# Chapter 11
# Microbiome in Embryonic Implantation and Implantation Failure

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## Introduction

### *The Human Microbiome*

The human microbiome is the sum of microorganisms, together with their genomes, which inhabit the human body, and represents a large entity. In fact, the human body is colonized with an order of magnitude more bacteria than human cells in the body [1]. Its impact and influence on the reproductive process existed even prior to a full understanding of its existence in the nineteenth century. The Hungarian physician Ignaz Semmelweis who lived from 1818 to 1865 intently studied "puerperal childbed fever"—a disease we know of today as postpartum endometritis. At the time, maternal mortality from the disease ranged from 7 to 15%. These studies led to his proposal in 1847 that hand washing in a hypochlorite solution could nearly

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eliminate the risk of puerperal fever. This along with germ theory findings proposed by Pasteur forever entwined reproductive health with the human microbiome.

However, the progression of our understanding of the role of the microbiome in both physiologic and pathophysiologic reproductive processes has been somewhat protracted. The advent of culture and microscopy were of great importance to a more complete characterization; however, the limits of these technologies have become apparent. Indeed, many microorganisms are not readily detected by traditional cultivation techniques, and thus their role in physiologic and pathophysiologic processes remains incompletely understood. A recent study in the surgical literature shows that more than 50% of the dominant pathogens and 85% of major pathogens in wound infections will not be identified by standard culture techniques [2]. However, new technologies and techniques have begun to revolutionize the way that we think of our microbiome.

The majority of published medical literature focuses on the subset of the microbiome which is involved in pathogenesis, while only a subset focuses on the physiologic role the microbiome plays. The importance of this physiologic role was prominently recognized as the human genome project was published in 2001 [3]. The scientists involved called for a "second human genome project" that would investigate the normal microbiome colonies at various sites in order to understand the synergistic interactions between the microbiome and its host [4, 5]. Several initiatives commenced worldwide, and in the United States the Human Microbiome Project (HMP) led by the National Institutes of Health (NIH) was launched in 2007 which utilized high-throughput sequencing technologies to characterize the human microbiome in normal, healthy volunteers at several different body sites which included the vagina [1].

This scientific revolution has been initiated by implementing new technologies such as DNA fingerprinting, microarrays, and targeted or whole genome sequencing that have in turn empowered the field of metagenomics—the study of genetic material recovered directly from environmental samples, in this case, the human reproductive tract. Indeed, work through the HMP and other investigators utilizing this technology have revealed that sites in the body traditionally thoughts to be sterile, such as the uterine cavity and the placenta, are in fact colonized with their own unique microbiome [6, 7]. These molecular techniques take advantage of the 16S rRNA gene which is unique to bacterial and contains a number of hypervariable regions which act like "fingerprints." These fingerprint sequences can then be used to identify genus and species based on a reference sequence. In addition to the sequencing technology, the field has seen great improvement in the bioinformatics that process this data. Indeed, bioinformatics research in the microbiome is at this point evolving faster than the molecular techniques which generate the data.

## *The Human Microbiome in Reproduction*

Much of the data surrounding the normal or healthy microbiome of the reproductive tract comes from the gynecology literature which characterized the vaginal microbiome as it changed through puberty, during the menstrual cycle, and in menopause [8]. There was further characterization of dysbiosis as seen in a number of

reproductive tract pathologies, as is seen in pelvic inflammatory disease caused by organisms such as *C. trachomatis*. The reproductive tract is dominated by *Lactobacilli* species, and this dominance is often altered in disease. These alterations in the microbiome may also be impactful on the reproductive potential of patients with implantation failure. Further, the physiologic alterations of the microbiome due to fluctuating estrogen levels have implications on controlled ovarian hyperstimulation in which supraphysiologic estrogen levels are achieved followed by a fresh embryo transfer. A greater understanding of this fluctuation in assisted reproduction may lead to more personalized treatment strategies.

It is important to note that the physiologic role of the microbiome in reproduction extends beyond the important implantation phase and into the health of the gestational phase as well. Thus, since our goal is healthy, full-term live birth, the role of the microbiome and its alteration in the pre- and peri-implantation phase may have much more wide-reaching implications. Indeed, dysbiosis in obstetrics has been linked to inflammatory states which result in spontaneous preterm birth, among other adverse obstetric outcomes [9].

As excitement for exploration of the "second human genome" has increased, our understanding of how the microbiome affects reproductive competence and implantation has evolved [6]. Data has been gathered on the microbiome at every stage of human reproduction from the ovary, follicle, and oocyte, to the testes and semen/spermatozoa, to the fallopian tube, uterus, cervix, and vagina. Both the male and female reproductive tracts exhibit complexity and diversity only realized within the last decade, and the microbiome is integrally involved in the process of human reproduction (Fig. 11.1).

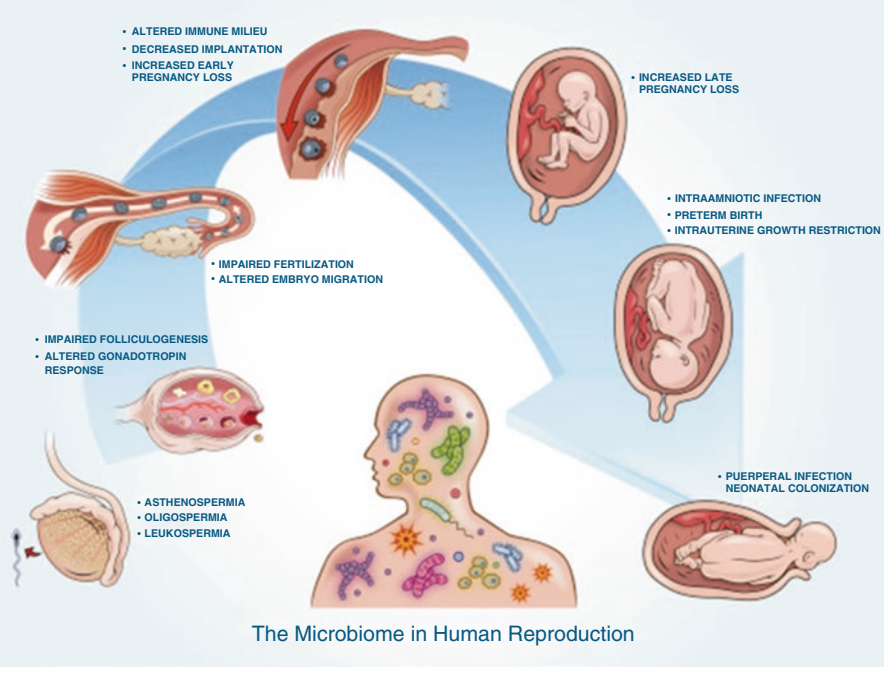


**Fig. 11.1** The microbiome's involvement in human reproduction. Used with permission [10]

## Characterization of the Reproductive Tract Microbiome

The human microbiome's definition—the totality of microorganisms and their collective genetic material present in or on the human body—was attributed to the American molecular biologist Joshua Lederberg in 2001 [11]. Of great importance to this definition is how the metagenomics data is procured. It is important to recognize that microbiome data are procured in one of two ways: culture-based or sequencing-based technology. Much of the early work describing the human microbiome comes from culture-based approaches utilizing the 16S rRNA analysis of highly conserved genes as a way to identify organisms in mixtures [12, 13].

However, data from cultivation-independent techniques suggests that many organisms cannot be identified utilizing culture-based techniques which results in an underestimate of the diversity of the ecosystem as well as failing to identify potentially important organisms when describing their relation to health and disease [14, 15]. Indeed, work which has followed in the wake of the HMP has utilized the advances of culture-independent approaches in order to confirm that places traditionally thoughts to be sterile, such as the uterine cavity and the placenta, are in fact colonized with their own unique microbiome. Thus, culture-based data, while still foundational and informative, must be interpreted within the limits of the technology.

The major goal of the HMP launched in 2007 by the NIH was to investigate the relationship between disease and changes in the human microbiome. It utilized high-throughput sequencing of the 16S rRNA gene. Specifically, the sequencing focuses on hypervariable regions within the gene which serves as a molecular fingerprint down to the genus and species level [16, 17]. Although data in regards to the microbiome of the reproductive tract has not utilized it extensively to date, metagenomics has also become an increasingly widespread approach to describing the microbiome [18]. Using this method, also termed community genomics, analysis of microorganisms occurs by direct extraction and cloning of DNA from a grouping of organisms. It allows for analysis which extends beyond phylogenetic descriptions and makes attempts as studying the physiology and ecology of the microbiome.

For the purpose of metagenomic analysis with high-throughput sequencing, biologic specimens can be simply collected. There is no need for complex care leading to specific culture conditions. DNA extraction and microbial DNA purification steps are performed. Subsequently, one of several molecular genetics techniques is then applied. The most common are fingerprinting, DNA microarrays, targeted sequencing, and whole genome sequencing (Fig. 11.2).

The various techniques available in metagenomics supply both strengths and weaknesses depending upon the primary purpose of the analysis. For example, fingerprinting, which utilizes the 16S rRNA gene to cluster bacterial communities, is relatively inexpensive, but lacks specificity. Targeted sequencing and microarray data focus on the hypervariable regions of the 16S rRNA and allow for greater specificity down to the genus and species level. However, this technique relies on bioinformatics processing which maps reads to a known or reference genome. Thus,

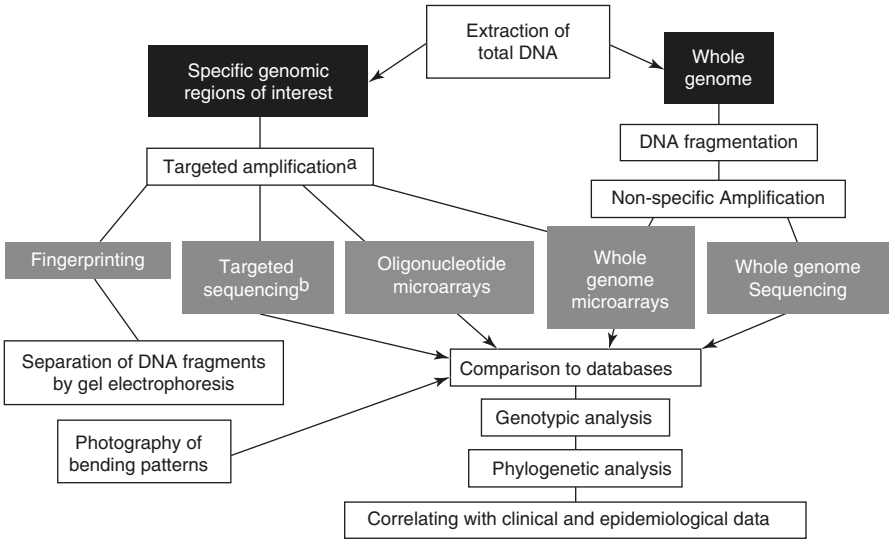


**Fig. 11.2** Molecular techniques utilized when characterizing the human microbiome. Used with permission [11]

they are reliant upon mapping to previously identified sequences or species. Although costly, whole genome sequencing allows for full discovery of an organisms genome and may yield information about functional differences of bacteria in a community.

Metagenomic sample sequencing produces read lengths of 200–300 bp paired-end reads up to 1000 bp reads depending on what sequencing platform is utilized. Read lengths and read depths—the number of reads per colony—are important in accurate characterization. The data generated by the sequencing must be processed and organized into clusters termed operational taxonomic units (OTUs). This is accomplished by mapping the 16S sequence to publically available taxonomic databases. OTUs are then utilized to determine sample composition and diversity. Several open-source software packages, for example, QIIME (Quantitative Insight Into Microbial Ecology), assist with the bioinformatics processing and analysis.

## *Microbiome Characterization: Limitations*

We have discussed the limitations of cultivation-dependent techniques as compared to cultivation-independent techniques in terms of accurate characterization of biodiversity. However, it is important to note some of the limitations of the technologies described above which are unique to the high-throughput sequencing approach.

Sequencing metagenomics samples allows the investigator to determine presence or absence of microbial genetic material. There is not data provided regarding the vitality of the microorganisms. Further, although read counts can be helpful in

this regard, quantification of a particular organism in a sample can be challenging. This read count clustering, also known as "binning," can be performed when known sequences exit; however it becomes much more challenging and less accurate when analyzing novel species [19].

Further limitations relate to clinical functionality. For example, while sequencing can give insight into the makeup of the microbiome, it does not give information about its biologic function, like resistance or susceptibility to antibiotics. Further there is a growing body of data which suggest that these microorganisms are not simply free-floating on the surface of tissue but form their own three-dimensional biofilms with inner and outer layers. This adds an additional complexity which could be of great importance but has been explored very little. The fact that these biofilms exist from the vagina to the fallopian tubes allows complex and dynamic interactions between the gametes and embryo as well as the maternal tissue interface [20, 21].

## The Female Reproductive Tract Microbiome in Health and Disease

### *The Microbiome in the Vagina and Uterus in Health*

The vast majority of data reporting the characterization of the normal state of the reproductive tract microbiome come from studies analyzing vaginal samples, due to the outdated belief that the uterine cavity was a sterile site. In this line, it has been widely reported that the normal vaginal microbiome in healthy women is generally dominated by *Lactobacilli* species [22], although it is subject to important variations along women's lifetime depending on age, changes in hormonal levels, as well as sexual activity and hygiene habits [23]. The vaginal microbiota during the infancy is characterized by a mixture of aerobic and anaerobic bacterial populations including *Prevotella*, *Peptostreptococcus*, *Enterobacteria*, *Streptococcus*, and *Staphylococcus* species [24]. In the pubertal period, the pH of the vagina decreases, and glycogen production increases in response to the estrogen rise, promoting the colonization of *Lactobacilli* species which are able to grow in acidic environments and displace other kinds of bacteria. A vaginal microbiota dominated by *Lactobacillus* genus has traditionally been associated with vaginal health during the woman's reproductive life, as the production of lactic acid by these bacteria would prevent the growth of potential pathogens that could produce vaginal or urinary infections, as well as sexually transmitted infections [25–27]. During menopause, estrogens levels decrease again together with the dominance of *Lactobacillus*, while high percentage of *Lactobacillus* is recovered in women receiving hormone replacement therapy [28, 29].

The analysis of the vaginal microbiome using molecular techniques has revealed that five vaginal community state types (CSTs) can be found in healthy reproductive-age women based on their bacterial composition. More than 70% of the women demonstrated vaginal microbiota dominated by *L. crispatus*, *L. gasseri*, *L. iners*, or

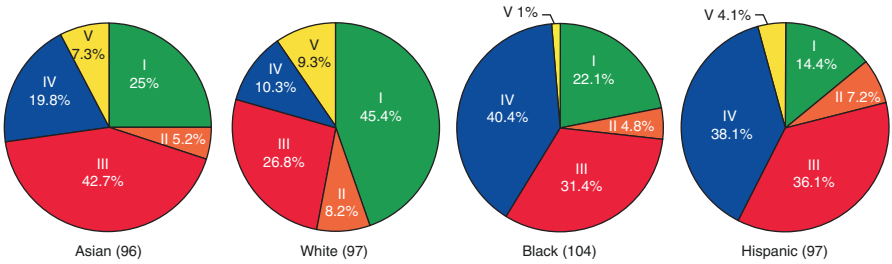


**Fig. 11.3** The vaginal bacterial community state types differ in women from different ethnicities. Number of women analyzed in each ethnic group is shown in parentheses. Used with permission [22]

*L. jensenii*, corresponding to CST-I, CST-II, CST-III, and CST-V, respectively. A small but yet important proportion of women presented CST-IV vaginal microbiota, characterized by lower percentage of *Lactobacilli* and dominance of anaerobic bacteria including *Aerococcus*, *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera*, *Prevotella*, and *Sneathia* species [22].

Interestingly, the vaginal microbiome is influenced by myriad factors and is dependent on its relationship with the host. One example is the influence of the ethnic background on the vaginal microbiota (Fig. 11.3); while Caucasian and Asian populations present a higher prevalence for *Lactobacilli*-dominated CST-I and CST-III, respectively, the non-*Lactobacilli*-dominated CST-IV microbiota is much more prevalent in Hispanic and African-American women [22].

Knowledge about the normal upper genital tract microbiome is much more scarce, as the uterine cavity has been historically considered to be sterile [30], and the isolation of bacteria from endometrial samples had been long considered to come from patients suffering overt uterine infections or through contamination of the sample [31, 32]. The existence of bacterial communities in the upper genital tract has been corroborated by qPCR detection of bacteria in 95% of endometrial samples obtained from asymptomatic women undergoing hysterectomy for benign indications [33]. Due to the limited number of targeted bacteria analyzed, no comprehensive endometrial microbiota data was available from these women, but it shows that the uterine cavity presents bacterial colonization that is quantitatively and qualitatively different from that of the vaginal microbiome from the same women [33].

Recently, a study conducted using next-generation sequencing of the 16S rRNA gene has compared the vaginal and endometrial microbiota of asymptomatic and fertile nonpregnant women [34]. Consistent with the work by Mitchell and coworkers, bacterial communities were detected in 100% of the subjects analyzed, showing that *Lactobacillus* was the most represented genus in endometrial fluid samples followed by *Gardnerella*, *Prevotella*, *Atopobium*, and *Sneathia*, which have been also identified in vagina. However, in approximately 20% of the women analyzed, the bacteria community identified in the vagina was dramatically different from the one in the endometrium, showing that, although closely related, endometrial and vaginal microbiota are not identical in each woman [34].

## *Pathological Shifts of the Female Reproductive Tract Microbiome*

The comprehensive understanding of the human microbiota in the reproductive tract, as well as other body sites, has revolutionized the traditional concept of bacterial pathogens. As mentioned above, *Lactobacillus*-deficient communities dominated by anaerobic bacteria, usually associated with a disease state, have been identified in the genital tract of otherwise healthy and asymptomatic women. In this scenario, the definition of a pathogenic microbiota should be revisited to evaluate not only the intrinsic virulence of a specific microorganism by itself but also its impact in the surrounding bacterial community and finally the impact on the host [35]. In this case, in the absence of symptoms, a non-*Lactobacillus*-dominated microbiota would be considered as "normal" even if it is made up of bacteria classically associated with human genital infections. Despite this, dysbiotic deviation from the "normal/healthy" *Lactobacillus*-dominated microbiota may produce imbalances in the homeostasis of the reproductive tract that may increase the susceptibility for acquiring bacterial or viral infections and other gynecological diseases [36].

## *Bacterial Vaginosis*

Bacterial vaginosis (BV) is a clinical microbiological syndrome caused by the shift from a *Lactobacillus*-dominated vaginal microbiota to a polymicrobial population including *Atopobium vaginalis*, *Gardnerella vaginalis*, *Dialister* spp., *Megasphaera* spp., *Prevotella* spp., *Sneathia* spp., and/or the so-called BV-associated bacteria (BVAB), among others. The prevalence of BV in the USA has been estimated to be 29.2% in the last decade [37]. Oral metronidazole in combination with vaginal clindamycin is the current treatment for BV, but relapse infection within a year is observed in 50% of the treated patients due to resistant bacterial strains [38].

BV has been associated with a higher risk of pelvic inflammatory disease [39], HIV-1 [40], and obstetrical complications such as late miscarriage and preterm delivery [41–43]. The implications of BV on infertility and IVF success remain unclear [44]. Of note, there is a high prevalence of BV in infertile patients occurring in as many as 40% of women receiving assisted reproductive treatment [42].

## *Chronic Endometritis*

Chronic endometritis (CE) is a persistent inflammatory condition of the endometrial mucosa produced by infection of the uterine cavity with common bacteria such as *Corynebacterium*, *Enterococcus faecalis*, *Escherichia coli*, *Gardnerella vaginalis*, *Klebsiella pneumoniae*, *Proteus* spp., *Pseudomonas aeruginosa*, *Staphylococcus*

spp., and *Streptococcus* spp.; genital pathogens as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Ureaplasma urealyticum*; and yeasts like *Saccharomyces cerevisiae* and *Candida* spp. [45–47]. The general prevalence of CE is 19%, but this percentage can be underestimated as it is often asymptomatic and thus, rarely suspected or diagnosed [48]. The current treatment for CE consists of a combination of ceftriaxone, metronidazole, and doxycycline (according to the Center for Disease Control), but relapse is a common feature in patients. Because of the frequent lack of symptoms and the fact that CE is not detectable through transvaginal ultrasound, the diagnosis is the most challenging feature its assessment. Traditional diagnosis methods include the histological observation of infiltrated plasma cells in endometrial stromal compartment, followed by classical microbiological culture, while observation of micropolyps, edema, and hyperemia through hysteroscopy has been lately accepted as a reliable method for the diagnosis of CE [49].

The prevalence of CE ranges from 2.8 to 29% in IVF patients depending on the diagnostic method used [49–56]. Although the impact of CE on IVF outcomes has been described to be minimal [50], retrospective studies have pointed out to an implication in repeated implantation failure (RIF) [55, 57, 58] and recurrent miscarriage (RM) [59]. These correlations have been corroborated in asymptomatic patients diagnosed by hysteroscopy that significantly improved their reproductive outcomes after receiving antibiotic treatment for CE [57, 59].

## Microbiome in Assisted Reproductive Technology

In order to give a full picture of the microbiome in reproduction, we have discussed the importance of the role of the microbiome in the physiology and pathophysiology of the gynecologic tract and will discuss its importance during gestation. Indeed, these areas have been foremost in the research to date. However, given the connections between the microbiome, host immunity, and infertility, it is quite clear that the vaginal and uterine microbiomes play a role in the physiology and pathophysiology of human reproduction.

### *Vaginal Microbiome in ART*

The vaginal microbiome has been characterized to a great degree through the HMP. Perhaps some of the most interesting data which came from this analysis was the analysis of diversity. The vaginal tract exhibited some of the lowest alpha (within samples from the same subject) and beta (comparison between subjects) diversity when classified using phylotypes compared to other sites such as the mouth or the skin [60] (Fig. 11.4). Indeed, when samples were taken at the vaginal introitus, midpoint, and posterior fornix, the variation of species was not great, and *Lactobacillus* spp. dominated all sites. The fact that vaginal communities is normal,

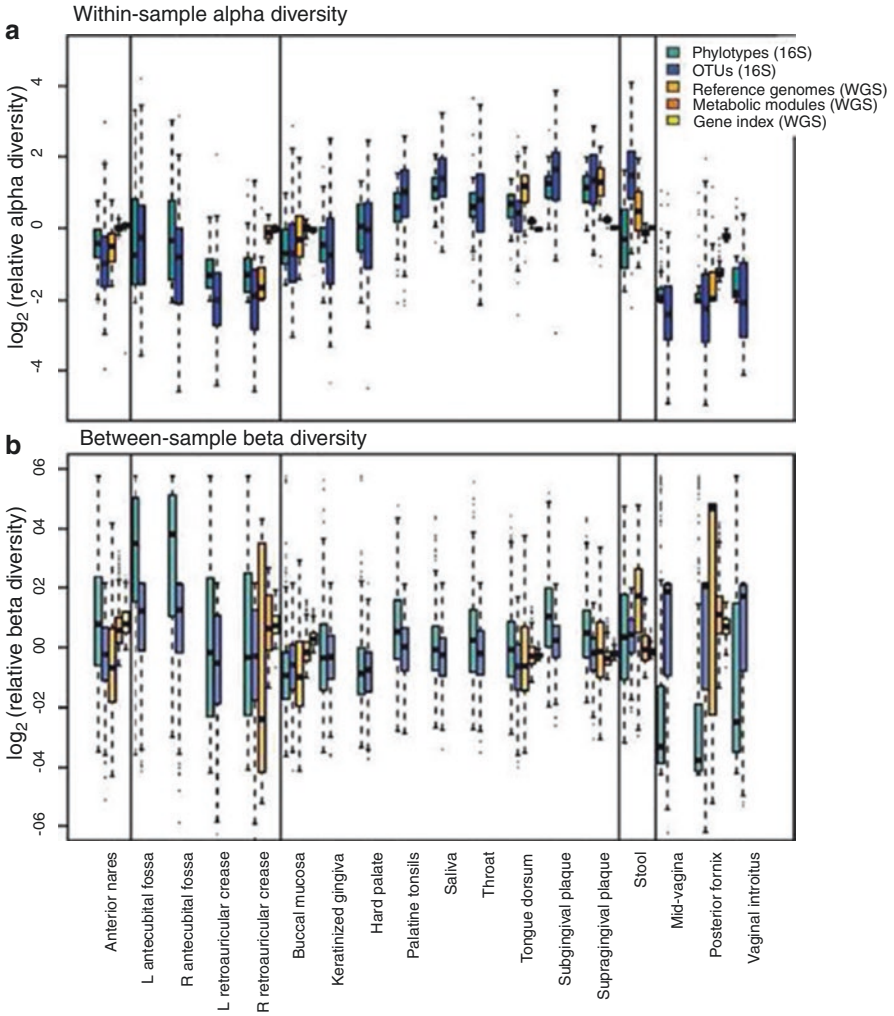


**Fig. 11.4** The Human Microbiome Project utilized 16S rRNA sequencing to identify diversity at various body sites. The alpha and beta diversity of the female reproductive tract is low when compared to other body sites. Used with permission [60]

healthy volunteers is relatively simple as compared to other sites of the body means that characterization of health and disease states could be informative in clearly defining shifts in the microbiome—in other words, simplicity of normality allows for easier identification of abnormality.

The vaginal microbiome as it pertains to ART has been investigated several ways. Utilizing culture-based technology, certain bacteria, such as *Enterobacteriaceae* and *Staphylococcus*, found at the time of embryo transfer on the transfer catheter were associated with poorer outcomes [61]. More robust studies utilizing sequencing

techniques and analyzing diversity indices found that lower diversity indices had better outcomes—as one would hypothesize given the fact the "normal state" has low diversity with *Lactobacilli* dominance [62].

Of note regarding stimulation, the vaginal microbiome has been shown to change during the normal menstrual cycle with varied estrogen levels in the physiologic range [17]. It is thus reasonable to assume the controlled ovarian stimulation required to achieve success in IVF would also impact the vaginal microbiome. This may represent yet another reason, in addition to embryo and endometrial synchrony and implantation failure discussed elsewhere in this book, that certain circumstances may dictate improved outcomes in terms of implantation when a physiologic state which more approximates nature is procured.

## *Endometrial Microbiome and Embryonic Implantation*

Although in present day the revelation is not so profound, it was only recently that the upper genital tract colonization could be deemed anything but pathologic [63–67]. There are a number of barriers in terms of cervical mucus and alterations of inflammatory milieu which may dictate that the microbiome in the upper tract would differ from the lower tract, but to think it was sterile would be difficult given that spermatozoa must traverse the same path. Indeed, studies which employed radiolabeled albumin spheres placed in the vagina found they ascended into the uterus in as little as 2 min [68].

The microbiological state of the endometrium at the time of embryo transfer has been long considered of particular interest as it could impact embryo implantation. Accumulated evidence from studies reporting bacterial isolates recovered upon microbiological culture of the embryo transfer catheter tip have linked the presence of endometrial pathogens to poor reproductive outcomes in IVF patients. Concretely, the isolation of *Enterobacteriaceae*, *Streptococcus* spp., *Staphylococcus* spp., *E. coli*, and Gram-negative bacteria from the transfer catheter tip is associated with significantly reduced implantation and pregnancy rates [61, 69–73].

In the "microbiome era," the attribution of negative IVF outcome to a specific isolated bacterium is not suitable anymore. The entire microbial community needs to be addressed in order to draw conclusions. To do so, all the efforts are now focused on the identification of an endometrial microbiome signature responsible for reproductive failure or success. In this regard, only few studies have been undertaken to characterize the endometrial microbiome in infertile patients.

Verstraelen and collaborators have reported the endometrial microbiome of 19 Caucasian patients with RIF, recurrent miscarriage (RM), or both [74]. The endometrial microbiota in those patients was formed by 183 bacterial phylotypes, being the *Bacteroides* and *Proteobacteria* phyla the most represented, although they found one patient with endometrial microbiota dominated by *Lactobacillus crispatus* and one patient presenting a polymicrobial community including *Prevotella*

spp., *A. vaginae*, *Mobiluncus curtisii*, *Porphyromonas*, *Dialister* spp., and *Peptostreptococcus* spp. phylotypes [74]. The results of this work are consistent with previous evidences showing dysbiotic shifts from a *Lactobacillus*-dominated microbiome in the reproductive tract are more frequent in subfertile population [44].

The endometrial microbiome of infertile patients and its functional impact on reproductive outcome have been recently assessed in two different studies. In the first study, 33 patients of different ethnicities (26 Caucasian, 5 Asian, 1 African-American, and 1 Hispanic) were interrogated for their endometrial microbiota at the time of embryo transfer of a single euploid embryo, and these results were correlated with their IVF outcomes [7]. The core endometrial microbiota in this patients was made of 278 genera, being *Flavobacterium* and *Lactobacillus* the most abundant genera in both patients with ongoing and non-ongoing pregnancies, and no other taxa was significantly identified as differential between women with or without ongoing pregnancies, mainly due to the large number of variables in the study that was not able to survive correction for multiple comparison in the statistical analysis [7]. The latest work has analyzed the impact of endometrial microbiome on reproductive outcome in endometrial fluid from 35 infertile Caucasian patients presenting RIF despite of having receptive endometrium assessed by molecular analysis [34]. The endometrial microbiota was made of 108 components being *Lactobacillus* spp. the most abundant bacteria detected. The results of this study show that endometrial microbiota profile can be classified according to the structure and relative abundance of the bacteria identified in endometrial fluid, as *Lactobacillus* dominated or non-*Lactobacillus* dominated with a cutoff value of *Lactobacillus* relative abundance ≥90% as the only significant variable able to predict reproductive success. Thus, a non-*Lactobacillus*-dominated (<90%) endometrial microbiota significantly correlates with adverse reproductive outcomes—measured as implantation, pregnancy, ongoing pregnancy, and miscarriage rates—when compared to subjects presenting a *Lactobacillus*-dominated (≥90%) endometrial microbiota (Fig. 11.5) pointing to the importance of endometrial bacteria in reproductive health [34].

## *The Immune System, the Microbiome, and Implantation*

A full detail of the immune systems interaction with implantation physiology and pathophysiology is discussed elsewhere in this book. It is important to note here however that the microbiome is integrally involved with the immune systems and thus the permissive environment required for successful implantation. Indeed, a complex microenvironment is created by the cytokines involved in both endometrial receptivity as well as embryo development and is influenced by nutrition, stress, injury, and infection and inflammation [75].

In addition to direct inhibition, production of $H_2O_2$ and bacteriocins, and modulation of epithelial receptivity, the microbiome has been implicated in directly modulating the immune system, in particular T lymphocytes [76]. T helper (Th) cells have also been shown to influence ART outcomes. In particular, there is a focus on

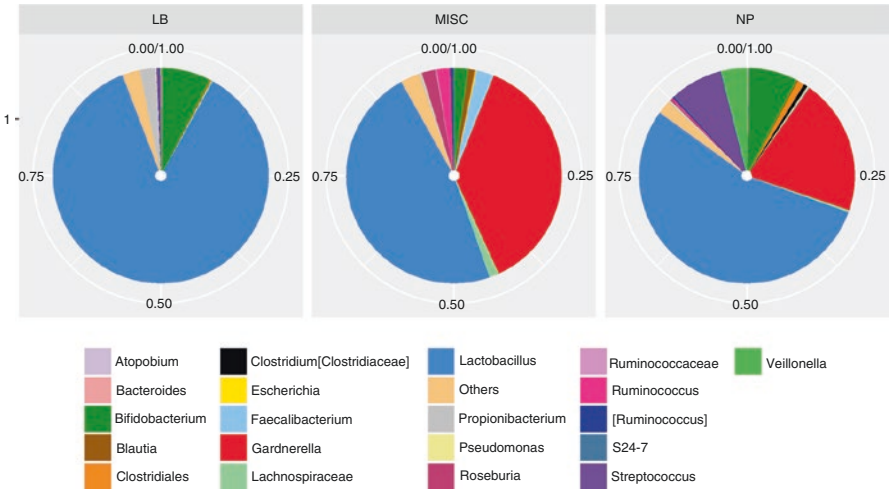


**Fig. 11.5** Low abundance of *Lactobacillus* in endometrial microbiota is associated with poor reproductive outcomes in IVF patients. *LB* live birth, *MISC* miscarriage, *NP* no pregnancy. Adapted from [34]

the ratio of Th1 cells, which produce interferon-gamma (IFN) and lymphotoxin, and Th2 cells, which produce IL-4, IL-5, IL-13, IL-25, and GM-CSF. Both cells produce GM-CSF, TNF, IL-2, and IL-3 [77]. The Th2 cells predominate during normal pregnancy, whereas Th1 is more predominate in women with pregnancy losses [78–81]. The Th1/Th2 ratio construct has been expanded to the Th1/Th2 as well as the Th17 and regulatory T cell construct. The Th17 cells secrete IL-17 which is pro-inflammatory and the T regulatory cells work to induce immune tolerance [82, 83]. Similar to Th1/Th2 ratios, studies have shown increased rates of unexplained spontaneous abortion with an increase in Th17 and decrease in regulatory T cells [84, 85].

The complex interaction between the microbiome, immune modulators, and implantation and reproductive competence is evolving rapidly. Once the physiologic state of the reproductive tract microbiome is better characterized, we will be able to determine more concretely how this microbiome changes the immune milieu and affects the process of immune tolerance.

## *Antimicrobials and ART*

Although the vaginal and uterine microbiome is incompletely understood in terms of its relationship to reproductive outcomes, there is a long history of attempting to influence it using prophylactic antibiotics at the time of procedures during ART. Given that antiseptics are often toxic, antibiotics have been utilized as a method of manipulating the microbiome since the studies in the late 1970s which

showed that contamination during ART procedures could negatively impact outcomes [86, 87]. Indeed, given concern for embryo transfer catheter tip contamination and inoculating of the upper tract, antibiotics are often prescribed leading up to the embryo transfer. This is of concern as wide-spectrum prophylactic antibiotics have the potential to interfere with the "healthy" microbiome which exists at the time of embryo transfer as well as impact those bacteria which are pathologic.

A recent Cochrane Database Systematic Review analyzed randomized controlled trials in the literature which investigated antibiotics at embryo transfer [88]. Only four potential studies were identified, of which three were excluded. The remaining study reported on clinical pregnancy rates as the primary outcome. Although administration reduced microbial contamination as defined by culture of embryo transfer catheter tips, the clinical pregnancy rate in those receiving antibiotics was 36%, and those not receiving was 35.5% (OR 1.02, 95% CI 0.66 to 1.58) [89]. The reviewers concluded more evidence is needed with live birth as the primary outcome [88].

One possible explanation for the lack of clear benefit of antimicrobial use at the time of embryo transfer is that, while the antibiotics successfully decrease the load of bacteria which are alive and can be cultured, it does not decrease the burden of bacterial remnants which still serve to modulate the immune system [89, 90]. This modulation of the immune system by the microbiome may indeed play the most critical role in the connection with ART outcomes.

Although at the present time, data on antimicrobial use has not shown clear benefit, there are other ways in which the microbiome might be altered. Rather than eliminating pathogenic bacteria, perhaps bacteria with beneficial profiles could be replaced. Probiotics have been investigated as a way to treat vaginal infections such as bacterial vaginosis with success [91]. This same approach may be a way to positively affect ART outcomes in the future, although more metagenomic data is needed to more fully characterize the physiologic state prior to intervention attempts.

## The Impact of the Microbiome on Pregnancy Outcomes

### *Non-gravid vs. Gravid Vaginal Microbiome*

The vaginal microbiome has been shown to be distinct in pregnant versus nonpregnant women in terms of structure and stability. Contrary to that observed in nonpregnant women, vaginal microbiota of pregnant women is very stable, and shifts in endometrial microbiota only occur between *Lactobacillus*-dominated CSTs. As a result gravid vaginal microbiota is most often dominated by *L. crispatus*, *L. jensenii*, and *L. gasseri* in women delivering at term, while taxa associated to CST-IV are very rarely observed in pregnant women regardless of their ethnicity [92]. When the spatiotemporal dynamics of the vaginal microbiota has been interrogated, results have shown that the diversity and richness of this microbiota decrease with gestational age and proximity to the uterus [93]. However, a destabilization of the vaginal

microbiota is commonly observed within few weeks preceding delivery and remains altered for approximately 1 year after delivery, showing certain similarities to the communities typically colonizing the gut [93, 94]. The relevance of *Lactobacillus* spp. in the vaginal microbiota during pregnancy and the mechanisms leading to this dominance remain unknown. However, some hypothesis points to the protective role that *Lactobacilli* could play in the reproductive tract against potential ascending infection which represents a risk factor for many obstetrical conditions [95].

## *Placental Microbiome*

The isolation of bacteria from placentas of healthy women delivering at term was reported for the first time in 1988 [96], challenging the general believe of a sterile onset of life. Nowadays, it is well accepted that the placenta harbors a low abundance but unique microbiome that is not the result of uterine infections or chorioamnionitis. The placental microbiome is composed of commensal bacteria belonging to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Tenericutes*, and *Fusobacteria* phyla [97]. Only a small set of taxa as *Burkholderia*, *Streptosporangium*, and *Roseovarius* are increased in placentas of women delivering preterm. Many different models have been proposed to explain the bacterial seeding of the placenta, from ascension from the lower genital tract to the contamination during delivery. However, the vast similarity observed between the community population of the placenta and the oral microbiome of nonpregnant women suggests that these bacteria may reach the placenta through hematogenous spread early in pregnancy, at the time of vascularization and placentation [97].

## *Preterm Birth*

Preterm birth (PTB) is defined as an early birth before 37 weeks of gestation. This very prevalent obstetrical complication has been linked to intrauterine infection with pathogenic microorganisms colonizing the fetal membranes, amniotic fluid, cord blood, placental, and fetus [98]. It is generally believed that this intrauterine infection could be originated in the lower genital tract by ascension of the pathogenic microorganisms producing the preterm premature rupture of membranes leading to PTB. This hypothesis is supported by evidences showing an association of BV with PTB [99]. Another hypothesis, given the placental microbiome's similarity to the oral cavity microbiome, is the hematogenous spread from periodontal infections. This would explain the high correlation observed between PTB and periodontal disease [100]. However, despite the mechanism of infection, the microorganisms causing PTB are well defined and include *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, *E. coli*, *Bacteroides* spp., *G. vaginalis*, *Sneathia*

*sanguinegens*, *Streptococcus* spp., and *Fusobacterium nucleatum* [101, 102] as well as with a decrease or lack of *L. crispatus* in the urogenital tract [94, 103].

## Summary

The microbiome in health and human disease, in particular in relation to the success or failure of human reproduction, is beginning to be unraveled. Given the abilities of new technologies and techniques for sampling and analyzing the microbiome in the reproductive tract, this knowledge is now growing at an unprecedented rate. As the reproductive tract dysbiosis is better characterized and understood, we may be better equipped to manipulate it more expertly and depart from the practice of broad-spectrum, indiscriminant antibiotic use which has been the mainstay of therapy.

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