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Insights Into Epidemiologic Assessments of the Microbiome and Challenges in Identifying Microbiome Relationships with Adverse Pregnancy Outcomes

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Abstract

Purpose of Review We describe different methods for microbiome assessment and analysis and highlight some of the challenges of using omics data in epidemiologic studies of adverse pregnancy outcomes.

Recent Findings Human microbiomes are dynamic and vary by ancestry and geography. The composition and dynamics of the vaginal microbiome have been associated with risk of preterm birth.

Summary There are several different methods for characterizing the microbiome. Choice of method depends on the research question and resources available. Added to known challenges of conducting and analyzing epidemiologic studies are the unique challenges associated with microbiome detection and analysis. The resulting omics assessments of human microbial communities have great potential to identify prognostics, diagnostics, and potentially therapeutics for adverse pregnancy outcomes.

Keywords Microbiome analysis · Adverse birth outcomes · Preterm birth · Vaginal microbiome

Introduction

Microbes are ubiquitous in the environment and on all living beings. How microbial we humans really are has recently been illuminated through the application of high-throughput genetic sequencing techniques not dependent on the ability to grow organisms (omics). Results of these studies estimate that humans have 10 times as many microbial genes as human genes [1]. Our microbial genes (the microbiome) perform many important functions including aiding in digestion, training our immune system, and protecting us from harmful microbes (pathogens). Microbial functions are possibly important for—or indicators of—normal fetal growth and development. Our purpose in this review is to highlight how epidemiologic studies of the microbiome might contribute to birth outcome research.

Like human organs, our microbiome responds rapidly to changes in the environment with detectable changes in function. For example, our pancreas regulates blood glucose levels by secreting products that either lower (insulin) or raise (glucagon) blood glucose levels. Similarly, the gut microbiome produces varying levels of short-chain fatty acids depending on the amount of fiber we consume. But this analogy is incomplete: pancreatic cells are not a dynamic ecologic community. Changes in the microbial environment may cause changes in overall microbial biomass or in the abundance of some microbes or in physical microbial structures (biofilms). Effects of human diet, hygiene, contact with other humans, and exposure to drugs, toxins, and pathogens can be read in the structure, composition, and ongoing functions of our microbial communities. Thus, learning to read the output of our microbes has high potential for increasing our understanding of normal and pathogenic processes that can lead to new diagnostics, prognostics, and therapies. We refer the reader to several excellent reviews that detail methods for collecting, processing, and analyzing microbiome data (see, for example, [2–4]) and focus here on some of the challenges associated with designing, conducting, and analyzing epidemiologic studies of the microbiome.

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Most birth outcome microbiome research has focused on preterm birth and neurological disorders. The vaginal microbiome becomes enriched for lactobacillus species over the course of a normal pregnancy, becoming less diverse [5, 6]. The observed increase in Lactobacillus during gestation may serve to enhance pathogen resistance—decreasing risk of ascending infection and initiating the development of a healthy infant gut microbiome. While more study is needed, this finding might be used to develop indicators of risk of preterm birth. The gut microbiome modulates immune response and is a regulator of nervous system function. Antibiotic use during pregnancy or labor and delivery or delivery by cesarean section might disrupt normal gut microbiome development leading to increased risk of autism spectrum and other neurological disorders [7, 8]. While large population-based studies do not support an association of infant microbiota with autism [9, 10], a recent mouse model suggests that the maternal gut microbiome may be an important mediator of the effects of viral infection during gestation on infant neurodevelopmental disorders [11].

Choice of Sample

Human microbial communities include bacteria, virus, archaea, and fungi. Note that in addition to virus that infect human cells, there are virus that infect bacteria, archaea, and fungi present in the microbiome; viral infection may be an important determinant of microbial population dynamics. For microbiome studies of birth outcomes, targeted microbiomes have included those in the vagina, gut, saliva, uterus, and placenta. Vaginal and salivary samples are easiest to collect. Uterine samples are more invasive, and there are substantial concerns regarding sampling the placental microbiome [12], because of potential contamination during collection and processing when samples have very low biomass [13]. The choice of sample depends on the research question and hypothesized biological mechanism. For example, for investigations of preterm birth, the vaginal microbiome may be most relevant. Infection is a major cause of preterm birth and the vaginal microbiome can be a source for ascending infection [14]. Furthermore, the vaginal microbiome changes throughout a normal pregnancy suggesting it may be a marker of normal fetal development [15, 16]. By contrast, the maternal gut microbiome is of interest for investigations of gestational diabetes [17, 18], as gut microbiota interact with the breakdown of food and subsequent metabolism. Furthermore, as the gut microbiota help prime and shape the immune response, infant or maternal gut microbiomes may be of interest for investigation of infant immune outcomes [19].

Choice of Omics Assessments

There are several different omics techniques available for describing the genotype and phenotype of microbial communities (Table 1). Genotypic measures identify what microbes are present, and their functional potential. Phenotypic measures capture ongoing processes and features by measuring transcripts, metabolites, and proteins. Different microbial community compositions can provide the same functions, for example, modulating pH. Therefore, the investigator needs to decide whether to measure the microbial community composition (which microbes are present), functional potential (genetic potential), or current function (what biologic functions are ongoing at time of sampling) when selecting an omics technique. Choice of technique also has implications for study conduct, as not all samples are appropriate for testing using every technique. Samples might need to be aliquoted and stored using different protocols if the intention is to test using multiple techniques. Collection protocols should consider if the microbial composition or response might change depending on time of day [20], gestational age [5, 6], or if the participant has engaged in specific behaviors, e.g., brushing their teeth prior to a saliva sample. If changes in microbiome composition or function are the outcome, collection of multiple samples from the same individual at specific time points using the same protocol is required. Table 1 provides an overview of omics techniques by whether they measure genotype or phenotype, the feature sets and microbial community characteristics measured, and requirements for sampling collection and storage, price, and ease of analysis.

To measure microbial community composition, many adverse birth outcomes researchers have used 16S rRNA amplicon sequencing [21–30]. This technique sequences one or more variable regions of the gene that codes for the ribosome, which is present in all bacterial cells. (Other amplicons can be used; e.g., 18S rRNA for fungi. Alternative methods are needed to identify virus.) Comparing observed variations in genetic sequence to reference databases allows determination of the phylogeny of the sequence. The low-cost per sample of amplicon sequencing enables testing of larger numbers of samples, increasing overall sample size and making it possible to test repeated measurements over the course of pregnancy. A disadvantage of amplicon sequencing is that taxonomic resolution depends on choice of variable region for sequencing and the quality of the reference databases. There are several ribosomal RNA sequence databases, including Greengenes [31] and Silva [32]; other reference databases are specifically curated for an environment of interest, such as the Human Oral Microbiome Database for oral microorganisms [33]. However, some sequence variants may not be resolved to the species level, and strain variants may not be reflected in the amplicon sequenced.

Table 1 Pros and cons of different omics techniques for describing the genotype and phenotype of microbial community characteristics, and examples of analytic packages

	Genotype		Phenotype	
Omic technique	16S/18S amplicon sequencing	Metagenomics	Metatranscriptomics	Proteomics/Metabolomics
Feature set	Operational taxonomic units (OTUs), amplicon sequence variants (ASVs)	Whole or partially assembled microbial genomes, genes, biochemical pathways	Transcripts	Proteins/Metabolites
Microbial community characteristic	Composition	Composition Functional potential	Current function	Current function
Brief description	Amplify and sequence a variable region of the 16S rRNA gene (or ITS 18S for fungal species). Allows for the assessment of the relative abundance of different bacterial (or fungal) taxa	Fragment and sequence of all the DNA in a sample. Allows for the assessment of the relative abundance of bacteria, fungal and viral microbes and of genes of interest.	Compare differences in microbiome gene expression (transcripts) under a variety of different conditions	Use nuclear magnetic resonance spectroscopy or mass spectrometry to identify proteins or metabolites
Pro	Relatively cheap, broad taxonomic survey	Greater taxonomic and strain specificity than amplicon sequencing. Measure of functional potential	Transcribed genes reflect the genotype of the microbiome and the expressed phenotype	Gives insight into interactions between the human host and microbial communities.
Con	Taxonomic resolution is limited	More expensive to sequence. Assembly can be computationally expensive and complex	Short half-life of transcripts requires care to avoid changes to the metatranscriptome during sampling	Disentangling proteins/metabolic products of the host vs the microbial community may not be possible
Examples of relevant software packages	QIIME, Mothur, DADA2, DEBLUR, Oligotyping	Short-read based: MEGAN, DIAMOND, bowtie2, Humann2, Metaphlann2 Assembly: IBAUD, MegaHit, MetaSpades	DEseq2	metaRbolomics toolbox, MetabNet
Important choices	Variable region of 16S or 18S gene, spike-ins to determine absolute abundance	Host DNA depletion; de novo vs reference based assembly	rRNA depletion	Selection of a targeted subset for analysis

Despite these limitations, amplicon sequencing can be very powerful. For example, in 2016, Callahan et al. observed that significant associations of the *Gardnerella* genus with preterm birth in a cohort of white women were driven by a single sequence variant of *Gardnerella vaginalis* [21]. Other sequence variants of *Gardnerella vaginalis* which differed at just one or two nucleotides in the V4 region of the 16S gene were not significantly associated with preterm birth [21]. Amplicon sequencing has also revealed that compositionally discrete communities can be functionally redundant. For example, a healthy vaginal microbiome was previously presumed to be dominated by Lactobacilli. A study using amplicon sequencing among 396 healthy white, black, Asian, and Hispanic women found 20–30% had non-Lactobacilli-dominated vaginal communities [34]. For these women, bacterial taxa capable of heterolactic or homolactic acid fermentation may be providing similar functions as Lactobacilli [35, 36]. To test this hypothesis, additional methods are needed as microbial functions cannot be determined using amplicon sequence data, although taxonomic information can be used analytically to guess at genetic potential (e.g., PiCRUST). Nonetheless, 16S rRNA amplicon sequencing is inexpensive, and resolution to the species—and even strain level—continues to improve with longer read length of genetic sequencing, enhanced analytics, and reference databases. Furthermore, there are user-friendly bioinformatics pipelines. These features make amplicon sequencing ideal for hypothesis-generating studies.

As the cost of sequencing and mass spectrometry has continued to fall, it has become increasingly feasible to use techniques more discriminatory than amplicon sequencing. Shotgun sequencing, in which all of the DNA in a sample is fragmented, amplified, and sequenced, simultaneously provides insight into both microbial community composition and functional potential ("metagenomics"). Shotgun sequencing can also be applied to RNA, after reverse transcription to cDNA, characterizing gene expression ("metatranscriptomics"). (Nanopore technology enables sequencing of all DNA and RNA without reverse transcription.) Like amplicon sequencing, metagenomics provides information on the composition of the bacterial community, but with increased resolution, including assessing the abundance of specific genes and biochemical pathways. Put succinctly, metagenomics enables characterization of the functional potential of a microbiome and metatranscriptomics the ongoing functions.

By applying metagenomics to the samples from the study by Callahan et al. described earlier, Goltsman et al. found that distinct strains of *G. vaginalis* exhibited differential functional enrichment, including in genes related to vitamin and cofactor metabolism and CRISPR Cas genes [37]. Increasingly, researchers incorporate multiple omics methods to capture not only the breadth but also the depth of microbial communities in relation to pregnancy progression and adverse pregnancy outcomes. For example, in 2019, Ghaemi et al. published an ambitious multi-omics analysis of longitudinal samples from 17 pregnant women, including measurements from the transcriptome, microbiome, proteome, and metabolome. They showed that by combining results from several omics measurements, their ability to predict gestational age improved [38]. In another example from 2019, Fettweis et al. used both metagenomics and metatranscriptomics to investigate the microbiome as a risk factor for preterm birth. They found that the overall transcriptional rate of *G. vaginalis* was higher in preterm vs term samples, suggesting that preterm birth risk varies by the transcriptional activity of *G. vaginalis* [39]. While incorporating multiple omics methods provides valuable information, metagenomics, transcriptomics, proteomics, and metabolomics (identified using mass spectrometry) are, however, 3 or more times as expensive and require more complex bioinformatics and statistical expertise than amplicon sequencing. Thus, amplicon sequencing is often applied first to identify samples for more in-depth analysis using other methods.

Challenges: Collection and Contamination of Microbial Samples

Regardless of the omics measurements used, incorporating omics assessments of microbial communities into epidemiologic studies can pose significant challenges. These include challenges arising from contamination, storage and batch effects, reagent and extraction biases, and compositionality and sparsity (reviewed elsewhere, see [3, 40]). Some of these challenges are exacerbated in adverse pregnancy outcomes research, such as in the case of collecting and processing placental samples. In 2014, Aagaard et al. published results showing the placenta, long thought to be sterile, harbored a unique microbiome [41]. While some other studies have documented examples of a placental microbiome, and even reported associations with preterm birth [42], other work suggested the microbial DNA detected in such studies is the result of contamination from other body sites [43], during collection [43, 44], or from extraction kits [43–45]. A recent study of more than 500 placental samples from a mix of preterm and term as well as C-section vs vaginal deliveries found no evidence of a resident, functional placental microbiome. The only microbial DNA the study identified from placental samples as a true signal, as opposed to contamination, was *S. agalactiae*, a pathogen [43]. Although placental pathogens could be important to preterm birth, the example of the purported placental microbiome underlines the challenges of contamination and the importance of good controls in conducting studies of the microbiome in prenatal epidemiology.

Challenges: Analysis of Microbial Omics Data

Once the samples are properly collected and processed, significant challenges remain stemming from the structure and type of data. Data from omics projects is high-dimensional, that is, each sample contains many features, such as many microbial taxa, many microbial genes, or many microbial metabolites. Additionally, microbiomes are highly individual and variable omics data are often sparse, i.e., many features are found in a low percentage of the overall sample. Finally, because sequencing effort varies across samples, microbial omics data are typically made to be compositional, that is, the sum of the features within a single sample is forced to sum to 1 or another constrained value. Although the high-dimensional nature of microbial omics studies is beneficial in that it provides a broad survey of the microbial community, it can be challenging to use in epidemiological analyses. Several approaches to address this challenge are available, based on individual features (i.e., individual taxa, genes, metabolites), data reduction (ecologic or diversity measures, ordination, and clustering methods), and machine learning (Table 2).

Feature-based approaches examine associations of selected features with an outcome or predictor of interest. Several statistical packages are available that test for the association of each feature independently with the outcome of interest and correct for the multiple-testing burden. Some of these techniques additionally exploit the availability of feature-wide information to model false discovery rates, infer abundance from counts, or shrink variance estimation. Alternatively, a researcher might select key microbial taxa, genes, or metabolites of interest for association testing in a candidate-feature approach. These key features may be selected a priori based on prior knowledge, or through a data-driven method. If several features are of interest, such as genes related to a similar metabolic pathway or taxa of known common pathogenicity, researchers might agglomerate the features into a weighted score.

Dimension reduction approaches assume that global microbial community characteristics, rather than individual features, are most relevant to disease processes. Ecological measures which characterize the number of species present (richness), their relative abundance (evenness), a joint measure of

Table 2 Feature-based, data reduction, and machine-learning methods used to compare microbiome characteristics in epidemiologic studies

Technique	Description	Examples
Feature-based approaches	Use individual features (e.g. taxa) to examine associations with outcome of interest	
Microbiome-wide association testing	Test for an association between each feature and the outcome of interest, correcting for multiple testing burden or otherwise exploiting information from the study to limit false positives	Many specific packages, such as Aldex2, DeSeq2, LefSe, MASLIN
Candidate microbe approach	Select for key features using either prior-knowledge or a data driven approach	Build networks and identify features of interest based on centrality metrics (Network hubs); select features based on prior knowledge
Data/dimension reduction approaches	Summarize all features into a smaller number of either continuous or categorical variables; examine associations of these summary variables with outcome of interest	
Ecological or diversity measures	Alpha diversity: measures of the number species present (richness) and relative abundance of species present	Alpha diversity: Chao1, Faith's phylogenetic diversity index, Simpson index, Shannon index, Rao's quadratic entropy
	Beta diversity: Measures of similarity between microbiome composition among samples	Beta diversity: Bray-Curtis, Euclidean distance, Jaccard, unifrac distance
Ordination methods	Order samples characterized by feature elements such that similar samples are grouped close together, and samples with dissimilar features are grouped further away.	Nonmetric multidimensional scaling (NMDS); principal components analysis (PCA); principal coordinates analysis (PCoA), Correspondence analysis (CA)
Clustering methods	Cluster samples together based on their features using a variety of methods	Community state typing; Hierarchical clustering
Machine learning approaches for feature-based classification	Split data into training and testing sets, and refine predictive models for classifying samples based on features	Support vector machines; random forest; k-nearest neighbors; neural networks

richness and evenness (alpha diversity), or the similarity between communities (beta diversity) can be informative. Microbiome features also can be grouped using ordination and data clustering methods, with the resulting groups used in data analysis. For example, grouping of the vaginal microbiome taxa into community states or vagitypes has provided important insights into vaginal microbiome dynamics [46] and comparisons by ancestry [34]. As noted earlier, the alpha diversity and vagitype classifications of vaginal microbiome samples from the same individual can be dynamic during pregnancy [5, 6].

Supervised machine learning approaches frequently develop prediction models to sort samples into groups, for example, disease and not diseased, based on available features. Some such approaches are random forests, neural networks, and support vector machines. Depending on the research question and sample set, researchers might screen features for inclusion in the algorithm. Machine learning can also be used to identify the importance of features for classification on the outcome of interest.

Challenges: Generalizability and Causality

Given the variability in microbiomes within and between populations [47], a particular concern is whether findings in one population can be generalized to others. Epidemiologic studies have identified differences in the microbiome by person, place, and time characteristics which inform the design and interpretation of additional epidemiology and clinical studies. The vaginal microbiome composition varies by ancestry [5, 34]. Foundational work in healthy, non-pregnant women has established that US African American and Hispanic women are more likely to have a diverse microbiome than women of European ancestry [34]. In cohorts of European ancestry, associations between low-diversity, non-lactobacilli-dominated microbial communities and preterm birth are well replicated [21, 26–30]. However, several studies in populations of other ancestry have not found an association between non-lactobacilli-dominated microbial communities and preterm birth [22, 23, 25] or between abundance of Lactobacillus species and preterm birth [6, 23]. Specific taxa, such as *G. vaginalis* and *L. iners*, are also associated with preterm birth in cohorts of European ancestry [21], but in a cross-study comparison, Fettweis et al. confirmed that associations between *L. iners* and *G. vaginalis* and preterm birth did not extend to populations of African American and Hispanic ancestry [39]. These findings suggest that ancestry could confound or modify the relationship between vaginal microbiomes and preterm birth. Therefore, ancestry should be taken into account in the design and analysis of the vaginal microbiome and preterm birth. As of yet, even larger studies often lack statistical power to test for associations within subgroups by ancestry [30].

In addition to ancestry, the microbiome can vary by geographic region. While the effect of geographic locale on the gut microbiome has been well documented [47], most omics studies of the vaginal microbiome have been conducted in the

USA or Canada, followed by Europe (reviewed in [24]). Few studies have described the vaginal microbiome of pregnant non-North American or European populations [48–51] or its potential relationship with preterm birth [23]. Thus, work in other populations is needed to elucidate if and how the association between the vaginal microbiome and preterm birth varies by ancestry and geography.

The microbiome also varies by time. Gut [52] and salivary [53] microbiomes are dynamic during infancy, and the gut microbiome has a circadian rhythm [54]. The vaginal microbiome varies by age [24], the menstrual cycle [46], and over the course of a pregnancy [5, 6]. When during pregnancy, the vaginal microbiome is sampled and can strongly influence whether there is an apparent association between the vaginal microbiome and preterm birth. In 2019, we reported effect modification of the association between vaginal community state type and preterm birth by timing of vaginal sampling among Peruvian women, suggesting that gestational age at sampling might modify observed associations between the vaginal microbiome and pregnancy outcomes [23]. This suggests that longitudinal study designs with multiple samples collected during pregnancy are optimal. Stout et al. found that women who went on to deliver preterm exhibited significant decreases in vaginal richness, diversity, and evenness between the first and second trimesters, while women who delivered at term showed no significant changes during that time period [6]. Similarly, Fettweis et al. observed that the trajectories of the relative abundance of key taxa of interest, including *G. vaginalis*, *A. vaginae*, and *L. crispatus*, varied by preterm birth status [39]. These temporal changes to the vaginal microbiome over gestation also appear to vary between ancestry groups [5, 39]. Importantly, timing of early life exposures impacts developmental systems. For example, the effects of viral infection during pregnancy on fetal development can vary by trimester of infection [55, 56]. Consideration of temporal variation of the vaginal microbiome over gestation is therefore important to consider both for epidemiologic and etiologic interpretations.

A fundamental question of all microbiome studies is causality. For preterm birth, the question is whether the vaginal microbiome causes preterm birth or whether the vaginal microbiome is responding to other physiologic changes associated with preterm birth or both. While the scientific literature does suggest an association between the vaginal microbiome and preterm birth [26–30, 57], multiple randomized control studies [58–60] and Cochrane reviews [61, 62] have found no significant effect of prophylactic antibiotic interventions on the incidence of preterm birth, and some evidence of increased risk of adverse outcomes [63]. Antibiotics only target bacteria; if virus, fungi, or bacteria not susceptible to the prescribed antibiotics are the cause, this treatment would be ineffective. Since many biologic systems regulate the progression of pregnancy, it is possible that a healthy vaginal microbiome is necessary but insufficient to achieve a term pregnancy. Under this scenario, both the vaginal microbiome and other, perhaps, host-related systems would need to be targeted to produce effective interventions. If the vaginal microbiome is only a corollary of a true cause for preterm birth, it may no longer be a target for interventions. However, the vaginal microbiome might still be a valuable prognostic or a diagnostic factor for preterm birth.

Conclusions

Microbiome research poses multiple challenges but has high potential to contribute to our understanding of adverse birth outcomes. One set of challenges stems from the dynamic nature of microbial communities, its measurement, and summarizing the resulting high dimensional data. A second set stems from the well-understood challenges of designing and implementing population-based studies of adverse pregnancy outcomes. A final challenge is in assembling a multidisciplinary research team with appropriate expertise and incorporating new methodologies as they rapidly arise. However, these challenges are not insurmountable and are well worth the effort. While it is unlikely that all the promise of microbiome research will ultimately be fulfilled, there is much to learn that will certainly generate new prognostics, diagnostics, and possibly treatments.

Declarations

Conflict of Interest The authors declare no competing interests.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors

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