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UNEXPECTED BEGINNINGS: THE HUMAN MICROBIOME IN PREGNANCY AND FOETAL DEVELOPMENT

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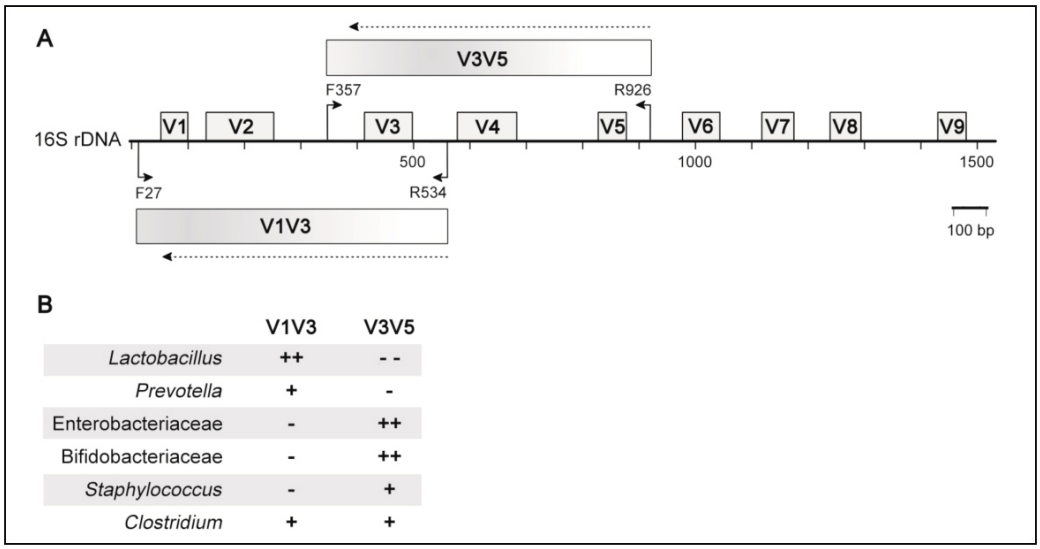
INTRODUCTION

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 in the United States. Together with the European MetaHIT metagenomics of the human intestinal tract, these landmark studies document bacterial diversity, niche specificity, and microbial gene carriage patterns far exceeding what was initially suspected (*Human Microbiome Project*, 2012a,b,c; *Aagaard* et al., 2012a,b; *Huse* et al. 2012; *Gevers* et al., 2012; *Li* et al., 2012). In the interval prior and the interval since, associations between "dysbiotic" (or variation among) microbiota 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; *Bäckhed* et al., 2007; *Cani* et al., 2007; *Turnbaugh* et al., 2008, 2009; *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 aetiologies for these common complex disorders have been suggested over the decades (*i.e.,* human single nucleotide polymorphisms and structural genomic variation, as well as epigenomic regulation and dysregulation). It is thus 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 occur in, and what other co-variates (such as host disease-susceptible genotype, host metabolic milieu and associated disorders, as well as age, gender, race/ethnicity, medications and diet) may contribute to any observed variation.

DOES THE MICROBIOME VARY DURING THE COURSE OF PREGNANCY?

Our laboratory and others have demonstrated that the vaginal microbiota vary during the course of normal pregnancy, thus providing a unique "signature" in



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

Figure 1: The 16S rRNA gene is an ideal target for classifying bacteria.
(A) The 16S rRNA 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 utilized 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.

pregnancy with relative altered abundance of multiple taxa (*Romero* et al., 2014; *Aagaard* et al., 2012b). But what impact does this have on either the pregnancy or the developing infant? Although it has long been suggested that intra-uterine infections, such as chorioamnionitis, are the sequelae of ascending microbiota from the upper vaginal tract (*Gonçalves* et al., 2002), the evidence supporting this are relatively sparse (Table 1). Moreover, evidence that 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 is not actually consistent with respect to representative microbiota (*Claud* and *Walker*, 2001; *Guthrie* et al., 2003; *Yee* et al., 2012). In this dawning era of metagenomic medicine and science we are questioning the notion of anatomically adjacent microbial migration, 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 which harbour unique microbiomes. These early observations challenge not only our assumed notions of "from whenst 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. This leads to a number of reflections on the rather unexpected beginnings of the neonatal and infant microbiome, which are themselves relatively low biomass microbiomes.

HOW DO WE DETECT LOW BIOMASS MICROBIOMES?

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 due to the nine hypervariable regions in this gene that can be used to distinguish species based on individual nucleotide polymorphisms (Figure 1A; *Klindworth* et al., 2013). However, 16S-amplicon based approaches are limited to a shorter read length as compared to 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 demonstrated 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). As a result, the resultant microbiome profile of any given body site will have subtle variations by virtue of the primer set used.

For example, comparison of the vaginal microbiome data from the HMP reveals that V1V3 will distinguish communities primarily by the relative predominant *Lactobacillus* species present, while 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 to V3V5 (*Huse* et al., 2012). However, unlike V3V5, V1V3 does not fully discriminate *Enterobacteriaceae* family projections (including *Escherichia* and *Proteus)*, and some genera (including *Staphylococcus)* (*Fettweis* et al., 2012) (Figure 1B). Few studies have directly compared the vaginal communities described by V6, V7, V8 and V9 to other primer sets, though a number of studies have utilized 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 to, and limitations when comparing to other published findings.

Whole genome shotgun (WGS) based metagenomics

Although 16S sequencing is a cost effective approach to perform metagenomics studies, WGS sequencing allows for deep and refined taxonomic classification to the species and strain level, as well as the capacity to capture total gene content and metabolic capacity (*Butler* et al., 2008; *Qin* et al., 2012; *Liu* et al., 2012; *Morgan* 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). Upon receiving sequence reads, quality filtering is performed to remove human contamination, which consists of more than 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

Table 1: Metagenomic studies pertaining to perinatal health

Authors	Year	Site	Technique(s)	Primers utilized	Study Design	Findings
Non-gravid 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, non- gravid vaginal microbiome
Gajer et al.	2012	mid-vagina (self-collected)	next-gen sequencing	V1V2	longitudinal	demonstrated temporal dynamics of the vaginal microbiome
Macklaim et al.	2013	vagina	meta-transcriptomics		cross-sectional	demonstrated potential or meta-transcriptomics on vaginal swabs
Gravid vaginal studies						
Aagaard et al.	2012	vaginal introitus, posterior fornix, and mid-vagina	next-gen sequencing	V3V5	cross-sectional	characterized healthy, gravid vaginal microbiome
Romero et al.	2014	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 1st and 3rd trimester stool
Beyond the vagina: the placenta						
Aagaard et al.	2014	placenta	next-gen sequencing	V1V3 and WGS	population based cross-sectional	the placenta harbours a unique micro-biome profile, most akin to the oral microbiome and varies by virtue of preterm birth and a remote history of antenatal infection

Table 1 (continued): Metagenomic studies pertaining to perinatal health

Authors	Year	Site	Technique(s)	Primers utilized	Study design	Findings
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
Dominiguez-Bello et al.	2010	oral, vagina skin, rectum	next-gen sequencing	V2	cross-sectional	characterized neonatal microbiome by mode of delivery
Koenig et al.	2011	stool	next-gen sequencing	V1V2	longitudinal	characterized the intestinal microbiome from birth to 2.5 years
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 months 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.	2014	colon, anus, stool	next-gen sequencing	V3V5	non-human primate	examined the role of maternal diet on juvenile microbiome

contigs, or genes. This is challenging due to 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, webtools 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.

WHAT MICROBES COMMENSALLY COLONIZE THE NEONATE?

Recently, the establishment of the neonatal microbiome has been the subject of debate and investigation. When directly examining the intestinal microbiome of infants born via caesarean or vaginal delivery, culture-based microbiological techniques have demonstrated 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 caesarean delivered infants in comparison to vaginally delivered infants. However, this study also demonstrated 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 to caesarean delivered infants. Again, infants born by caesarean delivery appeared to have an absence of *Bifidobacterium* in their intestinal microbiome (*Biasucci* et al., 2008).

Which microbiota first populate the infant?

Further studies into the establishment of the neonatal microbiome highlight this principle. Jost *et al.,* demonstrated 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 (*Jost* et al., 2012). 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 one year 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 years. 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 upon 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.

Is it vertical or horizontal transmission of microbes that populate the infant at birth?

An initial study by Schultz and colleagues examined the vertical transmission of a probiotic, *L. rhamnosus* GG, from mother to infant (*Schultz* et al., 2004). Gravid women took the probiotic twice daily from 30 to 36 weeks of gestation. The authors found that all infants born vaginally (4/4) contained this probiotic in their stool while the probiotic was only detected in the stool of half of the caesarean delivered infants (1/2) (*Schultz* et al., 2004). This study demonstrated 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 demonstrates that the maternal microbiome at birth and postpartum may be critical in the establishment and development of the neonatal microbiome via horizontal transmission. 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 has 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 to hospitalized, preterm infants using PCR-DGGE analysis (*Schwiertz* et al., 2003), and additional studies have confirmed that the microbiome of infants with conditions like PTB, very low birth weight, 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 due to early gestational age at delivery or hospitalization since neonatal exposure in early life is pertinent to the establishment of the microbiome.

Does it matter how the infant was delivered?

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 total, four giving vaginal birth and five having a caesarean delivery (*Dominguez-Bello* et al., 2010). This study used the V2 region of the 16S rRNA gene and demonstrated that the infant microbiome most closely resembled the mothers' vaginal microbiome following vaginal delivery. Similarly, if the infant was born by caesarean delivery, the infant microbiome most closely resembled the skin microbiome. Furthermore, a Canadian study using Next-Gen sequencing techniques for the V5, V6, and V7 regions of the 16S rRNA gene demonstrated that *Escherichia shigella* and *Bacteroides* were significantly diminished in the intestinal microbiome of infants born by caesarean-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 caesarean-section rather than by an elective caesarean-section or vaginally. However, when followed out to four months 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 to 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.

Furthermore, these exposures at delivery and early life may have lasting effects on the microbiome (*Ma* et al., 2014b; *Ding* and *Schloss*, 2014). For instance, a recent study demonstrated 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.

IS THE VAGINAL MICROBIOME A LIKELY FIRST SOURCE OF SEEDING THE NEXT GENERATIONS MICROBIOME?

As previously overviewed, it has been suggested by many, based on a paucity of data, that the infant microbiome is seeded at the time of vaginal birth (Table 1). Such a supposition would necessitate thorough understanding of the vaginal microbiome during pregnancy, as well as an appreciation for the vaginal microbiome constituent variation across gestational age.

Characterizing the vaginal microbiome

Prior to 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 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 anaerobic 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 causitave 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, 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 due to 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 demonstrated that *L. acidophilus* can protect from infection with *G. vaginalis*, *Bacteroides bivius*, and HIV through a peroxidase dependent mechanism (and *Coombs*, 1991; *Klebanoff* et *Klebanoff* al., 1991). Thus, while 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 demonstrated that the vaginal microbiome could be distinguished by ethnicity. Asian subjects had a higher prevalence of CST III (*L. iners*), non-Hispanic Caucasians had a higher prevalence of CST I (*L. 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 demonstrated the caution that must be utilized when performing analysis such as CSTs (*Koren* et al., 2013). In these investigations, Koren *et al.* demonstrated that these types of cluster analysis are sensitive to the distance metric used for analysis and that multiple distance metrics should be utilized to promote accuracy in data. In fact, this study utilized the data of Ravel *et al.* in their analysis and found varying support for the presence of CSTs based on the analysis used (*Ravel* et al., 2010; *Koren* et al., 2013). Thus, multiple methods of analysis must be used to establish a community state type. An additional method that may be useful to examine microbiome communities across ethnicities may be to utilize 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). While 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.

Variation of the vaginal microbiome during and across 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 hyperaemia 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). Oestrogen (namely oestradiol) 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 (*Paavonen*, 1983; *Gregoire* et al., 1971). This

metabolism of glycogen into lactic acid was historically thought to be performed by the vaginal epithelium, since the vaginal lumen is sufficiently 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 re-acidify the vagina *in vivo* following a neutralizing exposure (*i.e.* ejaculate) (*Boskey* et al., 1999; *Pybus* and *Onderdonk*, 1999). Afterwards, 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 oestrogen-induced increase in glycogen that contributes to the vaginal acidic environment, which is not only enhanced by lactobacilli but also fosters *Lactobacillus* growth.

Prior to the advent of metagenomics, the presence of lactobacilli were noted to increase as gestational age advanced during pregnancy (*Nieburgs*, 1947). Further, more recent studies demonstrated a high prevalence of *Lactobacillus* species in the vagina during either the first, second, or third trimester using PCR-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 demonstrated the presence of *Lactobacillus* species in the vagina during pregnancy. Our group initially published this 16S-based metagenomic characterization in a cross-sectional study employing V3V5 16S metagenomics (*Aagaard* et al., 2012b). When compared to the HMP non-pregnant 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 (*Aagaard* et al., 2012b) (Figure 2). When we examined this phenomenon further, we discovered that there was an overall enrichment of the orders Lactobacillus, Clostridiales, Bacteriodales, and Actinomycetales. And upon 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, L. jensenii,* and *L. johnsonii* (*Aagaard* et al., 2012b). The increase in lactobacilli may be explained by the increase in oestrogen that occurs during pregnancy. However, direct associations between specific species of *Lactobacillus* and oestrogen 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

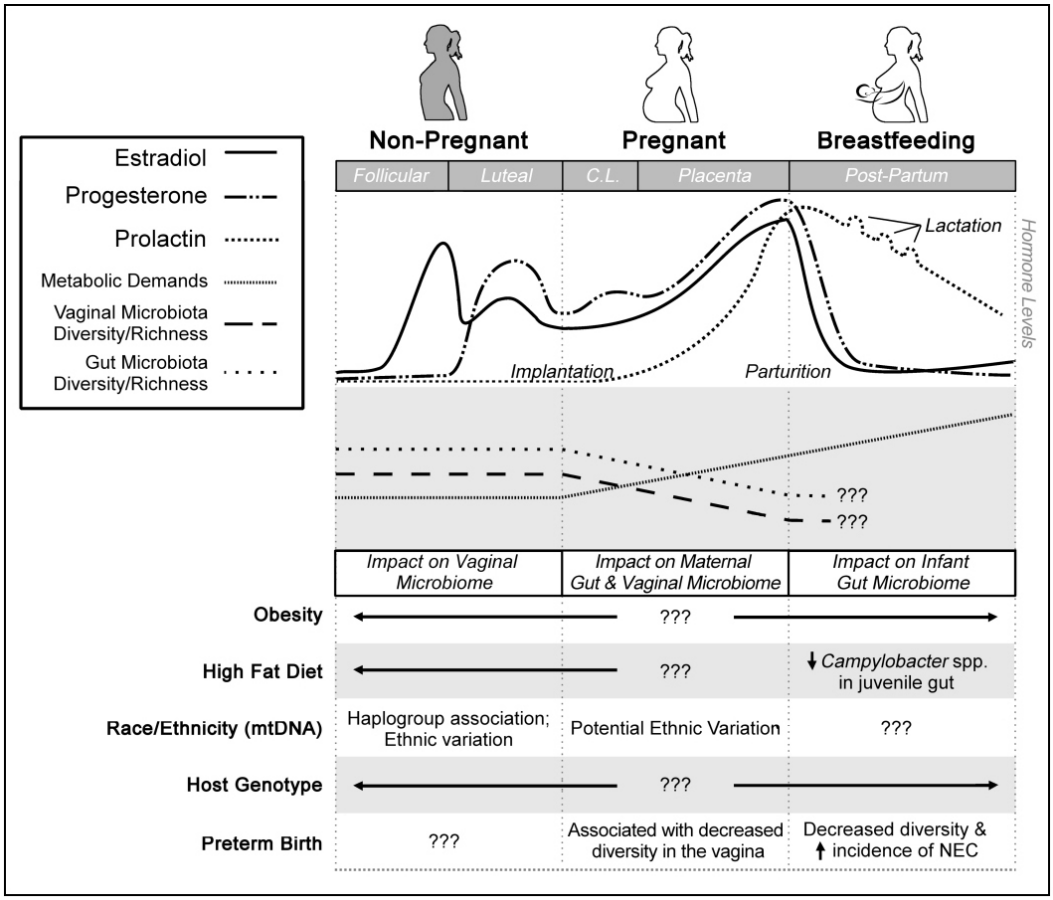


Figure 2: Potential influences on the microbiome during early development.
A number of hormonal changes, environmental exposures and genetic differences may impact the maternal microbiome before and during pregnancy that may alter the developing neonatal microbiome**.** During pregnancy, the maternal intestinal and vaginal microbiome have reduced alpha diversity and species richness. Metabolic demands increase throughout pregnancy and after parturition as the mother is lactating. Oestradiol, progesterone and prolactin levels gradually increase during pregnancy, though it is unclear how these changes affect the maternal microbiome. Increased oestrogen 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 demonstrated 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.

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 demonstrated that *L. crispatus* dominated during pregnancy and that prominence of *L.*

gasseri and/or *L. iners* during the first trimester may be associated with diminished lactobacilli as pregnancy progressed (*Verstraelen* et al., 2009). Recently, Romero *et al.* has 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., 2014). These studies utilized the self-collection of vaginal samples over 16 weeks of the non-pregnant 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., 2014). This study concluded that the vaginal microbiome of gravid women 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., 2014). 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., 2014). 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 towards CSTs dominated by lactobacilli as pregnancy progresses. Initial studies of the vaginal microbiome demonstrated that African-American women have a higher prevalence of vaginal CSTs consisting of *Lactobacillus* species (*Ravel* et al., 2010), which was further demonstrated by this recent study (*Romero* et al., 2014). 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 demonstrated that alpha diversity decreases as pregnancy progresses and that *L. crispatus* and *L. iners* dominate the vaginal microflora (*Walther-António* et al., 2014). (Figure 2) 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). While this insight should be kept in mind for future studies, these 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 utilized in these separate studies prevented in depth analysis (*Walther-António et al*., 2014; *Romero* et al., 2014). Despite these challenges, the authors found that while 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 while Caucasian gravidae did not (*Walther-António* et al., 2014). Thus, these studies highlight the need for further longitudinal studies with large subject enrolment and ethnic diversity. Additionally, studies are needed with a high enrolment 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.

How does the vaginal microbiome change among women destined for a preterm birth?

Knowing that term and preterm infants have a variant microbiome, if it were to be solely or even largely seeded by the maternal vagina, then the preterm and term vaginal microbiome would need to vary as well. Prior to the metagenomics era, the Preterm Prediction Study examined the association between BV and PTB (*Meis* et al., 1995). In this study, vaginal specimens were obtained at 24 and 28 weeks gestation, and an association with increased risk for spontaneous PTB at less than 35 weeks gestation was found in 19.8% of women with BV at 28 weeks gestation. However, conclusions could not be drawn regarding whether BV itself was causative of PTB (*Meis* et al., 1995). Additionally, while 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 weeks) 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.

A recent study by Hyman *et al.* 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 weeks) from preterm (< 37 weeks) subjects using Sanger sequencing methods. However, low-risk subjects had a higher prevalence of lactobacilli when compared to 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, while 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 non-pregnant (*Ravel* et al., 2010; *Aagaard* et al., 2012b; *Romero* et al., 2014). In sum, while promising, this study underscores the need for broader gestational age-specific, reference-based cohorts in order to define both the effect size and population variance. Prior to the publication of such studies, it would be premature to ascribe such microbiome profiling as indicative or heralding of preterm birth. Moreover, it does not explain the observed variation in the infants microbiome.

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

Maternal intestinal variation

In addition to changes described in the vaginal microbiome during pregnancy, Koren *et al.* investigated the intestinal microbiome during pregnancy using a prospective cohort study and found that as pregnancy progressed, the intestinal microbiome was altered (*Koren* et al., 2012). This study utilized the V1V2 region of the 16S rRNA gene and found that alpha diversity was decreased between the first and third trimesters (Figure 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 were a reflection of increases in Proteobacteria in the stool during the third trimester when compared to stool from the first trimester. When faecal 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 faecal 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 co-variates, such as body mass index (BMI), development of gestational diabetes mellitus, or multiparity (*Koren* et al., 2012).

Impact of the maternal diet

Additional alterations in the pregnant microbiome may also be regulated by diet. We have recently shown that diet is the main arbiter of the intestinal microbiome using a non-human primate model (*Ma* et al., 2014b), and a separate study demonstrated that consumption of a high fat diet during pregnancy resulted in impairment of the gut epithelial barrier integrity in a non-obese diabetic (NOD) mouse model (*Xue* et al., 2014). Intriguingly, this group found in two separate models, the NOD mouse model and a sheep model, that inflammation in the intestine of offspring was altered based on maternal diet (*Yan* et al., 2011; *Xue* et al., 2014). In fatal and postnatal life, offspring exposed to a maternal high fat diet had increases in the expression of inflammatory cytokines, Toll-like receptors (TLRs), and their respective signalling pathways (*Yan* et al., 2011; *Xue* et al., 2014). In relation to these studies, we found that maternal diet could persistently alter the intestinal microbiome of the offspring (*Ma* et al., 2014b) (Figure 2). Furthermore, these alterations in the intestinal microbiome resulted in differences in metabolic function using inferred analysis (*Ma* et al., 2014b). Thus, these aforementioned studies illustrate the need to not only expand the number of body sites across populations of women but to incorporate maternal comorbidities, such as diet, into analysis.

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 (*Onderdonk* et al., 2008a,b; *Olomu* et al.,

2009). 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 done 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 (*Stout* et al., 2013; *Cao* and *Mysorekar*, 2014). 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 the Murtha group showed that high levels of bacteria were strongly associated with premature rupture of membranes (PPROM) and membrane thickness, though there was no histological inflammation in half of the subjects. Although there were bacteria found in subjects of all groups including PPROM (both term and pre-term), PTB, and even normal gestation control, 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 over 300 microbial species in the chorioamnion and placenta (*Aagaard* et al., 2013). Interestingly, is was shown that this population was modifiable with a sterile intra-amniotic injection of IL-1ß 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 over 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

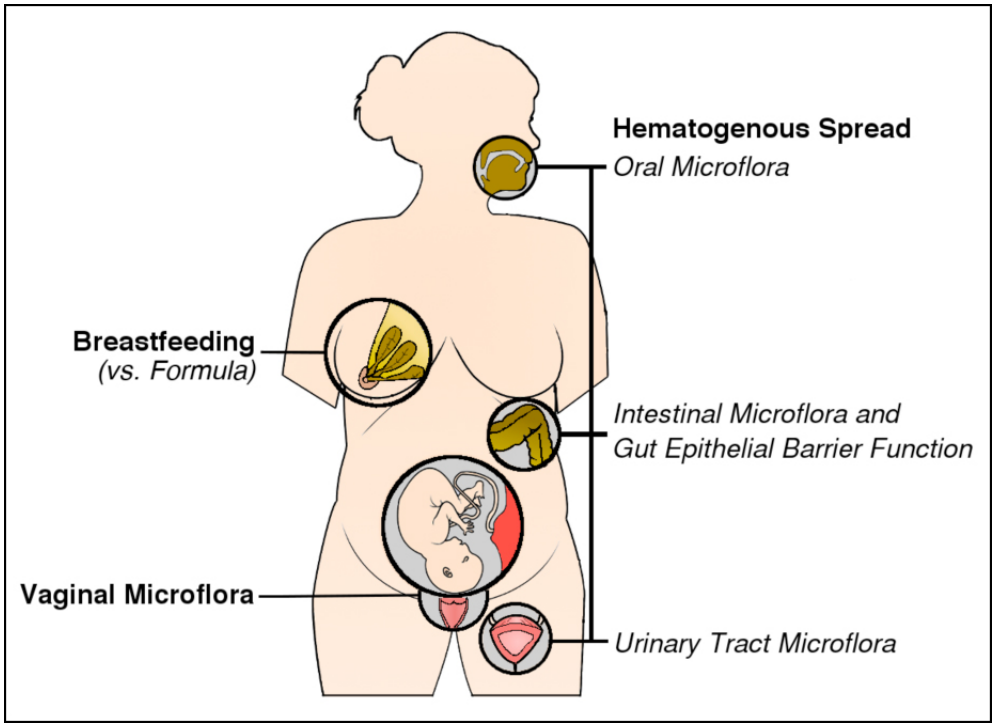


Figure 3: Speculated origins for microbiota colonizing the placenta and seeding the initial neonatal microbiome.
Vaginal microflora likely contribute to the initial seeding of the neonatal microbiome during vaginal deliveries, but the discovery that the uterine environment may not be sterile suggests 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 haematogenous route by which bacteria can seed the placenta and the developing foetus. Microbiota from maternal oral, vaginal, urinary tract and intestine are all potential sources for these colonizing bacteria. Microbiota from breast milk may be an important source of commensal bacteria during early infancy and must be considered when studying the microflora of the neonate.

instead, are quite possibly seeded largely from the oral cavity through haematogenous spread (Figure 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., 2000; *Michalowicz* et al., 2006; *Macones* et al., 2010). Animal models have demonstrated that bacteria may be spread haematogenously 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 haematogenous 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 haematogenous spread from mother to infant may be occurring via cord blood (*Kim* et al., 2009). Thus, haematogenous spread may promote colonization of the placenta and the foetus, but further studies are needed to examine this phenomenon. However, a potential mechanism to compromise the maternal-foetal 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 demonstrate definitively that haematogenous spread from the oral cavity is possible for colonization or infection of the placenta.

On-going 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 presence 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 which precedes clinical diagnosis (*Mai* et al., 2011, 2013). While 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 haematogenous 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. By the time this chapter reaches press, we do not doubt that more will be known and what we have described here will quickly be considered out-dated, archaic, and undoubtedly limited in scope and perspective. However, we would consider any such criticisms to be welcomed as they would be testament to the long-needed enthusiasm and interest in human reproductive biology from amongst the broader scientific community.

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 harbour 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. While 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 co-evolution of host and microbe. We are grateful to be a part of this highly collaborative and interrogative collective of metagenomic physician scientists, and look forward to the many discoveries ahead.

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