Maternal Microbiomes in Preterm Birth: Recent Progress and Analytical Pipelines

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Abstract

Worldwide, 10% of babies are born preterm, defined as birth before 37 weeks' gestation. We have had little success in developing strategies to prevent preterm births, the majority of which are due to infection or are idiopathic. An emerging hypothesis is that the maternal microbiome – the bacteria that inhabit the mother's body and play vital functions in normal health – contributes to the etiology of preterm birth. Here, we highlight the latest data revealing correlations between preterm birth and maternal intestinal, vaginal, cervical, and placental microbiomes. Additionally, we describe the most commonly used comparative microbiome analysis methods and highlight important issues to consider when conducting such studies.

Keywords

vagina; placenta; 16S rRNA profiling; cervical microbiome

Introduction

The World Health Organization estimates that each year, 15 million infants are born preterm, putting them at increased risk of morbidity and mortality[1]. Simplistically, preterm birth (PTB) occurs when normal term labor events – uterine contractions and cervical remodeling – occur early[2]. However, PTB is challenging to explain, predict, and prevent because up to 40 to 45% of cases are idiopathic (spontaneous), and numerous risk factors are known, including maternal history, demographics, nutritional status, stress, and infection[2].

Approximately 30% of PTB cases are caused by infection and inflammation[2]. Traditionally, infection-related PTB was thought to ensue from foreign microbes reaching the uterus via

ascending infection or hematogenous transfer. During ascending infections, microbes from the vagina travel upward through the cervix to reach the fetal membranes. For example, the presence of *Mycoplasma spp.* (*Ureaplasma nucleatum* and *Ureaplasma parvum*) and *Candida spp.*, in the vagina is associated with PTB[3]. Hematogenous infection occurs when bacteria travel through the blood stream from another site in the body and then traverse the placenta at the maternal-fetal interface[2].

Although foreign bacteria are important causes of PTB, current research in this area is building on the observation that, far from being sterile, the human body is home to millions of microorganisms[4]. Collectively, all of the bacterial genomes present in or on our body surfaces are known as the human microbiome, and each body niche has its own resident microbes. These bacteria contribute to human health in many ways, such as providing resistance to pathologic infection, breaking down nutrients, and educating the immune system[4,5]. In addition to contributing to physiology, microbiome communities respond to physiology. Pregnancy is a period of major physiological changes[6], such as immunological shifts and the vascular remodeling and metabolic changes needed to promote exchange of nutrients, gases, and wastes with the developing fetus[7]. Thus, the microbial community structure in various maternal niches has the potential to shift during pregnancy[6]. Although many of these changes may benefit or cause no harm to the mother and fetus, we are beginning to learn that dysbiosis of the maternal microbiomes is associated with adverse pregnancy outcomes such as PTB.

Here, we review human microbiome studies that define the microbiomes in key maternal niches and identify associations with both term and preterm birth. Additionally, we describe common technical, analytical, and statistical approaches used to conduct maternal microbiome studies. Rigorous studies together with curated microbiome data will provide an in-depth understanding of the maternal microbiomes and their impact on pregnancy and, hopefully, identify new therapeutic strategies to decrease the incidence and burden of PTB.

Maternal Intestinal Microbiome Changes During Pregnancy

A healthy gastrointestinal tract, dominated by *Bacteriodetes* and *Firmicutes*, contains numerous beneficial microbes that generate vitamins, break down complex foods, and synthesize products that can keep potentially harmful microbes at bay [8] (Figure 1). Because the intestinal microbiome regulates critical metabolic process, diseases, such as obesity and allergy, may arise when the community structure is abnormal [9].

Koren et al., used stool samples to characterize the maternal gut microbiome during the 1st and 3rd trimesters of pregnancy and post-partum[10]. They found that Proteobacteria was more abundant and *Faecalibacterium* was less abundant in the 3rd trimester compared to the other two stages of pregnancy. Although the underlying mechanisms and implications of this shift are not fully understood, the authors suggested that types of taxa present during these stages may be related to both anti-inflammatory (*Faecalibacterium*) and pro-inflammatory diseases (*Proteobacteria*).[10] The maternal gut microbiota may change as a result of shifts in the environment during pregnancy as a result the demand for the transfer of nutrients to the

fetus[11]. Future work will hopefully reveal the role of the gut microbiome during pregnancy and neonatal outcomes, which impact neonatal birth weight and infant gut colonization[12,13].

Vaginal Microbiome and Preterm Birth:

The hallmark of a healthy vaginal microbiome in non-pregnant, reproductive-age (12 to 45 years old) women of multiple ethnic groups is a preponderance of the *Lactobacillus* genera[14]. These bacteria thrive in anaerobic niches and contribute to the acidic vaginal environment by fermenting sugars and producing lactic acid[14–16]. Lactic acid-producing species, like *Lactobacillus,* are thought to help protect the uterus from ascending infections such as sexually transmitted infection and urinary tract infections[17,18]. For example, diminished lactic acid-producing bacteria is associated with greater susceptibility to human immunodeficiency virus[15,19].

The normal vaginal microbiome differs between pregnant and non-pregnant women[20]. Romero et al. found that whereas the community structure of the vaginal microbiota remained consistent across multiple gestational ages (and included species such as *L. vaginalis, L. crlspatus, L. gasserl,* and *L. jensenii)*, the vaginal microbiota of non-pregnant women was more variable across different time points, with differences in the dominating *Lactobacillus* species. Similarly, another study revealed that the vaginal microbiome six weeks post-delivery was more diverse than the vaginal microbiome during pregnancy, regardless of ethnic background[21]. However, the dominating *Lactobacillus* species in the vagina differed depending on the region from which the women originated[21], suggesting that region-specific factors such as diet affect the community structure of the vaginal microbiome. One possibility to explain the shifts before, during, and after pregnancy is that hormonal, nutritional, and immunological pressures alter the vaginal microbiota during pregnancy. Alternatively, these shifts in the vaginal microbiome may help maintain maternal and fetal health during pregnancy[20].

Thus far, evidence that the community structure of the vaginal microbiota plays a role in PTB is limited. Nonetheless, a number of studies found that PTB correlates with bacterial vaginosis, a dysbiosis of the vaginal microbiome characterized by the emergence of pathogens such as *F. nucleatum, Mycoplasma hominis,* and *Bacteriodetes urealyticus* and the loss of *Lactobacillus*[22], particularly during the 2nd trimester of pregnancy[23]. Prematurity in association with BV is also accompanied by low neonatal birth weight[23]. However, whether there is a casual relationship between BV and preterm birth has not been shown.

Cervical and uterine microbiomes

Vaginal microbes associated with PTB most likely reach the uterus by traveling through the cervix (Figure 1). Thus, more and more studies are beginning to address how microbes inhabiting this particular niche contribute to the PTB outcome. During the 2nd trimester of pregnancy, in women with a short cervix the preponderance of specific Lactobacillus species correlates with the gestational age at delivery—where increased vaginal *L. iners* is associated with preterm birth and *L. crispatus* is associated with term birth[24]. Further, the administration of vaginal progesterone, which normally used as a therapeutic for delaying

early delivery, does not alter the vaginal microbiota[24]. Recently, Ghartey et al. analyzed metabolites in the cervicovaginal fluid at two time points in women who went on to deliver either preterm or at term and identified several that differed between the two groups[25]. Although these authors did not assess the microbiome in these women, these alterations in the metabolic environment could make the cervix and vagina more or less hospitable to PTB-related microbes[26]. Additionally, metabolites produced by the cervical microbiome could induce cervical remodeling and thereby promote labor, but there is currently no evidence to support this hypothesis[25]. Further, the microbiota within the cervical fluid of women with premature rupture of membranes (PPROM) exhibited variations in inflammatory markers in association with specific bacterial community state types (CST)—namely cervical fluid with increased non-Lactobacillus CSTs had increased IL-6[27].

Less studied is the uterine microbiome in association with PTB outcomes because little is known about its origins. Uterine biopsies sampled from non-pregnant women exhibited the presence of *L. crispatus, L. iners, Prevotella spp.,* and other bacterial phylotypes[28]. Further, oral microbes, like *Fusobacterium nucleatum,* are thought to travel to the uterine cavity and contribute to PTB, as shown using a murine model[29,30]. Thus, many more studies must be conducted in order to understand the relevance of both the cervical and uterine microbiome in the etiology of PTB.

Placental Microbiome

Although the placenta was long thought to be germ-free, multiple histological and high-throughput sequencing studies have suggested that the placenta harbors its own microbiome[31] (Figure 1). For example, our group used a variety of staining methods to demonstrate the presence of intracellular microbes in the basal plate (the maternal-fetal interface) of term and preterm placentas[32]. Aagaard et al. used 16S sequencing and whole-genome shotgun sequencing to profile bacteria in term and preterm-cross placental biopsies. These authors identified *E. coli, Prevotella species, Bacteriodetes species, and Fusobacterium species*[33]. Notably, preterm placentas had a preponderance of *Burkholderia, Actinomycetales,* and *Alphaproteobacteria*. In a later study, the same group profiled the fetal membrane microbiome in patients with chorioamnionitis (infection of the fetal membrane) and spontaneous PTB and found that it contained species previously identified in PTB. These included *Ureaplasma* and species associated with the oral microbiome, such as *Fusobacteria*. Microbiome changes in the fetal membranes from PTB subjects with chorioamnionitis was accompanied by shifts related to glucose metabolism, inflammatory markers and the siderophore biosynthesis[34]. 16S rRNA sequencing has also been used to determine that there is spatial organization to the placental microbial communities with distinct differences noted in the basal plate, fetal membranes (which encapsulate the amniotic cavity), and placental villi which are bathed in maternal blood[35]. The study noted that the fetal membranes were dominated by *Firmicutes* including *Lactobacillus crispatus* and *Lactobacillus iners,* which have previously been detected in the human placenta[36]. Since these species are prevalent in the intestinal and vaginal flora, this study suggests that the fetal membranes may be exposed to microbial species ascending from the vagina. In contrast, the basal plate was dominated by *Proteobacteria,* including *Bacteriodetes,* which have been found in the non-pregnant uterus[28]. Given the location of the basal plate adjacent

to the uterine epithelium, the uterus may be a site from which the microbes in the basal plate originate. These findings provide new avenues for investigation into sites of origin of placental microbiota. Whether the spatial differences in microbial community membership impacts pathogenesis of PTB remains unknown.

The mechanisms by which microbes inhabit the placenta is far from understood. Recently, our lab reported that the cellular recycling pathway autophagy is an important mechanism for limiting infection in placental trophoblasts[37,38], as preterm placentas showed lower levels of autophagy and higher levels of infection-associated markers than term placentas[37]. Collectively, these data indicate that, in addition to the placental microbiome, dysfunction in placental levels of autophagy activity could contribute to PTB[38]. Further characterizing maternal microbiomes in association with PTB using clinical specimens is currently underway. These efforts, along with the advancement of technology with greater sensitivity for detecting microbes in low biomass niches, greater understanding of geographical ecology of the placenta, and the development of suitable models for testing the function of these communities in association with PTB may be available in the near future.

Methodology of Pregnancy Microbiome Research

The human maternal microbiome studies using high-throughput sequencing have revealed promising associations between microbial composition and health and PTB and have provided a wealth of sequencing datasets, some of which has been made publically available. The majority of associations noted thus far between maternal microbiomes and PTB are qualitative, which is a critical first step. A second priority is to develop the most complete and concise methodology for performing high-throughput maternal microbiome analysis in order to ensure both accuracy and validity of the datasets derived from clinical specimens. A standard pipeline for conducting human microbiome research is well-described by the Human Microbiome Project Consortium[39] and in a recent review by Goodrich et al.[40].

Here, for a clinical perinatology research audience, we present the key steps for conducting a high-throughput microbiome analysis of pregnant subjects including: 1) designing the study, 2) sampling the subjects, 3) extracting the DNA, generation 16S amplicons, sequencing on a high-throughput platform, 4) performing community analysis, and reporting findings. We have also addressed ways to control for contamination and have presented analytic limitations when using the generated sequencing dataset. Thus, fine-tuning high-throughput approaches to address the link between maternal microbiomes and PTB will strengthen the foundational evidence, lead to future studies that can address the function of the microbiota, and pave the way for the development of the microbiome-related diagnostics and therapeutics.

1. Designing the study

The study design is the most critical aspect of conducting a microbiome-based study. In a case-control study of pregnant subjects, the microbiota from the control group (e.g., women who went on to deliver spontaneously at term, >37 weeks) is compared to the microbiota from the cases (e.g., women who went on to deliver preterm). The inclusion criteria for the

control cohort most often include uncomplicated pregnancies and no evidence of maternal viral or bacterial maternal infection. Additionally, one should consider maternal factors that can affect the community structure of the microbiota. For example, Aagaard et al. found that chorioamnionitis is associated with a modified fetal membrane community[34]. Aagaard et al. also showed that the community structure of the placental microbiome correlates with weight gain during pregnancy[41]. Thus, one should record and consider as possible confounders, factors such as maternal infection history, weight, race/ethnicity, and even the hospitals from which samples are collected.

2. Sampling the subjects

To avoid contaminating samples, it is critical to use an appropriate sampling technique, which will vary depending on the niche of interest. The vagina, or a particular region in the vagina, is sampled by circumferential swabbing using sterile technique. The swabs, which are also used in the microbiology clinic for aerobic and anaerobic cultures, can either be stored long term at −80 °C or kept on ice for direct DNA extraction[42,43]. During sampling, most investigators also measure the vaginal pH[42]. In placental sampling, one can either take samples representing the entire placenta or target particular compartments (e.g., amniotic fluid, amniotic membrane, or basal plate). In one study, tissue was washed in sterile phosphate buffer saline [44] to remove blood contamination. Once sampled, tissue can be frozen in liquid-phase nitrogen[40] and then frozen at −80 °C or used immediately for DNA extraction. To sample the intestinal microbiome, investigators use stool, which can be stored between −80 °C and −20 °C without significantly altering the microbial abundance or diversity within samples[40].

3. DNA Extraction, Amplicon Generation, and Sequencing

In most cases, DNA is extracted directly from samples by using commercially available kits as shown in Table 1. Kits such as the QIAamp DNA Stool Mini Kit (QIAgen)[14,21,24,45], are used to extract microbial DNA from primarily from stool, but have been used to isolate microbial DNA from other human niches. However, since DNA extraction kits that target low abundance microbial communities like the placenta are not yet available, investigators have used kits such as the DNeasy PowerSoil Kit (MOBIO)[42,44], which can capture DNA from a variety of biological sources. Given samples with low microbial biomass, it is recommended that multiple kits be used along with extraction blanks and water, for microbiome analysis to minimize the possibility of contaminants and false positives[44].

Because DNA extracted directly from human samples will contain both bacterial and human genomes, one must specifically amplify the bacterial DNA. This is achieved by PCR amplifying regions of the small subunit ribosomal RNA gene sequence (16S)[46]. In addition to conserved regions, this gene contains nine variable regions that can be used to determine the individual species from which the sequence derived.[47] For many studies, primers are designed to amplify a sequence that spans one or multiple variable regions. Alternatively one can amplify and sequence random regions of the genome in a process called whole-genome shotgun sequencing[48]. In both cases, the most commonly used high-throughput amplicon sequencing platforms are Illumina Miseq and 454/Roche[49] (Table 1).

4. Performing community analysis: Analytic Considerations and Pitfalls

Based on a similarity threshold, quality filtered sequences are given taxonomic assignments or are ascribed to an operational taxonomic unit (OTU), a group of similar sequences[50], by using a reference database[48]. However, one must be cautious of comparing the taxonomic assignments derived from amplification of different rRNA regions because their relative abundances will not necessarily be correlated based on factors that are not fully understood, but may be related to the structure or function of the different regions of the ribosomal structure[51]. Assignments are commonly done using the software Quantitative Insights into Microbial Ecology (QIIME)[49], which requires basic programming experience. Other programs that can be used for taxonomic assignments and microbial community analysis are shown in Table 2.

An important step in analyzing maternal microbiomes is to determine the types and relative abundances of bacteria within a sample. This is usually done with two measures: first, alpha diversity, defines the diversity within each sample[49]. The most commonly used measure is the Shannon Diversity index; Chao1 and Simpson are also popular choices, and many publications report more than one measure. Alpha diversity of each sample can be computed by using a number of different programs and software including QIIME [49,52], R VEGAN package[53], and others listed in Table 2. Although some publications compare the diversity measures between groups with a t-test, this test requires that values be normally distributed, which is difficult with a small number of study subjects. Thus, Kruskal-Wallis and Mann-Whitney tests are usually more appropriate because they do not require normality and can be used to determine if at least one group out of many is different (Kruskal-Wallis) or if two groups differ from each other (Mann-Whitney). These tests can be performed in almost any programming environment (Table 3).

The second measure is beta diversity, which indicates how the microbial diversity differs between individuals or between cohorts[49]. Beta diversity calculations can be completed in the same programs used to calculate alpha diversity, but the outcome variable is more complicated. The QIIME output of beta diversity is a matrix comparing every sample to every other sample[49]. For example, the output of a beta diversity calculation for five samples will be a five-by-five matrix (or just the upper diagonal) of phylogenetic dissimilarity between species or OTUs. Several formulas can be used to calculate each measurement in the matrix, but publications usually only report one. Bray-Curtis and unweighted UniFrac are commonly used metrics. Each method has its own formula and calculation that must be considered when deciding which one to use for analysis. For example, Bray Curtis accounts for both the abundance of microbiota[54]. In contrast, unweighted UniFrac uses only the phylogenetic distance between the different taxa[55]. Each measure has its own set of assumptions that must be met before the results can be properly interpreted.

To test the null hypothesis that microbiomes from clinical groups are identical to one another, the current analytic convention is to run the statistical test, PERMANOVA[56], which can be used for any of the phylogenetic dissimilarity measures used for microbiome analysis. Flowever, for this test to be appropriate, the dispersions, or relative phylogenetic distances, of samples in one group to others in the same group must be statistically similar across groups.

Another method for analyzing beta diversity is to create a multi-dimensional scaling or principle components analysis plot[57]. This is a graphical representation of the similarity of individual subjects' microbiomes. Although there is no specific test to complete on the plot itself, the plot is useful for a few reasons. First, one can use different colored dots to distinguish subjects by a categorical or dichotomous variable (preterm premature rupture of membrane status, age group, pregnancy term, etc.) and then visualize where the different groups cluster, if at all. Second, most programs indicate the percentage of the difference between samples that is shown by each axis. The higher the percentage, the more one can conclude that samples cluster as a result of a handful of specific taxa within the microbiomes. Conversely, low percentages imply that many taxa are responsible for the differences. Such a finding indicates that no specific microbiota correlates with outcome of interest, such as PTB.

Notably, programs that use sequencing outputs, such as QIIME, will only provide OTU abundance measures relative to other taxa within a sample, not absolute abundance[58]. To compare the relative abundance microbial communities at the same number of reads, investigators often use rarefaction and normalize their samples to a single read depth[59].

Another approach to determining the absolute abundance of species detected within a sample is to perform quantitative PCR with species-specific primers targeting the 16S gene of the microbe of interest[60]. In this approach, one must generate a standard curve of the quantification cycle [61] values of a known quantity of serially diluted genomic DNA. Cq values derived from samples can then be used to calculate the 16S copy number per quantity of DNA. It is important to note that quantitative PCR will not distinguish between 16S rRNA from viable bacteria or from free nucleic acid within the tissue[62].

Reporting findings

One should always report the distance, measure, and index used for analysis, and explain the rationale behind the choices as there is no simple answer for choosing one method over the other. Each available method must be considered deliberately as reporting only the method that results in the "most significant findings" in a post hoc pick-and-choose process undermines the necessity for multiple analysis methods and formulae and, more alarmingly, raises ethical concerns about future clinical applications of the data.

Prospectus

The majority of associations noted thus far between microbiomes and PTB are qualitative. Long term, the goal of this field will be to identify unique bacteria or bacterial communities that can be targeted to prevent PTB. In such work, it will be important to consider the ecological dynamics of bacterial species and their interactions with the host environment. Currently, infection- and inflammation-related therapies to reduce PTB focus on either targeting the causal microbes, which requires timely and accurate diagnostic measures, or targeting cytokines and inflammatory pathways that promote labor[63]. Given that shifts in the maternal microbiota might contribute to PTB, we must consider how such therapeutics may alter the normal microbiota in various niches[64,65]. A leader in the field of gut microbiome research, Dr. Jeffrey Gordon, and colleagues have recently called for the establishment of

human-associated microbial communities, termed 'human microbial observatories'[66]. Development of a broad view of human development, health, physiology, and disease as being a composite result of the genome, epigenome, and the microbiome, is needed to truly understand etiologies of conditions such as PTB. We hope that studies such as those described above will use appropriate analytical methods and thereby elucidate the role of the maternal microbiome in PTB, leading to development of microbiome-based therapeutic strategies to prevent this outcome. Long term, this understanding can lead to refining our definitions of a healthy microbiome during pregnancy and may aid in development of strategies to restore the normal microbiota during pregnancy

Acknowledgements

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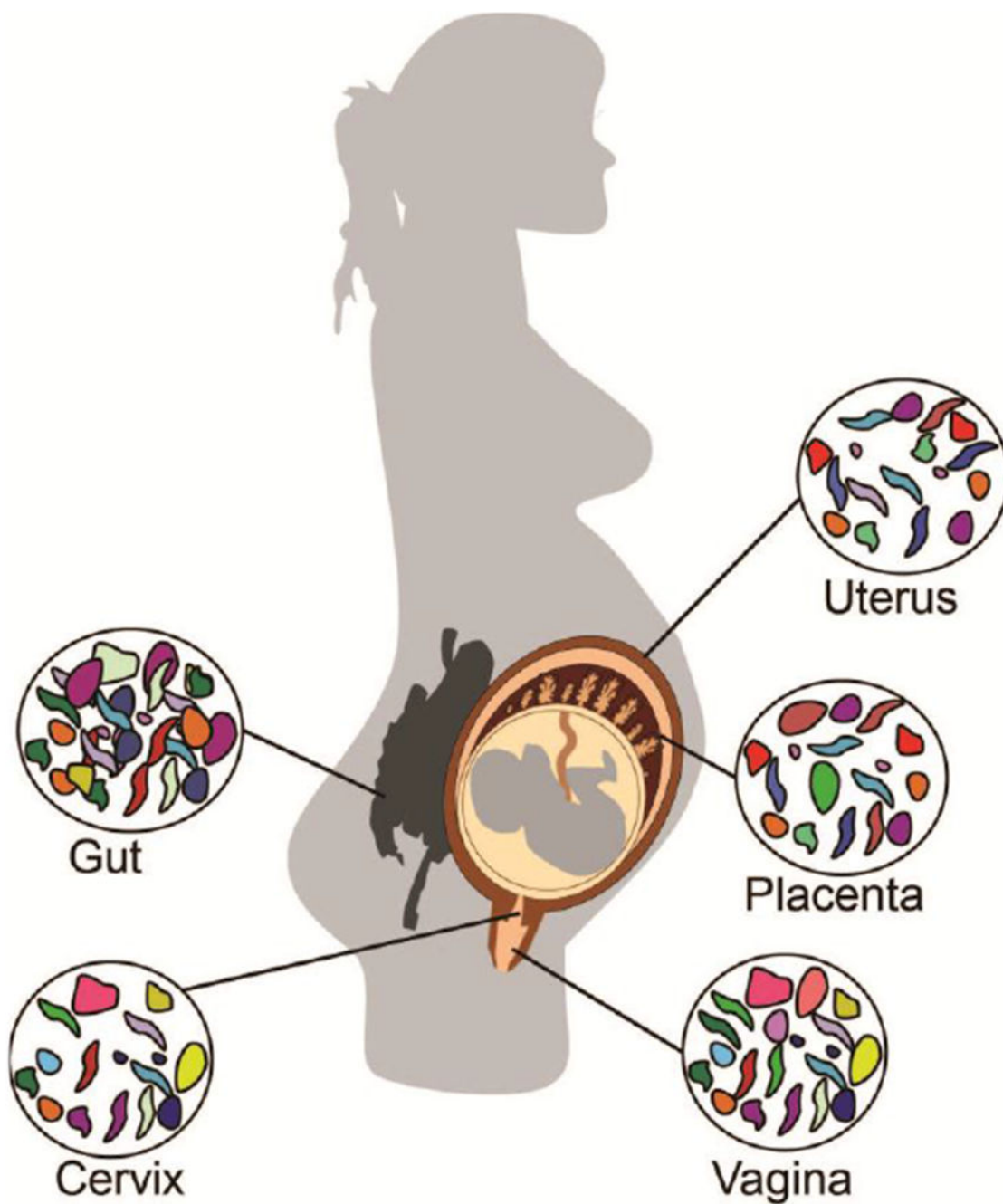
Figure 1: Schematic depicting the intestinal and reproductive tract microbial communities during pregnancy.

Adverse pregnancy outcomes may be associated with microbes that originate from reproductive tract (vaginal and cervical) and ascend to colonize the gestational compartment. However, mounting evidence suggests that other microbial communities including those in the gut and placenta may also impact pregnancy health.

Table 1:

Methods for maternal 16S sequencing studies. Universal primers F (F, forward primer) R (R, reverse primer); Ribosomal Database Project (RDP); Quantitative Insights into Microbial Ecology (QIIME); Linear discriminate analysis effect size (LEfSe).

Location	Sequencing Platform	Sampling	DNA Isolation Kit (Manufacturer)	Variable Regions (Primers)	rRNA reference database	Microbiome Analysis Program	Ref.
Placental	454-FLX Titanium Sequencer (Roche).	1-cm × 1-cm × 1-cm biopsy	PowerSoil DNA Isolation Kits (MOBIO)	V1-V3 (27F, 534R)	RDP	QIIME LefSe	33
Placental	Miseq (Illumina)	0.11 g – 0.66 g biopsy	SP Stool DNA Plus kit (STRATEC Biomedical) PowerSoil DNA Isolation Kits (MOBIO)	V1V2 (27F, 338R)	Greengenes v. 13_8	QIIME	44
Placental	Genome Sequencer FLX Titanium Series (454 Life Science)	10 mg biopsy	QIAamp DNA Stool Mini Kit (QIAgen)	V1-V3 (27F, 533R)	GreenGenes v 13_8	QIIME LefSe	45
Placental	Miseq (Illumina)	1 cm × 1 cm × 1 cm biopsy	E.Z.N.A. DNA Kit (Omega Bio-tek) AxyPrep DNA Gel Extraction Kit (Axygen Biosciences)	V3-V4 (338F, 806R)	RDP Classifier and SILVA (SSU115)	QIIME	67
Vaginal	454 FLX Titanium pyrosequencing	Swab	ZR Fecal DNA extraction kit (ZYMO Research,	V1-V2 27F, 338R	Version 0.2 of the vaginal community 16S rRNA gene reference tree (from pplacer)	pplacer version v1.1.alpha 08	20
Vaginal (introitus, posterior fornix, midvagina)	Genome Sequencer GS-FLX Titanium platform (Roche)	Swab	PowerSoil DNA Isolation Kit (MoBio)	V3V5 (354F, 926R)	RDP classifier v2.2	QIIME R packages (BiodiversityR; vegan) LefSe	42
Vagina	454 FLX pyrosequencing	Swab	QIAamp DNA Mini Kit (Qiagen)	V1-V2 (27F and 338R)	RDP	Ribosomal Database Project (RDP) Naïve Bayesian Classifier	14
Vaginal (posterior fornix)	Miseq (Illumina)	Swab	QIAamp DNA Mini kit (Qiagen)	V1-V3 (28F-519R)	Silva	Mothur	24
Vaginal (posterior fornix)	Miseq (Illumina)	Swab	QIAamp DNA Mini kit (Qiagen)	V1-V2 (28F-388R)	Silva	Mothur	21

Location	Sequencing Platform	Sampling	DNA Isolation Kit (Manufacturer)	Variable Regions (Primers)	rRNA reference database	Microbiome Analysis Program	Ref.
Vagina	454 pyrosequencing using 454 Life Sciences (Roche/454 Life Sciences)	Swab	CellFree500 kit	V1-V3 (27 F and 534R)	Pplacer speciateIT	QIIME	26
Stool	Illumina (HiSeq)	-	-	V1V2	Greengenes/LCA	QIIME	10

Table 2:

Common analysis tools used for microbiome analysis.

CloVR (Cloud Virtual Resource); LEfSe (Linear discriminate analysis effect size); MEGAN (MEtaGenome Analyzer); MG-RAST (MetaGenomic - Rapid Annotation using Subsystem Technology); PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States); QIIME (Quantitative Insights into Microbial Ecology); STAMP (Statistical Analysis of Metagenomic Profiles)

Analysis Tools	Access	Purpose	Ref.
CloVR	Web (can be downloaded onto Desktop)	Software used to analyze microbial communities from high-throughput sequencing outputs	68
Galaxy	Web-based	Software that uses various tools for metagenomic comparisons between communities.	45
LEfSe	Web-based	Online tool that incorporates statistical approaches to identify differential features between microbial communities.	34,42,45,69
MEGAN	Software	Downloadable bioinformatics software that constructs taxonomic hierarchical trees.	45
MG-RAST	Web-based	Performs microbial metagenomics analysis–both functional and phylogenetic.	10,33,34,70
Mothur	Command-line interface	Software used to analyze microbial communities from high-throughput sequencing outputs.	21,71
PICRUSt	Web-based	Online tool that uses 16S data to make metagenomic predictions about microbial communities.	45,72
QIIME	Command-line interface	Software used to analyze microbial communities.	33,34,44,52
R	Software	Programming software for statistical and computational analysis (commonly used packages include Phyloseq and Vegan).	21,73
STAMP	Software	Uses the output files from QIIME, PICRUST, MG-RAST, etc. to generate graphics and perform statistical tests and confidence intervals in order to test the null hypothesis.	24,45,74

Table 3:

Common statistical methods for microbiome analyses.

ANOVA (Analysis of variance); PERMANOVA (Permutational multivariate analysis of variance); PERMDISP (Permutational analysis of multivariate dispersion).

Statistical Methods	Purpose	Ref.
ANOVA	Compares the differences in the means of multiple groups to determine whether they are statistically significant.	[45]
Jensen-Shannon divergence	Used to predict the similarity between two groups of probability distributions	[20]
Kruskall-Wallis	Non-parametric; evaluates the statistical significance of two or more groups that do not meet the normality assumption	[42,44]
Mann-Whitney U-test	Non-parametric; evaluates the statistical significance of two groups that do not meet the normality assumption.	[34]
PERMANOVA	Statistical significance of beta diversity	[34]
PERMDISP	Statistical significance of beta diversity	[34]
Spearman's Correlation	Generates a coefficient that describes the degree to which two variables are negatively or positively correlated.	[10,14,67]
Student t-test	Parametric; evaluates the statistical significance of two groups that meet the normality assumption; can be either paired or unpaired.	[67]