Review
Microbial Programming of Health and Disease Starts during Fetal Life

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The pioneer microbiota of the neonatal gut are essential for gut maturation, and metabolic and immunologic programming. Recent research has shown that early bacterial colonization may impact the occurrence of disease later in life (microbial programming). Despite early conflicting evidence, it has long been considered that the womb is a sterile environment and human microbial colonization begins at birth. In the last few years, several findings have reiterated the presence of microbes in infant first stool (meconium) and pointed to the existence of in utero microbial colonization of the infant gut. The dominant bacterial taxa detected in meconium specimens belong to the *Enterobacteriaceae* family (*Escherichia* genus) and lactic acid bacteria (notably members of the genera *Leuconostoc*, *Enterococcus*, and *Lactococcus*). Maternal atopy promotes dominance of *Enterobacteriaceae* in newborn meconium, which in turn may lead to respiratory problems in the infant. This microbial interaction with the host immune system may in fact, originate during fetal life. Our review evaluates the evidence for an intrauterine origin of meconium microbiota, their composition and influences, and potential clinical implications on infant health.

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Key words: meconium; microbiota; fetal programming; infancy; childhood diseases

Introduction

The human body is colonized by a large number of bacteria, archaea, viruses, and eukaryotic microorganisms, whereby the gastrointestinal tract is the most heavily inhabited organ with microorganisms (Ley et al., 2006; Dietert and Dietert, 2015). The adult human gut microbiota consists of approximately 10^{12} bacteria and more than 1000 prevalent species (Marchesi, 2010), that collectively contain about 100 times more genes than the human genome (Qin et al., 2010; Dietert and Dietert, 2015). The first microbial colonization of the gut is a critical developmental stage, shaping health and disease risk through microbiota-host interactions. Maternal and early life exposures can contribute to the development of the gut microbiota and cause gut dysbiosis, known as an imbalance of the normal bacterial composition (Penders et al., 2006; Azad et al., 2013; Arrieta et al., 2014). Dysbiosis of the infant gut has been linked to the development of several chronic and metabolic disorders, including asthma, allergic diseases, and obesity (Kalliomaki et al., 2008; Candela et al., 2012; White et al., 2013). Furthermore, the classic dogma that a fetus resides in a microbiologically sterile environment has been questioned in the last decade with findings that gut colonization starts in utero. The following review summarizes the evidence for in utero microbial colonization of meconium (infant first stool) microbiota, as well as the clinical implications of meconium microbial changes on health and disease in infancy (Fig. 1 and Table 1).

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Gut Colonization Soon After Birth: The Pioneer Microbes

The process of infant gut microbiota development is complex and influenced by many early life exposures, including mode of delivery (vaginal versus cesarean section), infant diet (breastfeeding versus formula), use of antibiotics and/or probiotics, and birth and home environment of the newborn and infant (Fig. 1B) (Vallès et al., 2012; Azad et al., 2013; Arrieta et al., 2014; Jakobsson et al., 2014). This process, along with the postnatal factors that influence it, has been extensively described in recent reviews (Arrieta et al., 2014; Mueller et al., 2015; Rodríguez et al., 2015). Although a discussion on postnatal infant gut colonization is beyond the scope of the current review, a brief overview is presented to provide background information to our primary focus on the meconium microbiota.

During vaginal delivery, the infant gut becomes colonized by maternal vaginal and fecal bacteria (*Lactobacillus, Prevotella* of the Bacteroidetes phylum, *Sneathia* of the Fusobacteria phylum); in contrast, infants born via cesarean section have greater exposure to microbes from the skin and the hospital environment (*Staphylococcus,*

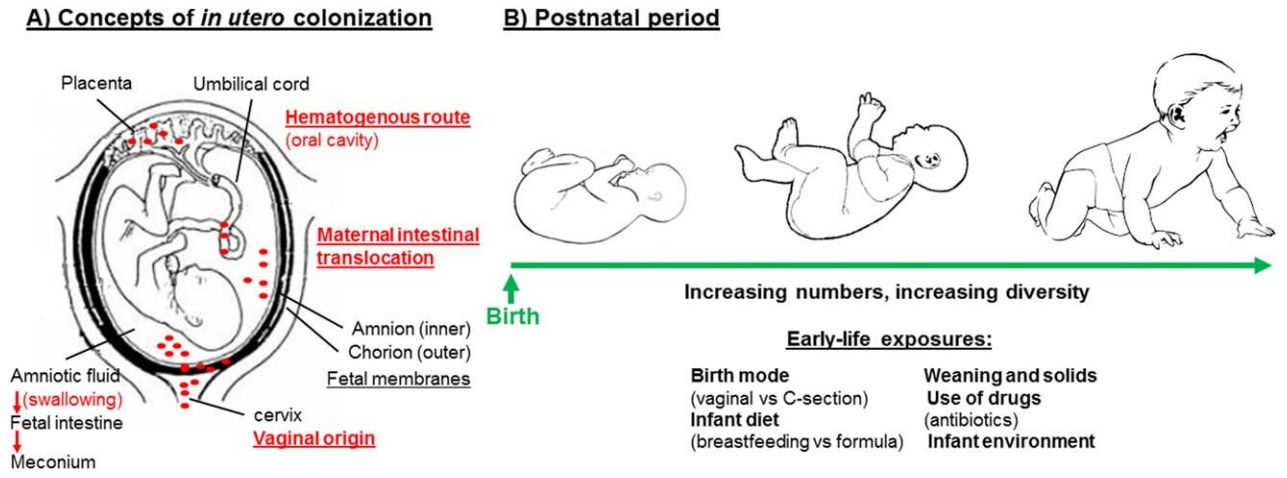

FIGURE 1. *Concepts of microbial origin in the in utero environment and their transmission meconium (***A***); early-life exposures demonstrated to influence infant gut microbiota development (***B***).*

Corynebacterium, Propionibacterium) (Dominguez-Bello et al., 2010). The pioneer colonizers of the infant gut are facultative anaerobes, such as members of the *Enterobacteriaceae*, that can tolerate the aerobic conditions of the newborn gut. Within the first few days of life, these first postnatal colonizers create an anaerobic environment in the intestinal lumen which allows strict anaerobes, such as members of the genera *Bifidobacterium, Clostridium*, and *Bacteroides*, to thrive (Pantoja-Feliciano et al., 2013). The gut microbiota of the newborn is characterized by lower bacterial diversity and higher inter-individual variability than that of adults (Yatsunenko et al., 2012). Moreover, gut microbiota of cesarean delivered infants are less diverse than those delivered vaginally (Jakobsson et al., 2014), and are enriched with *Clostridium difficile* and staphylococcal species (Penders et al., 2006). Infants delivered by cesarean section also harbor fewer *Bifidobacterium* and *Bacteroides* species compared with vaginally delivered infants (Penders et al., 2006; Biasucci et al., 2008; Azad et al., 2013).

The next major postnatal factor that influences the gut microbial composition of a growing infant is the diet (Morelli, 2008; Guaraldi and Salvatori, 2012). Comparisons between breast-fed and formula-fed infants have revealed that fecal samples from breast-fed infants generally contain larger populations of *Bifidobacterium* and *Lactobacillus*, whereas formula-fed infants are characterized by increased bacterial diversity and high prevalence of *C. difficile, Bacteroides, Streptococcus*, and *Veillonella* (Fallani et al., 2010; Bezirtzoglou et al., 2011; Azad et al., 2013). Human breast milk is abundant in bioactive ingredients required for healthy growth and development (Ballard and Morrow, 2013), including oligosaccharide polymers (human milk oligosaccharides, HMOs), which selectively stimulate the growth of bifidobacteria and lactobacilli (LoCascio et al., 2007; Marcobal et al., 2010).

In addition, human milk is thought to be a source of bacteria that further modify the acquisition and development of intestinal microbiota during infancy (Jeurink et al., 2013). Culture-dependent techniques have indicated the presence of mostly lactic acid bacteria, such as *Lactobacillus, Leuconostoc, Streptococcus, Enterococcus*, and *Weissella*, as well as *Bifidobacterium* and *Staphylococcus* (Jeurink et al., 2013). More comprehensive assessment of the human milk microbiota with culture-independent techniques and "omics" methods has further uncovered the presence of Gram-negative bacteria, including *Serratia, Pseudomonas, Veillonella*, and *Prevotella* (Hunt et al., 2011; Jeurink et al., 2013). Of interest, the complex ecosystem of human milk microbiota can be influenced by the same factors which affect gut microbiota, such as maternal overweight and delivery mode (Cabrera-Rubio et al., 2012).

Throughout the first year of life, gut bacterial diversity and richness continue to respond rapidly to changes in the infant diet (Fig. 1B). Introduction of solid foods marks another important shift in the gut microbiota composition, favoring the growth of polysaccharide fermenters such as *Bacteroides, Clostridium, Ruminococcus*, and *Faecalibacterium* (Vallès et al., 2014). By the end of the first year of life, the composition of the infant gut microbiota more closely resembles that of an adult; however, a typical adult microbial profile is not established until 2-3 years of age (Yatsunenko et al., 2012). Several new studies of fecal microbiota within a week after birth have shed additional light on the role of early microbial colonizers in this transition. Hesla et al. (2014) found a clear difference between gut microbial composition around 1 week of age and maternal microbiota during pregnancy, with Firmicutes microbes dominating maternal gut microbiota and Actinobacteria (bifidobacteria) as the dominant phylum in infants. Formula-feeding during the first week of life was a significant predictor of microbial composition 6 months

TABLE 1. *Summary of Studies on Meconium Microbiota*

Aim of study	Population studied	Method	Main significant findings	Reference
To culture and detect bacteria in the infant first specimen.	Healthy full-term infants (n = 100).	Culture-based	Bacteria were cultured from 38% of the specimens of meconium first passed.	Burrage, 1927
To investigate the bacterial presence in the infant first specimen.	Healthy full-term infants (n = 50).	Culture-based	Nineteen of the 50 tested meconium specimens (38%) contained bacteria. The majority of isolates were identified as *Micrococcus* spp.	Hall and O'Toole, 1934
To test the sterility of meconium.	Healthy full-term infants (n = 29).	Culture-based	6.3% to contain bacteria by direct culture and 36% by enrichment culture, the authors conceded the susceptibility of enrichment methods to contamination by skin microbes.	Snyder, 1936
To detect presence of bacteria in meconium.	Healthy full-term infants born by either vaginal delivery or cesarean section (n = 21).	Culture-based	Predominance of isolates belonging to *Enterococcus* and *Staphylococcus*.	Jiménez et al., 2008
To evaluate early intestinal microbial ecology in premature infants.	Infants born at 23 to 32 wk gestational age (n = 23).	PCR-based; DGGE profiling	Detection of microbial DNA in 91% of meconium samples (21/23). Detection of differences in meconium species diversity according to maternal chorioamnionitis and lower gestational age (<30 wk).	Mshvildadze et al., 2010
To study the impact of delivery mode on first bacterial communities in infant gut.	Healthy full-term infants born vaginally (n = 4) or by cesarean section (n = 6).	16S rRNA gene pyrosequencing	Meconium bacterial communities were undistinguishable from other body habitats, such as skin and oral mucosa, but similar to vaginal and skin microbiota depending on delivery mode.	Dominguez-Bello et al., 2010
To understand the gut microbial colonization in prematurity as a strategy to predict risk of sepsis.	Infant born at 24 to 27 wk gestational age (n = 6).	16S rRNA gene pyrosequencing (V6 region)	Meconium of all subjects was not sterile and was dominated with bacteria belonging to *Staphylococcus* and *Enterobacteriaceae*. Less diverse meconium microbiota was associated with the development of late-onset sepsis.	Madan et al., 2012

TABLE 1. *Continued*

Aim of study	Population studied	Method	Main significant findings	Reference
To characterize the meconium microbiota and determine if the bacterial community is affected by maternal diabetes status.	Full term infants born either vaginally (n = 13) or by cesarean section (n = 10).	16S rRNA gene SMRT sequencing (V3-V4 region)	Bacteria were detected in all the meconium samples and dominated by members from Proteobacteria phylum. Meconium microbiota was characterized with lower-species diversity and higher inter-individual variability compared with adult feces, and was affected by maternal diabetes status.	Hu et al., 2013
To characterize the meconium microbiota of term infants and determine the impact of maternal factors on meconium microbiota and subsequent consequences on child health.	Full-term infants born either vaginally (n = 14) or by cesarean section (n = 6).	16S rRNA gene pyrosequencing (V2-V3 region)	Bacteria were detected in all meconium samples and their profile resembled the fecal microbiota of young infants. Meconium microbiota was influenced by maternal eczema and was associated with respiratory problems in infants.	Gosalbes et al., 2013
To study the meconium microbiota and the development of fecal microbiota of preterm neonates during the first month of life.	Preterm infants with gestational age <32 wk, and delivered vaginally (n = 7) or by cesarean section (n = 7).	Culture-based; DGGE profiling; HITChip microarray	Meconium microbiota was characterized with inter-individual differences and dominated with bacilli, *Staphylococcus*, *Lactobacillus*, and *Streptococcus*. *Serratia* genus in meconium was strongly associated with higher degree of immaturity.	Moles et al., 2013
To determine any association between meconium microbiota and preterm birth.	Infants with gestational age ranging from 23 to 41 wk (n = 52).	16S rRNA gene Ion Torrent sequencing (V4 region)	Gestational age influenced meconium colonization. *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Photorhabdus* and *Tannerella* genera were negatively correlated with infant gestational age.	Ardissone et al., 2014
To confirm or refute sterility of meconium.	Infants with 37 to 40 wk gestational age, vaginally-delivered (n = 15).	FISH; PCR detection	FISH analysis revealed evidence of bacterial presence in 66% of meconium samples (10/15). Of these 10 samples, only 1 sample was positive with PCR amplification using generic bacterial primers.	Hansen et al., 2015

TABLE 1. *Continued*

Aim of study	Population studied	Method	Main significant findings	Reference
To determine if bacterial antibiotic resistance genes, such as β-lactam antibiotics and tetracycline, are present in meconium and in feces at 1 wk of age.	Healthy full-term infants (n = 20). Six born by cesarean section.	PCR screening	Bacterial antibiotic resistance genes were detected in 70% (14/20) of the meconium samples. High prevalence of resistance to β-lactam antibiotics and tetracycline was also detected in fecal samples from 1-wk-old infants.	Gosalbes et al., 2015
To characterize the gut microbiota establishment during the first year of life. Fecal samples were obtained at birth, 4 months and 12 months.	Healthy full-term infants born either by cesarean section (n = 15) or vaginally (n = 83).	Metagenomic shotgun sequencing	Meconium microbiota resembles the fecal microbiota at 4 months, whereas fecal microbiota composition at 12 months was more similar to the mothers' microbiota.	Bäckhed et al., 2015

PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; SMRT, single molecule real-time; HITChip, human intestinal tract chip; FISH, fluorescence in situ hybridization.

later. Dogra et al. (2015) identified several features of early colonization (within 3 days of birth) that predicted future microbial composition. Neonates with greater initial abundance of *Streptococcus* and *Enterobacteriaceae* had a lag in bifidobacterial colonization than those without this profile. Both papers reported delayed colonization with *Bacteroides* and/or *Bifidobacterium* species following a cesarean section delivery.

Meconium Microbiota: The First Colonizers of the Gut

Escherich (1886) first reported the microbiology of the neonatal gut using culture-based techniques to recover and classify bacteria. He noted that "pure meconium contains no trace of microbial elements" but that a rich microbial flora is present by the eighth day of life. From these observations, Escherich established the prevailing successional model of gut microbial development, in which the lumen of the gut is sterile at birth and progressively becomes colonized by bacterial species, whose abundance and variety change over time to culminate into a relatively stable microbial composition seen throughout adulthood.

For well over a century, Escherich's proposition that the fetal gastrointestinal tract is essentially absent of microbial colonization has remained generally accepted, despite early suggestions of microbially active meconium that conflicted with this hypothesis. Burrage (1927), for example, found bacteria present in 38% of meconium samples from nearly 100 infants. Hall and O'Toole (1934) observed positive bacterial cultures in the same fraction of aseptically collected meconium samples from 50 subjects. Using rectal cannulation and intra-rectal swabbing of newborns, Snyder (1936) found 6.3% of samples to contain bacteria by direct culture and 36% by enrichment culture, although he conceded the susceptibility of enrichment methods to contamination by skin microbes. One case, which he interpreted to be especially compelling of in utero colonization, involved the recovery of *Lactobacillus acidophilus* by culture without enrichment from a 9-min old baby whose perianal skin was sterile.

Further inquiries into the sterility of meconium essentially ceased until the work of Jiménez et al. (2008) who, using careful culture techniques, confirmed the nonsterility of infant meconium shown by earlier researchers. However, these authors further demonstrated in utero transmission of gut microbiota, using a mouse model in which a genetically distinctive strain of *Enterococcus faecium* administered maternally by oral instillation was detected by polymerase chain reaction (PCR) in the intestinal lumen of pups delivered 1 day prematurely by cesarean section. Using non-culture-based techniques, Mshvildadze et al. (2010) provided further evidence on the presence of bacteria in meconium obtained from neonates born at 22 to 32 weeks gestational age. These

authors detected microbial DNA in 91% of meconium samples (21 of 23), and denaturing gradient gel electrophoresis (DGGE) profiling revealed an association between prematurity and reduced meconium microbial diversity (Mshvildadze et al., 2010). A later study by Moles et al. (2013) aimed to characterize the evolution of gut microbiota during the first month of life of preterm neonates, including the meconium microbiota. The bacterial diversity and taxonomy of 14 meconium specimens were examined by culture and molecular techniques, such as DGGE and Human Intestinal Tract Chip (HITChip) microarray. Interindividual differences were detected in meconium microbial profiles; bacilli, *Staphylococcus*, *Lactobacillus*, and *Streptococcus* members of the Firmicutes phylum predominated in the infants' first stools.

The application of next generation DNA sequencing techniques has cast further doubt on meconium sterility. In a prospective longitudinal study, Madan et al. (2012) applied high throughput pyrosequencing of the hypervariable V6 region of the 16S rRNA gene to study gut microbial colonization in prematurity. The authors found that the meconium specimens obtained from six preterm neonates were not sterile, and the predominant bacterial genera were *Lactobacillus*, *Staphylococcus*, and *Enterobacteriaceae*. The association of bacterial colonization of meconium with prematurity was also studied by Ardissone et al. (2014). The analysis of meconium collected from 52 infants with gestational age ranging from 23 to 41 weeks displayed low bacterial diversity, and microbial colonization was correlated with gestational age (Ardissone et al., 2014). This study provided further evidence for intrauterine origin of meconium microbiota, by revealing a high similarity between meconium and amniotic fluid microbial profiles.

Using 16S rRNA gene pyrosequencing, Dominguez-Bello et al. (2010) studied the impact of birth mode on bacterial communities in a rectal swab obtained at birth. They found that the meconium microbiota composition of full-term infants was influenced by the mode of delivery. Genera *Lactobacillus, Prevotella, Atopobium*, and *Sneathia* were common in vaginally-born infants, whereas staphylococci predominated after cesarean delivery. Gosalbes et al. (2013) also employed pyrosequencing to examine the meconium microbiota of 20 full-term newborns from a Spanish birth cohort. They found that meconium microbiota were dominated by lactic acid or enteric bacteria, and resembled the microbiota of infants' fecal samples (Gosalbes et al., 2013). Hansen et al. (2015) investigated the meconium microbiota of 15 healthy full-term vaginally-delivered infants using a different molecular approach, fluorescent in situ hybridization (FISH) with 16S rRNA-targeted probes. The FISH results revealed evidence of microbial presence in 66% (10 of 15) of the infants. Members belonging to *Bifidobacterium*, *Enterobacteriaceae*, *Enterococcaceae*, and *Bacteroides-Prevotella* predominated in meconium.

The existence of a diversified meconium microbiota in full-term infants ($n = 23$) but less so than in adult fecal samples and with higher sample-to-sample variation, was also reported by Hu et al. (2013) using next generation sequencing methods. In a recent pilot study of our group, 57 meconium samples from the Winnipeg site of the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort were characterized by Illumina 16S rRNA sequencing of the hyper-variable V4 region (Koleva et al., 2015). We found that 23% of meconium specimens obtained from healthy, full-termed infants were colonized with bacteria. Similar to Gosalbes et al. (2013), samples were dominated by enteric or lactic acid bacteria. Further analyses on a larger sample size of the CHILD cohort are underway to reproduce these findings and test the association of meconium microbiota with childhood health outcomes.

In summary, culture and molecular techniques have provided preliminary evidence for diverse assemblages of bacteria in meconium from both term and preterm infants. The origin of detected microbes in meconium, however, remains unclear. Similar to early fecal microbiota, meconium microbial communities have low species diversity and high interindividual variability (Hu et al., 2013; Moles et al., 2013). At the phylum level, meconium microbiota more closely resemble the gut microbes of infants than of adults (Dominguez-Bello et al., 2010). On the other hand, when compared with fecal samples at 3 and 12 months, meconium was found to be less abundant with *Bacteroides* and *Bifidobacterium* species but more likely to be colonized with *Escherichia-Shigella* and *Enterococcus* (Bäckhed et al., 2015). Moreover, the high similarity between meconium and amniotic fluid microbes (Ardissone et al., 2014), and the fact that large quantities of amniotic fluid are swallowed by the fetus in the last trimester of pregnancy (Gilbert and Brace, 1993), points to the notion that meconium microbiota have an intrauterine origin (Fig. 1A). As reviewed in detail in the next section, these findings contradict the classic dogma of the newborn emerging from a sterile environment and suggest that the establishment of intestinal microbiota is initiated in the prenatal gut.

Evidence for in Utero Origins of Meconium Microbiota

Until recently, the in utero environment has been considered sterile under normal conditions, and the establishment of our gut microbiota was thought to start at birth when the infant is exposed to the mother's microbiota and the environment. Contrary to the classic "sterile womb" dogma, microbes have been found in placental tissue (Aagaard et al., 2014), umbilical cord blood (Jiménez et al., 2005), fetal membranes (Jones et al., 2009), amniotic fluid

(DiGiulio, 2012), and meconium (Moles et al., 2013). The presence of microbes in the in utero environment is viewed as a potential danger to the developing fetus, since microbes have been cultured in preterm delivery and/or intrauterine infection. On the other hand, recent studies have provided evidence of the presence of uterine microbiota in healthy, term infants without any indications of infection or inflammation (Pettker et al., 2007; Jiménez et al., 2008; Jones et al., 2009; Gosalbes et al., 2013), suggesting the functionality of bacterial exposure pathways in the developing fetus.

PLACENTA

Bacterial colonization of placental tissue is not unexpected with intrauterine infection and preterm birth (Pararas et al., 2006; Stout et al., 2013), but a commonly-held belief is that the placenta protects the fetus from microbes throughout a healthy pregnancy. Yet, bacteria have also been detected in the placenta in full-term deliveries and in the absence of infection. Positive placental cultures have been documented in women with no intra-amniotic infection or inflammation, and interestingly, microbes have been isolated from the placenta of women who delivered by elective cesarean section with a low risk of contamination of the placenta (Pettker et al., 2007). Using culture-dependent and species-specific PCR, Satokari et al. (2008) characterized the presence of *Bifidobacterium* and *Lactobacillus* DNA in 34 placenta samples obtained from women following full-term delivery. DNA from bifidobacteria and lactobacilli was detected in most placental samples, and no differences in the abundance of microbial DNA were observed between vaginal and cesarean section deliveries. More recently, investigations into the presence of microbiota in placental specimens have used 16S ribosomal RNA and whole-genome techniques (Aagaard et al., 2014). The Aagaard et al. (2014) study of 320 women revealed that the placenta harbors a low-abundant but metabolically rich microbiota, with members belonging to the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria.

AMNIOTIC FLUID

Another compartment of the in utero environment, amniotic fluid, also frequently contains significant levels of microbes, even in the absence of amniotic sac ruptures (DiGiulio et al., 2008; Romero and Yergeau, 2014). Microbial presence in the amniotic fluid, determined by cultivation and PCR-based techniques, has been linked to prematurity and intra-amniotic infection (DiGiulio, 2012). Common species cultivated from the amniotic fluid with intra-amniotic infection or prematurity are *Ureaplasma* spp. and *Streptococcus* spp., *Mycoplasma* spp., *Fusobacterium* spp., *Bacteroides* spp. and *Prevotella* spp. (DiGiulio, 2012). Other uncultivated or difficult-to-cultivate bacterial taxa are also commonly found in the amniotic fluid, using taxon-specific PCR techniques based on the 16S ribosomal DNA. Overall, the majority of bacteria in the intra-amniotic cavity in preterm labor with intact membranes belong to two different phyla: Fusobacteria, non-spore forming Gram-negative bacilli, and Tenericutes, microorganisms distinguished by the absence of a cell wall (DiGiulio et al., 2008; Han et al., 2009; Marconi et al., 2011; DiGiulio, 2012). The rest are classified as bacteria belonging to the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria phyla, that predominate in most human-associated habitats. In his review, DiGiulio (2012) summarized that amniotic fluid microbial profiles differ between preterm labor with intact membranes and preterm labor with rupture of the membranes. The phylum Fusobacteria is the most abundant (31%) in preterm labor with intact membranes. Relative proportions of the Bacteroidetes and Firmicutes phyla also appear to vary quite significantly across various clinical phenotypes (DiGiulio, 2012).

Of interest, the bacteria of the intra-amniotic cavity of women in spontaneous labor at term are similar to the microbes found in the amniotic fluid of women in preterm labor (Romero et al., 1992, 1993). The most common microbes isolated from the amniotic fluid in spontaneous term labor are *Ureaplasma* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Fusobacterium* spp. These studies published by Romero et al. (1992, 1993) used culture-based methods for detection; identification by novel 16S rRNA-based techniques is required to further interrogate the microbial profile of the amniotic fluid in spontaneous term labor. It is still not clear whether the presence of microbes in the amniotic fluid always causes preterm delivery or some of those detected microbes are normal residents of the in utero environment.

CORD BLOOD (REPRESENTING FETAL TISSUE)

Very few studies have investigated the presence of bacteria in umbilical cord blood obtained at birth (Jiménez et al., 2005; Goldenberg et al., 2008a). Goldenberg et al. (2008a) detected *Ureaplasma urealyticum* and/or *Mycoplasma hominis* in 23% of umbilical cord blood samples from neonates delivered at 23 to 32 weeks. Neonates with a positive cord blood culture for *U. urealyticum* and/or *M. hominis* were significantly more likely to have positive placental cultures. However, they did not report the presence of other bacteria. In contrast, Jiménez et al. (2005) detected commensal microbes in their study of healthy full-term neonates born by elective cesarean section (Jiménez et al., 2005). Isolates identified from cord blood were species belonging to *Enterococcus, Streptococcus*, *Staphylococcus*, and *Propionibacterium* genera; no Gram-negative bacteria were cultured. Further research with the use of high throughput sequencing techniques is needed to comprehend fully the microbial composition of umbilical cord blood, ideally with sampling from the umbilical

artery to more closely represent the colonization status of the fetus.

SOURCE OF MICROBES IN THE PLACENTA, AMNIOTIC FLUID, AND FETUS

Altogether, these findings strongly suggest that the first contact with bacteria for many infants is not at birth, but begins in utero (Fig. 1A). How colonization of the placenta, amniotic fluid and fetus occurs, remains unclear. Microbes potentially gain access to the in utero environment through ascent from the vagina, and/or originate from the intestine and oral cavity through the blood stream. One concept of in utero colonization suggests that microbes found in the amniotic fluid translocate through the fetal membranes from the vagina (Romero et al., 2007; Goldenberg et al., 2008a,b). Recent studies have reported on the presence of bacteria in both preterm and full term intact fetal membranes (Steel et al., 2005; Jones et al., 2009), which potentially originated from the cervix. Another hypothesis suggests that bacteria in the in utero environment originate from the maternal intestine through the blood stream (hematogenous route). Generally, gut epithelial cells act as a physical barrier to separate the mammalian host from the external environment and prevent microbial entry into the blood stream (Peterson and Artis, 2014). One possible route for translocation may be via the dendritic cells in the lamina propria, which can penetrate the gut epithelium, phagocytize live bacterial cells on the luminal side of the gut, and mediate their translocation through blood and lymph (Vazquez-Torres et al., 1999; Rescigno et al., 2001).

Perez et al. (2007) documented microbial translocation during pregnancy in C57/BL6 mice model. Using culture-based methods, the study examined bacterial translocation to extra-intestinal tissues in conventional non-pregnant, pregnant, and lactating mice. Pregnant animals were 60% more likely to harbor microbes in their mesenteric lymph nodes compared with nonpregnant animals. Evidence of bacterial translocation from the maternal gut to that of the neonate has been provided in another two experimental studies with mice by Jiménez et al. (2005, 2008). Using culture-dependent and PCR-based techniques, a genetically labeled strain of *Enterococcus faecium* was detected in the amniotic fluid and meconium of term offspring after sterile cesarean section (Jiménez et al., 2005, 2008). Another potential source of intrauterine bacteria may arise from hematogenous transport of microbes from the maternal oral cavity. Taxa common to the human mouth have been frequently reported in amniotic fluid cultures of women with intact amnions (Hill, 1998; Bearfield et al., 2002; DiGiulio, 2012). In fact, it has been also reported that placental microbiota contain more species common to the oral cavity than the urogenital tract (Han et al., 2006; Fardini et al., 2010). A recent study has further revealed a similarity between the placental taxonomic profile and the human oral microbiota (Aagaard et al., 2014). Taken together, these observations are intriguing, but require additional research to reveal the origin of microbes in the in utero environment, and determine their role in infant gut colonization and maturation of the immune system.

Role for in Utero Microbes in Shaping the Fetal Immune System

Akin to historic beliefs that the fetal environment is maintained sterile, so was the old view of an immature and inactive fetal immune system (Brugman et al., 2015). It is becoming increasingly apparent that the fetus is exposed to environmental antigens through its mother; the fetal immune system interacts closely with, and is influenced by, the maternal immune system, both physiologically and metabolically (Hsu and Nanan, 2014; Romano-Keeler and Weitkamp, 2015). Even though the placenta acts as a physical barrier between the mother and the fetus, various maternal factors, such as hormones, cytokines, maternal gut bacteria, and possibly their metabolites, can also be transmitted to the intrauterine environment and impact the fetal immune system (Hsu and Nanan, 2014). Please see Kollmann et al. (2012) for an overview on the development of the immune system during infancy, and Brugman et al. (2015) for a comprehensive review on early influences of this development.

Direct interaction between in utero microbes and the fetal immune system could potentially occur through Toll-like receptors (TLRs). TLRs are a class of pattern recognition receptors present on macrophages, mast cells, and dendritic cells that are critical components of the innate immune system (Kawai and Akira, 2010). Various types of TLRs can recognize distinct pathogen-associated molecular patterns and trigger an inflammatory response. Signaling and expression of TLRs in newborns is well established (Strunk et al., 2011; Kollmann et al., 2012); however, production of innate immune effector molecules in response to TLRs stimulation is diminished in early life (Kollmann et al., 2012). Additionally, there are variations in innate immune responses within different populations of infants (Smolen et al., 2014). TLRs, particularly TLR2 and TLR4, recognizing lipopylsaccharides on the cell wall of bacteria, have also been detected in the fetal small intestine (Fusunyan et al., 2001). Further, expression of various TLRs is up-regulated in fetal lung tissues from the early stages of lung development (Petrikin et al., 2010).

Maternal gut bacteria can also influence development of the fetal immune system through their short chain fatty acids (SCFA) metabolites. SCFA have been shown to induce T-regulatory cells in the gut (Atarashi et al., 2013; Smith et al., 2013), and may indirectly affect T-regulatory cell homeostasis by modulating IL-10 production (Meijer et al., 2010; Liu et al., 2012). SCFA are carried across epithelial cells via monocarboxylate transporters (MCTs), which are proton-linked plasma membrane transporters located on

gut epithelial cells. A study by Nagai et al. (2010) has reported that MCTs are abundantly present in a rodent placenta, suggesting that SCFA could be also transported across the placenta.

Finally, intrauterine bacteria have the capacity to interact with immune system of the fetus via other metabolites. Recently, mucosa-associated invariant T cells (MAIT) have been discovered in fetal tissues in the second trimester (Corbett et al., 2014; Leeansyah et al., 2014). They recognize microbial-derived riboflavin metabolites and are found to be highly abundant in the small intestine, lung, and liver of the fetus. In response to microbial stimulation, MAIT cells are able to produce interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-17 (IL-17) (Kjer-Nielsen et al., 2012; Corbett et al., 2014). Enhanced proliferative capacity of the MAIT cells have been reported in the fetal small intestine compared with adult MAIT cells (Leeansyah et al., 2014). The fact that fetal immune tissues possess MAIT cells which can recognize microbial-derived metabolites further provides evidence for in utero microbial activity.

In Utero Influences on Meconium Microbiota

Maternal health conditions and risk factors, as well as medical interventions can contribute to the initial colonization and shaping of the meconium microbial community. We have already described the dysbiosis of gut microbiota seen in infants born by cesarean section (Azad et al., 2013; Hesla et al., 2014; Dogra et al., 2015). In our new publication describing 198 infants from the CHILD study, maternal intrapartum antibiotic prophylaxis for cesarean section or group B *Streptococcal* vaginal colonization significantly lowered *Bacteroides* spp. abundance and elevated genus *Clostridium* and *Enterococus* at 3 months (Azad et al., 2015a). Using DGGE profiling to detect microbes in meconium, Mshvildadze et al. (2010) assessed meconium microbial diversity according to pre- and perinatal factors. Lower microbial diversity was reported in meconium samples obtained from infants whose mothers received intrapartum antibiotics (Mshvildadze et al., 2010). Of interest, Hu et al. (2013) found no global differences in meconium microbial composition between vaginal and cesarean delivery in a small sample of healthy pregnancies ($n = 9$).

Independent of birth method, maternal overweight during pregnancy can also alter gut microbial composition in offspring. Using FISH and qPCR methods, Collado et al. (2010) found that infants born to mothers with excessive weight gain during pregnancy had lower bacterial counts of genus *Bacteroides-Prevotella* at 1 month of age, compared with infants born to mothers with normal weight gain. Gestational diabetes is common in overweight women during pregnancy. Using a culture-independent sequencing approach, Hu et al. (2013) evaluated meconium microbiota composition and diversity according to maternal gestational and pre-gestational type 2 diabetes. The bacterial diversity of meconium was elevated following prenatal diabetes, as was the abundance of *Bacteroides, Parabacteroides*, and *Lachnospiraceae* microbiota, especially among infants born to mothers with pre-existing diabetes (Hu et al., 2013). Gosalbes et al. (2013) studied the meconium microbial community, according to maternal history of atopic diseases, including asthma, rhinitis and eczema. In their study, shifts in meconium microbial composition were linked to prenatal maternal eczema, but not to maternal asthma or rhinitis. Notably, higher abundance of members belonging to *Enterobacteriaceae* family, and lower meconium microbial diversity and richness were associated with maternal eczema (Gosalbes et al., 2013).

Meconium Microbial Dysbiosis and Future Health

Several recent reviews have been published on the establishment of gut microbiota during the postnatal period and on the health outcomes of microbial dysbiosis (Arrieta et al., 2014; Muller et al., 2015; Rodríguez et al., 2015). This year, our group reported on two infant gut microbial predictors of food sensitization at age 1: low microbial diversity and a higher ratio of *Enterobacteriaceae/Bacteroidaceae* abundance at age 3 months (Azad et al., 2015b). Similar evidence for associations with meconium microbiota is sparse.

It has been long suspected that a relationship exists between intrauterine microbes and preterm delivery (Mshvildadze et al., 2010; Madan et al., 2012; Moles et al., 2013; Ardissone et al., 2014). As noted earlier, the Ardissone et al. (2014) group found an association between bacterial colonization of meconium and newborn gestational age. The *Enterobacter, Enterococcus, Lactobacillus, Photorhabdus*, and *Tannerela* genera were present more often in the meconium of preterm infants. In addition, Madan et al. (2012) documented that low species diversity in meconium specimens of preterm infants elevated the risk for neonatal sepsis. The meconium of infants who developed sepsis was dominated with species belonging to Proteobacteria and Firmicutes (*Staphylococcus*).

To our best knowledge, only the study by Gosalbes et al. (2013) has tested associations between microbial composition of meconium and disease in older children. Comparisons of meconium microbiota were conducted according to atopy-related problems among children in the first and fourth year of life. Meconium samples dominated by *Leuconostoc, Enterococcus, Lactococcus, Streptococcus*, and *Staphylococcus* genera, and members from the *Enterobacteriaceae* family, were more common in 1-year-old infants with mucus congestion (Gosalbes et al., 2013).

Conclusions and Future Research Directions

Studies on the meconium microbiota are accumulating to provide new insights on how initial colonization shapes

microbial composition of the infant gut, and future health and disease. Intrauterine influences on the development of meconium microbiota are largely unknown and speculative. Prenatal microbial colonization may play an important role in the adaption of the fetus for life after birth, and as such, requires rigorous testing to detect the presence of microbes and confirm their role. In this review, we have provided evidence for the existence of a fetal gut microbiome which can interact with the fetal immune system. As evident by changes in meconium composition, maternal immune and metabolic status during pregnancy has the capacity to shape the fetal microbiome. Newer metagenomic studies, such as those by Gosalbes et al. (2015), also tell us that the fetal microbiome can be easily influenced by maternal antibiotic treatment. Their study detected the presence of bacterial β-lactam or tetracycline resistance genes in 70% (14 of 20) of meconium specimens obtained from 20 full-term term healthy infants using PCR methods.

Historically, the investigation of the "fetal microbiome" was accomplished by direct cultivation, enumeration and identification of microbes on growth media. Though informative, these approaches tended to over-represent highly prolific microbes, notably saprotrophic contaminants, while failing to report fastidious autochthonous microorganisms. The advent of culture-independent, high-throughput sequencing has revolutionized our ability to describe entire communities comprising the "fetal microbiome" irrespective of their viability or culturability (Furrie, 2006; Hamady and Knight, 2009; Ignys et al., 2014). Sequence analysis of the 16S ribosomal RNA rRNA gene has emerged as a preferred culture-independent approach to better identify poorly described and rarely isolated bacterial strains. Furthermore, the development of next-generation sequencing has vastly simplified and improved the sequencing depth for 16S rRNA gene sequencing (Sanschagrin and Yergeau, 2014). However, limitations of the culture-independent methods, and particularly the next generation sequencing techniques, are their computational complexity and high cost. Caution is needed in the interpretation of results from low biomass samples such as placenta, as allochthonous contaminants may be erroneously interpreted to be indigenous microbiota. Aseptic sampling and preparatory techniques are of paramount importance in handling these specimens to reduce the potential for contaminant introduction. Another limitation of 16S rRNA gene sequencing is that it only provides insight into the taxonomic composition of a microbial community. An alternative approach to study the microbial biodiversity, as well as the metabolic functions of bacterial taxa in a sample, is the relatively new shotgun metagenomic DNA sequencing technique (Sharpton, 2014). Due to the large and complex structure of the data, and alignment with less well annotated metabolic pathways, metagenomic sequencing is also challenging to conduct (Sharpton, 2014).

Many questions remain unanswered regarding microbial colonization of the intrauterine environment of healthy pregnancies. We still know very little about the number and identity of commensal microbes that traverse the placenta, where they originate, whether they are alive or persist in the newborn infant, whether they prime fetal immune and metabolic development, and whether their presence has long-term health consequences for the child. Further interrogation of meconium bacteria associated with preterm deliveries and maternal health complications is needed to inform strategies for the prevention of diseases in later childhood.

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