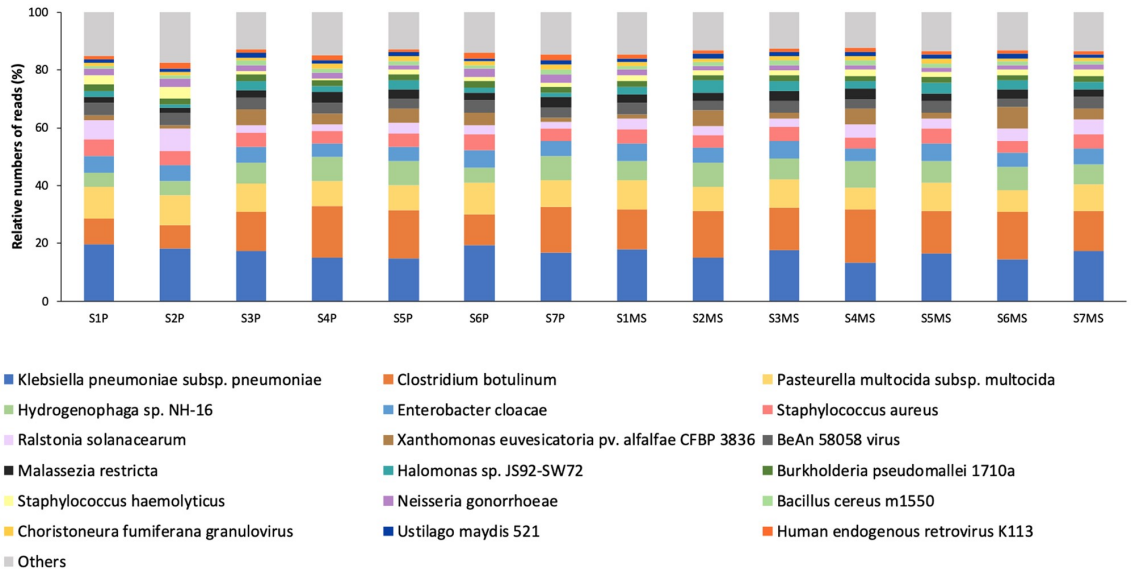

Figure 2. The most abundant microorganisms (bacteria, viruses and fungi) in the endometria of healthy reproductive-aged women. Percent-stacked barchart of those taxa whose relative abundances were higher than 1% are represented. S1–S7 indicate the samples while P and MS indicate proliferative and mid-secretory phases, respectively.

Next, we explored whether there were meta-transcriptome differences between the mid-secretory and proliferative phase endometria. Out of 5326 transcriptionally active microorganisms detected in the endometrial samples, 33 microbial species that included bacteria, viruses and archaea were differentially expressed in the mid-secretory *vs*. proliferative phase endometria (Table I). The mid-secretory phase endometria presented more transcriptionally active microorganisms (25 species) than the proliferative phase samples (8 bacteria), being in line with the general trend of transcriptional activation of endometrial genes in the host at the receptive phase (Altmäe *et al.*, 2014, 2017).

Table 1. Differentially expressed microbial species between the mid-secretory and proliferative phase endometria. Positive fold change (FC) value denotes microorganisms that were up-regulated in the mid-secretory phase endometria and negative value indicates up-regulated bacteria in the proliferative phase.

Species	logFC	P value	Adjusted P value*
Bacteria			
Muricauda ruestringensis DSM 13258	2,49	<0.001	<0.001
Burkholderia sp. CCGE1003	2,26	<0.001	<0.001
Pseudomonas corrugata	2,02	<0.001	<0.001
Ectothiorhodospira haloalkaliphila	1,78	<0.001	0,001
Providencia rustigianii	1,76	<0.001	0,008
Flavonifractor plautii	1,74	<0.001	0,001
Ignavibacterium album JCM 16511	1,69	<0.001	0,01
Methylocella silvestris BL2	1,68	<0.001	0,002
Blautia hansenii DSM 20583	1,66	<0.001	0,002
Borrelia recurrentis A1	1,66	<0.001	0,04
Brachybacterium sp. VR2415	1,65	<0.001	0,002
Nostoc sp. NIES-3756	1,57	<0.001	0,02
Candidatus Pelagibacter sp. HIMB1321	1,56	<0.001	0,03
Lachnospiraceae bacterium GAM79	1,45	<0.001	0,01
Neorhizobium sp. SOG26	1,39	<0.001	0,03
Obesumbacterium proteus	1,36	<0.001	0,04
Lachnospiraceae bacterium oral taxón 500	1,34	<0.001	0,03
Fluviicola taffensis DSM 16823	1,30	<0.001	0,04
Brevibacterium aurantiacum	-1,30	<0.001	0,04
Staphylococcus sp. M0911	-1,37	<0.001	0,03
Mycobacterium kansasii	-1,40	<0.001	0,04
Megasphaera hexanoica	-1,49	<0.001	0,02
Gardnerella vaginalis	-1,56	<0.001	0,03
Burkholderia plantarii	-1,62	<0.001	0,01
Achromobacter insolitus	-1,66	<0.001	0,04
Sneathia amnii	-2,15	<0.001	0,001
Viruses			
Bovine gammaherpesvirus 4	2,70	<0.001	0.005
Simbu orthobunyavirus	2,25	<0.001	<0.001
Shamonda orthobunyavirus	1,74	<0.001	0,001
Ecklonia radiata-associated virus 5	1,65	<0.001	0,002
Lactobacillus virus ATCC8014	1,52	<0.001	0,008
Archaeas			
Methanococcus voltae A3	1,76	<0.001	0,0025
Methanocella paludícola SANAE	1,60	<0.001	0,02

*P-values were adjusted using the FDR method of Benjamini and Hochberg to control the False Discovery Rate , the expected proportion of false discoveries amongst the rejected hypotheses (Benjamini and Hochberg, 1995).

Validation of functional microbiota mapping on independent samples

In order to validate our meta-transcriptome findings, we repeated the microbial transcriptome analysis process on three independent endometrial samples from mid-secretory endometria from fertile control women (BioProject ID: PRJNA379542 (Huang *et al.*, 2017)). Also in the validation set, different functionally active bacteria, fungi, viruses and archaea were detected demonstrating similar pattern as in our original data set (Figure 3A). Further, correlation analysis based on the microorganisms identified confirmed that the validation samples from the mid-secretory endometria grouped closer to our experimental set of mid-secretory endometria when compared to proliferative phase endometria (*t* test p-value = 0.027) (Figure 3B).

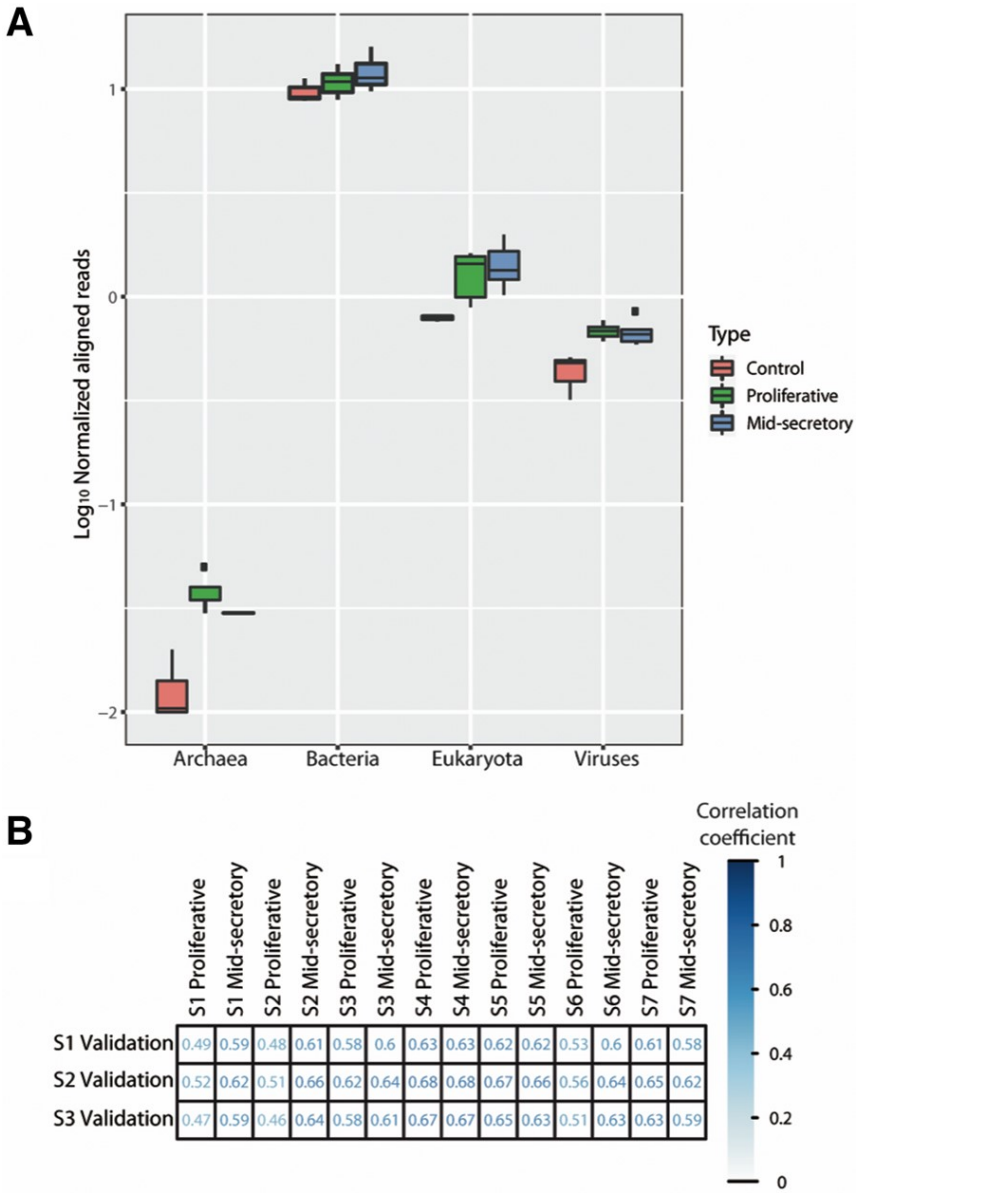

Figure 3. Validation analyses. A. Box plot of the taxonomic distribution and relative abundance of taxa in the experimental samples from proliferative (green) and mid-secretory (blue) phase samples together with validation (red) samples. A box denotes values from the first quartile to the third quartile (25–75%), while horizontal line in the box corresponds to the median value (50%) and the whiskers mark the minimum up to the maximum values (0–100%). B. Correlation plot of microbial abundances among experimental and validation samples.

Enrichment analysis of metabolic pathways: possible host-microbiota interplay

To investigate the possible role of the microorganisms identified in the endometrium, we compared the metabolic pathways obtained from the host

transcriptome and microbial meta-transcriptome analyses, since both were run ultimately using MetaCyc.

In the human host, out of total 21 365 mRNA identified, 1454 genes were differentially expressed ($FDR < 0.05$ and log2-fold change $\geq |1.5|$) between the mid-secretory and proliferative phase endometria (Supplementary Table SIII). Performing a functional enrichment analysis from differentially expressed genes of human RNA-seq, we obtain nine pathways statically enriched in the mid-secretory endometria: C20 prostanoid biosynthesis (PWY66-374) ($P = 0.02$), putrescine degradation III (PWY-0) ($P = 0.02$), superpathway of steroid hormone biosynthesis (PWY-7305) ($P = 0.02$), mucin core 1 and core 2 O-glycosylation (PWY-7433) ($P = 0.03$), urate biosynthesis/inosine 5′-phosphate degradation (PWY-5695) ($P = 0.03$), purine nucleotide degradation (PWY-6353) ($P = 0.03$), androgen biosynthesis (PWY66-378) ($P = 0.04$), guanosine nucleotides degradation (PWY-6608) ($P = 0.04$) and superpathway of tryptophan utilisation (PWY66-401) ($P = 0.04$) which includes, among others, pathways L-tryptophan degradation via tryptamine (PWY-6307), serotonin and melatonin biosynthesis (PWY-6030) and serotonin degradation (PWY-6313) (Figure 4).

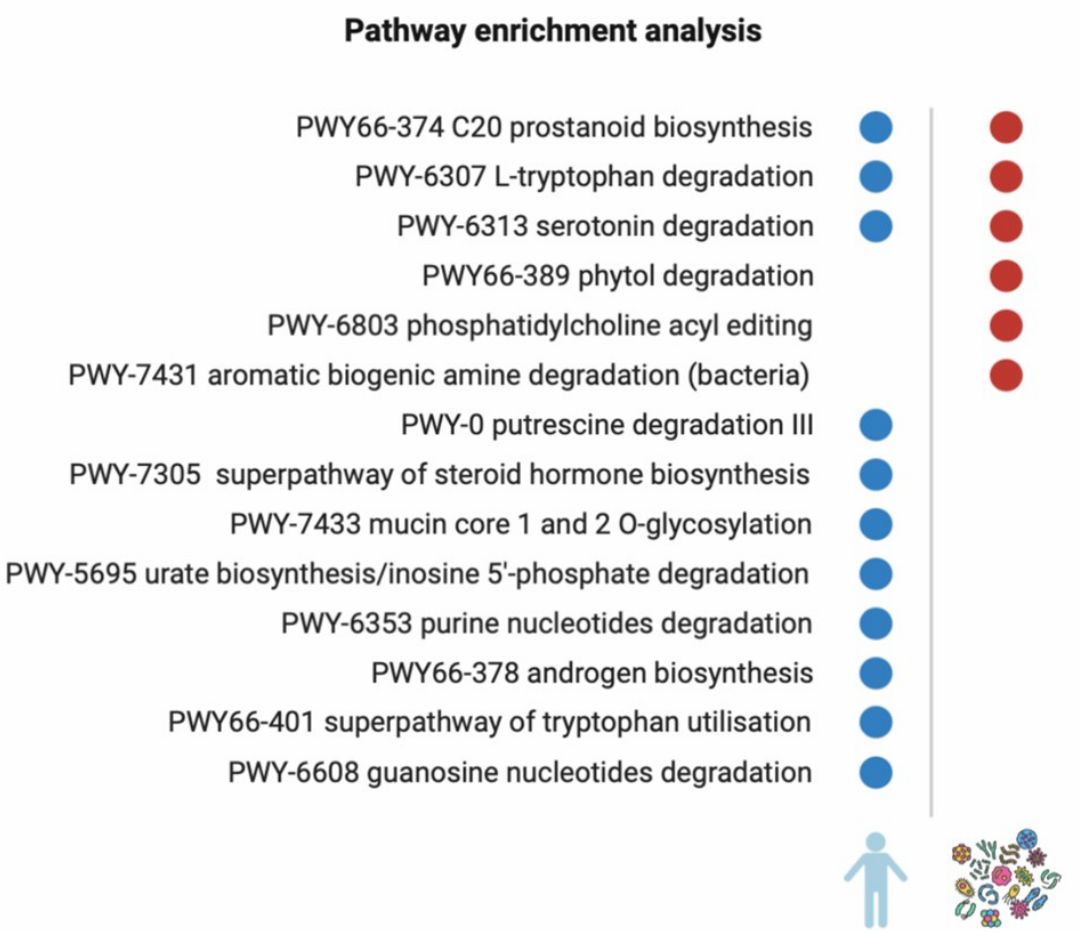

Figure 4. The host–microbiota metabolic pathways enriched in the mid-secretory endometrium. In blue are highlighted the human pathways and microbial pathways are indicated with red. Created with BioRender.

Among the detected microorganisms, a total of six metabolic pathways were significantly enriched in the mid-secretory phase endometria when compared with metabolic pathways identified in the proliferative phase endometria (Figure 5).

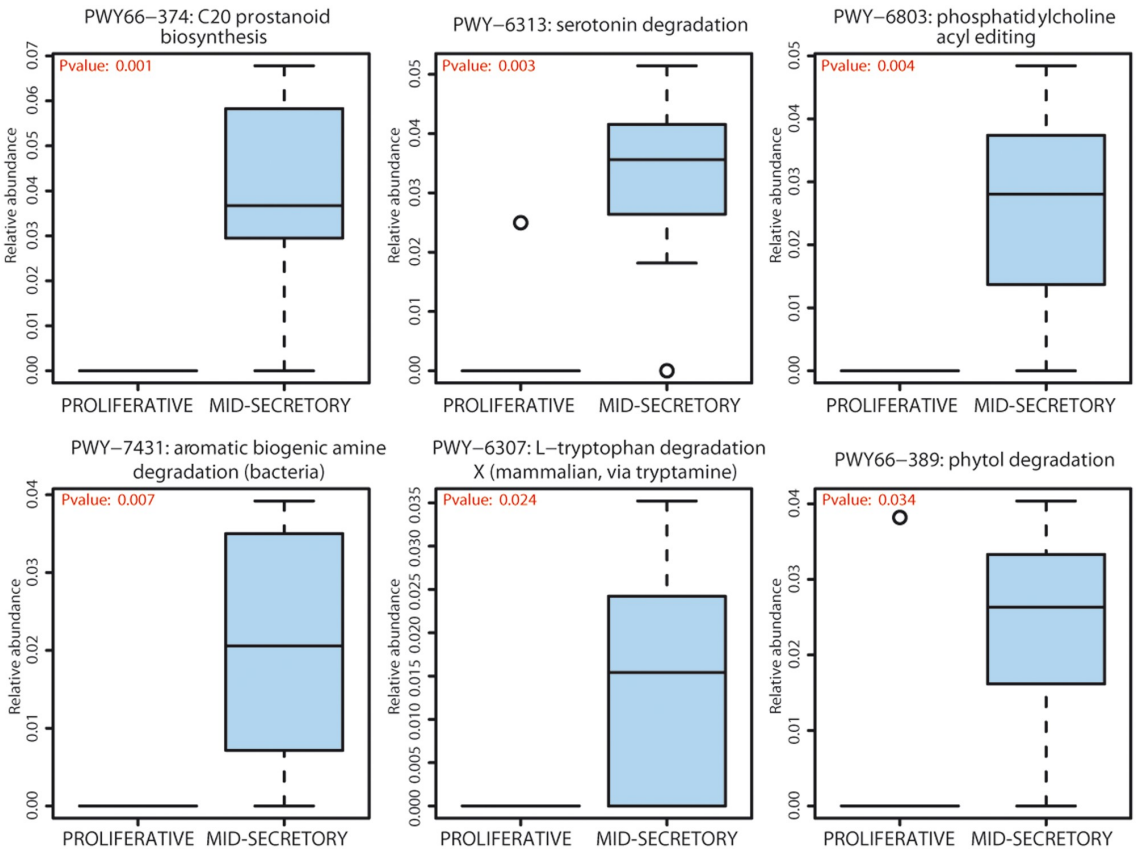

Figure 5. Microbial pathways significantly enriched in the mid-secretory phase ($P < 0.05$). The post-hoc analysis was performed using ANOVA statistical models with the pathways identified by HUMAnN2.

The common host–microbiota metabolic pathways activated in the mid-secretory endometrium are highlighted in Figs 4 and 6. Metabolic pathway C20 prostanoid biosynthesis (PWY66–374) was enriched in both human and microorganisms, while microbial pathways L-tryptophan degradation via tryptamine (PWY–6307) and serotonin degradation (PWY–6313) intertwined with the human superpathway of tryptophan utilisation (PWY66-401) that unites L-tryptophan degradation, serotonin and melatonin biosynthesis, and serotonin degradation routes (Figure 6).

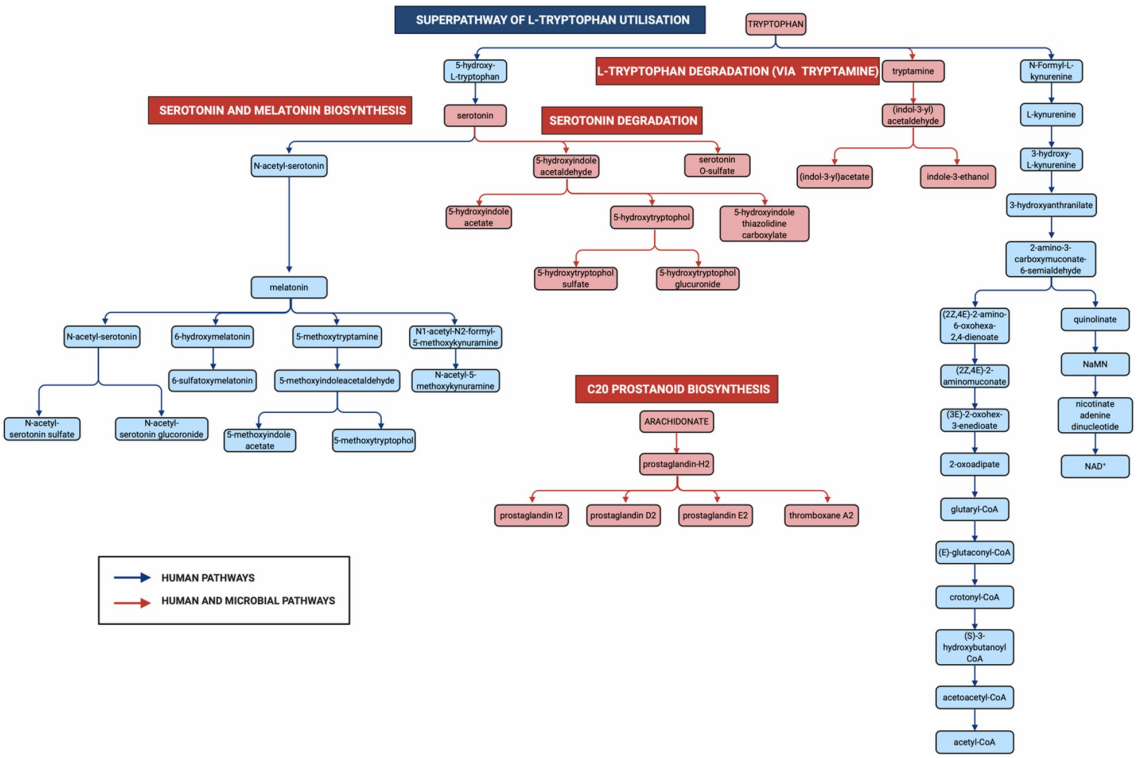

Figure 6. Functional enrichment of metabolic pathways between the human host and microbiota in the mid-secretory phase endometria. The boxes represent the metabolic products within a pathway. The pathways enriched in human are highlighted in blue and the routes enriched both in human and microbiota are coloured in red. Metabolic pathways were obtained with Pathway Collage Tools (BioCyc), and the diagram was adapted to a figure with BioRender.

DISCUSSION

To the best of our knowledge, we are the first to map the whole active microbial composition of endometrium and assess whether there are cyclical changes along the menstrual cycle in its composition, and to identify possible microbiota-host functions in endometrial receptivity.

DNA is a very stable molecule, contrary to RNA that is degraded rapidly, and is capable of persisting for decades, thus it is not surprising that evidence of bacterial existence, when analysing bacterial DNA sequences with 16S rRNA gene sequencing and metagenome methods, can be found in almost all body sites (Glassing *et al.*, 2016), including the endometrium. However, whether endometrial microbiome contains truly active (i.e., alive) microbes is so far

unclear. Additionally, given the difficulty in obtaining uterine samples from healthy young women, the core endometrial microbial composition has not yet been established. Up to today, only five endometrial samples in total from women with endometriosis and implantation failure have been analysed for endometrial metagenome, where viruses, fungi, and archaea in addition to big part of the bacteria were detected (Li *et al.*, 2018; Garcia-Grau *et al.*, 2019; Moreno *et al.*, 2020). These works provide the first glimpse into the potential whole microbial composition, though only DNA sequences were analysed. With our novel focus on analysing microbial mRNA sequences, we were able to identify the full cartography of the functionally active microbiota composing of over 5300 microorganisms within the endometrium from healthy women, confirming that live bacteria, viruses, fungi, and archaea seem to exist in the endometrium, though on low abundances, and are functionally active. When comparing the identified functionally active bacteria with previous 16S rRNA gene studies of endometria from healthy control women, *Clostridium* and *Staphylococcus* were prevalent (8–13%), while all other microbes, including *Lactobacillus* were less than 1% abundant (see Supplementary Table SII for the list of 'core' bacteria identified in previous 16S rRNA gene sequencing studies). Altogether, the identified core microbiota composed the majority of bacteria (85%), while active viruses (5%), fungi (10%) and archaea (0.3%) were also present. Our meta-transcriptome findings are proportionally in line with a recent meta-transcriptome study conducted in human gut samples, where bacteria comprised the major part of the microbiota (90%), and viruses (7%), fungi (0.6%) and archaea (1.4%) the minor part (Abu-Ali *et al.*, 2018).

For microbiota, location is everything. Factors such as temperature, oxygen and nutrients determine what kind of microorganisms can thrive in a particular

place (Woo, 2018). Some studies have suggested *Lactobacillus* dominance in the endometrium (Moreno *et al.*, 2016; Kyono *et al.*, 2019), however other studies have not detected *Lactobacillus* predominance (Chen *et al.*, 2017; Li *et al.*, 2018; Leoni *et al.*, 2019; Winters *et al.*, 2019; Younge *et al.*, 2019) and considering the uterine pH being 7.1 (Parrat *et al.*, 1995; Tomaiuolo *et al.*, 2020) other microbes could be favoured. Our study results confirm, at least on the level of alive microbes, that *Lactobacillus* does not seem to dominate in the endometrium from healthy young women.

Another ongoing debate is whether the endometrial microbiota changes throughout the menstrual cycle (Chen *et al.*, 2017; Pelzer *et al.*, 2018b) or is maintained stable (Khan *et al.*, 2016; Moreno *et al.*, 2016; Cregger *et al.*, 2017; Kyono *et al.*, 2019). In our study, we detected different bacteria, viruses, and archaea as differentially regulated between the proliferative and mid-secretory endometria, supporting the hypothesis of microbiota cycle-dependence. It is well established that microbiota is hormone-dependent, and that the endometrium is constantly exposed to hormonal changes. Already decades ago, Sonnex et al. observed that genital tract infections appeared in a cycle-dependent manner, suggesting that the changes in the oestradiol and progesterone concentrations might favour the growth of some microorganisms (Sonnex, 1998). In fact, hormonal fluctuations are shown to modulate antimicrobial peptides in the uterine mucosa and endometrial fluid (Agostinis *et al.*, 2019; Crha *et al.*, 2019), and the number and phenotype of immune cells in the endometrium changes throughout the menstrual cycle (Agostinis *et al.*, 2019). Endometrial mucosa, being under hormonal influence, is an important tissue barrier that offers protection against pathogens, while supporting a symbiotic relationship with commensal microbes (Agostinis *et al.*, 2019).

The effect of commensal microbes on host functions is being extensively studied (Ruff *et al.*, 2020), while in the endometrium this aspect is unexplored. In this study, we focussed on the metabolic pathways that could play a role in the host–microbiota interplay in the endometrium. Of special interests are the C20 prostanoid biosynthesis pathway and L-tryptophan metabolism, where microbial and host metabolic routes were elegantly intertwined (Figure 6). Prostanoid biosynthesis pathway produces prostaglandins I2, D2, E2 and thromboxane A2. Prostaglandins have an important role in the endometrial functions (Catalano *et al.*, 2011; Vilella *et al.*, 2013), and prostaglandin E2 has been proposed as a sensitive biomarker of endometrial receptivity, being particularly abundant in the endometrial fluid during the window of implantation (Vilella *et al.*, 2013).

Tryptophan metabolism, on the other hand, is well known to be in symbiosis with microbes, where different bacteria such as *Clostridium*, *Ruminococcus*, *Blautia* and *Lactobacillus* are involved in the degradation of this essential amino acid (Williams *et al.*, 2014). Tryptophan 2,3-dioxygenase, the first enzyme that catalyses the oxidation of L-tryptophan is expressed in the endometrium in a cycle-dependent manner and is induced at the time of embryo implantation as demonstrated in a murine model (Doherty *et al.*, 2011). Tryptophan is also a precursor in the biosynthesis of serotonin and melatonin, and serotonin degradation, where host–microbiota crosstalk seems to play a role. Melatonin has been shown to promote uterine functions, where it regulates different pathways associated with endometrial receptivity, and irregular uterine melatonin production has been related to recurrent spontaneous abortion (Chuffa *et al.*, 2020). Serotonin has newly been linked to microbiota in the gut mucosa, where microbes can regulate peripheral serotonin production and serotonin can

modulate the levels of specific bacterial species (Jones *et al.*, 2020). This bi-directional role of serotonin in human endometrial mucosa, though, needs to be investigated.

Endometrium is a site of low microbial biomass, and technical difficulties in analysing endometrial microbes have to be acknowledged. The biggest limitation is the high microbial contamination risk from the cervix and/or vagina when sampling the endometrium. Indeed, we detected the common cervical microbiota taxa such as *Gardnerella*, *Megasphaera* and *Pseudomonas* (Ata *et al.*, 2019; Cheong *et al.*, 2019) in our samples, comprising in total 0.2% abundance of all microorganisms, while *Lactobacillus* that is 97–99% predominant in the cervix and vagina (Chen *et al.*, 2017) was detected at 0.1% level. Nevertheless, *Lactobacillus iners* that is the dominant vaginal bacteria (Ravel *et al.*, 2011) was not detected in our study samples. In short, the bacterial contamination from the vagina and cervix is present, though on very low levels, and we cannot rule out the ascendance of these bacteria into the uterus in normal physiological conditions.

The novelty of this study is to demonstrate that although uterus is a low microbial biomass site, the identified microorganisms are alive, cycle-dependent, and with possible metabolic activity in the host–microbiota crosstalk in supporting endometrial functions in the receptive phase. Here we provide a new methodological pipeline to analyse the microbial composition, its transcriptional and metabolic activities in human samples, which could serve to inspire new analysis approaches in this new field of research in reproductive medicine.

SUPPLEMENTARY MATERIAL

Supplementary material may be found online in the Supplementary data section: https://academic.oup.com/humrep/article/36/4/1021/6141565?login=true

In addition, the supplementary files can be downloaded in this link: https://osf.io/yrbqh/

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STUDY IV: Dysregulated metabolically active endometrial microbiota in women with recurrent implantation failure

INTRODUCTION

Embryo implantation is a crucial and tightly regulated step in human fertility that occurs in a very short period of time. It is known as window of implantation (WOI) and happens in the mid-secretory menstrual phase about 7 days after the LH hormone peak (Day LH+7). Successful embryo implantation requires a good-quality embryo as well as receptive-phase endometrium and a timely crosstalk between them. Recurrent implantation failure (RIF) is a common and burdening diagnosis in infertility treatment that affect about 10-15% of women undergoing in-vitro fertilization (IVF) treatment (Busnelli *et al.*, 2020). RIF is characterised based on unsuccessful IVF cycles (two to six), the number of embryos transferred (three to ten) or a combination of both factors (Tan *et al.*, 2005). Despite scientific advances in the field, the cause of RIF remains unclear and the treatment options ambiguous.

Microorganisms play an important role in human physiology and pathology, and their potential involvement in endometrial functions and health are gaining support (Koedooder *et al.*, 2019; Molina *et al.*, 2020). Recent studies of microbial communities in RIF patients have revealed compositional alterations in bacterial taxa like *Gardnerella*, *Burkholderia*, *Atopobium*, *Delftia*, *Prevotella* and *Sneathia* (Kitaya *et al.*, 2019; Diaz-Martínez *et al.*, 2021; Ichiyama *et al.*, 2021). Despite the increasing efforts trying to identify the core endometrial microbiome, numerous factors influenced the microbial population including methodological complication and individual factors (Altmäe, 2018; Molina *et al.*, 2020) have made the task difficult. Our previous RNAseq-based-study of microbiota mapping of healthy endometrium identified over 5000 functionally active microorganisms (bacteria, viruses, fungi, archaea) that participate in key metabolic pathways for embryo implantation, specifically in the prostanoid biosynthesis pathway and L-

tryptophan metabolism (Sola-Leyva *et al.*, 2021). In the present work we focus on microbiota mapping in pre-receptive (LH+1-3) and receptive endometria (LH+7-9) of fertile women as well as RIF patients using a meta-transcriptome analyses to determine the potential role of microorganisms in the endometrial maturation process and impairment in endometrial functions in achieving pregnancy.

MATERIALS AND METHODS

Study population

Healthy fertile and RIF women from the NOTED study (Non-invasive Tests for Endometrial Dysfunction, EU41564) were included in the study. A total of 22 fertile women with at least one live-birth (1.5 ± 1.0) in the last 10 years (5.3 ± 2.6 years), aged 30.2 ± 3.3 years, and with body mass index (BMI) 23.1 ± 4.2 kg/m^2. The RIF patients group consisted of 21 women, aged 35.1 ± 3.9 years, BMI 22.3 ± 2.3 kg/m^2 (mean ± standard deviation) (Rekker *et al.*, 2018). RIF patients had at least three failed IVF treatment cycles (4.0 ± 1.5), no donor eggs were used in the cycles, and they were not undergoing hormonal stimulation at the time of sampling. The reasons for IVF treatment of this patients were tubal and male factor, endometriosis, and unexplained infertility. Two participants had secondary infertility with 6.5 ± 0.5-years evolution.

The present study was approved by the Research Ethics Committee of the University of Tartu, Estonia (No 221/M-31).

Sampling and study material

Study participants monitored LH levels in urine by using commercial kits (BabyTime® hLH urine cassette, Pharmanova, Beit Shemesh, Israel) and LH+0 was considered when LH surge was detected. Samples were collected in LH+7

to LH+9 corresponding to the mid-secretory phase for both fertile (MS-C) and RIF (MS-RIF) women, whereas LH+1 to LH+3 (early-secretory menstrual phase, ES-C) samples were sampled only for fertile women. Endometrial samples were obtained using Pipelle catheter and were placed into RNA*later* solution and stored and frozen at −80 °C.

RNA extraction

RNeasy MinElute kits (Qiagen, Hilden, Germany) were used to extract RNA from up to 30 mg of endometrial tissue followed by DNase I treatment using RNase-Free DNase Set (Qiagen). Quantity of purified RNA was determined with Bioanalyzer 2100 Small RNA kit (Agilent Technologies, Santa Clara, CA, USA).

mRNA sequencing, analysis, and data preparation

TruSeq Stranded technology (Illumina, USA) was used to generated libraries from 4 ug of total RNA as described in (Suhorutshenko *et al.*, 2018). The total RNASeq data from healthy women is deposited in Gene Expression Omnibus (GEO) database with accession number GSE98386. To identify potential non-biological experimental variation (batch effect) because of experimental condition principal component analysis (PCA) was conducted.

Meta-trancriptomics analysis

miARma-seq was used for quality study, removing adapter sequences, aligning against a reference genome, obtaining the raw counts, and doing a statistical study (Andres-Leon *et al.*, 2016) to obtain those differentially expressed genes in endometrium between the pre-receptive from control (ES-C) or mid-secretory phase endometrial from RIF (MS-RIF) versus receptive endometria

from fertile women (MS-C). Briefly, FastQC was used for quality step (Andrews, 2010) and Cutadapt to remove the adapter sequences (Martin, 2011). Sequences were aligned against human genome GRCh38 from Gencode v34 by HITSAT2 (Kim *et al.*, 2015) and rRNA contamination were filtered with SortmeRNA (Kopylova *et al.*, 2012). Subsequent, Kraken2 was used to align microbial sequences (i.e., those that did not align to human genome) to determine microbial composition including bacteria, archaea, viruses, and fungi include in the National Centre for Biotechnology Information (NCBI-Genbank). Krona software was used to visualise taxonomical results (Ondov *et al.*, 2011). Specifically, metagenomeSeq R package was used to determine microbial composition differences among study groups (Paulson *et al.*, 2013).

Functional enrichment analyses of microbial metabolism

In order to study microbial metabolism by related protein-coding sequences and clustering them in families with comparable function, the functional enrichment was performed with HUMAnN3 (Beghini *et al.*, 2021). The resulting metabolic pathways were afterward compared between study groups using one-way ANOVA (FDR < 0.05).

Human transcriptome

miARma-Seq calculated the gene expression values by using FeatureCounts (Liao *et al.*, 2014), and the resulting raw counts were studied with edgeR (Nikolayeva and Robinson, 2014) between study groups. A gene was established as differentially expressed between groups when the false discovery rate (FDR) value was under 0.05 and the log2-fold change $\geq |1.5|$. Enrichment analyses were carried out by using GSEA (gene set enrichment analysis), and the

GSEAPreranked test (Subramanian *et al.*, 2005) was performed against the human metabolic pathways contained in MetaCyc (Caspi *et al.*, 2020), a database for all domains. Also, Gene Ontology (GO) enrichment was carried out as provides the potential biological processes, molecular functions, and cellular components of detected targets.

RESULTS

Active microorganisms in the endometrium

Interactive microbial atlas for each sample and the controls and RIF groups are available at http://bioinfo.ipb.csic.es/Metatranscriptome/. The relative abundance analysis revealed that in endometria from fertile women and RIF patients (Figure 1, Supplementary Table SI) the most abundant microbial taxa was *Klebsiella pneumoniae* subsp. *pneumoniae* followed by *Streptomyces lividans* 1326, *Pasteurella multocida* subsp. *multocida*, *Staphylococcus simulans Staphylococcus aureus*, *Fusarium pseudograminearum* CS3096, *Enterobacter cloacae Burkholderia pseudomallei* 1710a, BeAn 58058 virus and Human endogenous retrovirus K113.

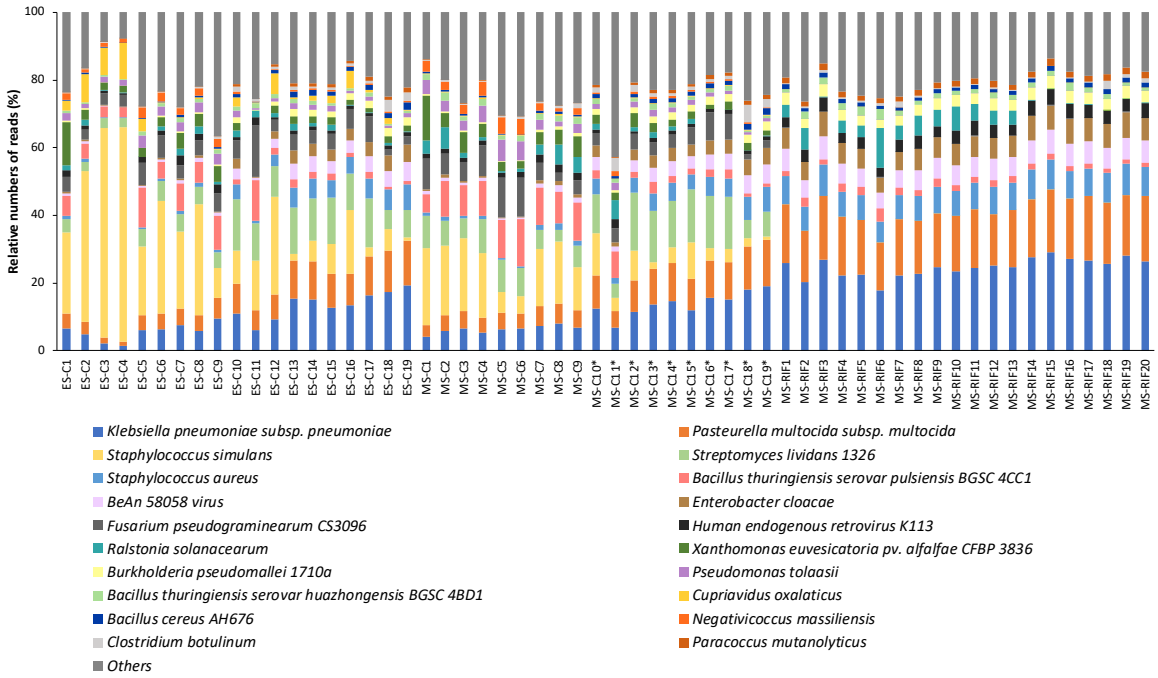

Figure 1. The most abundant microorganisms (bacteria, viruses, and fungi) in the endometria of fertile (C) and RIF patients in receptive (MS) and pre-receptive receptive (ES) endometrial samples. Percent-stacked bar chart of top 20 taxa. *Controls samples included in the comparison with RIF women.

The next step was to determine microbial composition differences between (1) mid-secretory endometrial samples from control women (MS-C, receptive) and early-secretory samples from control women (ES-C, pre-receptive), and (2) MS-C and MS-RIF (samples from mid-secretory phase RIF women). Since batch effect was detected after PCA (Supplementary Figure SI), 9 MS-C samples (C1, C2, C3, C4, C5, C6, C7, C8, and C9) were discarded from the comparisons with MS-RIF samples for avoiding undetected RIF effect on microbial population. The analyses revealed that out of all the transcriptionally active microbes detected, 105 microbial taxa including bacteria, archaea, viruses, and fungi were expressed significantly different (adjusted p-value < 0.05) when ES-C and MS-C (1) were compared (Supplementary Table SII). The top 20 differentials expressed microbes are shown in Table 1. When we compared MS-C versus MS-RIF patients (2), up to 180 microorganisms were detected with different expressions (Supplementary Table SII, Table 1).

Table 1. Differentially expressed microbial species analysing both mRNA and miRNA between the mid-secretory (MS) and early-secretory (ES) phase endometria in fertile women (C) and between MS in fertile versus RIF women. Positive fold change (FC) value denotes microorganisms that were up-regulated in the ES phase endometria or RIF patients (depending on comparison) and a negative value indicates up-regulated in MS-C.

Species	*logFC*	*P value*	*Adjusted P value*
ES-C vs. MS-C (TOP 20)			
Providencia_rustigianii	4.09	<0.001	<0.001
Myroides_odoratus_DSM_2801	2.67	<0.001	<0.001
Salmonella_phage_FelixO1	2.63	<0.001	<0.001
Christensenella_massiliensis	2.44	<0.001	<0.001

Rhodopseudomonas_palustris_TIE-1	2.26	<0.001	<0.001
Borrelia_recurrentis_A1	2.01	<0.001	<0.001
Zobellella_denitrificans	1.64	<0.001	<0.001
Mycobacteroides_abscessus	1.63	<0.001	<0.001
Listeria_ivanovii_subsp._londoniensis	1.54	<0.001	<0.001
Pedobacter_steynii	1.53	<0.001	<0.001
Pseudomonas_aeruginosa	1.46	<0.001	<0.001
Salmonella_enterica_subsp._salamae_serovar_55:k:z39_str._1315K	1.41	<0.001	<0.001
Bovine_gammaherpesvirus_4	1.37	<0.001	<0.001
Paenibacillus_sp._FSL_R7-0273	-1.31	<0.001	<0.001
Saimiriine_gammaherpesvirus_2	-1.48	<0.001	<0.001
Gloeobacter_kilaueensis_JS1	-1.62	<0.001	<0.001
Mycobacterium_tuberculosis_str._Beijing/NITR203	-1.77	<0.001	<0.001
Kitasatospora_aureofaciens	-2.37	<0.001	<0.001
Cupriavidus_oxalaticus	-3.23	<0.001	<0.001
Klebsiella_variicola	-3.24	<0.001	<0.001
RIF-C vs. MS-C (TOP 20)			
Streptomyces_lividans_1326	-13.14	<0.001	<0.001
Xanthomonas_euvesicatoria_pv._alfalfae_CFBP_3836	-11.04	<0.001	<0.001
Staphylococcus_simulans	-8.63	<0.001	<0.001
Pseudomonas_tolaasii	-8.52	<0.001	<0.001
Negativicoccus_massiliensis	-8.16	<0.001	<0.001
Fusarium_pseudograminearum_CS3096	-6.92	<0.001	<0.001
Lactobacillus_lindneri	-6.31	<0.001	<0.001
Cupriavidus_oxalaticus	-5.73	<0.001	<0.001
Staphylococcus_lugdunensis	-5.50	<0.001	<0.001
Rhodobacter_sphaeroides	-5.22	<0.001	<0.001
Alteromonas_australica	-5.08	<0.001	<0.001
Streptomyces_alboflavus	-4.94	<0.001	<0.001
Bordetella_avium_197N	-4.38	<0.001	<0.001
Brenneria_sp._EniD312	-3.79	<0.001	<0.001
Enterobacteria_phage_ID2_Moscow/ID/2001	-3.77	<0.001	<0.001
Streptomyces_hygroscopicus	-3.67	<0.001	<0.001
Shamonda_orthobunyavirus	-3.61	0.003	0.04
Actinoalloteichus_hymeniacidonis	-3.40	<0.001	0.003
Tsukamurella_paurometabola	-3.35	<0.001	0.004
Enterobacteria_phage_WA13_sensu_lato	-3.28	<0.001	<0.001

Enrichment analysis of microbial metabolic pathways

Comparing microbial metabolic activities involved in 'pre-receptive' (ES) and 'receptive' (MS) fertile endometria, two microbial metabolic pathways were

significantly differentially expressed (Figure 2A). In ES-C endometrium PWY-5138: fatty acid & beta-oxidation IV was enriched whereas PWY-6313: serotonin degradation was more active in receptive (MS-C) endometrium. When we compared the microbial activities regarding fertile versus RIF women, up to 93 metabolic pathways were differently enriched (Figure 2B). Interestingly the overlap between the comparison (1) and (2) resulted in PWY-6313: serotonin degradation was more enriched in receptive endometrium from fertile women in both cases, revealing the important role of microbes in the establishment of this metabolic pathway for a receptive phase endometrium.

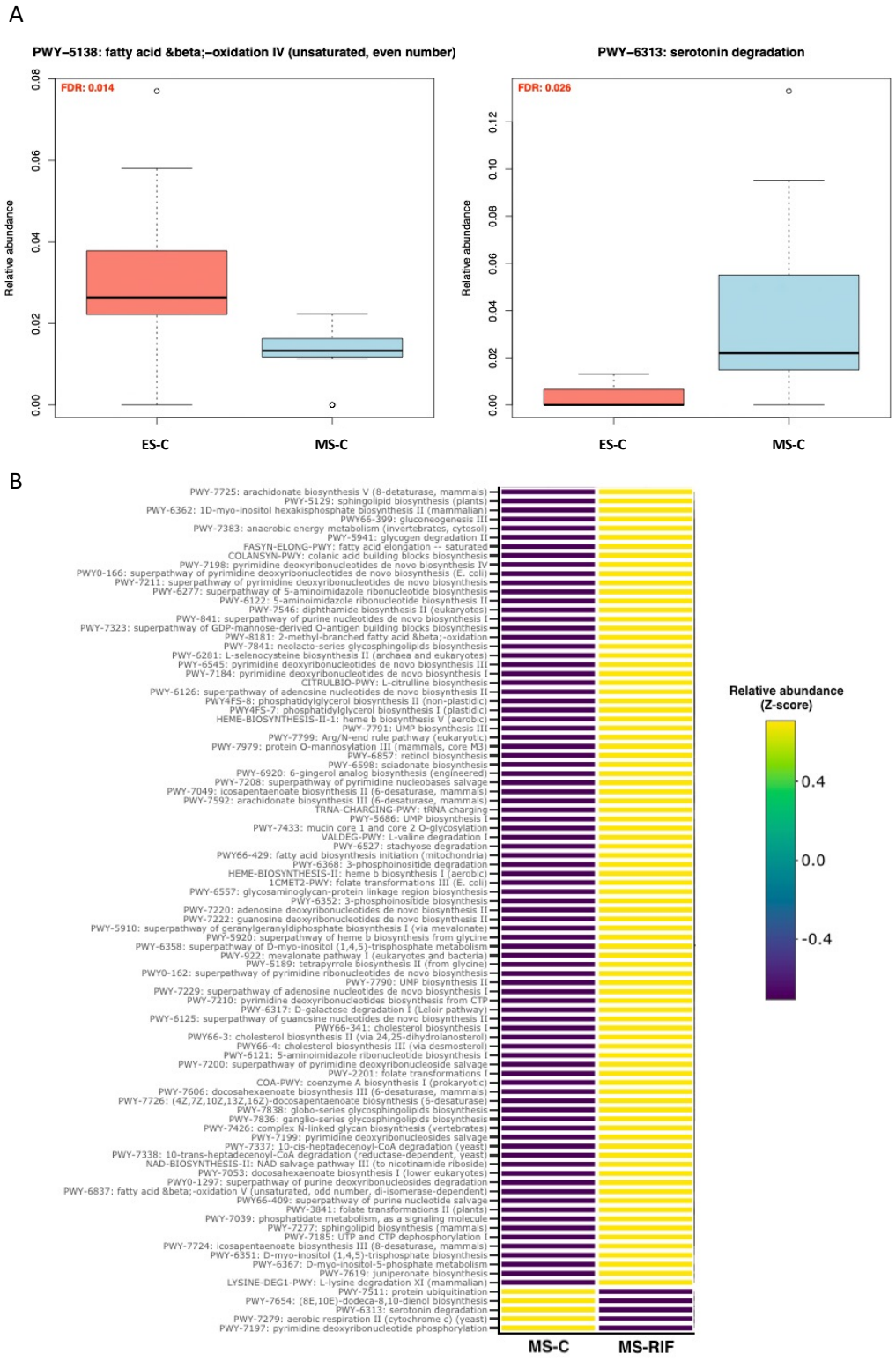

Figure 2. Microbial pathways significantly enriched between (A) early-secretory fertile endometria (ES-C) versus mid-secretory fertile women endometria (MS-C), and (B) mid-secretory RIF patient endometria (MS-RIF) versus MS-C. The

post-hoc analysis was performed using analysis of variance (ANOVA) statistical models with the pathways identified by HUMAnN3. Purple bars mean upregulation while yellow bars represent downregulation based on Z-score.

Differential gene expression between fertile and RIF women

We identified a total of 4077 significantly up-regulated genes (with maximal |log2FC| 9.74) and 5767 down-regulated genes (with maximal |log2FC| 6.68) in pre-receptive endometria of fertile women (ES-M) when compared with receptive endometrial phase (MS-C) (Supplementary Table SIII). When MS endometrial samples from RIF patients were compared with MS fertile women, 222 significantly down-regulated genes (with maximal |log2FC| 5.83) and 147 up-regulated genes (with maximal |log2FC| 6.04) in MS RIF women samples (Supplementary Table SIII).

When the enrichment was carried out using GSEA (gene set enrichment analysis), after FDR correction PWY-6857 Retinol biosynthesis (FDR=0.043) was positively enriched in receptive endometria of fertile (MS-C) women when compared with pre-receptive endometria within the same cohort (ES-C). Regarding RIF patients (MS-RIF), PWY-6261 Thyroid hormone metabolism ii (via conjugation and/or degradation) was positively enriched in RIF women compared with controls (FDR=0.01).

The biological functional analyses of differentially expressed genes (DEGs) by using GO in terms of biological processes, cellular components, and molecular functions showed that the DEGs in receptive endometria of fertile women were involved in different biological processes like blood vessel morphogenesis and angiogenesis, regulation of ion transport and immune processes; molecular functions such as transmembrane signaling; and cellular components such as extracellular matrix, cell surface and actin cytoskeleton when compared with pre-receptive endometria (Figure 3A, 3B and 3C). However, RIF women DEGs

belonged to biological processes such as hormone metabolic, xenobiotic metabolism, and cellular and retinoic acid metabolic process; and molecular function involving retinoic acid, retinoid, isoprenoid, iron, and hemo bindings were negatively affected (Figure 3D and 3E).

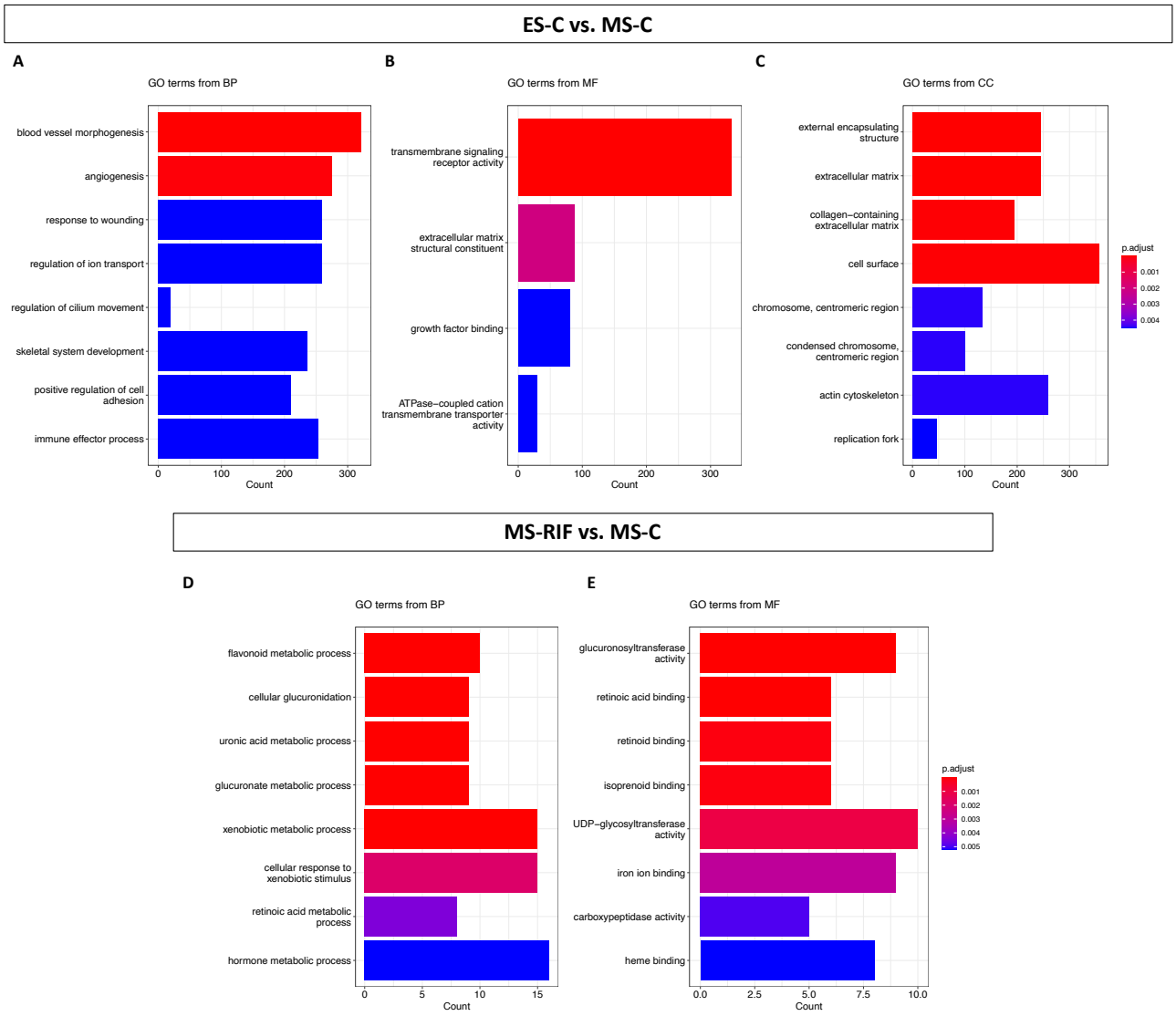

Figure 3. Gene Ontology (GO) analysis of genes associated with endometrial receptivity (ES-C vs. MS-C) and with recurrent implantation failure (RIF) (MS-RIF vs. MS-C) in terms of biological process (A, D), molecular function (B, E) and cellular components (C).

Integrated analysis of host-microbe interactions

To determine the potential function of the microorganisms in endometrial receptivity and embryo implantation, we compared the metabolic pathways

from transcriptome and the meta-transcriptome analyses, both obtained latest via MetaCyc (Figure 4). The metabolic pathway of serotonin degradation (PWY-6313) emerged as a key player in the microbe-host interaction. A significant increase in gene-related serotonin degradation pathway was found in the receptive endometrium of fertile patients (MS-C) compared to pre-receptive endometrial (ES-C) and mid-secretory endometria from RIF patients (MS-RIF) gene expression.

On the other hand, retinol biosynthesis pathway (PWY-6857) was differentially expressed when comparing pre-receptive (ES-C) versus receptive (MS-C) endometrial cell transcriptome of fertile women. While this pathway did not show significant alterations in RIF patients (MS-RIF), suggesting the same related-gene activity in RIF conditions, a significant increase in this pathway was observed in microbial gene level, showing a higher microbial metabolism of retinol biosynthesis in the endometrium of patients with RIF.

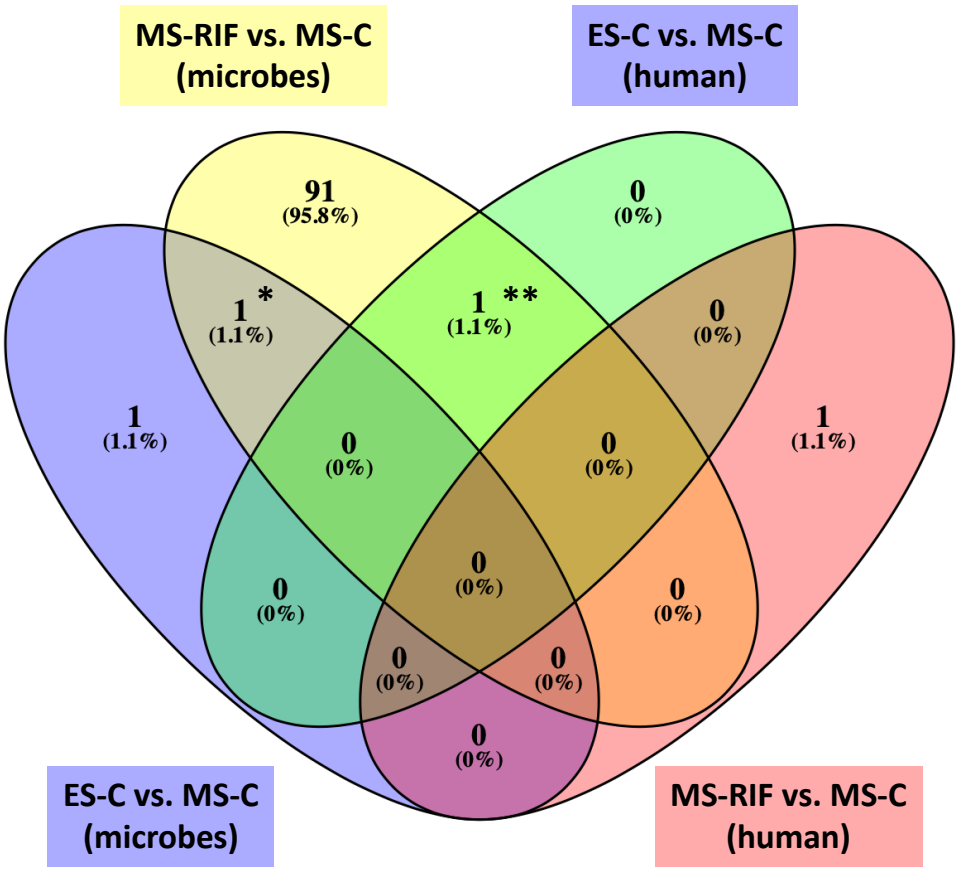

Figure 4. Number of differentially microbial and human metabolic pathways enriched using Gene Set Enrichment Analysis (GSEA) in pre-receptive and receptive fertile endometria (ES-C vs. MS-C) and mid-secretory endometria from fertile control (MS-C) versus recurrent implantation failure mid-secretory endometrial samples (MS-RIF). *PWY-6313 serotonin degradation. **PWY-6857 Retinol biosynthesis

DISCUSSION

Increasing evidence indicates that endometrial microbiota has a fundamental role in female physiology and pathology (Molina *et al.*, 2020, 2021). Here we aimed to clarify the compositional and functional microbiota changes occurring within the acquisition of endometrial receptivity in fertile women, and the differences with RIF patients. The analysis of microbial and host mRNA sequences allows us to identify the functional and metabolically active microbes in human endometrium and to investigate potential host-microbe interactions. Taxonomic designation by using mRNA sequences revealed a total of 5661

microbes functionally active within the cohorts, among them 76.3% where taxa shared our previously published endometrial meta-transcriptome study (Sola-Leyva *et al.*, 2021). Our study results confirm that human endometrium possessed metabolically active microbes in health and disease. Also, our study data continuously indicate that Lactobacillus is not the dominating bacteria, at least on the functionally active level, in the human uterus.

Embryo implantation depends on a combination of the genomic constitution and embryo quality, and it has been suggested disruption of endometrial receptivity induced by inflammatory state (Moore *et al.*, 2000). Endometrial microbiome imbalance could drive to metabolically and inflammatory alterations limiting endometrial functions (Benner *et al.*, 2018; Molina *et al.*, 2020). We identified considerable microbial differences in the RIF women when compared to controls. RIF cohort microbiota composition and function differed from fertile controls and, up to 180 microorganisms were detected with significant different expression level.

According with our results, microbial dysbiosis has been recently linked to RIF. In 2016, Moreno et al. demonstrated that high numbers of *Lactobacillus* spp. whereas high percentage of *Gardnerella vaginalis* and Streptococci resulted in adverse reproductive outcome (Moreno *et al.*, 2016). Posterior work on the endometrial microbiota with 145 RIF patients comparing to 21 controls discovered no differences regarding diversity indexes between groups. However, RIF group showed significant enrichment of 14 genera *Atopobium*, *Burkholderia*, *Delftia*, *Gardnerella*, and *Prevotella* (Ichiyama *et al.*, 2021). Recently, it was shown that patients with RIF had an endometrial microbiome dominated by greater *Lactobacillus helveticus*, *Sneathia amnii*, and *Prevotella* but less *Lactobacillus iners*, *Lactobacillus jensenii*, and the genus *Ralstonia* (Diaz-Martínez *et al.*, 2021).

In the analysis of the metabolic pathways, we found that the differential microbial functions in pre-receptive and receptive endometria were mainly associates with fatty acids degradation; highly active in early-secretory (ES-C) endometria and serotonin degradation more active in receptive endometria (MS-C). Fatty acids β-oxidation pathway consists in the metabolization of fatty acids in the mitochondria to produce acetyl-coA and ATP. During the β-oxidation process, acetyl-CoA molecules are released from the fatty acid inside the mitochondrial matrix and can be incorporated in the mitochondrial Krebs cycle and in electron transport chain to generate ATP. This process has been established as crucial for oocyte maturation and embryo development (Hewitson *et al.*, 1996; Downs *et al.*, 2009; Sturmey *et al.*, 2009; Dunning *et al.*, 2010, 2011). In fact, the supplementation with L-carnitine that increase the β-oxidation rate has been associated with good quality oocyte, follicle growth, fertilisation, and embryo development (Dunning *et al.*, 2010, 2011). Also, fatty acids beta oxidation is key to implantation and endometrial stromal cell decidualisation in both human and mice (Tsai *et al.*, 2014). Our study demonstrated that endometrial microbiota could have an important role in this metabolic phenotype in early stages of endometrial maturation process.

Results obtained in serotonin degradation by microbes, here up-regulated in MS-C, are consistent with our previously published meta-transcriptome study (Sola-Leyva *et al.*, 2021). Interestingly, in RIF patients we detected this microbial pathway down-regulated. Serotonin has newly been linked to microbiota in the gut mucosa, where microbes can regulate peripheral serotonin production and serotonin can modulate the levels of specific bacterial species (Jones *et al.*, 2020), and in general, tryptophan related-compounds like melatonin and serotonin have been shown to promote uterine functions, where it regulates different

pathways associated with endometrial receptivity, and irregular uterine melatonin production has been related to recurrent spontaneous abortion (Chuffa *et al.*, 2020).

Next, our results demonstrate upregulation in fertile receptive endometria of retinol biosynthesis pathway, but RIF patients also showed a microbial enhancement of this metabolic pathway may result in an excess of retinoids-related compounds and an impairment for embryo implantation process. Retinoids-related compounds such as retinol or vitamin A have been arisen as important molecules during embryogenesis and embryo development (Kam *et al.*, 2012). However excessive levels of retinoic acid are toxic requiring a tight metabolic regulation between maternal tissues and embryo (Geelen and Peters, 1979; Collins and Mao, 1999).

Our result suggest that endometrial microbiota function and composition is variable in the establishment of endometrial receptivity, changing between early- to mid-secretory endometrial phases and also, RIF patients showed their own microbial signature with altered metabolic functions.

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STUDY V: Microbial composition across body sites in PCOS: a systematic review and meta-analysis

ABSTRACT

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous endocrine disease in women of reproductive age, whose aetiology remains still unclear. Recent evidence is linking microbial composition in different body sites with PCOS, nevertheless the studies are barely comparable and results inconsistent. The aim of this systematic review and meta-analysis was to clarify the relationship of the microbiome of different parts of the human body with PCOS. For this purpose, a systematic search in main databases such as PubMed, Web of Science, Scopus, Cochrane Library, PROSPERO, medRxiv and bioRxiv was carried out up to April 2022. Although the evidence associates some changes in the microbiome with PCOS, the heterogeneity of the studies, the small sample size, the lack of adequate controls, and the possible effect of confounders, make difficult to establish a clear relationship. Based on our meta-analysis of the gut microbiome data from 1868 women (737 women with PCOS and 631 controls) demonstrate decreased gut microbiome diversity compared to controls, which may contribute to PCOS development. Future studies are needed to determine the mechanisms by which microbes may alter/modulate the symptomatology and progression of this metabolic disorder.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous endocrine disorder that affects up to 20% of reproductive-aged women worldwide (Goodarzi *et al.*, 2011), being one of the most prevalent gynaecological disorders. Several diagnosis criteria have been suggested but clinical and/or biochemical hyperandrogenism, oligoanovulation and polycystic ovaries (the presence of ≥12 follicles with maximum diameter of 2–9 mm or any ovarian volume >10 mL) are the key criteria, and the presence of two of the above conditions are considered sufficient for the diagnosis (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Additionally, other features such as hirsutism, acne, alopecia, menstrual dysfunction, metabolic syndrome, cardiovascular disease, hypertension, endometrial cancer, and endometrial receptivity alteration could be related to PCOS (Escobar-Morreale, 2018). Despite greats efforts to PCOS diagnosis and its high prevalence, the aetiology of PCOS remains widely unknown (Dokras *et al.*, 2017).

Recent evidence is linking microbial composition in different body sites with various diseases, including PCOS (Molina *et al.*, 2020; Hill and Round, 2021). Several microbiota (collection of microorganisms) and microbiome (genomes of the microorganisms) studies are highlighting the role of microorganisms in human physiology and pathology by both the direct or indirect interactions with host cells, modulating our metabolism, immune system and therefore our state of health (Power *et al.*, 2017; Altmäe *et al.*, 2019; Laniewski *et al.*, 2020).

Microbial communities may be influenced by several factors such as diet, physical activity, cultural habits, host genetics and hormones, among others (Molina *et al.*, 2020). Hormones are crucial in microbial function and composition

(Wilson *et al.*, 2007; Baker *et al.*, 2017). Likewise, microbes could influence the microenvironment by the production of several metabolites like bile acids, ceramides, short-chain fatty acids, branched-chain amino acids and trimethylamine N-oxide (Chen and Pang, 2021). Therefore, given the endocrine aetiology of PCOS, it seems reasonable to investigate the potential role of microorganisms in this pathology. The aim of this systematic review and meta-analysis was to gather the knowledge and raise the power for analysing the relationship between the microbiome composition in different body sites with PCOS.

METHODS

Bibliography search strategy

The search strategy was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page *et al.*, 2021). The review protocol has been registered in the International Prospective Register of Systematic Reviews (PROSPERO, CRD42020180191). We performed a systematic search of the literature available in PubMed, Web of Science and Scopus up to 19 April 2022. The terms were also indexed in Cochrane Library, PROSPERO, medRxiv and bioRxiv to ensure the up-to-date data. The search approach was performed pairing/combining the terms "polycystic ovary syndrome", "polycystic ovary disease", "PCOS", "PCOD" with terms "microbiome", "microbiota", "microorganism", "microbes", "infection", "bacteria", "virus", "flora", "microflora".

Selection criteria

The study population consisted of women with and without diagnosed PCOS. The inclusion criteria were as follows: all case-control studies that compared the microbiome between women with and without PCOS. The exclusion criteria were: 1) conference abstracts, letters to editors, study protocols or review articles and 2) studies written in any language other than English and Spanish. The outcomes of interest were microbial diversity, abundance/richness, and changes in specific taxa of any human tissue/body site.

The resulting studies from the systematic search were screened by title and abstract by two independent researchers (ASL and NMM) and possible discrepancies were discussed and solved by a third independent researcher (SA). For every eligible study, data extraction was performed including the following information: 1) authors' name and bibliographic reference; 2) cohort's characteristics (number of study subjects, condition, age, ethnicity, exclusion criteria, and PCOS criteria); 4) sample collection (body niche, type of sample); 5) detection of microorganisms (DNA extraction, detection method); 6) main findings; and 7) raw data availability.

Quality assessment and risk of bias

To evaluate the internal quality and possible bias in the study design of the selected works, two researchers (ASL and NMM) independently used the Joanna Briggs Institute Critical Appraisal Tool for Systematic Reviews (Moola *et al.*, 2015). Specifically, checklist for case-control studies was used, which consist of 10 items assessing the potential risk of bias for each study: 1) Were the groups comparable other than the presence of disease in cases or the absence of disease in controls?; 2) Were cases and controls matched appropriately?; 3) Were the same criteria used for identification of cases and controls?; 4) Was exposure

measured in a standard, valid and reliable way?; 5) Was exposure measured in the same way for cases and controls?; 6) Were confounding factors identified?; 7) Were strategies to deal with confounding factors stated?; 8) Were outcomes assessed in a standard, valid and reliable way for cases and controls?; 9) Was the exposure period of interest long enough to be meaningful?; 10) Was appropriate statistical analysis used? (Aromataris and Munn, 2020). Each researcher considered each item in detail and reported an overall evaluation. Possible inconsistencies were resolved through common agreement. A risk score was calculated by dividing the number of positively scored criteria by the total number of criteria. Low risk of bias was considered when the study achieved at least 75% of the items listed, otherwise the study was categorised as high risk of bias.

Meta-analysis of the microbiome studies

A meta-analysis was conducted to assess the associations of microbial composition with PCOS. While microbiome studies of all body sites were included into the systematic search, meta-analysis was only performed on the gut microbiome studies, as studies on other body sites were scarce. Based on the available data of microbial diversity metrics, our meta-analysis focussed on Shannon diversity and Chao1 indexes from different gut microbiome studies. A random-effect model was performed using the Comprehensive Meta-Analysis software (version 3; Biostat Inc.,1385, NJ, USA). Statistical heterogeneity across studies was assessed using the I^2 value, considering 25%, 50%, and 75% as low, moderate, and high heterogeneity, respectively (Higgins *et al.*, 2003). The effect size was calculated as standardised mean difference (SMD) based on Cohen's d and 95% confidence intervals (CIs). Specifically, when SMD<0, the control group

showed a higher alpha-diversity compared to PCOS group, and when SMD>0, a higher alpha-diversity was detected in the PCOS group.

It is important to highlight the following considerations in our meta-analysis: 1) only those studies that compared PCOS patients with body mass index (BMI)-matched controls or adjusted by BMI were included in the meta-analysis for discarding the effect of weight on microbiome data; 2) at least three studies were required to perform the meta-analysis as this is the minimal number recommended for a meta-analysis (Higgins *et al.*, 2003); 3) the Web-PlotDigitizer 4.4 software (Ankit Rohatgi [https://automeris.io/WebPlotDigitizer/]) was used to calculate the effect size for those studies that did not report alpha-diversity indexes in their manuscript (i.e., mean and standard deviation [SD]) and did not provide the data after request. This software allowed us to estimate the mean and SD from graphs reported in the article or, otherwise, the median, interquartile range (IQR) and maximum and minimum values, which were subsequently transformed into mean and SD by Wan method (Wan *et al.*, 2014). Web-PlotDigitizer is a web-based plot digitizing tool for extracting data from plots and has proven valid and reliable (Knowles *et al.*, 2016; Drevon *et al.*, 2017; Dhakal *et al.*, 2018; Bjørnelv *et al.*, 2022). The Wan method allows to estimate the mean and SD by incorporating the sample size, median, IQR and maximum and minimum values, demonstrating more accurate estimations when compared to other methods (Weir *et al.*, 2018).

RESULTS AND DISCUSSION

Overall characteristics of selected studies

A PRISMA flowchart of search strategy and selection of studies included in this work is shown in Figure 1. A total of 2839 studies were found across the

databases and were examined by tittle and abstract. After exclusion, a full-text review was carried out for 52 studies. Finally, 33 studies met the inclusion criteria. Of these, 17 studies were selected for the meta-analysis (see Supplementary Table SI).

Regarding the risk of bias, over half of the studies identified in the systematic literature search (18/33, 54.4%) presented high risk of bias, and out of the 17 studies included into the meta-analysis 10 categorised as with high risk of bias (Supplementary Tables SII). This quality assessment clearly highlights that a big part of the microbiome studies conducted lack rigorous study design, especially in the aspect of properly matched cases and controls and consideration and controlling for confounders (Supplementary Table SII). It has been shown that body weight can alter gut microbial composition (Dominianni *et al.*, 2015), thus, BMI-matched controls would provide clearer information of the microbial changes in association to PCOS excluding the effects of obesity/overweight on microbiome. Confounders, other important points to consider in microbiome studies, is shown to result in spurious relations between the condition and the results if not properly taken into account (Lv *et al.*, 2021).

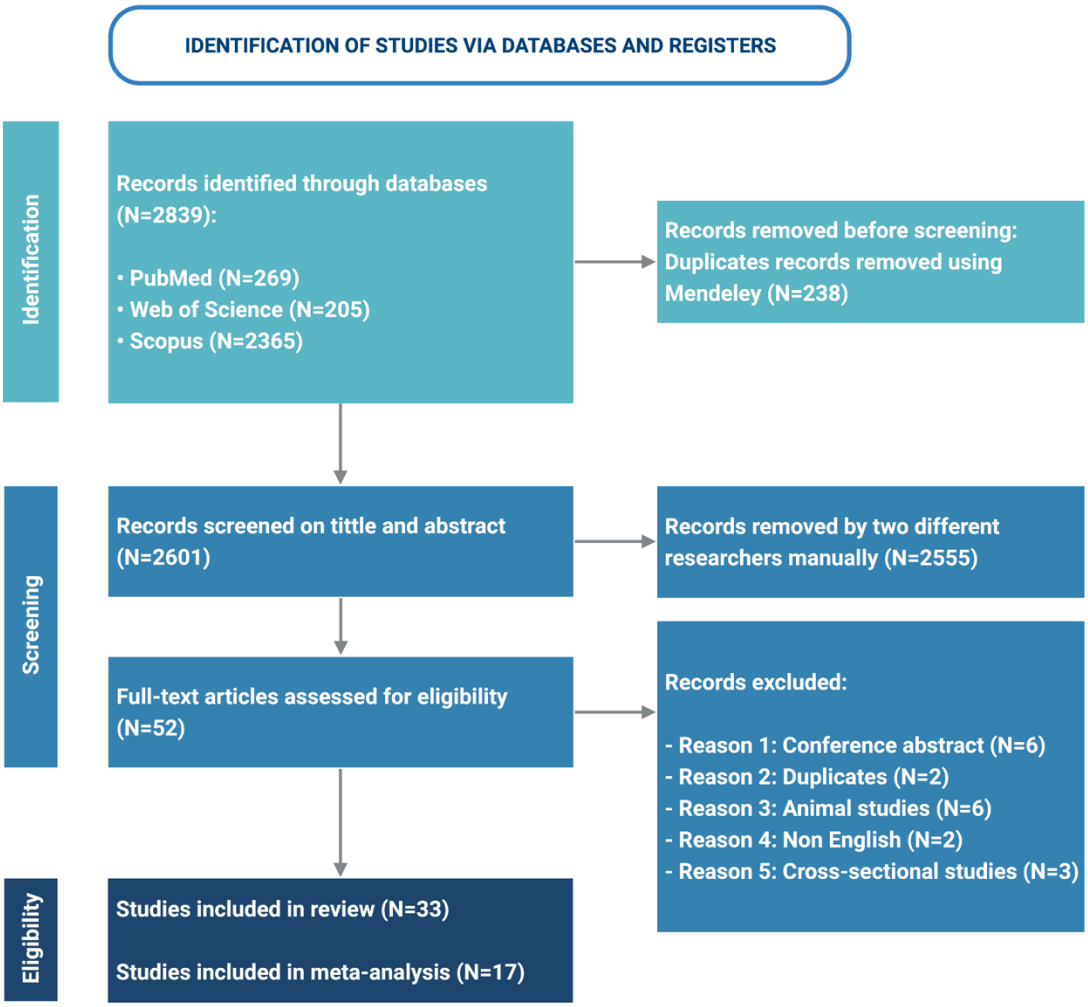

Figure 1. PRISMA flowchart of the systematic literature selection. Created with BioRender.

Microbiome of the oral cavity

Oral microbiome constitutes an important component of the microenvironment in human body and recently, oral cavity has been established as a potential source of microbes that could affect intestinal homeostasis and lead to inflammatory diseases (Atarashi *et al.*, 2017; Read *et al.*, 2021). Growing evidence links oral and salivary microbiome with PCOS. According to our systematic search, 4 studies have described the microbiome of the oral cavity in relation to PCOS (Akcali et al., 2014; Lindheim et al., 2016; Belkova et al., 2020; Wendland et al., 2020; Li et al., 2021) (Supplementary Table SI, Figure 2). The female sex

hormones have been associated with the composition of oral microbiome linked to oral pathology, such as periodontal diseases (Kumar, 2013). The study of the most common bacterial pathogens that specifically cause gingivitis in women with and without PCOS revealed that there were not differences in microbial abundances directly linked to PCOS (Akcali *et al.*, 2014). However, the study of the oral microbiome composition in relation to PCOS by using metagenomic approaches reported that PCOS women had a decreased relative abundance of *Actinobacteria* (Lindheim *et al.*, 2016) and increased *Fusobacterium* (Li *et al.*, 2021). In terms of diversity metrics, there is no consensus so far about the differences between PCOS and controls women. Interestingly, the variation of the oral microbiome over time in patients with PCOS has been recently analysed and it revealed that PCOS and controls could be differentiated by their oral microbiome at different time-points (Li *et al.*, 2021). For a comprehensive understanding of the influence of oral microbiome in PCOS bigger-size studies are required, and exhaustive oral analysis including oral health factors should be included as potential confounders.

Blood microbiome

Actual evidence is showing that blood harbours its own microbiome and that variation in blood microbial composition could be linked to non-infectious-diseases (Amar *et al.*, 2013; Potgieter *et al.*, 2015; Lelouvier *et al.*, 2016; Païssé *et al.*, 2016). We found one study where the relationship between blood microbiome and PCOS was assessed (Wang et al., 2022). These preliminary results detected a decreased alpha diversity of blood microbiome in PCOS women and beta-diversity analysis showed dissimilarities between microbial communities of PCOS and controls women. The relative abundance of *Proteobacteria*, *Firmicutes*,

and *Bacteroidetes* decreased significantly, while *Actinobacteria* increased significantly in PCOS women compared to controls (Wang *et al.*, 2022). Deeper understanding of blood microbiome related to PCOS aetiology should be addressed in bigger studies.

Lower genital tract microbiome

Altered lower genital tract microbiome in PCOS could be driven by the changes related to menstrual cycle and hormone levels (Song *et al.*, 2020). Based on our research, 4 studies have analysed the relationship between female reproductive tract microbiome and PCOS (Yeow *et al.*, 2016; Hong *et al.*, 2020, 2021; Tu *et al.*, 2020; Lu *et al.*, 2021) (Supplementary Table SI, Figure 2). Overall, these studies reported that PCOS women presented a vaginal microbiome dominated by *Mycoplasma* (Hong *et al.*, 2020; Tu *et al.*, 2020), *Prevotella* (Hong *et al.*, 2020; Tu *et al.*, 2020), *Gardnerella* (Tu *et al.*, 2020), *Actinomyces*, *Enterococcus* and *Atopobium* (Lu *et al.*, 2021). Regarding Lactobacilli species, women with PCOS showed less abundance when compared with controls (Hong *et al.*, 2020; Tu *et al.*, 2020). Also, a cross sectional study evaluated the presence of bacterial vaginitis and vulvovaginal candidiasis in a cohort of 89 women with PCOS revealed that approximately 15% of women presented microbial pathologies (Hong *et al.*, 2021).

Gut microbiome

The pivotal presence of insulin resistance and chronic inflammation in most of women with PCOS prompted Tremellen and Pearce in 2012 to propose a new paradigm for PCOS related to dysbiosis of the intestinal microbiome (Tremellen and Pearce, 2012). Since then, several studies have analysed the association

between intestinal microbiome and PCOS (Supplementary Table SI, Figure 2). Nevertheless, a clear cause-effect relationship has not been established yet and the gut microbial diversity indexes in PCOS is still controversial. Although different studies have demonstrated a decreased alpha diversity and differences in beta diversity analyses, many other studies have not detected significant changes (Supplementary Table SI). Since the lack of consensus results could be influenced, in addition to study protocol, by the study size, it is worthy to mention that the biggest study up to now with 102 PCOS women and 201 age- and BMI-matched control women did not detect any significant differences in diversity indexes neither in microbial composition (Lüll *et al.*, 2020). Despite the discrepancies across studies, there seem to be some consensus on several microbial taxa being more prevalent among PCOS women: *Bacteroides* (Torres *et al.*, 2018; Qi *et al.*, 2019; Zeng *et al.*, 2019; Chu *et al.*, 2020; Haudum *et al.*, 2020), *Parabacteroides* (Zhang *et al.*, 2019; Chu *et al.*, 2020), *Prevotella* (Zhang *et al.*, 2019; Liang *et al.*, 2020), *Megamona* (Haudum *et al.*, 2020; Liang *et al.*, 2020), *Megasphaera* (Haudum *et al.*, 2020), *Escherichia* (Liu *et al.*, 2017; Chu *et al.*, 2020) and *Shigella*, while *Bifidobacterium* (Zhang *et al.*, 2019), *Lactobacillus* (Liu *et al.*, 2017) and *Faecalibacterium* (Zhang *et al.*, 2019; Chu *et al.*, 2020) genera seem to be less prevalent (Figure 2).

Among the clinical manifestation of PCOS, obesity raises as the most prevalent and some studies have shown that abdominal obesity is associated with clinical parameters of PCOS (Chen and Pang, 2021). In terms of microbial diversity in the gut, no differences between obese PCOS patients and lean PCOS women were found (Liu *et al.*, 2017; Insenser *et al.*, 2018; Zhou *et al.*, 2020). However, several studies are proving that gut microbiome in women with PCOS could be affected by obesity, increasing the abundance of *Bacteroides* spp.,

Candidatus (Insenser *et al.*, 2018)*, Lachnoclostridium*, *Fusobacterium*, *Coprococcus*_2, and *Tyzzerella* (Zhou *et al.*, 2020). To determine the association between PCOS and microbiome composition, it is recommended to use BMI-matched controls to exclude the potential alteration driven by obesity on microbial composition.

Insulin resistance in terms of fasting glucose and insulin levels also conform an important alteration in PCOS patients. Microbial dysbiosis could be involved in insuline resistance via endotoxemia, some gut-brain peptides, hyperandrogenism and some abnormal metabolites (He and Li, 2020). Some trials performed in humans have analysed the influence of insulin resistance revealing that microbial families such as *Lachnospiraceae* and *Ruminococcaceae* had higher abundance in PCOS patients without insuline resistance. Also, Zeng and coworkers reported that women with insuline resistance and PCOS showed lowest number of observed bacterial taxa and a lower Shannon index (Zeng *et al.*, 2019).

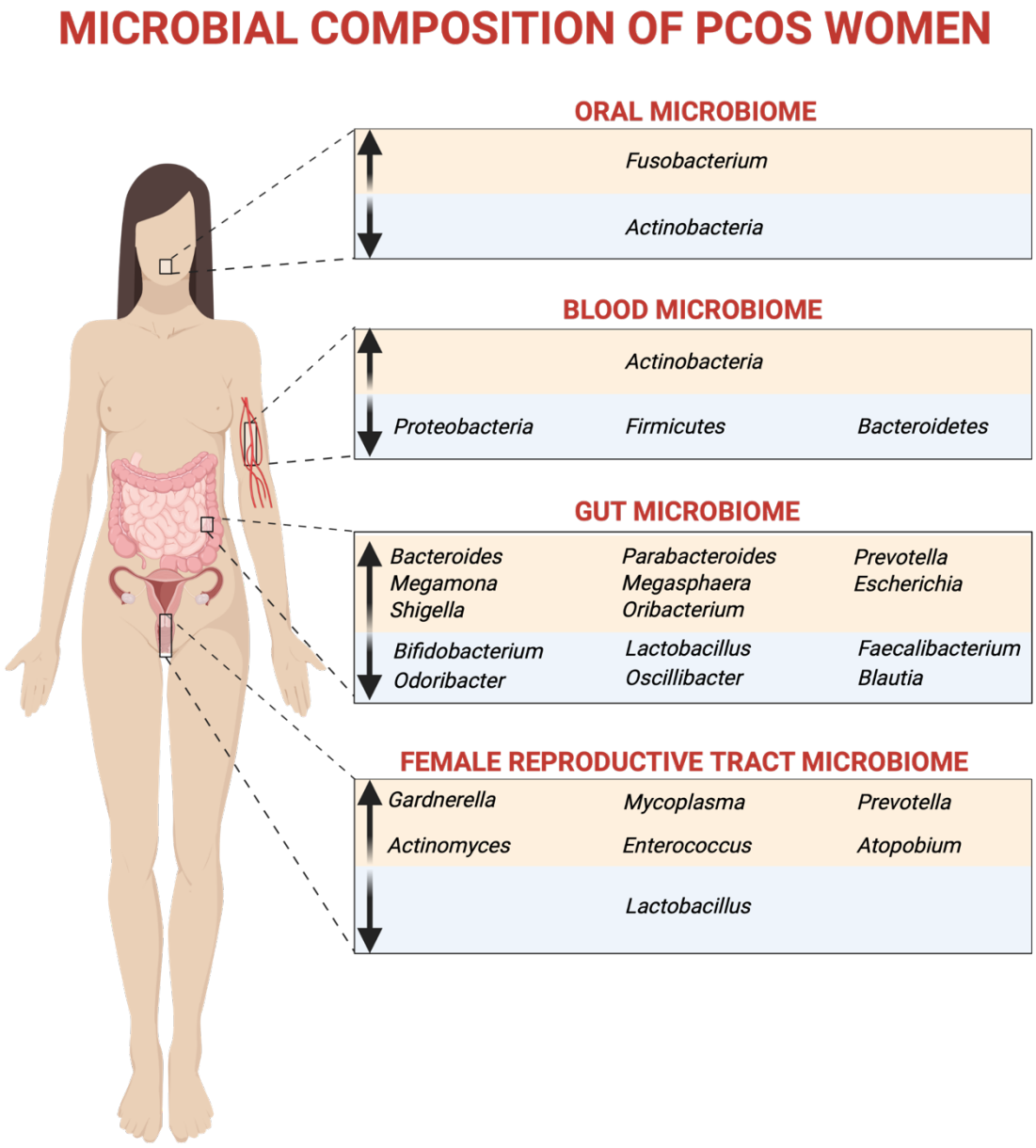

Figure 2. Microbial composition in women with PCOS in oral cavity, blood, gut and female reproductive tract. Created with BioRender.

Meta-analysis of the gut microbial diversity in PCOS

Alpha diversity is considered a good indicator of the gut microbiome health and, particularly, PCOS has been associated with a decrease in alpha diversity (Thackray, 2019). So far, many studies have reported a decreased alpha diversity of the gut microbiome in women with PCOS, however, some studies have not detected such differences (Guo *et al.*, 2022). The studies are with different study design and often conducted in a small sample size, therefore a meta-analysis could increase the power in clarifying whether there are differences in microbial composition in PCOS.

Figure 3 illustrates the meta-analysis of the PCOS *vs.* BMI-matched controls' effect on both alpha diversity metrics, i.e., Shannon diversity and Chao1 indexes. Specifically, 14 studies (624 cases and 573 controls) were eligible for the first meta-analysis focussed on Shannon diversity index, while 9 (394 cases and 228 controls) were included in the second meta-analysis based on Chao1 index. A significant pooled SMD was found for Shannon diversity index, indicating a significantly higher richness in the control group compared to PCOS group (SMD= -0.204; 95% CI -0.360- -0.048; p= 0.010; I^2= 5.508). Contrastingly, no significant differences were found for Chao1 index, although a tendency to a higher Chao1 index favouring controls was observed (SMD= -0.153; 95% CI -0.475- 0.170; p= 0.353; I^2= 0.000).

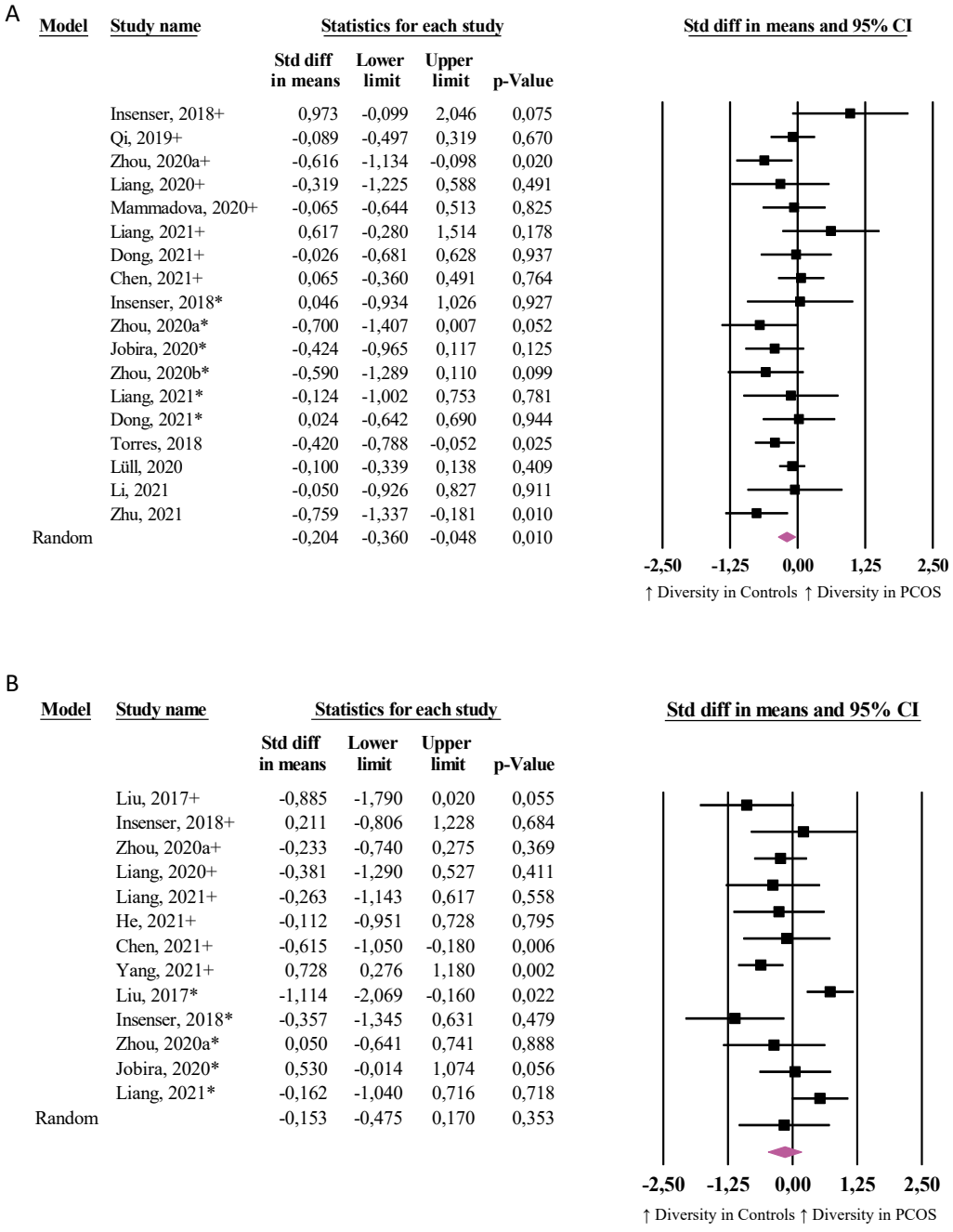

Figure 3. Forest plots of alpha-diversity metrics including Shannon diversity (A) and Chao1 (B) indexes in PCOS patients and healthy controls. Pooled effect size was estimated using a random-effects model. Each point represents standardised mean difference (SMD) and 95% confidence interval (CI). +PCOS patients with normal-weight *versus* healthy controls with normal-weight; *PCOS patients with overweight/obesity *versus* controls with overweight/obesity. Torres *et al*. 2018, Lüll *et al*. 2020, Li *et al*. 2021 and Zhu *et al*. 2021 included participants of different BMI categories, however, there were no significant differences between groups, or adjusted by BMI if applicable.

Inflammatory state, microbiome and PCOS

Supporting the hypothesis presented in 2012 by Tremellen and Pearce, disorders in the gut microbiota of women with PCOS could increase mucosal permeability and lead to increased surrounding lipopolysaccharides (LPS) levels in the blood resulting in systemic inflammation mediated by C reactive protein (CRP), interleukin-6 (IL-6), tumour necrosis factor α (TNFα), among others. The increased inflammation can interfere with insulin signalling promoting ovarian hyperandrogenism (Tremellen and Pearce, 2012). Therefore, PCOS has been acknowldeged as part of the systemic chronic low-grade inflammation syndromes (Duleba and Dokras, 2012). The study conducted by Zeng et al. demonstrated that the levels of CRP, IL-6 and TNFα were higher in PCOS patients compared with healthy controls, and levels of these biomarkers were negatively associated with the abundance of *Prevotella* (lower abundant in PCOS women) (Zeng *et al.*, 2019). Another study correlated the levels of zonulin (i.e., modulator of intercellular tight junctions) with microbial alpha diversity, pointing out that changes in the integrity of the intestinal epithelium is directly connected with the microbiome and with the inflammatory state produced by PCOS (Lindheim *et al.*, 2017). Even the evidence prompts to confirm the effect/cause PCOS-microbiome relationship, there are not enough studies in the field.

CONCLUSIONS AND FUTURE PERSPECTIVES

It is clear that microbes have important role in human health and disease and there is growing body of evidence that microbes play a role in the aetiolgoy of PCOS. Different body sites have been analysed for the microorganismal composition, such as oral cavity, whole blood, vagina and the gut, and although novel, the studies across different sites in women with PCOS are scarce, except

for the gut. The studies possess different limitations, such as a low number of participants and differences in age, ethnicity, and BMI as well as other non-controlled variables that need to be taken into account for the study of the microbiome (Molina *et al.*, 2020), making difficult to reach any solid conclusions whether the microbiome is different in women with PCOS *vs.* controls.

With our systematic review, we gathered microbiome data of 1868 women, 737 women with PCOS and 631 control women in order to raise the power for clarifying whether there are changes in the microbial diversity and composition in PCOS. As the majority of studies in the field have analysed the gut microbiome, we were able to meta-analyse microbiome diversity data from 624 cases and 573 controls for Shannon diversity index, and 394 cases and 228 controls for Chao1 index, making it the biggest analysis conducted. Our study results support the concept of the decreased gut microbial diversity in PCOS, which may contribute to PCOS development. Although the cause and/or causality need to be established in the future studies. The results here presented support the hypothesis previously proposed by Larsen et al. which argues that greater diversity leads to greater stability of the microbial system that is associated with redundancy (Larsen and Claassen, 2018). The protective/damaging role of the gut microbiome in metabolic functions, and therefore in PCOS, was evident when transplants of the gut microbiome from obese mice into normal mice induced an increase in body fat and resistance to insulin (Turnbaugh *et al.*, 2008). Also, as letrozole model causes hyperandrogenism in the mice, it is likely that the changes in microbiome are linked to steroid hormone functions as has also been shown in other animal models, linking androgens and gut microbiome in the occurrence of diabetes (Markle *et al.*, 2013).

Our study also demonstrates the shortcomings in the study design in the microbiome analyses, highlighting the need for well-planned and conducted studies with bigger sample size, proper negative and positive controls, control for important confounders and proper case-control matching. Future studies are needed to determine the mechanisms by which microbes may alter/modulate the symptomatology and progression of this common metabolic disorder.

SUPPLEMENTARY MATERIAL

The supplementary files can be downloaded at this link: https://osf.io/yrbqh/

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5. General discussion

The present Doctoral Thesis aimed to provide current knowledge in the microbiome studies in female reproductive health and to identify the functionally active microbes in the uterus together with their potential role in the host-microbe interactions. The results obtained in **Study I** reveal that there are several studies demonstrating that the endometrial microbiome has an important role in female reproductive physiology and pathology and provides a novel promising therapeutic tool for improving the uterine microenvironment in reproductive health. **Study II** emphasises the importance of experimental design in obtaining results and interpretation when analysing active microbiota using meta-transcriptomic tools. In **Study III,** a comprehensive map of functionally active microbes in the endometrium together with their possible role in endometrial functions is proposed. Further, the functionally active endometrial microbiota in women with RIF was assessed and compared to healthy control women in **Study IV**, and the possible host-microbe interactions in health and disease are determined. Ultimately, a systematic review and meta-analysis were performed in **Study V** to gather the knowledge and to analyse the relationship between the microbial composition in different body sites with PCOS.

Endometrial microbiome and methodological consideration in meta-transcriptomic analysis for identification of functionally active microbes

The review of endometrial microbes analysed in women with different gynaecological disorders and healthy women demonstrates that endometrium harbours diverse compositions of microbes, which could have a role in endometrial functions (**Study I**). Nevertheless, there is no consensus on the endometrial core microbiome composition and different studies identify diverse

bacteria related to gynaecological diseases. There is, in fact, an active debate ongoing on whether the endometrial microbiome composes mainly of *Lactobacillus* spp. or not, as several studies demonstrate Lactobacilli dominance (>90%) within the sample (Moreno *et al.*, 2016; Hashimoto and Kyono, 2019; Kyono *et al.*, 2019) while other studies do not detect *Lactobacillus* majority (Chen *et al.*, 2017; Leoni *et al.*, 2019; Winters *et al.*, 2019; Younge *et al.*, 2019). Thus, a common approach in endometrial microbiome studies is to classify patients into *Lactobacillus*-dominant (*Lactobacillus* spp. abundance higher than 90%) or non-*Lactobacillus* dominant (*Lactobacillus* spp. abundance lower than 90%) groups (Mitchell et al., 2015; Moreno et al., 2016, 2022; Verstraelen et al., 2016; Tao et al., 2017; Kyono et al., 2018; Carosso et al., 2020). Lactobacilli dominance has been positively associated with increased implantation rates, pregnancy, ongoing pregnancy, and live birth rates, while *Lactobacillus* species reduction has been linked to clinical miscarriage and no pregnancy (Moreno *et al.*, 2016, 2022; Kyono *et al.*, 2019). Since most of these studies obtained samples for analysis transcervically, there is the possibility of microbial contamination from the vagina and cervix, where *Lactobacillus* spp. form most of the microbial communities (Molina *et al.*, 2021). In fact, endometrial samples obtained via hysterectomy, laparoscopy, and/or during caesarean section surgery have revealed microbial dominance of *Pseudomonas*, *Acinetobacter*, *Vagococcus*, and *Sphingobium* (Chen *et al.*, 2017; Leoni *et al.*, 2019; Winters *et al.*, 2019; Younge *et al.*, 2019). Furthermore, in several studies there are endometrial samples that do not seem to harbour any detectable microbiome, which adds to the general concerns in the microbiome studies in female reproductive health: whether all women have endometrial microbes, what is the core microbial composition in health, and disease, whether these detected microbes are functionally active, do they have

any role in the endometrial functions, is there any change throughout the menstrual cycle, and whether these microbes are tourists, residents or invaders.

All the previous microbiome studies performed in human endometrium so far have focussed on analysing microbial composition at DNA level, 16S rRNA gene sequencing, or metagenome analysis, meaning that whether the detected microbes are alive remains an open issue. Additional analyses based on microbial RNA transcripts analysis (meta-transcriptome analysis) could provide new knowledge about the functionally active microorganisms in the endometrium. This novel approach was applied in **Study III** and **Study IV**, where the active microorganisms mapping was performed in healthy women at different cycle phases (proliferative and mid-secretory phases) and in RIF women with implantation failure (mid-secretory phase). Methodological considerations for applying this analysis technology were summarised in **Study II**. We discuss that human microbiota is composed of bacteria, viruses, fungi and other eukaryotes, and archaea. Many microorganisms can generate polyadenylated transcripts (poly(A) tails) with regulatory functions in the transcription process, like in the eukaryotic host cells. RNA sequencing protocols use either oligo (dT) primers or random hexamer primers for amplifying the cDNAs, meaning that when using oligo (dT) primers, only transcripts with poly(A) tails will be fished out for further analysis, and thereby the microbial transcripts not possessing the poly(A) tails in their mRNA molecules will be discarded. Therefore, depending on the exact sequencing protocol of choice, the non-polyadenylated microbial RNA sequences could be detected or discarded, which need to be taken into account when interpreting the obtained results.

Mapping the functionally active microorganisms in endometrium related to healthy and RIF conditions

Applying the meta-transcriptomic analysis approach, a cartography of the functionally active endometrial microbiota of healthy women and RIF patients was provided (**Study III** and **Study IV** respectively). Over 5000 active microbes were detected in the endometria of healthy women, where bacteria formed 80%, 10% of fungi, 5% of viruses, and 0.3% of archaea. The most abundant active microbes identified in the endometrial of healthy women were *Klebsiella*, *Clostridium*, *Pasteurella* and *Hydrogenophaga*, while most of them had relative abundance below 1%, highlighting endometrium as the site of low microbial biomass and non-*Lactobacillus* microbial composition since this microbial taxon accounted for less than 1% of the relative microbial abundance. Our studies on 2 different cohorts (**Study III and IV**) demonstrate 76% similarity in microbial composition, supporting the identification of the core endometrial microbial map in healthy endometrium. Further, the reliability of this methodological approach was tested in a different cohort (Huang *et al.*, 2017), and the results confirmed that the validation samples of mid-secretory endometrium clustered close to our experimental data set (**Study III**).

When predicting the possible metabolic activities of the functional microbes in the host-microbiota crosstalk in the endometrium, the prostanoid biosynthesis pathway and L-tryptophan metabolism seemed to have important role in the endometrium. Prostaglandins have an important role in the endometrial functions and prostaglandin E2 has been proposed as a biomarker of endometrial receptivity (Vilella et al., 2013). Tryptophan, on the other hand, is a precursor in the biosynthesis of serotonin and melatonin. Melatonin has been shown to promote uterine functions, where it regulates different pathways associated with

endometrial receptivity, and irregular uterine melatonin production has been related to recurrent spontaneous abortion (Chuffa *et al.*, 2020). Altogether, this *in silico* metabolic modelling highlights the possible host-microbe interactions in the endometrial functions.

Along the menstrual cycle, several hormonal changes occur that can drive microbial composition fluctuation. Indeed, significant differences in the microbial abundances in the mid-secretory *vs.* proliferative phases were detected in our study (**Study III**). Specifically, 33 microbial species were differentially expressed in the mid-secretory endometria in comparison to the proliferative phase and most of the microbial transcripts (75%) of them were transcriptionally more active in the mid-secretory phase. Although there are studies demonstrating no changes in the microbial composition throughout the menstrual cycle (Khan *et al.*, 2016; Moreno *et al.*, 2016; Cregger *et al.*, 2017; Kyono *et al.*, 2019), our study results support the hypothesis of the microbiota cycle-dependence. It is well established that the microbial composition is hormone-dependent, and that the endometrial functions are under hormonal control. Endometrial mucosa, being under hormonal regulation, functions as an important tissue barrier against pathogens and forms a symbiotic relationship with commensal microbes (Agostinis *et al.*, 2019).

Endometrial microbial composition has been associated with different gynaecological diseases, including RIF (Toson *et al.*, 2022). Our next study focussed on analysing functionally active microbiota differences in the mid-secretory endometria in women with RIF *vs.* healthy controls (**Study IV).** At the taxonomic level, a total of 180 microbes were differentially abundant between the RIF patients and healthy controls, where *Streptomyces*, *Xanthomonas*, *Fusarium* and *Burkholderia* were significantly less detected in the RIF group, meaning that

women with endometrial pathology demonstrate less richness in microbial composition than healthy controls. Among the possible metabolic routes, the results demonstrate that the microbes in the endometrium of RIF women have increased metabolic activity in the biosynthesis of retinol, which is essential for the establishment of pregnancy (Kam *et al.*, 2012). On one hand, the high retinol production might help to promote the initial steps of embryo implantation, while on the other hand, an excess of retinol-related compounds in the uterine microenvironment may counteract the process, since excessive levels of retinoic acid are toxic, requiring a tight metabolic regulation between maternal tissues and embryo (Geelen and Peters, 1979; Collins and Mao, 1999). As we detected in **Study III**, an important metabolic pathway in the possible host-microbes interplay related to the receptive endometrium is the serotonin degradation pathway. In RIF patients, the microbial metabolic activity prediction identified the endometrial serotonin degradation pathway to be down-regulated, which could interfere with the receptive-phase endometrial functions. The current Doctoral Thesis provides novel aspects of potential host-microbe interactions in the endometrium, which require further investigation.

Microbiome in PCOS

Another gynaecological disease where microbes could play an important role is PCOS. Several studies have detected changes in microbial composition and diversity along the human body in women with PCOS. However, the study results are hard to compare and there is no consensus on the microbial composition in PCOS due to the different methodological limitations, starting with a small sample size. We performed a systematic literature search and comprehensive meta-analysis with the aim to bring more clarity to the microbial

composition throughout the body in PCOS (Study V). Our meta-analysis, composed of the gut microbiome data of 1868 women, detected significant differences in microbial diversity, indicating a diminished richness in PCOS women when compared to controls. The results of our study indicate that the decreased abundance of some bacteria may be associated with PCOS, which supports the hypothesis previously proposed by Larsen et al. which argues that greater diversity leads to greater stability of the microbial system that is associated with redundancy (Larsen and Claassen, 2018).

6. Conclusions

In conclusion, this Doctoral Thesis provides new insights into the composition and possible functions of microbes in female reproductive health. The new knowledge generated could lead, in not-too-distant future, to the therapeutic interventions in female reproductive health, ranging from endometrial functions in receptivity and embryo implantation to gynaecological disorders such as PCOS. Microorganisms may prove to be important allies in improving uterine microenvironment through possessing a huge potential of its composition being modifiable.

SPECIFIC CONCLUSIONS

- **Study I**: Uterus harbours its own microbial composition that is dysregulated in different gynaecological conditions like infertility, endometriosis, endometritis, endometrial polyps, dysfunctional menstrual bleeding, and endometrial cancer. Nevertheless, the core/consensus endometrial microbiome has not been established. Modulation of endometrial microbiome

by antibiotics, pro- and prebiotics is a promising field with high clinical relevance, but it is too early to offer this treatment options for patients.

- **Study II**: Many microorganisms are able to generate poly(A) tails in the process of transcription (similar to the host), while several microbes may lack poly(A) tails, therefore the wide application of microbial RNA sequence analysis (meta-RNA-seq) must be supported by a well-prepared protocol for a comprehensive understanding of the entire microbial atlas.
- **Study III**: The analysis of the functionally active endometrial microbiota shows that >5000 microorganisms (bacteria, fungi, viruses and archaea) are present in the endometrium of healthy women and changes in composition and function along the menstrual cycle are detected. Microbes have possible metabolic activity in the host-microbiota crosstalk in receptive phase endometrium related to prostanoid biosynthesis pathway and L-tryptophan metabolism. Our study confirms the presence of active microbes in the human endometrium with implications in receptive phase endometrial functions, meaning that microbial dysfunction could impair the metabolic pathways important for endometrial receptivity.
- **Study IV**: Women suffering RIF have significantly different functionally active microbial profile, where retinol biosynthesis and serotonin degradation metabolic pathways in the host-microbe interactions were dysregulated when compared to healthy controls. Our study confirms the presence of the core microbiota in the human endometrium in health and that in women with implantation failures the microbial composition demonstrates less richness which could impair the metabolic pathways important for endometrial functions.

- **Study V**: The relation between the microbial composition and the aetiology of PCOS is an active field of research. Most of the studies performed in the field focus on gut microbiome analysis, nevertheless the studies are barely comparable and findings inconsistent. Our meta-analysis gathers 17 studies and a total of 1368 women (737 women with PCOS and 631 controls) of individuals and demonstrates that women with PCOS possess lower richness in the gut microbial composition when compared to control women. These findings support the potential importance of microbiome in PCOS development with possible future biomarker/treatment options.

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