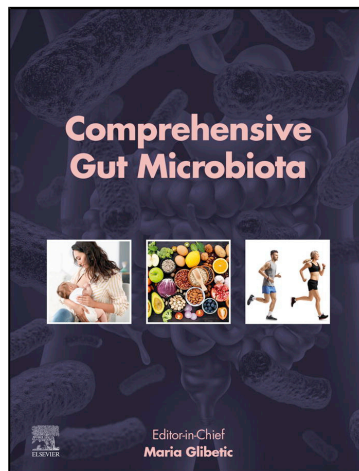


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2.02 Microbiota *in utero*? When and Where Microbial Establishment Starts?

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Glossary

16S hypervariable region One of a series of hypervariable sequence regions within the 16S rRNA, designated 5' to 3' as V1–V9 as a basis to distinguish bacterial taxa (e.g., V4 as the fourth variable region).

16S rDNA The transcript of the 16S ribosomal subunit gene, the smaller RNA component of the prokaryotic ribosome, used as the most common taxonomic marker for microbial communities.

Actinobacteria Phylum of bacteria.

Amnion Membranous sac which surrounds and protects the embryo.

Amniotic fluid Liquid contained in the amniotic sac that protects the growing fetus composed mainly by water and some nutrients.

Archaea Domain of single-celled prokaryotes organisms different from Bacteria.

Bacteroidetes Phylum of bacteria.

Chorion The outermost fetal membrane around the embryo in mammals.

Decidua The modified endometrium during pregnancy.

DNA Deoxyribonucleic acid that contains the instructions needed for an organism to develop, survive and reproduce.

Dysbiosis Alteration of the microbial balance of the microbiota.

Firmicutes Phylum of bacteria.

Fluorescent in situ hybridization (FISH) Molecular technique that uses fluorescent probes that bind to complementary nucleic acid sequences and allows to detect and localize the presence of DNA sequences.

Germ-free gnotobiotic A host animal containing no microorganisms.

Gnotobiotic A host animal containing a defined set of microorganisms, either synthetically implanted or transferred from another host; often used to refer to model organisms with humanized microbiota.

Gram stain Traditional bacterial staining technique that differentiates bacteria into gram-positive and gram-negative groups by detecting peptidoglycan in the cell wall of gram-positive organisms.

Group B streptococci *Streptococcus agalactiae* characterized by the presence in the cell wall of the group B antigen of the Lancefield classification. GBS can cause serious illness and sometimes death, especially in newborns, the elderly, and people with compromised immune systems.

Meconium First stool of infants after birth.

Metagenome The total genomic DNA of all organisms within a community.

Metagenomics The study of uncultured microbial communities, typically relying on high-throughput experimental data and bioinformatic techniques.

Microbiome The total microbial community, genomic content, and biomolecules within a defined environment.

Microbiota The total collection of microbial organisms within a community, typically used in reference to an animal host.

PCR Polymerase Chain Reaction. A molecular technique used to amplify DNA sequences.

Premature rupture of membranes (PROM) Rupture of the amniotic sac before 37 weeks of gestation.

Preterm infant Infant born before 37 weeks of gestation.

Proteobacteria Phylum of Gram-negative bacteria.

2.02.1 Microbes and Life

In the last years it has been recognized the great benefits that microorganisms confer to living beings. We should admit, despite our generally anthropocentric view of the World, that the microbial population dominates life on the Earth, both in diversity and in quantitative terms (Whitman et al., 1998).

The human body hosts a multitude of bacteria, archaea, viruses and eukaryotic microbes, which globally, make up our microbiota (Huttenhower et al., 2012). Most of the internal and external surfaces of our body during adult life—including the gastrointestinal tract (Eckburg et al., 2005; Gill et al., 2006; Costello et al., 2009; Arumugam et al., 2011; Koenig et al., 2011; Segata et al., 2012; Yatsunenkov et al., 2012; Schloissnig et al., 2013; Huang et al., 2020), skin (Costello et al., 2009; Grice et al., 2009; Grice and Segre, 2011; Rosenthal et al., 2011; Bay et al., 2020; Flowers and Grice, 2020), and vaginal mucosa (Ravel et al., 2011; Huttenhower et al., 2012; Mendling, 2016; Gupta et al., 2020)—are colonized by microorganisms, with the largest concentration within the colon (Zhou et al., 2013).

This microbiota is directly related with our health status (Cénit et al., 2014) supporting many functions in the human body, helping in the digestion of some nutrients that would otherwise be inaccessible for us (Flint et al., 2012; Wasielewski et al., 2016), the training and modulation of the immune system (Chow et al., 2010; Kau et al., 2011; Gensollen et al., 2016; Thaïss et al., 2016), and protection against pathogenic organisms (Buffie and Pamer, 2013). Any alteration of the microbial balance or dysbiosis of this symbiotic relationship could lead to an infection or disease in adults, including inflammatory bowel diseases (IBDs) (Manichanh et al., 2006; Morgan et al., 2012; Parekh et al., 2015), type 2 diabetes (Qin et al., 2012; Upadhyaya and Banerjee, 2015), colorectal cancer (Zeller et al., 2014; Vogtmann et al., 2016) and obesity (Ley et al., 2006; Turnbaugh et al., 2009; Parekh et al., 2015). Similarly, in infancy and childhood it is associated with IBD (Kolho et al., 2015; Maukonen et al., 2015; El Mouzan et al., 2018), Crohn's disease (Dicksved et al., 2008; Gevers et al., 2014; Wang et al., 2016), type 1 diabetes (Vatanen et al., 2018; Goodwin, 2019; Harbison et al., 2019), necrotizing enterocolitis (Elgin et al., 2016; Ward et al., 2016; Rozé et al., 2017; Lindberg et al., 2020), obesity (Karlsson et al., 2012; Riva et al., 2017; Shin and Cho, 2020) and asthma (Attar, 2015; Yamamoto-Hanada et al., 2017; Ni et al., 2019).

2.02.2 Initial Colonization

Microbial colonization of the human body in early life is essential for the development of host immunity (Gensollen et al., 2016) and metabolism (Cox et al., 2014). But when the microbial colonization of our body starts is still under discussion.

The traditional paradigm of initial colonization postulates that fetuses are sterile *in utero* (Tissier, 1900; Escherich, 1989) and become colonized with microbes after birth vertically (from the mother) through the process of delivery (Dominguez-Bello et al., 2010; Neu, 2017; Sakwinska et al., 2017; Imoto et al., 2018) and type of feeding (Harmsen et al., 2000; Jiménez et al., 2008a; Bezirtzoglou et al., 2011; Bäckhed et al., 2015; Corona-Cervantes et al., 2020), and horizontally from other humans or other environmental exposures (Shin et al., 2015; Derilus et al., 2020).

The microbes-free placental condition may arise from the fact that most of the adverse pregnancy outcomes such as preterm deliveries or premature rupture of membranes (PROM) were related to the presence of bacteria in intrauterine environment. Actually, samples from placenta, amniotic fluid, and meconium were only microbiologically analyzed in cases where an intrauterine infection was evident or suspected (Rodríguez et al., 2015). Different species of bacteria such as *Mycoplasma* spp, *Ureaplasma urealyticum*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Corynebacterium* spp, *Propionibacterium* spp, Coagulase-negative *Staphylococcus*, *Gardnerella vaginalis*, *Fusobacterium nucleatum* have been clearly related to preterm labor (Bobitt and Ledger, 1977; Hillier et al., 1988; Hitti et al., 1997; Pettker et al., 2007; Onderdonk et al., 2008a, 2008b; Romero et al., 2008, 2015; Han et al., 2009; Doyle et al., 2014; Yoneda et al., 2016). Although, it has also been shown that the mere presence of bacteria in this type of tissue may not provoke an immune system response that causes preterm labor (Onderdonk et al., 2008a). It has been postulated that the presence of bacteria in low levels may be an important stimulus for the developing fetal immune system and only when the intrauterine immune system loses the control over bacteria in these tissues an inflammatory process starts, leading to a preterm birth (Onderdonk, 2008; Sullivan, 2008). How the placenta controls microbes is still under research. Different studies have shown a diverse barrier capacity depending on the placental compartment (Parnell et al., 2017b). It has been suggested that the cellular recycling pathway autophagy of the syncytiotrophoblasts of the placental villi may be an important mechanism for limiting infection (Parnell et al., 2017a). In agreement with that hypothesis, a disfunction in autophagy has been reported in preterm placentas compared to term placentas (Cao et al., 2016, 2017).

Numerous studies had provided knowledge about the effect of potential pathogenic bacteria during pregnancy on fetal and neonatal health finding a strong correlation between preterm deliveries and intrauterine infections (DiGiulio et al., 2008; Goldenberg et al., 2008), but unfortunately very little data are available on non-pathogenic bacteria in healthy pregnancies (Collado and Segata, 2020). Obtention without risk of intrauterine samples in healthy pregnancies for the mother and the fetus avoiding environmental contamination has been a difficult task. Additionally, the nonculturable nature of many organisms may have contributed to the lack of the assessment of the microbial composition of the fetal microbiome.

2.02.3 Presence of Bacteria in Placenta, Umbilical Cord Blood and Amniotic Fluid in Healthy Individuals

In the last decades the sterile womb hypothesis has been challenged and a large number of independent research groups have repeatedly reported on the detection of intra-uterine bacteria in healthy pregnancies (Jiménez et al., 2005; Steel et al., 2005; Satokari et al., 2009; Rautava et al., 2012; Stout et al., 2013; Aagaard et al., 2014; Cao and Mysorekar, 2014; Collado et al., 2016; Prince et al., 2016; Parnell et al., 2017b; Seferovic et al., 2019; Younge et al., 2019; He et al., 2020).

Classical culture techniques allowed the isolation of life bacteria from umbilical cord blood of healthy neonates born at term after caesarean section, although in a very low concentration (Jiménez et al., 2005). In this study, 9 of 20 samples, 45%, showed bacterial growth after an enrichment step in anaerobic BHI broth (Jiménez et al., 2005). The species *Enterococcus faecium*, *Cutibacterium acnes* (formerly *Propionibacterium acnes*), *Staphylococcus epidermidis* and *Streptococcus sanguinis* were the most abundant (Jiménez et al., 2005). In a different way, using fluorescent in situ hybridization (FISH) the presence of bacteria was shown in more than 73% of amnion, chorion, and decidua samples without direct relationship with preterm labor, PROM or a systemic fetal inflammatory response (Steel et al., 2005). Later, Pettker et al. (2007) reported the occurrence of positive placenta culture in 80% of subjects studied, although without amniotic fluid infection or pathologic consequences and who had delivery by elective caesarean (Pettker et al., 2007).

With the use of new molecular and next generation sequencing techniques the presence of bacterial DNA in placenta, amniotic fluid and the fetus of healthy individuals has been widely detected (Satokari et al., 2009; Rautava et al., 2012; Aagaard et al., 2014; Collado et al., 2016; Prince et al., 2016; Parnell et al., 2017b; Seferovic et al., 2019; Younge et al., 2019; He et al., 2020).

A placental microbiome niche is composed of nonpathogenic commensal microbiota from different phyla was characterized from 320 samples (Aagaard et al., 2014). As in other mucous membranes and tissues of our body, the placental microbiome seems to have a variable inter-individual profile although *Escherichia coli* appears to be the most abundant bacterium in most individuals. This finding coincides with what had been described about the meconium microbiota (Jiménez et al., 2008b; Gosalbes et al., 2013), reinforcing the idea that there is prenatal colonization of the fetal intestine.

Comparing the microbiota of the placenta with that of other distant mucosa, a great similarity has been observed between the placental and the oral microbiota (Aagaard et al., 2014). In this regard, the influence of the bacterial composition of the oral microbiota on the outcome of pregnancy is well known, establishing a clear relationship between the presence of certain oral pathogens and preterm birth (Dixon et al., 1994; Dasanayake, 1998; Offenbacher et al., 1998; Goldenberg et al., 2008; Han, 2011; Ren and Du, 2017). Some bacteria such as *Actinomyces naeslundii* or *Porphyromonas gingivalis* have been detected and even localized in trophoblasts, decidua cells, cells in the amniotic fluid and vascular cells (Dasanayake et al., 2005; Katz et al., 2009). It has been suggested that the oral bacterial composition can be used as a predictor of pregnancy outcome after observing in 300 women that the presence of *A. naeslundii* was negatively associated with birth weight and gestational age, while the presence of *Lactobacillus casei* in the oral microbiota was positively associated with term deliveries and higher birth weights (Dasanayake et al., 2005).

To investigate prenatal and neonatal microbial transfer and gut colonization, Collado et al. (2016) studied the microbiota of the placenta, the amniotic fluid, feces, colostrum meconium and infants feces of 15 mother-infant pairs with deliveries at full term by elective caesarean section to ensure the sterility of the collection of the samples (Collado et al., 2016). In this work, various complementary microbiological techniques were used, such as culture, qPCR, DGGE, and next generation sequencing of the 16S rDNA gene. It was observed, in agreement with other studies, the predominance of the *Enterobacteriaceae* family with *E. coli* as the main species (Aagaard et al., 2014; Prince et al., 2016). In these samples, the second most prevalent genus was *Propionibacterium*, a genus also commonly described in meconium samples from healthy infants and even in the placental parenchyma in the second trimester of pregnancy and in umbilical cord blood (Jiménez et al., 2005; Onderdonk et al., 2008b; Gosalbes et al., 2013). Other genera detected, although in smaller quantities, were *Streptococcus* and *Staphylococcus*, also previously detected in umbilical cord blood and meconium of healthy individuals (Jiménez et al., 2005, 2008b; Gosalbes et al., 2013). In a lower concentration, the presence of *Lactobacillus* was detected in the amniotic fluid and in the placenta. The presence of bacterial DNA—most often belonging to the common gut bacteria *Lactobacillus* spp. and *Bifidobacterium* spp.—in placenta and amniotic fluid samples obtained after sterile elective caesarean section deliveries at term without signs of infection, rupture of membranes or onset of labor, has been corroborated by other authors who also detected the presence of lactobacilli by PCR, more specifically the *L. rhamnosus* species, in the placenta (Satokari et al., 2009; Rautava et al., 2012).

In most of the studies carried out to determine the microbiota of the placenta, biopsies or samples of the whole placenta were taken without discriminating between the different tissues that compose it. The placenta is subdivided into three main components: the fetal amniotic membranes that encapsulate the fetus in the amniotic cavity; the placental villi with trophoblast and cytotrophoblasts layers that are in direct contact with maternal blood during pregnancy; and on the maternal side of the placenta - the basal plate, responsible for immune tolerance, defense, and maternal-fetal dialog. The basal plate harbors various types of cells, including immune cells of the mother, uterine endothelial cells, and extra-villous trophoblasts derived from the fetus, which extravasates from placental villi to invade the basal plate and promote spiral artery remodeling to facilitate blood flow to the fetal side of the placenta (Parnell et al., 2017a). Detection of the presence of intracellular microorganisms in the decidual basal plate had been made by histological examination of the placenta using different stains (Stout et al., 2013) and more specifically in the fetal extra-villous trophoblasts of the placental basal plate in women without clinical symptoms or chorioamnionitis or premature childbirth (Cao and Mysorekar, 2014). With this technique, the presence of intracellular individual bacteria and bacteria forming biofilms has been described (Cao and Mysorekar, 2014). Extra-villous trophoblasts are believed to be more susceptible to colonization due to their low basal autophagic activity. Similarly, but using next generation sequencing, it has been possible to detect different bacteria present in different tissues of the placenta, such as *Ralstonia insidiosa* in the basal plate, or *Lactobacillus* in the fetal membranes (Parnell et al., 2017b).

2.02.4 Meconium: The Reflex of a Fetal Gut Microbiota

The fetus, during the development in the uterus, acquires the nutrients provided by the mother and eliminates the waste products through the umbilical cord. For the initiation of the digestive tract maturation, the fetus begins to swallow amniotic fluid around weeks 10 or 11. Ingested amniotic fluid, along with some liquid secretion from the lungs, passes into the stomach, and almost all of the water is reabsorbed in the fetal colon, returning to the uterine cavity through urine. By week 14, gastric peristalsis is clearly observed (Sase et al., 2005). At the end of pregnancy, the daily volume of amniotic fluid swallowed by the fetus ranges between 500 and 1000 mL, with gastric emptying cycles every 80 minutes (Mann et al., 1996; El-Haddad et al., 2004). The fetus, when swallowing amniotic fluid, “cleanses” it of cellular debris, mucus, lanugo, etc., accumulating these wastes in the intestine. At the end of gestation, the intestine is filled with meconium, which is the medical term used to refer to the first feces of the newborn and is usually excreted between the first 12 and 48 h after birth (Clark, 1977).

The detection of bacteria in meconium, traditionally considered sterile, supports the idea of an *in utero* exposure to bacteria (Jiménez et al., 2008b; Gosalbes et al., 2013; Hu et al., 2013; Moles et al., 2013; Ardissonne et al., 2014; Del Chierico et al., 2015; Hansen et al., 2015; Martin et al., 2016; Nagpal et al., 2016; Shi et al., 2018; He et al., 2020; Yang et al., 2020).

Culture-based techniques have shown that *Staphylococcus* spp. are the most prevalent bacteria in meconium samples followed by *E. coli*, *Enterococcus*, *Lactobacillus*, and *Bifidobacterium* (Jiménez et al., 2008b; Moles et al., 2013). Similarly, the meconium from 52 preterm and full-term infants analyzed by culture-independent techniques was characterized by a generally low diversity of bacteria from dominant phyla (*Firmicutes* and *Proteobacteria*) (Ardissonne et al., 2014). In a study conducted in Japan in 151 children born at term by cesarean section and vaginal delivery, the same type of bacteria were found by RT-qPCR (Nagpal et al., 2016). In this study, 95% of the samples showed the presence of bacteria while 5% appeared sterile. The most abundant genera, found in more than 50% of the infants, were *B. fragilis* group, *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* followed by bifidobacteria, lactobacilli, *C. coccoides* group, *C. leptum* subgroup, *C. perfringens*, *Atopobium* cluster, and *Prevotella* (Nagpal et al., 2016).

In general, no differences had been found between meconium samples of preterm and full-term infants (Jiménez et al., 2008b; Moles et al., 2013) although a greater percentage of meconium samples had been described as non-sterile in infants of <33 weeks gestational age compared to infants >33 weeks gestational age, but not in a statistically significant way (Ardissonne et al., 2014).

Different bacterial profiles of meconium have been related to different health conditions. Meconium samples from infants that developed later respiratory problems were dominated by the family *Enterobacteriaceae*, mainly represented by the *Escherichia/Shigella* genus (Gosalbes et al., 2013). In contrast, *Firmicutes*, and particularly lactic acid bacteria of the families *Leuconostocaceae*, *Enterococcaceae* and *Streptococcaceae*, predominated in meconium samples from infants with mothers suffering eczema (Gosalbes et al., 2013). In another study, multi-barcode 16S rRNA gene sequencing was used to assess the bacterial composition of meconium samples from 23 newborns stratified by maternal diabetes status (Hu et al., 2013). The results obtained in this study showed a microbiome with higher alpha diversity in meconium samples from mothers with diabetes compared to the healthy ones. In addition, the phyla *Bacteroidetes* and the genus *Parabacteroides* were enriched in the meconium samples of the diabetes group (Hu et al., 2013).

Recently, a study about the significant role of prenatal maternal mood in the early-life bacteria colonization of their offspring has been published (Hu et al., 2019). Evidence shows that if the pregnant mother is stressed, anxious, or depressed, her child is more likely to have an abnormal development that maybe linked with a range of physical and mental health problems (van den Bergh et al., 2005; Entringer et al., 2010; Field, 2011). Animal models have suggested that newborn gut microbiota can explain the relationship between maternal stress in pregnancy and infant health and neurodevelopment (Jašarević et al., 2015a, 2015b). The meconium microbiome assessment of 75 infants showed that greater pregnancy-related anxiety was significantly associated with a less diverse bacterial community and lower abundance of the *Enterococcaceae* family (Hu et al., 2019). These findings indicate that maternal antenatal stress may be associated with the diversity and the composition of infants' initial microbiota.

Mode of delivery may also affect the meconium bacterial composition. Different authors have found the main differences in *Lactobacillus* genus abundance (Nagpal et al., 2016; Shi et al., 2018). In Japanese infants born by vaginal delivery *L. gasseri* subgroup was the most prevalent (Nagpal et al., 2016). Similarly, Chinese infants showed higher diversity and abundance of lactobacilli in the vaginal group than in the C-section group (Shi et al., 2018). Additionally, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* species were more commonly found in meconium samples from C-section deliveries and *Cutibacterium acnes* species predominated in meconium samples obtained after vaginal deliveries (Shi et al., 2018).

The use of antibiotics during childbirth is a common clinical practice in case of cesarean section or recto-vaginal colonization of mothers by Group B streptococci. Prenatal or perinatal antibiotics have been associated with numerous subsequent health problems, such as childhood obesity (Rasmussen et al., 2018; Wong et al., 2020) or mastitis in the lactating mother (Kinlay et al., 2001; Foxman et al., 2002; Amir et al., 2007). The latter may affect the development of colonization of the gastrointestinal tract of the newborn (Mueller et al., 2015), since breast milk has been described as the main source of bacteria for the infant (Martín et al., 2003; Jiménez et al., 2008a; Lyons et al., 2020). The use of prenatal and intrapartum antibiotics has recently been shown to affect the bacterial diversity of meconium (Wong et al., 2020). More specifically, C-section and the use of antibiotics during delivery were associated with greater alpha diversity in the meconium samples and a lower relative abundance of the genus *Escherichia* (Wong et al., 2020).

In contrast to previously discussed studies, the bacterial composition of meconium in other studies did not differ based upon vaginal or C-section birth (Hu et al., 2013). The analysis of a total of 550 samples from 36 mother-neonate pairs with vaginal births and 42 mother-neonate pairs with caesarean sections showed no differences in bacterial composition because of the delivery

Table 1 Microorganisms identified at genus or species level in intrauterine and fetal tissues of healthy individuals

Main microorganisms identified	Sample	Technique	Reference
<i>E. faecium</i> , <i>C. acnes</i> , <i>S. epidermidis</i> , <i>Str. sanguinis</i> .	Umbilical cord blood	Culture and 16S rDNA partial sequencing	(Jiménez et al., 2005)
<i>E. coli</i> , <i>Escherichia</i> spp., <i>Prevotella tannerae</i> , <i>Bacteroides</i> spp., <i>Streptomyces avermitilis</i> , <i>C. acnes</i> , <i>Rhodococcus erythropolis</i> , <i>Neisseria polysaccharea</i> , <i>Neisseria lactamica</i> , <i>Fusobacterium</i> spp.	Placenta	16S ribosomal DNA-based and whole-genome shotgun (WGS) metagenomic study	(Aagaard et al., 2014)
<i>S. epidermidis</i> , <i>S. caprae</i> , <i>S. aureus</i> , <i>E. faecalis</i> , <i>E. faecium</i> , <i>Str. mitis</i> , <i>Str. oralis</i> , <i>L. mesenteroides</i> , <i>B. bifidum</i> , <i>R. mucilaginosa</i> , <i>Enterobacter</i> spp., <i>E. coli</i> , <i>Klebsiella</i> spp., <i>P. distasonis</i> , <i>B. dorei</i> .	Meconium	Culture and 16S rDNA partial sequencing	(Jiménez et al., 2008b)
<i>Escherichia/Shigella</i> spp., <i>Leuconostoc</i> spp., <i>Enterococcus</i> spp., <i>Lactococcus</i> spp., <i>Staphylococcus</i> spp., <i>Streptococcus</i> spp., <i>Bacteroides</i> spp., <i>Parabacteroides</i> spp., <i>Chryseobacterium</i> spp.	Meconium	16S rDNA amplification and pyrosequencing	(Gosalbes et al., 2013)
<i>L. rhamnosus</i> , <i>Bifidobacterium</i> spp.	Placenta	Species-specific PCR	(Satokari et al., 2009)
<i>E. coli</i> , <i>Propionibacterium</i> spp., <i>Streptococcus</i> spp., <i>Staphylococcus</i> spp., <i>Lactobacillus</i> spp.	Placenta, amniotic fluid, maternal feces, colostrum meconium and infants feces	Culture, qPCR, DGGE, next generation sequencing of the 16S rDNA gene	(Collado et al., 2016)
<i>B. fragilis</i> group, <i>Enterobacteriaceae</i> , <i>Enterococcus</i> spp., <i>Streptococcus</i> spp., and <i>Staphylococcus</i> spp., <i>Bifidobacterium</i> spp., <i>Lactobacillus</i> spp., <i>C. coccoides</i> group, <i>C. leptum</i> subgroup, <i>C. perfringens</i> , <i>Atopobium</i> cluster, <i>Prevotella</i> spp.	Meconium	RT-qPCR	(Nagpal et al., 2016)

References: Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., Versalovic, J., 2014. The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6 (237), 237ra65. <https://doi.org/10.1126/scitranslmed.3008599>; Collado, M.C., Rautava, S., Aakko, J., Isolauri, E., Salminen, S., 2016. Human gut colonization may be initiated *in utero* by distinct microbial communities in the placenta and amniotic fluid. *Sci. Rep.* 6, 23129. <https://doi.org/10.1038/srep23129>; Gosalbes, M.J., Llop, S., Vallès, Y., Moya, A., Ballester, F., Francino, M.P., 2013. Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. *Clin. Exp. Allergy* 43 (2), 198–211. <https://doi.org/10.1111/cea.12063>; Jiménez, E., Fernández, L., Marín, M.L., Martín, R., Odriozola, J.M., Nueno-Palop, C., et al., 2005. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr. Microbiol.* 51 (4), 270–274. <https://doi.org/10.1007/s00284-005-0020-3>; Jiménez, E., Marín, M.L., Martín, R., Odriozola, J.M., Olivares, M., Xaus, J., et al., 2008. Is meconium from healthy newborns actually sterile? *Res. Microbiol.* 159 (3), 187–193. <https://doi.org/10.1016/j.resmic.2007.12.007>; Nagpal, R., Tsuji, H., Takahashi, T., Kawashima, K., Nagata, S., Nomoto, K., Yamashiro, Y., 2016. Sensitive Quantitative Analysis of the Meconium Bacterial Microbiota in Healthy Term Infants Born Vaginally or by Cesarean Section. *Front. Microbiol.* 7, 1997. <https://doi.org/10.3389/fmicb.2016.01997>; Satokari, R., Grönroos, T., Laitinen, K., Salminen, S., Isolauri, E., 2009. Bifidobacterium and Lactobacillus DNA in the human placenta. *Lett. Appl. Microbiol.* 48 (1), 8–12. <https://doi.org/10.1111/j.1472-765X.2008.02475.x>.

method (Liu et al., 2019). This is consistent with the idea that colonization of the gut occurs prior to delivery independently of delivery mode despite that it strongly influences over the development of the gut colonization (Martin et al., 2016).

In summary, the results obtained by different authors show that *E. coli*, *Propionibacterium* spp. *Streptococcus* spp. *Staphylococcus* spp. *Lactobacillus* spp. and *Bifidobacterium* spp. are common species in intrauterine and meconium samples corroborating an *in utero* colonization of the fetal gut (Table 1).

2.02.5 Weakness of Evidence Supporting the “*in utero* Colonization Hypothesis”

One of the main criticisms of the hypothesis of the colonization *in utero* is that the techniques used to demonstrate the presence of bacteria *in utero* can introduce many biases (Perez-Muñoz et al., 2017). In next generation sequencing techniques, contamination of commercial reagents and other sources can be a problem when analyzing samples with a low amount of biomass, such as the placenta or amniotic fluid (Laurence et al., 2014; Salter et al., 2014; de Goffau et al., 2018; Eisenhofer et al., 2019). DNA sequences related to contaminants typically correspond to water- and soil-associated bacterial genera, including *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Bradyrhizobium*, *Herbaspirillum*, *Legionella*, *Leifsonia*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Novosphingobium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas*, *Stenotrophomonas*, and *Xanthomonas* (Milani et al., 2017). Interestingly, these microbial groups have been considered as “the placenta microbiome” (including core members) (Aagaard et al., 2014).

In a well-designed study, the microbiota present in oral, vaginal, and placental samples from six women were compared with air controls, the technique's reagents, and a sterile swab identical to that used to collect the samples (Lauder et al., 2016). The results obtained showed that the vaginal and oral samples contained their own microbial profile while the microbiota detected in the placenta samples could not be differentiated from that observed in the negative controls (Lauder et al., 2016). Later, these authors compared 20 placentas from term births with 20 placentas from premature births. Their hypothesis was based on the fact that even if the placentas of healthy women were not colonized, perhaps those from preterm births would be (Leiby et al., 2018). Again, the results obtained did not allow them to differentiate between the microbial profile of all placenta samples from the negative controls (Leiby et al., 2018). Similar results were obtained in another study in which different microbiological techniques (culture, qRT-PCR and 16S rRNA gene sequencing) were combined to describe the microbiota of placentas obtained from 29 women who gave birth by cesarean section (Theis et al., 2019). Thus, these findings cannot contribute to the idea of a consistent placental microbiome, but quite the opposite.

Because of the criticism of the techniques used, numerous researchers have insisted with their work on demonstrating the presence of a fetal microbiota during healthy pregnancy with different techniques or improving the already used ones with new protocols avoiding external contaminations (Stout et al., 2013; Cao and Mysorekar, 2014; Seferovic et al., 2019; Stinson et al., 2019; Younge et al., 2019; He et al., 2020). Recently, Seferovic et al. (Seferovic et al., 2019) used fluorescent in situ hybridization (FISH), conventional histological staining, culture, and sequencing of the V4 region of the 16S rRNA gene to localize bacterial RNA in the placenta. Their results allowed the visualization and localization of a low concentration of bacteria in most of the samples analyzed. Bacteria were mainly located in the villous parenchyma and syncytiotrophoblasts (Seferovic et al., 2019). Furthermore, with application of the massive sequencing technique it was possible to differentiate the taxonomic profiles of the cohort of premature infants and those born at term and negative controls (Seferovic et al., 2019).

Similarly, Younge et al. (2019) (Younge et al., 2019) combined bacterial DNA sequencing, FISH, and bacterial culture to study the microbiota of human and mouse dyads, and contribute to the understanding these relationships, localizing bacteria in the fetus, and demonstrating bacterial viability. They used the Divisive Amplicon Denoizing Algorithm (DADA2) to infer 16S rRNA gene amplicon sequencing variants, therefore discriminating fine-scale sequence variation. In this study, the microbial communities within the uterus and placenta were distinct from the amniotic membrane microbiota that shared more similarity with the plasma microbiota (Younge et al., 2019). Additionally, the placental and uterine bacterial profiles differed from the maternal fecal microbiota but no significant differences were observed with the microbiota of the vagina, infant oral cavity, or infant meconium (Younge et al., 2019). The microbial communities of the infant meconium and oral cavity varied between subjects in their relationship to other sites but were distinct from the microbiota of the maternal feces and plasma. Again, as shown by other authors, the genus *Escherichia* was one of the most abundant in the *in utero* environment and infant samples' sites (Younge et al., 2019).

Another argument against fetal colonization is the existence of gnotobiotic animals. The generation of sterile newborns by cesarean section had been shown in numerous species of mammals, including humans, transferring them to a sterile environment (Perez-Muñoz et al., 2017). Therefore, it could be accepted that the placenta may be colonized, and the fetus may not. Perhaps gnotobiotic newborns produce antibacterial substances that kill bacteria from the uterus (Bushman, 2019). In this case, microbial cellular walls or postbiotics would remain playing an important role in the development of the newborn's immune system (Seferovic et al., 2019). But gnotobiotic animals show an architecture of undeveloped intestinal tissues that leads to a deficiency in the absorption of nutrients and vitamins and a great susceptibility to gastrointestinal infections (Dethlefsen et al., 2007; Smith et al., 2007; Fiebigler et al., 2016). If we think that natural evolution leads us to a continuous improvement of the human species and functioning of our body, it is difficult to assume that we remain sterile during our development, with structural and functional deficiencies, just waiting to be born.

Our immune system faces a great challenge, and it must learn to actively and selectively tolerate its own microbiota at steady state but at the same time be capable of generating an inflammatory response in case of infection or disease (Chow et al., 2010). By the end of the first trimester of pregnancy the mucosal immunity in the developing human fetal intestine is able to respond to microbial stimuli and initiate robust T cell responses (Howie et al., 1998; McGovern et al., 2017). Recently the influence of the present microorganisms within the fetal intestine in the mucosal immune development has been examined (Rackaityte et al., 2020). Firstly, the authors visualized by scanning electron microscopy (SEM) cellular structures similar to bacterial cocci in isolated pockets of meconium embedded with mucin structures in fetal terminal ileum sections. Subsequently, using next generation techniques, 18 taxa were detected in fetal meconium with *Lactobacillus* and *Micrococcaceae* identified as the most abundant. Other bacteria like *Bacteroides*, *Bifidobacterium* and *Prevotella* were also identified. Additionally, mimicking the fetal intestinal environment, the authors recovered from cryopreserved fetal meconium samples bacteria identified as *Micrococcus luteus*. The immune profiling data generated showed that the presence of *Micrococcaceae* in the fetal intestines lead to different patterns of T cell composition (Rackaityte et al., 2020). This study supports the hypothesis of a low abundance colonization of our body during the uterine stage, controlled by our mother's immune system, that will prepare us for a life in the outside world.

2.02.6 Colonization of Intrauterine Tissues

The mechanisms by which bacteria are capable of colonizing intrauterine tissues remain unknown. There are several suggested hypotheses (Goldenberg et al., 2000, 2008; Romero et al., 2019): (i) the ascent of bacteria from the vagina through the cervix; (ii) hematogenous spread from the intestine or oral cavity through the placenta; (iii) colonization of the peritoneal cavity through the fallopian tube and (iv) introduction of microorganisms in procedures such as amniocentesis.

In full-term pregnancies, without any complication or infection, the first two options are believed to be the most plausible. On one hand, the fact that the genus *Lactobacillus*, a common colonizer of the vaginal mucosa in both pregnant and non-pregnant women (Ravel et al., 2011; Aagaard et al., 2012), is regularly detected in intrauterine samples could reinforce the path ascending bacteria from the vagina (Fox and Eichelberger, 2015). Other authors argued that most of the identified intrauterine bacteria are not usually found in the urogenital tract but in the digestive tract (Satokari et al., 2009; Neu, 2016), which would reinforce the hypothesis of the hematogenous dissemination of bacteria from distant mucosa (Milani et al., 2017). It has been suggested that the bacteria could reach the placenta through the bloodstream after translocating in the intestine. The mechanism by which dendritic cells could select bacteria from the intestinal lumen, without compromising the epithelial barrier, and transport them alive to distant mucosa has been described (Donnet-Hughes et al., 2010; Rodríguez, 2014). To investigate this potential transfer of mother-child bacteria through the placenta, two studies were performed in an animal model (Jiménez et al., 2005, 2008b). A genetically labeled strain of *Enterococcus faecium* was administered to pregnant mice during gestation. Before delivery, a cesarean section was performed to collect amniotic fluid or meconium samples and to analyze them. The genetically labeled *E. faecium* strain could be detected in the amniotic fluid and meconium samples of mice whose mothers had ingested the strain, but not in the control group (Jiménez et al., 2005, 2008b).

On the other hand, as previously described, various species from the oral cavity have been detected in the placental microbiota. The increase in immune tolerance reported during pregnancy could be associated with the greater permissiveness of translocation of bacteria from the gastrointestinal tract into the bloodstream (Pelzer et al., 2017). Various studies in animals have also demonstrated the existence of the hematogenous pathway through the transmission of bacteria from the oral cavity to the placenta (Han et al., 2004, 2006; Fardini et al., 2010).

We must not forget that the uterus of non-pregnant women is not sterile (Mitchell et al., 2015; Moreno et al., 2016; Kyono et al., 2018). In recent years, the uterine microbiota and its important role in fertility have been described (Moreno et al., 2016; Verstraalen et al., 2016; Kyono et al., 2018; Bardos et al., 2019; Moreno and Simon, 2019; Tomaiuolo et al., 2020). In healthy women, the most commonly found phyla in the uterus are Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, and the most commonly detected genus has been *Lactobacillus* (Bardos et al., 2019). Previously in this article, the detection of Proteobacteria and Bacteroidetes in the basal plate of the placenta has been mentioned. Given the location of the basal plate adjacent to the uterine epithelium, the uterus could be the origin of the bacteria detected in the basal plate (Parnell et al., 2017b), and possibly other intrauterine tissues.

2.02.7 Final Remarks

Over the last few years, numerous authors have detected the presence of microorganisms both in intrauterine membranes and in fetal and meconium samples. It is obvious that the limitations of all the techniques used must be considered, but this does not lead to denial of the possibility of a prenatal colonization. The great role that microorganisms play in the life of living beings should convince us that the sooner the symbiotic life begins, the better prepared we will be for life outside the protection of the maternal uterus.

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