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The Perinatal Microbiome and Pregnancy: Moving Beyond the Vaginal Microbiome

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The human microbiome, the collective genome of the microbial community that is on and within us, has recently been mapped. The initial characterization of healthy subjects has provided investigators with a reference population for interrogating the microbiome in metabolic, intestinal, and reproductive health and disease states. Although it is known that bacteria can colonize the vagina, recent metagenomic studies have shown that the vaginal microbiome varies among reproductive age women. Similarly, the richness and diversity of intestinal microbiota also naturally fluctuate among gravidae in both human and nonhuman primates, as well as mice. Moreover, recent evidence suggests that microbiome niches in pregnancy are not limited to maternal body sites, as the placenta appears to harbor a low biomass microbiome that is presumptively established in early pregnancy and varies in association with a remote history of maternal antenatal infection as well as preterm birth. In this article, we will provide a brief overview on metagenomics science as a means to investigate the microbiome, observations pertaining to both variation and the presumptive potential role of a varied microbiome during pregnancy, and how future studies of the microbiome in pregnancy may lend to a better understanding of human biology, reproductive health, and parturition.

Completed in 2012, the Human Microbiome Project (HMP) characterized the microbiome composition of multiple body sites in healthy individuals of different ethnicities located in two separate cities (St. Louis, Missouri and Houston, Texas) in the United States. This multicenter effort showed that bacterial diversity, niche specificity, and microbial gene car-

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riage patterns far exceeded what was initially suspected (Aagaard et al. 2012a,b; Human Microbiome Project 2012a,b,c; Huse et al. 2012; Gevers et al. 2012; Li et al. 2012). These initial studies on the "healthy" reference human microbiome laid the foundation for a burgeoning wealth of investigations of the potential role of the microbiome in a spectrum of health and disease states. Indeed, associations between dysbiotic microbiota, or a microbial imbalance, and disease have been suggested for obesity, type II diabetes mellitus, ulcerative colitis, Crohn's disease, and colorectal cancer (Mangin et al. 2004; Ley et al. 2005; Gophna et al. 2006; Manichanh et al. 2006; Turnbaugh et al. 2006, 2008, 2009; Bäckhed et al. 2007; Cani et al. 2007; Willing et al. 2009; Larsen et al. 2010; Schwiertz et al. 2010; Wu et al. 2010; Joossens et al. 2011; Lepage et al. 2011; Marchesi et al. 2011; Sobhani et al. 2011; Qin et al. 2012; Wang et al. 2012; Devaraj et al. 2013). However, causation has yet to be established, and a multitude of other etiologies for these common, complex disorders have been suggested over the decades. Thus, it is critically important to first discriminate when in the course of the lifespan there is normal and anticipated variation in the human microbiome, in which body niches such variation occurs, and what other covariates (such as host disease-susceptible genotype, host metabolic milieu and associated disorders, as well as age, gender, race/ethnicity, and medications and diet) may contribute to any observed variation.

Our laboratory and others have shown that the vaginal microbiota vary in association with normal pregnancy, thus providing a unique "signature" in pregnancy with relative altered abundance of multiple taxa (Aagaard et al. 2012b; Romero et al. 2014b). This is intriguing, as the vaginal microflora influences gestational and postnatal health. It has long been suggested that intrauterine infections, such as chorioamnionitis, are the sequelae of ascending microbiota from the upper vaginal tract (Gonçalves et al. 2002), and the development of highly morbid neonatal conditions, such as neonatal sepsis and necrotizing enterocolitis, are potentially attributable to anatomical displacement of these flora with subsequent inflammation and neonatal acquisition (Claud and Walker 2001; Guthrie et al. 2003; Yee et al. 2012). However, in this dawning era of metagenomic medicine and science, we are questioning these notions, and are coming to appreciate that many so-called "sterile" niches—notably in and among the female reproductive tract (such as the placenta)—may function as active low biomass ecologic niches that harbor unique microbiomes. These early observations challenge not only our assumed notions of "from when and where" our earliest microbiomes are colonized (or seeded), but our concepts of inflammatory mediators, reproductive immunity, and whether microbes in such niches may constitute more friend than foe.

Here, we will review the literature on how the microbiome is studied and how metagenomic science tools have been used in characterizing microbial communities and their genomic repertoire (Table 1). We will thereafter discuss findings by our group and others pertaining to the microbial ecology of pregnancy, with a focus on the potential role of the microbiome in mediating parturition and notably preterm birth. Finally, we will close with a discussion on future implications and applications as metagenomic medicine emerges as a frontier for both understanding and managing the most common and complex disorders of our current time.

METAGENOMIC SCIENCE: METHODS, APPLICATIONS, AND CHALLENGES IN THE FIELD

Mapping the human microbiome in reproduction requires that we not only characterize which microbes are present and what each taxon contributes to the biology of the host, but what their genetic repertoire entails and encodes for (so-called "gene carriage patterns"). Numerous methods have been developed to address these questions over the years, and with the advent of Next Generation (Next-Gen) sequencing, investigators have been able to relatively efficiently describe the composition of a microbial community. Additionally, other multi'omic techniques (e.g., metabolomics and metatranscriptomics) have been developed to attribute potential functions and metabolic capacity.

Table 1. Metagenomic studies pertaining to perinatal health

Reference	Site	Technique(s)	Primers used	Study design	Findings
Nongravid Vaginal Studies					
NIH HMP Consortium 2012	Skin, nares, oral, vagina	Next-Gen sequencing	V1V3 V3V5	Longitudinal	Characterized healthy reference population
Ravel et al. 2010	Mid-vagina (self-collected)	Next-Gen sequencing	V1V2	Cross-sectional	Characterized healthy, nongravid vaginal microbiome
Gajer et al. 2012	Mid-vagina (self-collected)	Next-Gen sequencing	V1V2	Longitudinal	Showed temporal dynamics of the vaginal microbiome
Macklaim et al. 2013	Vagina	Metatranscriptomics		Cross-sectional	Showed potential for metatranscriptomics on vaginal swabs
Gravid Vaginal Studies					
Aagaard et al. 2012b	Vaginal introitus, posterior fornix, and mid-vagina	Next-Gen sequencing	V3V5	Cross-sectional	Characterized healthy, gravid vaginal microbiome
Romero et al. 2014b	Posterior fornix	Next-Gen sequencing	V1V2	Longitudinal	Characterized healthy, gravid vaginal microbiome throughout pregnancy
Walther-António et al. 2014	Posterior fornix, cervix	Next-Gen sequencing	V3V5	Longitudinal	Characterized healthy, gravid vaginal microbiome throughout pregnancy
Beyond the Vagina: Intestinal Microbiome					
Koren et al. 2012	Stool	Sequencing	V1V2	Longitudinal	Characterized first and third trimester stool
Beyond the Vagina: The Placenta					
Aagaard et al. 2014	Placenta	Next-Gen sequencing	V1V3 and WGS	Population-based, cross-sectional	The placenta harbors a unique microbiome profile, most akin to the oral microbiome and varies by virtue of preterm birth and a remote history of antenatal infection
Beyond the Vagina: Neonatal Studies					
Schultz et al. 2004	Stool	Sequencing	Strain-specific	Longitudinal	Vertical transmission from mother to infant
Palmer et al. 2007	Stool	Sequencing, Microarray, PCR	Universal 16S rRNA	Longitudinal	Characterized healthy neonatal microbiome
Dominguez-Bello et al. 2010	Oral, vagina, skin, rectal	Next-Gen sequencing	V2	Cross-sectional	Characterized neonatal microbiome by mode of delivery

Continued

Table 1. *Continued*

Reference	Site	Technique(s)	Primers used	Study design	Findings
Koenig et al. 2011	Stool	Next-Gen sequencing	V1V2	Longitudinal	Characterized the intestinal microbiome from birth to 2.5 yr
Jost et al. 2012	Stool	Sequencing	Sanger, V5V6	Longitudinal	Characterized healthy neonatal microbiome
Wang et al. 2013	Cord blood, amniotic fluid	Bacterial culture and sequencing	Universal 16s rRNA	Cross-sectional	Neonates with necrotizing colitis had predominantly one bacteria dominating
Milisavljevic et al. 2013	Gastro-esophageal	Sequencing	Universal 16S rRNA	Longitudinal	Characterized the microbiome in VLBW infants
Azad et al. 2013	Stool	Next-Gen sequencing	V5, V6, V7	Longitudinal	Characterized the neonatal microbiome from birth to 4 mo while examining mode of delivery and feeding
Rogier et al. 2014	Stool	Microarray		Murine	Examined the role of maternal IgA on intestinal microbiome
Ma et al. 2014b	Colon, anus, stool	Next-Gen sequencing	V3V5	Nonhuman primate	Examined the role of maternal diet on juvenile microbiome

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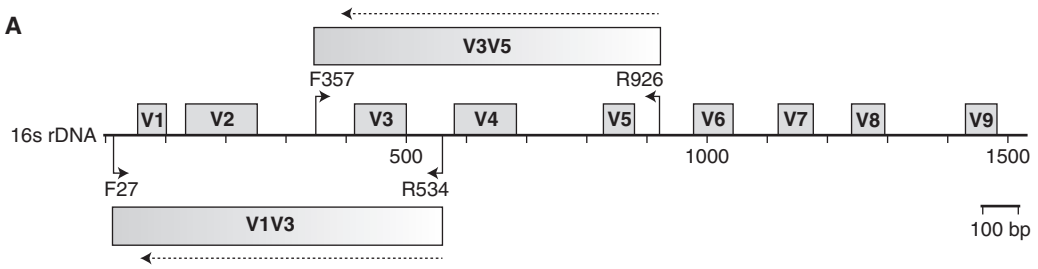
16S-Based Metagenomics

Sequencing of the 16S rRNA gene using Next-Gen technology has recently been widely exploited to characterize the human microbiome (Jonasson et al. 2007; Liu et al. 2007). The 16S rRNA gene is an ideal target to classify bacteria because of the nine hypervariable regions in this gene that can be used to distinguish species based on individual nucleotide polymorphisms (Fig. 1A). Ergo, Next-Gen sequencing characterizes both coarse (phylum level) as well as fine (genus and limited species and strain) differences using universal primers to the adjacent conserved regions (Klindworth et al. 2013). However, 16S-amplicon-based approaches are limited to a shorter read length as compared with Sanger sequencing, and as a result, only a few hypervariable regions can be contiguously sequenced at a time. Initial work on approach and validation by the HMP Consortium showed that there is variation in the taxonomy profile identified based on sequencing different variable regions. For example, V1V3 amplicons may underestimate *Acinetobacter* and *Escherichia* genera, but V3V5 provides both breadth and depth of communities dominated by these genera. Furthermore, V6V9 may underestimate *Bacteroides* but provides good coverage for *Pseudomonas* and *Escherichia* (Human Microbiome Project 2012b).

Comparison of the vaginal microbiome data from the HMP reveals that V1V3 will distinguish communities primarily by the relative predominant *Lactobacillus* species present whereas, V3V5 amplicons will reveal either lactobacilli-dominant or lactobacilli-diminished groups. Furthermore, the number of *Lactobacillus* species detected (and thus relative abundance) will vary depending on the 16S region sequenced, with V1V3 revealing more unique *Lactobacillus* operational taxonomic units (OTUs) as compared with V3V5 (Huse et al. 2012). However, unlike V3V5, V1V3 does not



B

	V1V3	V3V5
Lactobacillus	+ +	– –
Prevotella	+	–
Enterobacteriaceae	–	+ +
Bifidobacteriaceae	–	+ +
Staphylococcus	–	+
Clostridium	+	+

Figure 1. The 16S rDNA gene is an ideal target for classifying bacteria. (*A*) The 16S rDNA gene of bacteria contains nine hypervariable regions (V1–V9) that are flanked by conserved regions, which makes this gene an ideal target for PCR amplification and bacterial classification. The V1V3 and V3V5 primers sets used by the HMP consortium are outlined. The dotted line indicates the direction of amplification. (*B*) Advantages and disadvantages to characterizing the vaginal microbiome with V1V3 (includes V2) and V3V5 (includes V4) primer sets. Species identification of *Lactobacillus* is enabled using a V1V3 primer set; however, V3V5 primers sets are better suited to identify Enterobacteriaceae and Bifidobacteriaceae. Thus, experimental design is essential when examining the pregnant microbiome.

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fully discriminate *Enterobacteriaceae* family projections (including *Escherichia* and *Proteus*), and some genera (including *Staphylococcus*) (Fig. 1B) (Fettweis et al. 2012). Few studies have directly compared the vaginal communities described by V6, V7, V8, and V9 to other primer sets, although a number of studies have used these regions with good coverage (Hummelen et al. 2010; Ghartey et al. 2014). Taken together, these data emphasize the need for prudent consideration when choosing primer sets for sequencing with appreciation of both the body site to be characterized, the reference data set to be compared with, and limitations when comparing to other published findings.

Numerous bioinformatics suites, such as Quantitative Insights into Microbial Ecology (QIIME), Mothur, and Genboree, have been developed specifically to handle the taxonomic information gathered from 16S studies (Schloss et al. 2009; Caporaso et al. 2010; Riehle et al. 2012). These tools streamline the process of quality filtering, operation taxonomic unit (OTU) picking, chimeric sequence removal and taxonomic assignment. For rapid and accurate taxonomic assignment, several reference databases, including Ribosomal Database Project (RDP), SiLVA, and Greengenes, have curated full length 16S sequences for more than 700 distinct taxa (DeSantis et al. 2006; Pruesse et al. 2007; Wang et al. 2007). Lastly, these bioinformatics tools can perform basic microbial community analysis, including measuring alpha diversity (within sample variation) and beta diversity (between sample variation).

Whole Genome Shotgun (WGS)-Based Metagenomics

Although 16S sequencing is a cost effective approach to perform metagenomics studies, whole genome shotgun (WGS) sequencing allows for deep and refined taxonomic classification to the species and strain level, as well as the

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capacity to capture total gene content and metabolic capacity (Butler et al. 2008; Liu et al. 2012; Morgan et al. 2012; Qin et al. 2012; Aagaard et al. 2014). However, the sheer volume of data generated by this approach poses significant bioinformatics challenges (Prakash and Taylor 2012). On receiving sequence reads, quality filtering is performed to remove human contamination, which consists of >90% of reads in vaginal or placental samples (Human Microbiome Project 2012c; Aagaard et al. 2014). To get species assignment to provide potential gene expression information, sequence reads are first assembled into contigs, or genes. This is challenging because of the lack of reference genomes, which results in de novo assembly of microbial genomes with the potential to distort the species abundance and generate chimeric genomes (Pop 2009). Following assembly, gene prediction is then possible by analyzing molecular characteristics of existing open reading frames of sequenced genomes (Zhu et al. 2010). Given the complexity of assembly and low efficiency, taxonomy classification can also be achieved by alignment of sequence reads to clade-specific markers identified from integrated microbial genomes (IMG) without prior assembly (Segata et al. 2012). The reconstruction of functional profile is achieved by mapping reads onto pathway collections such as Kyoto Encyclopedia of Genes and Genomes (KEGG), with additional interference steps for pathway coverage and abundance (Abubucker et al. 2012). Further, web tools have recently been developed to perform the tasks described above to facilitate the analysis of WGS data (Glass et al. 2010). However, regardless of approach, WGS and 16S sequencing are limited to describing community composition and its potential metabolic or functional capability.

Multi'omics Data Integration

To truly understand how the microbiome impacts human health, metabolomic and metatranscriptomic approaches will be required to characterize their precise roles as symbionts (reviewed in Morgan and Huttenhower 2014). Metatranscriptomics, unlike its DNA-based counterparts, reveals dynamic gene expression patterns and nonprotein-coding small RNAs present in microbial communities. Therefore, metatranscriptomics can elaborate more precisely how bacteria are contributing to host biology. However, the current challenge in this area of research is how to remove host nucleotides and rRNA while preserving the shorter microbial transcripts (Booijink et al. 2010; Gosalbes et al. 2011; Schmieder et al. 2012). Once this challenge is overcome, the analysis of metatranscriptomics could use previous statistical methods developed for RNA-seq studies.

Metabolomics will be similarly critical for linking bacterial function to host health. Although studying the collection of metabolites associated with a microbial community is not unlike studying single organisms, associating individual metabolites with the microbe of origin is a significant challenge. Because microbes provide metabolic capabilities otherwise unavailable to the host, observing alterations in metabolites through changes in diet or the use of antibiotics in correlation with the microbiome may help us decipher the interrelationships between bacteria and their corresponding metabolomics (Zhang et al. 2010; Sellitto et al. 2012; McHardy et al. 2013). Although it is currently challenging to fully integrate multi'omics data, meaningful associations are beginning to be established through correlations, network analysis, and co-occurrence analysis (Shi et al. 2011; Faust et al. 2012; McHardy et al. 2013; Morgan and Huttenhower 2014).

THE VAGINAL MICROBIOME IN REPRODUCTIVE AGED WOMEN

Before the advent of Next-Gen sequencing, the characterization of the vaginal microbiome through traditional microbiological techniques (culture-dependent) revealed a predominance of *Lactobacillus* species (Redondo-Lopez et al. 1990; Larsen and Monif 2001). These early characterizations of the vaginal microbiome resulted in the delineation of "normal" flora (defined as *Lactobacillus* predominant), and "abnormal" or "aberrant" vaginal flora (non-lactobacillus predominant). Early descriptions

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also attempted to characterize abnormal vaginal flora, most notably in conjunction with bacterial vaginosis (BV). BV is a common and complex alteration of vaginal flora, but the description of the bacteria involved in the vaginal dysbiosis has changed over time. The association between anerobic cocci and abnormal vaginal discharge was first described by Curtis in 1914, and *Gardnerella vaginalis* was first described as a causative agent for BV in 1955 (Eschenbach 1993; Ledger 1993). However, by the 1990s, multiple other species were found in anaerobic cultures of vaginal discharge from subjects with symptoms consistent with BV (Faro et al. 1993; Hillier et al. 1993). Despite the multiplicity of causative agents, one common finding is that women experiencing clinically symptomatic BV tend to be deficient in lactic acid producing species of bacteria that also convert oxygen to H_2O_2 (Eschenbach et al. 1989; Hillier et al. 1992, 1993). This observation has led to several decades of data demonstrating that adverse reproductive health outcomes accompany "abnormal flora" associated with BV (Gravett et al. 1986; Martius et al. 1988; Krohn et al. 1991; Hillier et al. 1995a; Martin et al. 1999; Wiesenfeld et al. 2003; Ness et al. 2005; Brotman et al. 2010). For instance, the incidence of BV has been associated with preterm birth and with increased risk for acquiring sexually transmitted diseases, such as *Neisseria gonorrhea, Chlamydia trachomatis,* and human immunodeficiency virus (HIV) (Gravett et al. 1986; Martius et al. 1988; Krohn et al. 1991; Kurki et al. 1992; Hillier et al. 1995b; Martin et al. 1999; Wiesenfeld et al. 2003; Ness et al. 2005; Brotman et al. 2010; Perla et al. 2012). This increased risk of infection is thought to be because of the deficiency of *Lactobacillus* species that produce lactic acid and H_2O_2 to provide protection from pathogenic bacteria and viruses (Eschenbach et al. 1989). Along these lines, previous in vitro studies have shown that *Lactobacillus acidophilus* can protect from infection with *G. vaginalis*, *Bacteroides bivius*, and HIV through a peroxidase dependent mechanism (Klebanoff and Coombs 1991; Klebanoff et al. 1991). Thus, although BV may indicate vaginal dysbiosis in a clinical setting, there has been historically a lack of data regarding whether vaginal dysbiosis occurred as "normal" flora in women asymptomatic for BV. Currently, Next-Gen sequencing techniques have enabled the vaginal microbiome to be more thoroughly characterized to determine the bacterial flora of the "normal" versus "abnormal" vagina.

One of the first studies to reveal the complexity of the vaginal microbiome using Next-Gen techniques was performed by Ravel et al. This study recruited nearly 400 women of mixed ethnicities. Samples were prepared for either V1V2 16S sequencing and were scored for BV using Nugent criteria (Ravel et al. 2010). Five distinct community state types (CSTs) were revealed, and the majority of these CSTs were dominated by species of lactobacilli. The fourth CST included women with a vaginal microbiome deficient in *Lactobacillus* species, and interestingly, this group had increased incidence of BV. Also, this study showed that the vaginal microbiome could be distinguished by ethnicity. Asian subjects had a higher prevalence of CST III (*Lactobacillus iners*), non-Hispanic Caucasians had a higher prevalence of CST I (*Lactobacillus crispatus*), and African-American and Hispanic subjects had a higher prevalence of CST III (*L. iners*) and IV (decreased *Lactobacillus* species) (Ravel et al. 2010). However, recent studies have shown the caution that must be used when performing analysis such as CSTs (Koren et al. 2013). In these investigations, Koren et al. (2013) showed that these types of cluster analysis are sensitive to the distance metric used for analysis and that multiple distance metrics should be used to promote accuracy in data. In fact, this study used the data of Ravel et al. (2010) in their analysis and found varying support for the presence of CSTs based on the analysis used (Koren et al. 2013). Thus, multiple methods of analysis must be used to establish a CST. An additional method that may be useful to examine microbiome communities across ethnicities is to use single nucleotide polymorphisms (SNPs) in mitochondrial DNA (mtDNA), which provides more precision in analysis (Ruiz-Pesini et al. 2007). We have recently used this method in conjunction with analysis of the microbiome through data leveraged from the HMP to examine associations

between mtDNA haplotypes and microbiome communities (Ma et al. 2014a). Although we did see similar microbiome communities as the Ravel group, our analysis has provided a molecular basis in which to describe the structure of microbiome communities (Ma et al. 2014a).

Although this initial study revealed the complexity of the vaginal microbiome, it did not determine the stability of the vaginal microbiome. This aspect was queried in a separate study in which 32 subjects, mostly African-American and non-Hispanic Caucasian, self-collected vaginal samples over a 16 wk time frame (Gajer et al. 2012). Intriguingly, the stability of the vaginal microbiome over time appeared to vary by individual. In other words, some women tended to have high variability in their vaginal microbiome, even to the extent in which the CST classification of their vaginal microbiome was altered up to three times over the course of the study (Gajer et al. 2012). However, other women tended to have an incredibly stable vaginal microbiome throughout the study (Gajer et al. 2012). Because this study had a limited number of subjects and ethnic diversity, it is challenging to determine how host genetics may be associated with variability in the stability of the vaginal microbiome between subjects. Thus, utilizing the mtDNA haplogroups described above may be of great utility in future studies that examine the stability of any microbiome community over time. Additionally, fluctuations in alpha diversity were associated with the menstrual cycle, such as increased levels of estradiol and progesterone corresponded to decreased diversity (Gajer et al. 2012). Increases in these hormones promote the presence of lactic acid producing bacteria, which maintain low pH and low diversity in the vagina.

Alterations in the vaginal microflora attributed to pH have been described to have functional consequences as well. Using inferred function analysis obtained from WGS sequencing data, the HMP consortium noted that a decrease in the vaginal pH corresponded to an increase in metabolic activity, specifically when examining the phosphate transport system (Human Microbiome Project 2012c). Even further, a recent study examined the vaginal metatranscriptomics profile of two women with healthy vaginal profiles and two women diagnosed with BV. This group found that there were distinct differences in expression of CRISPR-related genes, butyrate production, and glycogen metabolism (Macklaim et al. 2013). In women with healthy vaginal microflora, glycogen metabolism resulted in the production of lactic acid although subjects with BV had an increase in succinate production (Macklaim et al. 2013). What is even more intriguing is that some of these differences appeared to be attributable to gene regulation by *L. iners*. Thus, this study exemplifies the need for further metatranscriptomic and metabolomic studies involving large cohorts to understand the role of the reproductive microbiome in health and disease.

THE VAGINAL MICROBIOME IN PREGNANCY

With the demonstration that the vaginal microbiome fluctuates based on the menstrual cycle, with intercourse, and (to a much more limited degree) with clinical symptoms of BV, we and others sought to characterize the vaginal microbiome during pregnancy. Nearly every organ system changes during pregnancy to promote pregnancy maintenance or prepare for parturition. In the vagina, increased vascularity and hyperemia develop in the skin of the vulva and the mucosa of the vagina. Additionally, the vaginal mucosa increases in thickness and cervical secretions increase, which causes the underlying smooth muscle cells to hypertrophy and relax the connective tissue. At the epithelial surface, the vaginal epithelium hypertrophies and causes a crowding of the epithelial cells, which are rich in glycogen (Nieburgs 1947). Estrogen (namely estradiol) rises across gestation, and further leads to increases in glycogen levels; glycogen is metabolized into lactic acid resulting in the decrease pH (acidity) of the vagina (Gregoire et al. 1971; Paavonen 1983). This metabolism of glycogen into lactic acid was historically thought to be performed by the vaginal epithelium, because the vaginal lumen is suffi-

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ciently distant from the oxygen supply to become anaerobic. However, the primary source of lactic acid became debated when Boskey et al. reported that vaginal lactobacilli were capable of producing lactic acid in vitro at a rate sufficient to reacidify the vagina in vivo following a neutralizing exposure (i.e., ejaculate) (Boskey et al. 1999; Pybus and Onderdonk 1999). Afterward, the specific lactate structures in the vagina were explored and it was discovered that the majority of the vaginal lactic acid was of the D-isoform, which cannot be produced by human metabolism (Boskey et al. 2001). Thus, vaginal bacteria, namely lactobacilli, appear to be the primary source of lactic acid in the vagina. In pregnancy, the preponderance of *Lactobacillus* species appears to be aided by the estrogen-induced increase in glycogen that contributes to the vaginal acidic environment, which is not only enhanced by lactobacilli but also fosters *Lactobacillus* growth.

Before the advent of metagenomics, the presence of lactobacilli were noted to increase as gestational age advanced during pregnancy (Nieburgs 1947). Further, more recent studies showed a high prevalence of *Lactobacillus* species in the vagina during either the first, second, or third trimester using PCR-denaturing gradient gel electrophoresis (DGGE) for the V3 region of the 16S rRNA gene, and the species with the highest prevalence were *L. acidophilus* and *L. iners* (Hernández-Rodríguez et al. 2011). The utilization of Next-Gen sequencing techniques to study the vaginal microbiome further showed the presence of *Lactobacillus* species in the vagina during pregnancy. Our group initially published this 16S-based metagenomic characterization in a cross-sectional study (Aagaard et al. 2012b). We used V3V5 16S sequencing and examined the posterior fornix, mid-vagina, and vaginal introitus of 24 gravid and 60 nongravid subjects. Vaginal samples collected from a cross-sectional survey of gravid subjects from 18 wk gestation to term or delivery, and all samples were collected by a single physician who also conducted the HMP sampling for 50% of subjects. All samples were extracted in a manner identical to that used by the HMP, and sequenced on the same pipelines. When compared with the HMP nonpregnant reference subjects, we found that pregnancy was associated with an altered vaginal microbiome and marked by a decrease in alpha diversity at the subgenus level (Fig. 2) (Aagaard et al. 2012b). When we examined this phenomenon further, we discovered that there was an overall enrichment of the orders Lactobacillus, Clostridiales, Bacteriodales, and Actinomycetales. And on probing at the species level using supervised machine learning approaches, such as linear discriminate analysis (LDA) effect size (LEfSe) and Boruta feature selection, we discovered that the vaginal microbiome during pregnancy was enriched in *L. iners, L. crispatus, Lactobacillus jensenii,* and *Lactobacillus johnsonii* (Aagaard et al. 2012b). The increase in lactobacilli may be explained by the increase in estrogen that occurs during pregnancy. However, direct associations between specific species of *Lactobacillus* and estrogen levels are lacking and warrant further investigation.

However, the aforementioned studies were cross-sectional, which allows for the characterization of a pregnancy and gestational age-common microbiome signature, but lacks capacity for description of the dynamic changes, which may occur in an individual over time. Longitudinal analysis of the vaginal microbiome in a small cohort of women across gestation has been performed using terminal restriction fragment length polymorphism (tRFLP) in 100 gravid women (Verstraelen et al. 2009). This study categorized gravidae into two cohorts during the first trimester: lactobacilli-dominant and lactobacilli-diminished. Interestingly, only 16.9% of gravidae that had a lactobacilli-dominant vaginal microbiome in the first trimester decreased the prevalence of lactobacilli during pregnancy. On the other hand, 56.5% of gravidae with a lactobacilli-diminished vaginal microbiome during the first trimester gained prevalence of this genus. In contrast to the cross-sectional study, these longitudinal cohorts showed that *L. crispatus* dominated during pregnancy and that prominence of *Lactobacillus gasseri* and/or *L. iners* during the first trimester may be associated with diminished lactobacilli as pregnancy progressed (Verstrae-

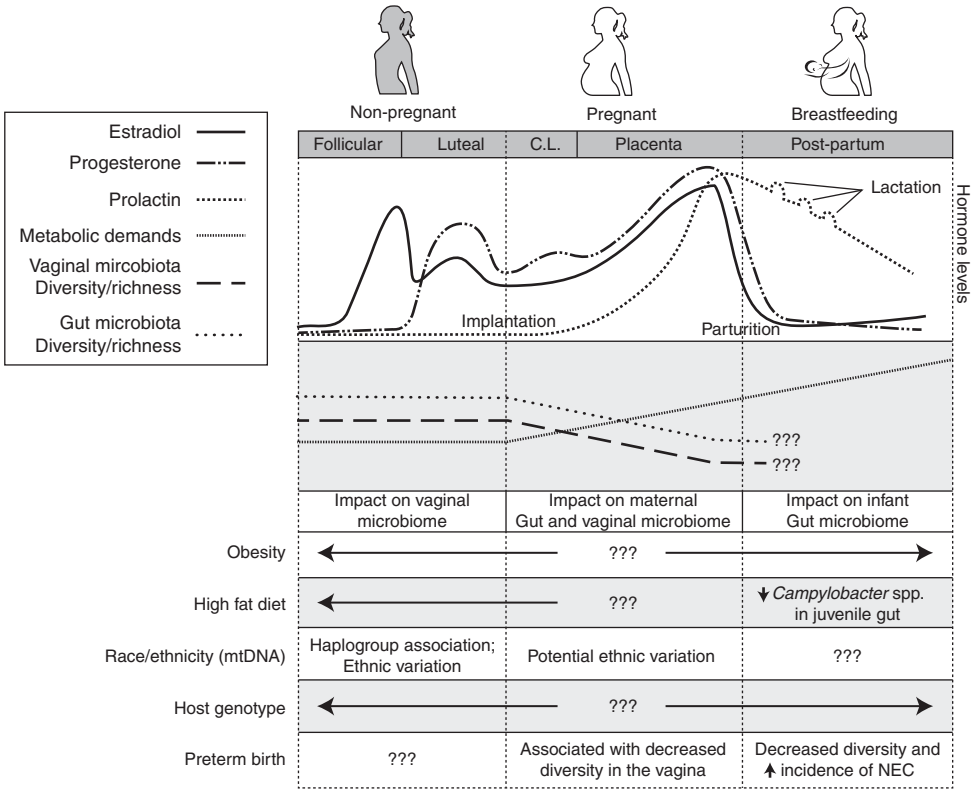

Figure 2. Influences on the pregnant microbiome. A number of hormonal changes, environmental exposures and genetic differences may impact the maternal microbiome before and during pregnancy that may alter the developing neonatal microbiome. During pregnancy, the maternal intestinal and vaginal microbiome have reduced alpha diversity and species richness. Metabolic demands increase throughout pregnancy and after parturition as the mother is lactating. Estradiol, progesterone and prolactin levels gradually increase during pregnancy, although it is unclear how these changes affect the maternal microbiome. Increased estrogen raises glycogen production in the vagina, but how the availability of this substrate structures the vaginal microbiome is unknown. The effect of host genetics on the maternal microbiome throughout pregnancy is relatively unknown. Different ethnicities, which can be inferred by mitochondrial DNA (mtDNA) haplotypes, have been shown to have varied vaginal microbiomes before and after pregnancy. Further studies are needed to understand these differences, and to explore the effect of host genotype on the maternal microbiome. The impact of diet and obesity on the pregnant microbiome is just beginning to be explored. A primate model of maternal high fat diet showed that diet alone can persistently alter the juvenile microbiome at one year of age regardless of juvenile diet. However, how diet alters the maternal environment during pregnancy and how this affects the vertical transmission of bacteria is unknown.

len et al. 2009). Recently, Romero et al. have performed longitudinal studies of the posterior fornix of the vaginal microbiome during pregnancy using Next-Gen sequencing of the V1V2 region of the 16S rRNA amplicon (Romero et al. 2014b). These studies used the self-collection of vaginal samples over 16 wk of the nonpregnant cohort and swabbing of the posterior fornix at four prenatal visits from the gravid cohort with 22 subjects. Additionally, the gravid cohort consisted of mainly African-American ethnicity. In their analysis, the authors used the CSTs established by Ravel et al. to interrogate the vaginal microbiome during pregnancy (Ravel et al. 2010; Romero et al. 2014b). This study concluded that the vaginal microbiome of gravid wom-

en mostly consisted of the CST I or III with odds ratios of 2.986 and 2.136, respectively, and that the vaginal microbiome of gravid women shift toward these two CSTs as their pregnancy progressed (Romero et al. 2014b). When the stability of the vaginal microbiome was examined during pregnancy, the authors noted that the vaginal microbiome of gravid subjects shifted between CSTs dominated by lactobacilli but rarely shifted to CST IV, which is marked by a diminished abundance of *Lactobacillus* species (Romero et al. 2014b). However, with a low number and low ethnic diversity of subjects in the study, further studies are needed to confirm these shifts of the vaginal microbiome toward CSTs dominated by lactobacilli as pregnancy progresses. Initial studies of the vaginal microbiome showed that African-American women have a higher prevalence of vaginal CSTs consisting of *Lactobacillus* species (Ravel et al. 2010), which was further shown by this recent study (Romero et al. 2014b). In a separate study, the vaginal microbiome was examined longitudinally during gestation using the V3V5 amplicon of 16S rRNA in 12 subjects that were mostly Caucasian (Walther-António et al. 2014). In agreement with previous studies, these authors showed that alpha diversity decreases as pregnancy progresses and that *L. crispatus* and *L. iners* dominate the vaginal microflora (Fig. 2) (Walther-António et al. 2014). Intriguingly, these authors suggest that maternal age may be important for the dominance of *L. crispatus* or *L. iners,* with, *L. iners* being dominant in older gravidae (Walther-António et al. 2014). Although this insight should be kept in mind for future studies, this study had only two subjects with advanced maternal age (34–36) (Walther-António et al. 2014). To take these studies further, this group attempted to analyze their data in conjunction with the Romero et al. study; however, differences in primer sets and sequencing platforms used in these separate studies prevented in depth analysis (Romero et al. 2014b; Walther-António et al. 2014). Despite these challenges, the authors found that, although alpha diversity of the vaginal microbiome decreased with gestational age in both African-American and Caucasian subjects, African-American subjects had increased beta diversity between gravid subjects whereas Caucasian gravidae did not (Walther-António et al. 2014). Thus, these studies highlight the need for further longitudinal studies with large subject enrollment and ethnic diversity. Additionally, studies are needed with a high enrollment of BV subjects or women with a dysbiotic vaginal microbiome to lend further insight into vaginal microbiome shifts and stability that are associated with pregnancy.

In addition to better understanding shifts in the vaginal microbiome during pregnancy, investigating the microbiome of subjects with BV may lend insight into the role of the vaginal microbiome in preterm birth. It is known that BV is associated with preterm birth (Martius et al. 1988; Krohn et al. 1991; Hillier et al. 1995a,b), and therefore, it is logical to question the potential association of the vaginal microbiome with preterm birth (Ganu et al. 2013). The Preterm Prediction Study examined the association between BV and preterm birth (PTB) (Meis et al. 1995). In this study, vaginal specimens were obtained at 24 and 28 wk gestation, and an association with increased risk for spontaneous PTB at $<$35 wk gestation was found in 19.8% of women with BV at 28 wk gestation (Meis et al. 1995). However, conclusions could not be drawn regarding whether BV itself was causative of PTB (Meis et al. 1995). Additionally, although treatment of BV during pregnancy does eradicate infection, it does not reduce the risk of PTB (Hillier et al. 1995b; Brocklehurst et al. 2013). Therefore, given the lack of benefit, screening of asymptomatic women in pregnancy is not recommended (Nygren et al. 2008). Even more concerning are the findings of two studies that found an increase in preterm delivery ($<$34 wk) among women who tested negative for BV but were treated (Hauth et al. 1995; Vermeulen and Bruinse 1999). Thus, the relationship of BV and preterm birth is complicated and the benefit of treatment is questionable. These issues regarding BV and PTB warrant further investigation into these associations, and the examination of the microbiome using Next-Gen sequencing techniques will be of great utility for these studies.

Despite the need for further investigation into the role of the vaginal microbiome in association with BV and preterm birth, a recent study by Hyman et al. (2014) has examined the vaginal microbiome in preterm birth. This group used a prospective cohort study with 46 high-risk (previous unexplained PTB) and 42 low-risk (all other gravid) subjects for PTB gravidae enrolled (Hyman et al. 2014); however, only 14 subjects were able to be sampled in each trimester. Intriguingly, the investigators found that the presence of lactobacilli did not distinguish term (>37 wk) from preterm (<37 wk) subjects using Sanger sequencing methods. However, low-risk subjects had a higher prevalence of lactobacilli when compared with high-risk subjects (Hyman et al. 2014). Despite the lack of association of lactobacilli with preterm birth, measured alpha diversity was reported as diminished when comparing Caucasian term and preterm subjects (Hyman et al. 2014). Among the two longitudinal subjects that ultimately delivered preterm, their vaginal microbiomes were dominated by *L. crispatus* (Hyman et al. 2014), which is in contrast to a previous study demonstrating that *L. crispatus* is dominant in healthy term pregnancies (Verstraelen et al. 2009; Aagaard et al. 2012b). Moreover, although two subjects in the Hyman et al. study had outgrowths of *Bifidobacterium* and *Ureaplasma* genera, separately, these genera are reported in both normal pregnant subjects as well as nonpregnant (Ravel et al. 2010; Aagaard et al. 2012b; Romero et al. 2014b). Similar findings were found in an independent study by Romero et al. In this prospective study, term (38–32 wk) versus preterm (<34 wk) subjects were sampled at the posterior fornix by an obstetrician and sequenced using V1V3 primers (Romero et al. 2014a). The authors found no association between the CSTs of the vaginal microbiome and preterm birth, nor did they find any CST of the vaginal microbiome associated with preterm subjects having acute chorioamnionitis (Romero et al. 2014a). In contrast to the study by Hyman et al., this study found no significant difference in the alpha diversity of the vaginal microbiome of term and preterm subjects (Hyman et al. 2014; Romero et al. 2014a). In summary, although promising, these studies underscore the need for broader gestational age-specific, reference-based cohorts to define both the effect size and population variance. Before the publication of such studies, it would be premature to ascribe such microbiome profiling as indicative or heralding of preterm birth.

VARIANCE OF THE HUMAN MICROBIOME IN PREGNANCY: BEYOND THE VAGINAL COMMUNITY

In addition to changes described in the vaginal microbiome during pregnancy, Koren et al. (2012) investigated the intestinal microbiome during pregnancy using a prospective cohort study and found that as pregnancy progressed, the intestinal microbiome was altered. This study used the V1V2 region of the 16S rRNA gene and found that alpha diversity (within sample variation) was decreased between the first and third trimesters (Fig. 2). Further, stool samples collected from gravidae in the first trimester clustered separately from stool samples collected in the third trimester (Koren et al. 2012). These differences in beta diversity (between sample variation) were a reflection of increases in Proteobacteria in the stool during the third trimester when compared with stool from the first trimester. When fecal transplants of first and third trimester stool into germ-free mice were performed, mice receiving third trimester stool had increases in inflammatory cytokines and adiposity, similarly to fecal transplants involving obese subjects (Turnbaugh et al. 2006; Koren et al. 2012; Ridaura et al. 2013). These alterations in the intestinal microbiome did not associate with any other covariates, such as body mass index (BMI), development of gestational diabetes mellitus, or multiparity (Koren et al. 2012).

BEYOND THE VAGINAL MICROBIOME: EARLY INFANT COLONIZATION

Recently, the establishment of the neonatal microbiome has been the subject of debate and investigation. As shown with vaginal micro-

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biome studies, there has been wide variability between the use of techniques and the variable region of 16S used for studies. An initial study by Schultz and colleagues examined the vertical transmission of a probiotic, *Lactobacillus rhamnosus* GG, from mother to infant (Schultz et al. 2004). Gravid women took the probiotic twice daily from 30–36 wk of gestation. The authors found that all infants born vaginally (4/4) contained this probiotic in their stool whereas the probiotic was only detected in the stool of half of the cesarean delivered infants (1/2) (Schultz et al. 2004). This study showed that vertical transmission did occur between mother and infant. However, this study included a small subject number and did not examine the maternal stool postpartum, which would be relevant to determine the persistence of the probiotic in the maternal microbiome that would provide an opportunity for horizontal transmission. The maternal microbiome during infancy, and not solely delivery mode, may be an important factor in establishing the neonatal microbiome. In fact, a recent study examining the establishment of the intestinal microbiome of healthy, term neonates using qPCR found that bacterial loads between maternal and infant stool were remarkably similar (Jost et al. 2012). This study shows that the maternal microbiome at birth and postpartum may be critical in the establishment and development of the neonatal microbiome via horizontal transmission.

When directly examining the intestinal microbiome of infants born via cesarean or vaginal delivery, culture-based microbiological techniques have shown differences in the colonization of the neonatal intestinal microbiome, particularly of *Bifidobacterium*-like bacteria, *Lactobacillus*-like bacteria, and *Bacteriodes fragilis* (Grönlund et al. 1999a). However, a separate study by this group also determined that bacterial enzymes were not altered in the stool of infants based on mode of delivery (Grönlund et al. 1999b). Using culture-independent, PCR-based techniques, Penders et al. also showed that *B. fragilis* and *Bifidobacterium* were decreased in cesarean delivered infants in comparison to vaginally delivered infants. However, this study also showed differences in the intestinal microbiome of infants based on formula-feeding or breastfeeding (Penders et al. 2006). An Italian study further investigated the intestinal microbiome of infants based on mode of delivery using the V6V8 region of the 16S rRNA gene in PCR-DGGE and PCR-temperature gradient gel electrophoresis (TGGE) assays (Biasucci et al. 2008). This group determined that infants born vaginally had increased diversity in their intestinal microbiome when compared with cesarean delivered infants. Again, infants born by cesarean delivery appeared to have an absence of *Bifidobacterium* in their intestinal microbiome (Biasucci et al. 2008).

To date, few studies on this issue regarding mode of delivery have used Next-Gen sequencing techniques. An initial study was performed in Venezuela with nine gravid subjects, four giving vaginal birth and five having a cesarean delivery (Dominguez-Bello et al. 2010). This study used the V2 region of the 16S rRNA gene and showed that the infant microbiome most closely resembled the mothers' vaginal microbiome following vaginal delivery. Similarly, if the infant was born by cesarean delivery, the infant microbiome most closely resembled the skin microbiome (Dominguez-Bello et al. 2010). Furthermore, a Canadian study using Next-Gen sequencing techniques for the V5, V6, and V7 regions of the 16S rRNA gene showed that *Escherichia shigella* and *Bacteroides* were significantly diminished in the intestinal microbiome of infants born by cesarean section (Azad et al. 2013). This study also found that infants with the highest species richness and diversity of their intestinal microbiome were born by emergency cesarean section rather than by an elective cesarean section or vaginally (Azad et al. 2013). However, when followed out to 4 mo postpartum, infants' intestinal microbiome could be differentiated primarily based on mode of feeding. Specifically, infants that were formula-fed had a higher prevalence of Peptostreptococcaceae and Verrucomicrobiaceae when compared with breastfed infants (Azad et al. 2013). Thus, mode of feeding may be more crucial than mode of delivery in regards to the long-term establishment of the microbiome. These findings suggest that the establishment of a stable

microbiome is not only a question of what bacteria are present at birth, but also what factors, either host-derived or environmental, influence the species that persist.

Which Microbiota First Populate the Infant?

Further studies into the establishment of the neonatal microbiome highlight this principle. Jost et al. (2012) showed that the intestinal microbiome of infants born vaginally and exclusively breastfed decrease the amount of the Firmicutes phylum, of which *Lactobacillus* belongs, over time while increasing the prevalence of *Bacteroides* species. Thus, although these infants were born vaginally, a high presence of Firmicutes did not persist. Intriguingly, these vaginally delivered infants could be classified into two cohorts in this study: those that had species of *Bacteriodes* present in their intestinal microbiome and those that did not (Jost et al. 2012), and this trend was also seen in a separate study (Palmer et al. 2007). This is in contrast to previous studies finding that *Bacteroides* relative abundance differed based on mode of delivery (Grönlund et al. 1999a; Penders et al. 2006). However, Palmer et al. also determined that variations in the *Bacteroides* species seen early in neonatal life were less variant and more consistent in the abundance by 1 yr of age. Additionally, the neonatal intestinal microbiome also appeared more adult-like near the end of the first year of life (Palmer et al. 2007). This finding was confirmed in a study by Koenig et al. in which the intestinal microbiome of an infant was monitored for 2.5 yr. Here, the authors found that the diversity of the intestinal microbiome increased over time and with the introduction of foods (Koenig et al. 2011). Also, *Bacteroides* species were found to increase on the introduction of vegetables (Koenig et al. 2011). Altogether, this data indicate that the neonatal microbiome is highly variable within the first year of life. Therefore, various exposures during this time may have a significant impact on the developing microbiome.

Although it is unclear if the health and microbiome of the offspring is persistently influenced by mode of delivery, gestational age at delivery appears to be the greater arbiter for the developing microbiome. For instance, differences in the intestinal microbiome of preterm and term neonates have been described (Schwiertz et al. 2003). In this study, the authors found that healthy, term neonates that were breast fed had increased diversity in their intestinal microbiome when compared with hospitalized, preterm infants using PCR-DGGE analysis (Schwiertz et al. 2003). Additional studies have confirmed that the microbiome of infants with conditions like PTB, very low birth weight infants, or necrotizing enterocolitis, also have an altered microbiome (Schwiertz et al. 2003; Hällström et al. 2004; Milisavljevic et al. 2013; Wang et al. 2013). However, it is unclear if these alterations are caused by early gestational age at delivery or hospitalization because neonatal exposure in early life is pertinent to the establishment of the microbiome.

Furthermore, these exposures at delivery and in early life may have lasting effects on the microbiome (Ding and Schloss 2014; Ma et al. 2014b). For instance, a recent study showed that breastfeeding has an impact in the long-term enterotype of an individual (Ding and Schloss 2014). This study is bolstered by murine studies suggesting that maternal antibodies transferred via breast milk may have a persistent impact on the intestinal microbiome (Rogier et al. 2014). However, retrospective analysis of the literature determined that mode of delivery may influence obesity in adulthood (Darmasseelane et al. 2014). Thus, these aforementioned associations with mode of feeding and delivery may be attributable to differences in the seeding of the neonatal microbiome. Therefore, future studies are needed to entail how and why microbes remain in distinct body sites. In other words, it may not be a question as to who is there and from whenst and where do they arise, but rather why do certain microbes take up and retain residence? As the maternal diet and microbiome has been shown to influence the establishment and development of the infant microbiome, these future studies may reveal early mechanisms of adult metabolic disorders, which may allow for the early treatment and/or prevention of associated diseases.

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THE PLACENTAL MICROBIOME

The placenta has long been considered sterile in normal gestation, where the presence of bacteria in clinical cultures is diagnostic for intrauterine infection and a significant risk for PTB (Hillier et al. 1988). The ELGAN studies constituted a large effort that systematically identified bacteria from PTB placentas under this assumption (Olomu et al. 2009; Onderdonk et al. 2008a,b). However, there is increasing recognition of a large discordance between the presence of bacteria as per culture-based diagnoses and clinical outcome (Watts et al. 1992; Pettker et al. 2007; Buhimschi et al. 2009; Han et al. 2009; Leviton et al. 2010; Stout et al. 2013; Combs et al. 2014; Fortner et al. 2014). In fact, the presence of placental or membrane bacteria in the absence of histological infection has been discovered repeatedly over the last few decades (Hillier et al. 1988; Steel et al. 2005; Redline 2007; Stout et al. 2013; Fortner et al. 2014). This has led to the recognition of the need to study and redefine our understanding of the role of intrauterine bacteria in gestation.

One of the earliest studies to recognize the absence of pathogenicity of intrauterine bacteria was performed using histological analysis. It was shown that membranes of normal pregnancies often contain bacteria, yet show no signs of histological infection (Steel et al. 2005). Later studies using similar methodologies discovered intracellular bacteria localized to the trophoblast of the basal plate of the maternal decidua in the absence of chorioamnionitis although there was an association with PTB (Cao and Mysorekar 2013; Stout et al. 2013). Seemingly in agreement, it was also shown that intra-amnionic invasion is relatively benign in the absence of inflammation with associations between PTB and inflammation alone rather than PTB and bacterial invasion (Combs et al. 2014). Intriguing work by Murtha and colleagues showed that high levels of bacteria were strongly associated with premature rupture of membranes (PPROM) and membrane thickness, although there was no histological inflammation in half of the subjects. Although there was bacteria found in subjects of all groups including PPROM (both term and preterm), PTB, and even normal gestation controls, there was no inflammation detected in the majority of subjects (Fortner et al. 2014). Thus, these studies suggest that it is not the occurrence of bacteria in the placenta, but the bacterial populations present that may initiate intrauterine infection.

Along these lines, a metagenomic study using a Rhesus macaque model identified more than 300 microbial species in the chorioamnion and placenta (Aagaard et al. 2013). Interestingly, it was shown that this population was modifiable with a sterile intra-amniotic injection of IL-1β that induced histological inflammation (Aagaard et al. 2013). This suggests that chorioamnionitis may be caused by microbial dysbiosis rather than the presence of bacteria per se. The potential translational implications of this model to human pregnancy was emphasized recently by our description of a vibrant and diverse commensal placental microbiome in normal pregnancies (Aagaard et al. 2014). Analysis of more than 300 human samples using both 16S and WGS sequencing revealed a low abundance complex community dominated by the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria, found in nearly all samples. Subjects with a remote history of antenatal infection and antibiotic treatment, or who developed PTB, had discrete statistically significant groupings of taxa (Aagaard et al. 2014). It was also found that the placental microbiome most closely resembles the oral microbiomes of the supragingival plaque and the dorsum of the tongue; however, the placental microbiome did not closely resemble the stool or the vaginal microbiomes. This implies that the bulk of the low level placental bacteria are likely not ascending nor are contaminants of the stool or the vagina, and instead, are quite possibly seeded largely from the oral cavity through hematogenous spread (Fig. 3).

The entering of oral microbes into the blood stream as a result of periodontitis or dental procedures is well established (Han and Wang 2013), and it has been known for decades that periodontal disease is linked to PTB (Offenbacher et al. 1996, 2009; Goldenberg et al.

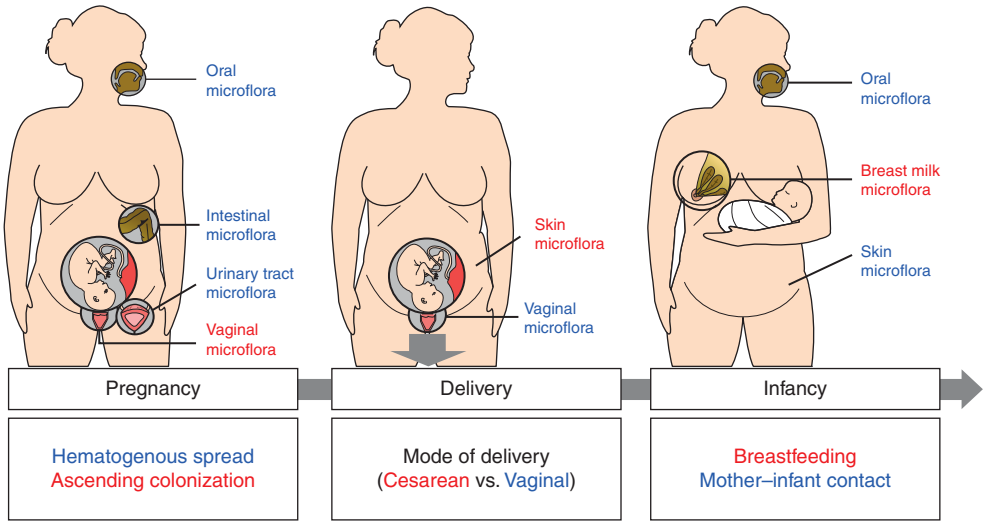

Figure 3. Speculated origins for microbiota colonizing the placenta and seeding the initial neonatal microbiome. Vaginal microflora likely contribute to the initial seeding of the neonatal microbiome during vaginal deliveries, but the discovery that the uterine environment may not be sterile suggests that colonization of the infant may happen before birth. Recent data demonstrating that the placenta has its own unique microbiome most closely resembling the oral microbiome suggests a potential hematogenous route by which bacteria can seed the placenta and the developing fetus. Microbiota from maternal oral, vaginal, urinary tract, and intestine are all potential sources for these colonizing bacteria. Microbiota from breast milk and maternal contact may be an important source of commensal bacteria during early infancy and must be considered when studying the microflora of the neonate.

2000; Michalowicz et al. 2006; Macones et al. 2010). Animal models have shown that bacteria may be spread hematogenously to the placenta (Han et al. 2004; Fardini et al. 2010), and reports have suggested that oral bacteria may be associated with pregnancy complications (Katz et al. 2009; Han et al. 2010; Swati et al. 2012). *Fusobacterium nucleatum* is an oral pathogen that is frequently found in diagnostic cultures following PTB, PPROM, and stillbirth (Romero et al. 1989; Watts et al. 1992; Han et al. 2004, 2010; Cahill et al. 2005). Our finding of Fusobacteria to be a relatively abundant taxon in the placenta supports the hypothesis of hematogenous spread from the oral cavity to the placenta (Aagaard et al. 2014), and this theory is further buttressed by the finding of bacteria in cord blood of normal pregnancies (Jiménez et al. 2005). Along these lines, bacteria can spread from the intra-amnion outward to the chorion, which indicates that hematogenous spread from mother to infant may be occurring via cord blood (Kim et al. 2009). Thus, hematogenous spread may promote colonization of the placenta and the fetus, but further studies are needed to examine this phenomenon. However, a potential mechanism to compromise the maternal–fetal barrier has been illuminated. In a mechanism akin to *Listeria monocytogenes* (Bakardjiev et al. 2006; Le Monnier et al. 2007), *F. nucleatum* expresses adhesin FadA that interacts with E-cadherin, which compromises cell–cell adhesion and membranes (Lecuit et al. 2004; Ikegami et al. 2009; Fardini et al. 2011). However, further studies are necessary to show definitively that hematogenous spread from the oral cavity is possible for colonization or infection of the placenta.

Ongoing and future study of the complex origins of neonatal bacteria should consider oral and placental microbiomes in addition to the vaginal microflora. This need is exemplified by studies involving infants born preterm with early-onset neonatal sepsis showing the pres-

ence of *Fusobacterium, Ureaplasma,* and *Mycoplasma* in the cord blood, amniotic fluid, and neonatal blood (Wang et al. 2013). Additionally, neonates with late onset sepsis or necrotizing enterocolitis have been found to have intestinal microbial dysbiosis that precedes clinical diagnosis (Mai et al. 2011, 2013). Although the vaginal microbiome may be implicated in these neonatal diseases, significant differences in the abundance of placental bacteria have been found when comparing preterm and term placentas (Jones et al. 2009; Stout et al. 2013; Aagaard et al. 2014). Thus, we speculate that seeding of the neonatal microbiome with bacteria from the placenta, which arose through hematogenous spread, may facilitate early colonization. Thus, this early colonization in combination with variable, modifying host factors (King et al. 2007; Zeldovich and Bakardjiev 2012) may conceivably provide the initial seeding of a dysbiotic microbiome that may render susceptibility to neonatal disease in a preterm or stressed infant.

CONCLUSIONS AND CLINICAL SIGNIFICANCE

Here we have described the current state of the science on several aspects of the female reproductive microbiome, as well as their current association with perinatal disorders of both the mother and her offspring. What we understand today is far more complex and confounded than was appreciated less than a decade ago, and is much simpler than what we will come to realize in coming years. The vaginal microbiome varies from one woman to the next, across the lifespan, and in association with both health and disease states. Simplified views of "less diversity and less rich vaginal microbiomes are equivalent to disease states" have been challenged and discounted, and concepts of clearly delineated CSTs remain to be fully validated. Previously assumed to be "sterile" reproductive tract tissues have been shown to harbor low biomass microbiomes, and yet we remain unclear as to what, how and when the infant is colonized. What will serve as decisive determinants of community structure is still unknown, and the relative influence of antibiotics, prebiotics, and probiotics (as well as early in life diet and exogenous exposures) has yet to be robustly characterized. A systemic analysis of the microbiome across the reproductive health spectrum (adolescence, pregnancy, postnatal and perimenopausal/menopausal/postmenopausal) will undoubtedly shed light on the most significant and perplexing common disorders of our time. Although this is a challenging area of research, the advent of metagenomics, combined with integrative multi'omics, will enable reproductive scientists and physician scientists to unravel the mysteries plaguing not only our generations' health and disease, but will likely shed light on human and primate coevolution of host and microbe.

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