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Authors	O'Neill, Ian J.;Sanchez Gallardo, Rocio;Saldova, Radka;Murphy, Eileen F.;Cotter, Paul D.;McAuliffe, Fionnuala M.;van Sinderen, Douwe
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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Maternal and infant factors that shape neonatal gut colonisation by bacteria

Ian J O'Neill[1], Rocio Sanchez Gallardo[1], Radka Saldova[3,7], Eileen F. Murphy[4], Paul D. Cotter[1,5] and Fionnuala M. McAuliffe[6], Douwe van Sinderen[1,2,*]

Affiliations

1. APC Microbiome Ireland, National University of Ireland, Cork, Ireland.
2. School of Microbiology, National University of Ireland, Cork, Ireland.
3. The National Institute for Bioprocessing, Research, and Training (NIBRT), Dublin, Ireland.
4. Alimentary Health Group, Cork Airport Business Park, Cork, Ireland.
5. Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland.
6. UCD Perinatal Research Centre, School of Medicine, University College Dublin, National Maternity Hospital, Dublin, Ireland.
7. UCD School of Medicine, College of Health and Agricultural Science, University College Dublin, Ireland.

* Corresponding author: d.vansinderen@ucc.ie

Key words: , Microbiota, Infant, Pregnancy, Breastmilk, Delivery, Antibiotics.

Running title: Maternal and infant factors that shape neonatal gut colonisation by bacteria

Abstract

Introduction: Early life is a critical development window which coincides with the establishment of a community of neonatal gut microbes which are vitally important for immune development. The composition of this microbial community is affected by multiple factors.

Areas covered: The effect of pre-pregnancy and pregnancy maternal health, maternal nutrition, pregnancy disorders such as gestational diabetes, maternal antibiotic usage, delivery mode, infant feeding, and infant antibiotic usage on microbial composition are outlined along with the potential impact of these differences on infant health.

Expert opinion: Recent developments in understanding what shapes our microbiota indicates that the greatest impact on infant gut microbiota composition during the first year of life is seen with mode of delivery, infant feeding and infant antibiotic usage. Current data is insufficient to fully establish the role of less important factors such as maternal health on microbiota development although their impact is likely smaller. Technological advances will allow for greater understand of underlying mechanisms by which specific microbes impact infant health leading to greater appreciation of the role microbes play in early life development.

Keywords: Microbiome, Microbiota, Pregnancy, Infant, Breastmilk, Delivery, Antibiotics, Gut microbiota, **Infant health**

1 Introduction

The human gut is home to a vast and diverse community of bacteria, fungi, archaea and viruses collectively known as the gut microbiota [1]. This gut microbiota has profound effects on our health and well-being throughout life, for example by contributing metabolic functions such as digestion of fermentable dietary fibers and production of vitamins such as vitamin K and B group vitamins [2]. Furthermore, the microbiota shapes and provides guidance to the immune system during a critical developmental window in early life (extensively reviewed in [3–5]). Notably, multiple studies of mother-infant pairs have indicated the existence of vertical transmission of microbes from mother to infant that can contribute to this colonizing microbiota [6–9]. Indeed, the composition of the infant microbiota is dynamic and dependent on multiple factors, including maternal microbiota composition, delivery mode, infant feeding mode, antibiotic usage and environmental factors, such as the presence of pets and siblings. Disturbance of the microbiota composition during infancy is also associated with atopic disease, asthma and obesity in later life [10]. This review will focus on recent developments in understanding the factors in infant microbial colonization and the impact of these factors on infant development and risk of later life disease. It is noted that the majority of studies relate the relative abundance of microbes in fecal samples to the composition of the gut microbiota, thus any changes or differences in microbial content discussed herein are based upon changes/differences in relative abundance of microbes unless otherwise stated.

2 Microbiota colonization *in utero*

Traditionally, the uterus and fetus are considered sterile environments unless clinical infection occurs, such as in chorioamnionitis; however, the development of DNA sequencing technologies that can detect unculturable microbes has led to studies that question this dogma [11]. Most of these studies have focused on determining the presence of microbes in amniotic fluid, the placenta and the meconium in either vaginally or caesarean section born infants [12–18]. There are multiple technical challenges in detecting microbes in these environments, such as ensuring sterility when sampling placenta or amniotic fluid, and contamination in DNA extraction and sequencing, which has led to questions regarding the validity of the obtained results [11,19]

In many microbiota studies, the presence of bacteria in samples is detected using either quantitative PCR (qPCR) for specific species of bacteria, or PCR amplification of a variable part of the bacterial 16S ribosomal RNA (rRNA) gene, followed by high-throughput sequencing of the amplification product to identify the bacterial genera present in the sample. There are a number of technical challenges that must be considered and if possible overcome when employing these techniques to study low bacterial abundance samples such as amniotic fluid, placenta and meconium [16,18,20]. The main drawback is that, unless additional measures are taken, these techniques will amplify any DNA in the sample regardless of the presence of viable bacteria, therefore the results may not reflect the actual microbiota present in the sampled environment. Furthermore, laboratory reagents and kits used to extract DNA can themselves be contaminated with environmental DNA, the so-called "kitome" [21–24]. When appropriate controls are not used in such studies, it is difficult to

distinguish reads from contaminating DNA versus that from bacteria that were present in the tested sample, and thus the findings from such studies must be interpreted with caution.

Some sequencing-based studies on the microbial composition of amniotic fluid have indicated that the placenta harbors its own microbiome [12,13,25,26], though these findings have received a serious level of skepticism [11,16,27]. Proteobacteria, and specifically the genera *Enterobacter* and *Escherichia*, have been commonly identified as the most abundant phylum in the amniotic fluid [12,13], but these latter two taxa also happen to be the commonly identified contaminants in laboratory and extraction kit reagents [21,22]. A recent study using extensive controls for contamination showed that bacterial DNA detected in the amniotic fluid is no more abundant than that contaminating the employed reagents [20]. Using principal component analysis the authors demonstrated that any bacterial DNA detected in the amniotic fluid clustered with that found in reagent controls indicating the absence of an amniotic fluid-specific microbiota [20].

Whether or not the placenta harbors a microbiome is still under debate [12,13,16–18]. Some studies have shown by either cultivation or DNA sequencing that the placenta is dominated by Proteobacteria and contains species such as *Escherichia* and *Enterococcus faecalis* [12,13,17,26]. More recently, however, a very thorough examination of the placental microbiome which incorporated multiple controls for contamination showed that the bacteria detected were contaminants or species acquired during labor [18]. One exception was Group B *Streptococci*, a common infection that can lead to pregnancy complications.

A recent study reported on the presence of bacteria in human fetal intestines *in utero* [28]. These bacteria were identified by scanning electron microscopy and 16S rRNA sequencing in a small number of fetal meconium samples obtained from terminated pregnancies. Despite the very low number of bacteria in the meconium, *Lactobacillus* and

Micrococcus were identified as the most abundant taxa in some of the samples and *Bacteroides, Bifidobacterium* and *Prevotella* in others. In addition, *Micrococcus luteus* was successfully cultivated from meconium [28]. *Micrococcus* spp. is often found in the cervicovaginal microbiome, however, in this study bacteria were only isolated from meconium so it is unclear if this exact strain originated from the mother's vagina [28]. These findings in a small cohort suggest that colonization of the meconium *in utero* is highly restricted both taxonomically and spatially. Detection of microbes in the meconium soon after birth has been used as an indication that microbes colonize the infant gut before birth [13,17]. Longer labor and time after labor before the meconium is passed impact on whether bacteria can be detected in the meconium suggesting that bacteria detected in the meconium are acquired after birth rather than *in utero* [11].

No doubt, debate will continue on whether or not microbial colonization occurs *in utero* and well controlled, large cohort studies are essential to ensure that the data produced is robust and reliable.

3 Maternal factors that contribute to the infant gut microbiota

3.1 Maternal diet and health status before and during pregnancy.

Maternal diet and health has been proposed to play an important role in shaping the infant and maternal gut microbiota [29–34] with maternal obesity [30,35], gestational diabetes mellitus (GDM) [32,36,37], gestational weight gain [38,39], and maternal diet [31,33] all associated with changes in the neonatal microbiota.

The maternal gut microbiota changes during pregnancy, with in particular an expansion of beta-diversity (diversity in microbes between two environments, i.e. differences

in diversity between different samples) and community composition between trimester one and trimester three that could not be attributed to other factors such as diet or GDM [37]. In trimester one the gut microbiota composition, as inferred through DNA sequencing of stool samples, is enriched with butyrate producers of the order Clostridiales, specifically *Faecalibacterium* and *Eubacterium.* By trimester 3, an increase is observed in the relative abundance Proteobacteria and Actinobacteria [37], which are highly abundant in the early infant gut, again raising the intriguing question if microbial changes enhance transfer opportunities of these taxa to infants.

Maternal weight can also impact the microbes present in the infant gut. Infants born to obese or overweight mothers are three times more likely to be overweight by one year of age, which increases to five times in infants born by caesarean section [34]. An increase in infant associated *Lachnospiraceae* was associated with overweight mothers but the genus of *Lachnospiraceae* was different between vaginally born and caesarean section born infants. This suggests that it is the maternal microbiome rather than delivery mode that is a factor for obesity in early life. Maternal obesity impacts on the alpha diversity (diversity within a given environment i.e. the diversity of microbes within a given infant fecal sample) of vaginally born but not caesarean born infants at 6 weeks of age [38]. Furthermore, changes in beta diversity associated with maternal obesity were only seen in vaginally and not caesarean born infants [29]. Vaginally born infants with overweight mothers have been shown to possess a gut microbiota with a higher relative abundance of *Escherichia*, *Enterococcus*, *Klebsiella* and *Ruminoccocus*, while infants born to obese mothers carry a gut microbiota with a higher relative abundance of *Parabacteroides* and *Staphylococcus* [38]. These observed differences have been shown to vary between studies as another investigation found that *Enterococcus* was depleted in infants born to obese or overweight mothers [29], while *Bacteroides*

abundance in infants of obese mothers was shown to be increased in one study [29], it was reported to be decreased in another [30]. These disparities in the results from independent studies highlights the lack of information on the effect of maternal obesity on infant microbiota composition. Furthermore, it appears that maternal BMI does affect the infant microbiota but such effects are likely less impactful than those associated with birth mode (see section on delivery mode below).

Weight gain during pregnancy is a normal physiological response, however, excessive gestational weight gain (defined as 16 kg or above for women with BMI 19.8-25 or above 11.5 for women with BMI > 25 [40]) has been shown to result in increased relative abundance of *Escherichia* and *Dorea* in vaginally born infants [38]. Metagenomics analysis has shown that gestational weight gain impacts on the function of the infant microbiome [39]. Enrichment of bacterial glucose pathways and phenylalanine, cysteine/serine, folate, thiamine, biotin, and pyridoxine synthesis pathways in infants were shown to be associated with maternal gestational weight gain [39]. These effects are seen up to 8 months post-birth, highlighting a longer-term impact on microbiota function. The relative abundance of *Bifidobacterium*, a key genus in the healthy early infant gut, was decreased in infants of mothers with excessive weight gain [30] and those with insufficient weight gain (< 11.5 kg for women with BMI 19.8-25 and < 7.0 kg for women with BMI > 25) [38], suggesting that this genus is sensitive to maternal factors in pregnancy.

GDM results in abnormal glucose tolerance and can affect up to 20 % of pregnant mothers [36]. An increase in alpha diversity from second to third trimester is seen in women with GDM [41]. An increased relative abundance of Firmicutes and decreased relative abundance in Bacteroidetes and Actinobacteria from the first to third trimester was observed in women with GDM [41]. In a separate study of healthy women without GDM, Actinobacteria

increased in relative abundance from the first to third trimester [37]. Differences in genera associated with GDM compared to healthy women included increased *Faecalibacterium* and *Anaerotruncus* and decreased *Clostridium* and *Veillonella* [42]. Another study showed that *Klebsiella*, *Clostridium*, *Eggerthella*, *Megamonas*, *Slakia* are enriched in women with GDM, while the relative abundance of *Bifidobacterium*, *Eubacterium*, *Alistipes* and *Roseburia* are depleted compared to healthy controls [43]. These studies show that diabetes mellitus influences the maternal microbiota, which may in turn affect mother-to-infant microbial transmission events during and after birth.

A limited number of studies have suggested that there is an impact of GDM on infant microbiota in very early life [32,36,44]. These studies focused on the microbial composition of infant stool samples within 24 h of birth when the microbiota is unstable, so the longer-term effects are not fully understood. An increase in *Corynebacterium*, *Bacteroides* and *Bevundimonas* was seen in stool of infants of mothers with GDM in one study [36], while another study showed that Bacteroides was decreased in stool of infants from mothers with GDM, while an increase in Proteobacteria and Actinobacteria was observed when compared to healthy controls [32]. It is apparent that there is a large variation in results from studies on microbiota in GDM and it is currently unclear if the observed changes have any long-term health effects on the infant.

Diet can profoundly impact on gut microbiota composition [45,46] and the maternal diet during pregnancy may also influence the infant gut microbiota. A maternal high-fat diet is associated with an enrichment of *Enterococcus* and depletion in *Bacteroides* in the infant microbiota compared to a normal fat diet [31]. A study on a Danish cohort found associations between maternal fruit intake and higher levels of *Streptococcus* and *Clostridium* in breast-

fed infants and maternal dairy intake and higher levels of *Clostridium* in caesarean-born infants [33].

The above studies show that maternal lifestyle and health status influences both the maternal as well as the infant gut microbiota composition, as microbes are transmitted from mother to infant, although what the long-term impact of these changes are on infant health is yet to be determined.

3.2 The vaginal microbiome

The vaginal microbiome plays an important role in preventing urogenital diseases, such as bacterial vaginosis, yeast infections, sexually transmitted infections including HIV and urinary tract infections [47]. It is also an important factor in a woman's reproductive health as it helps to prevent colonization of pathogens by (i) lowering the pH of the vagina through lactic acid production [48], (ii) production of antimicrobial compounds that inhibit or kill pathogens [49], and (iii) outcompeting pathogenic bacteria [50].

The composition and characterization of the vaginal microbiome has been studied using culture-dependent [51,52] and culture-independent methods [53]. The diversity of the cervicovaginal microbiota is generally low, dominated by one or more species of the genus *Lactobacillus* [54]. Several studies reported that the most frequently identified species are *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners* and *Lactobacillus jensenii.* [55–58]. Genera that are present in lower relative abundance are *Enteroccoccus, Prevotella, Bifidobacterium, Dialister, Atopobium, Gardnerella, Megasphaera, Peptoniphilus, Sneathia, Eggerthella, Aerococcus, Finegoldia,* and *Mobiluncus* [47,59]. Because the vaginal microbiome profile is very variable amongst individuals and over time, some researchers have suggested

to categorize or cluster microbiome profiles into a number of community state types (CSTs) or vaginotypes [60,61]. These are commonly used to interpret vaginal microbiome data, however, it has not been established whether or not CSTs represent biological states or have a clinical relevance.

The cervicovaginal microbiome is a very dynamic ecosystem, influenced by many factors and physiological changes such as sexual development, sexual intercourse, personal hygiene, menstruation cycle, pregnancy, menopause and hormonal changes [62]. It is worth noting that one of the factors that has a major influence is racial background, where large differences are observed in terms of diversity and abundance. Several studies have shown that African women and women of African descent have a lower *Lactobacillus* abundance and higher ecological diversity than Caucasian women [63–67], associating this fact with higher risk of HIV infection [68,69]. Similar studies were carried out in Japanese and Chinese populations, showing high similarity with those previously described for Caucasian women [56,70].

Pregnancy affects the entire body and gestational changes in the vaginal microbiome are especially relevant due to its implication in the vertical transmission of microbes to the newborn during vaginal delivery [71,72]. The vaginal microbiome structure of non-pregnant women differs from that of pregnant women; the latter possess a vaginal microbiota that is even more dominated by *Lactobacillus* species with lower richness and diversity than that of non-pregnant woman. This simplified ecosystem also seems to be more stable [58,62]. Compositional changes may be a consequence of the physiological state of pregnancy. In non-pregnant women, during the menstrual cycle, the bacterial communities are more stable when estrogen concentrations are high due to the maturation of the vaginal epithelium resulting in the accumulation of glycogen in the upper epithelial layer [73]. Fermentation of

glycogen by *Lactobacillus* spp. produces lactic acid generating an acidic environment that prevents the growth of pathogens and opportunistic microbes in the vaginal cavity [62,74]. Disruption of the vaginal microbiome can lead to bacterial vaginosis (BV), which is one of the most common genital disorders in women of reproductive age and has been associated with negative effects during pregnancy, such as preterm birth. [75].

The impact of the vaginal microbiome on the infant before birth remains unknown, however gestational changes that happen during the pregnancy could be part of an adaptive response to promote the correct development and health of the fetus [72]. The composition of the vaginal and gut microbiota are related to each other, as studies have shown that many bacteria are shared between rectum and vagina, including *Lactobacillus* and *Bifidobacterium* [76,77]. Moreover, some of the earliest colonizers of the infant gut are species present in the vagina [78]. The most dominant genus in the vagina, *Lactobacillus*, has been shown to persist in the infant gut long after birth [8,79,80], although one study suggests that specific species of *Lactobacillus* from the maternal vagina cannot be detected in the infant gut by one month, while other vaginal species such as *Atopobium vaginae* and *Gardnerella vaginalis* were still detected, although whether these strains are inherited from the mother is unknown [6].

While the vaginal microbiome does contribute bacteria to the infant microbiome, it may be that this contribution is minimal, in terms of overall abundance, due to low diversity of the vaginal microbiome during pregnancy. Differences in microbiota composition between vaginally and caesarean born infants are well documented (see section below), although such differences may be due to multiple other factors such as antibiotic usage in the caesarean section procedure.

4 Microbial colonization of the neonatal gut following birth

Microbial colonization of the infant gut occurs rapidly after birth and is influenced by a number of factors including delivery mode, infant diet (i.e., breast vs formula milk), intrapartum and infant antibiotic usage, and maternal health status. It is generally accepted that the maternal microbiota is a major contributor to the neonatal microbiota [6,7,80–84]. The infant microbiota composition changes rapidly in the first weeks following birth and then stabilizes until the infant is weaned off an exclusive milk diet and on to solid foods, as a result of which the gut microbiota becomes more adult like [9,85–87]. Below we outline the microbial succession following birth in a full term, vaginally born infant who is breastfed and has not received antibiotics. The effects of changes to this "normal" scenario are outlined in the sections on delivery mode, infant diet and antibiotic usage.

Following birth, infants appear to share roughly 50 % of microbial species in their gut with those found in the maternal gut, oral, vaginal or skin microbiota indicating vertical transmission of microbes from mother to infant [6]. One-week post birth, the contribution of maternal oral, vaginal and skin microbes to the infant gut microbiota decreases, with a concurrent increase in the influence of the maternal gut microbiota. This is likely due to decreasing oxygen availability in the infant gut leading to loss of facultative anaerobes such as *Escherichia* and an increase in strict anaerobes such as *Bifidobacterium*. *Escherichia* is a pioneering species of the neonatal gut and along with other facultative anaerobes create an environment that is ready for colonization of strict anaerobes such as *Bacteroides* and *Bifidobacterium* [6,7,88,89]. Metagenomic and metatranscriptomic analyses support this scenario as genes required for the tricarboxylic acid cycle of aerobic metabolism are enriched in the first week but not afterwards [9,90,91]. Furthermore, microbes from the maternal gut

are expected to be better equipped to colonize the gut than those from other body sites.

Alpha diversity decreases during the first week of life as microbes establish in the gut and expand. From month one to three of life alpha-diversity increases again and the microbiota is dominated by *Bifidobacterium* and *Bacteroides* [6,7,87]. Minor taxa include *Lactobacillus*, *Prevotella*, *Staphylococcus* and *Escherichia* [9,80,81,87,92–94]. Strains of *Bifidobacterium*, *Bacteroides* and *Escherichia* can be traced back to the maternal gut microbiota using genomic sequencing of infant and maternal fecal samples consistent with the hypothesis that bacteria are transmitted from mother to infant [6,7,80,82,83].

Infant-associated *Bifidobacterium* species include *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium longum* subsp. *infantis* which are known to metabolize human milk oligosaccharides (HMOs, [95] - see section on breastfeeding below for further details) which in part accounts for their dominance in infants. Multiple metagenomic studies have revealed that *Bacteroides vulgatus* and *Bacteroides dorei*, *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* are commonly found in the infant gut [9,80–82]; some of these species have been shown to metabolise HMOs using the same metabolic pathways used to digest mucus [96]. This may account for the ability of certain *Bacteroides* species to be resident in both the infant and adult gut [82].

Once weaning occurs there is a dramatic shift in the microbiota with a further increase in alpha diversity. The gut microbiota now becomes dominated by members of the phyla Firmicutes and Bacteroidetes similar to an adults [9,86,87]. This major shift in microbiota composition is accompanied by a substantial shift in the deduced functionalities of genes carried by members of the microbiota to reflect the changing diet. For example, at 12 months, the infant microbiome is enriched with *Bacteroides thetatiotaomicron* genes involved in the degradation of complex carbohydrates and starch reflecting increased fermentation of plant-

derived dietary sugars [9]. Similarly, there is a decrease in relative abundance of infant-associated bifidobacteria, such as *Bifidobacterium longum* and *B. breve*, while relative abundance of *B. adolescentis*, which apparently can digest a wider range of dietary carbohydrates, remains stable [87]. The observed changes in microbiota composition is accompanied by an immunological response and increase in regulatory T-cells leading to low susceptibility to pathological inflammation in animal models [97]. It is likely that a similar response occurs in humans.

4.1 Factors that affect microbial composition after birth

4.1.1 Delivery mode

It is not surprising that birth mode has a significant role to play in influencing the neonatal microbiota composition. Children born by caesarean section can have altered immune development and are at a higher risk for numerous non-communicable diseases such as obesity, allergy, asthma and atopy [98]. Given the role of the microbiota in immune programming [4,5], various studies have reported on the differences in fecal microbiota composition in infants born either vaginally or by caesarean section [8,34,78,80,92,99]. These studies have highlighted that there are substantial differences in the composition of the microbiota between vaginally delivered neonates as compared to those born by caesarean section. How long these differences remain is somewhat unclear with some studies indicating that the differences are no longer significant after 6-weeks [8], and others that show differences may last up to two years [92].

Vaginal birth exposes the infant to microbes of the birth canal such as *Lactobacillus*, *Prevotella* and *Sneathia*, which can then be detected in the infant gut in samples taken soon after delivery. Other taxa that are present in early stool samples of vaginal birth infants

include *Bacteroides*, *Escherichia*, *Bifidobacterium* and *Parabacteroides* [9,80,81,92]. These genera dominate the infant microbiota and comprise up to 68 % of all microbes at 4 days after birth [80]. In contrast, early samples taken from neonates born by caesarean section were higher in relative abundance of *Enterococcus*, *Staphylococcus, Streptococcus, Klebsiella, Enterobacter and Propionibacterium* [8,9,80,81]. These taxa are commonly associated with the skin and the hospital environment and many are sources of nosocomial infections. In caesarean section infants, these taxa can account for 68 % of infant microbiota four days after birth [80]. It is less than ideal that newborns with an immature immune system are colonized with opportunist pathogens which could take advantage of a blunt immune response, furthermore, formula fed infants are more prone to infection [100]. The microbial provenance in caesarean section born infants is distinct between infants born by elective or emergency caesarean section [8]. The source of the gut microbiota in infants born by emergency caesarean section is presumed to be the skin and vagina, whereas the skin was thought to be the predominant microbial origin of the gut microbiota in infants born by elective caesarean section [8]. These differences may be due to fetal membrane rupture which commonly occurs before emergency caesarean section leading to infiltration by vaginal microbes.

Shotgun metagenomic sequencing allows researchers to identify strain level transmission of bacteria from mother to infants. Interestingly, two studies employing this technique showed that transmission of strains of *Bifidobacterium* spp., *Bacteroides* spp., *Parabacteroides* spp. and *E. coli* is higher in vaginally born infants compared to that observed in caesarean born infants [80,81]. These results are confounded by the use of perinatal antibiotics in caesarean section which can impact the maternal microbiota. This is apparent in and consistent with the observed low abundance of *Bacteroides* spp. in infants born vaginally to mothers that received perinatal antibiotics [80,101]. The loss of the key early life

genus *Bifidobacterium* may have longer terms effects including an impaired response to vaccines later in life [102].

Diversity of the microbiota increases after the first week following birth in both vaginally born and caesarean section born infants [93]. Despite this, multiple studies indicate that birth mode is the primary factor accounting for differences in the microbiota composition between the two groups throughout the first year of life [80,87,92], although one study suggests there is no appreciable difference by six weeks [8]. In vaginally born infants, *Bifidobacterium* and *Bacteroides* continue to dominate the gut microbiota, yet tend to be present in lower relative abundance in caesarean section infants which typically have higher relative abundance of *Clostridiaceae* and *Enterobacteriaceae*. While *Bifidobacterium* relative abundance in caesarean section infants can recover, potentially due to transmission from breast milk, the relative abundance of *Bacteroides* does not recover in a similar manner (Bäckhed *et al.*, 2015a; Bokulich *et al.*, 2016; Shao *et al.*, 2019). This is significant as *Bacteroides* is a dominant member of in the infant gut which expands after weaning likely due to members of this taxa encoding genes involved in digestion of complex carbohydrates.

Delivery mode clearly has a major impact on the composition of the infant microbiota, however, how long term these effects are is still matter of debate and confounded by other factors such as breastfeeding and antibiotic usage in mother and infant. Importantly, many of the taxa that are in higher abundance in cesarean section infants compared to vaginally born infants contain opportunistic pathogens and taxa responsible for nosocomial infections. This may lead to increased risk of infection which may have to be treated with antibiotics further damaging the normal development of the infant microbiota.

4.1.2 Breast milk microbiota and infant diet

4.1.2.1 Breast milk composition and microbial content

Human breast milk is a complex fluid able to satisfy all nutritional requirements of the newborn by providing essential nutrients and bioactive compounds that promote the development of the infant as well as support its immune system [103]. Breastfeeding confers protection against respiratory and gastrointestinal infections and decreases the risk of sudden infant death syndrome and certain inflammatory diseases such as dermatitis, asthma, obesity, type 1 and 2 diabetes [104,105]. The benefits of prolonged breastfeeding for the infant, especially during the first six months following birth, are many, while it also positively impacts on the appropriate development of the infant gut microbiota [93].

The composition of the milk varies and depends on different factors: stage of lactation period; degree of breast fullness; infant feeding; mother-infant health status; maternal diet and maternal genetics [106,107]. Breast milk is composed of carbohydrates, fats, proteins/amino acids, iron/lactoferrin and antibodies that are important for infant nutrition and health. Lactose is the primary carbohydrate and provides an efficient energy source for the growing infant. In addition, breast milk also contains human milk oligosaccharides (HMOs) that can only be digested by certain members of the gut microbiota. HMOs can prevent infection by pathogenic bacteria by promoting growth of competitive commensal bacteria [108,109]. The presence of certain HMOs in human milk is correlated with increased relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. in the infant gut contributing to the development of a bifidobacteria-rich gut microbiota that is believed to support health and well-being of the infant [110,111].

Breast milk itself is also a source of commensal bacteria that are naturally present in this secretory fluid. It has been estimated that a breastfed (BF) infant ingests between 1 x 10^4

and 1 x 10^6 bacteria daily [112]. Over the past two decades studies have sought to characterize the full diversity of these milk-associated bacterial communities and their relative stability over time. To date, certain bacterial communities in the milk have been identified by both culture-dependent and culture-independent analysis [104,113–115]. As with the composition of breast milk itself, its associated microbiome changes during the lactation period. Different factors are thought to be responsible for its composition and diversity, such as breastfeeding practices, infant gender and maternal factors (BMI, parity and delivery mode) [116]. As previously discussed, obesity influences the maternal gut microbiota but it also affects the milk microbiota and a study in a Kenyan population shows that obese mothers tend to have a less diverse milk microbiota compared with that of normal weight mothers [35,117].

The most frequently found bacteria in human milk are those belonging to the genera *Staphylococcus, Streptococcus, Lactobacillus, Pseudomonas, Bifidobacterium, Corynebacterium, Enterococcus, Acinetobacter, Rothia, Cutibacterium, Veillonella and Bacteroides* [118]. Some genera, like *Staphylococcus, Corynebacterium* or *Propionibacterium* can be isolated from the skin and are also frequently found in human milk [106,119]. It is worth noting that *Bifidobacterium* is present in breast milk, while it is also one of the first and most prevalent and abundant colonizers of the infant gut [115,120]. There is evidence that *Bifidobacterium* genus has a high potential for vertical mother-neonate transfer via breast milk [121]. The high abundancy of bifidobacteria in exclusively breastfed infants is at least partly a result of the ability of these infant-associated bacteria to metabolize HMOs, and the presence of *Bifidobacterium* in breast milk may be the result of and/or contribute to this effect [83,122].

Several studies have shown that not only the nutritional composition, but the microbiota composition differs between colostrum and regular breast milk. Species like *Weisella, Leuconostoc, Staphylococcus, Streptococcus* and *Lactococcus* have predominantly been detected in colostrum samples, while typical inhabitants of the oral cavity such as *Veillonella* and *Prevotella* were shown to be significantly increased in later stage milk samples [117]. To date, the origin of the human milk-associated bacteria remains unknown, leading some authors to suggest that bacteria in human milk are contaminants from the skin or are being regurgitated by the infant during feeding. A recent study showed that the microbiota in pre-colostrum samples that had been collected before delivery and that of the infant oral cavity are similar, indicating that there is no contamination from the infant, and suggesting that an infant receives certain oral bacteria through breastfeeding [123]. Furthermore, two studies have shown that bacteria consumed orally by lactating women or mice can later be isolated from the milk [124,125] suggesting that bacteria reach the mammary gland through an internal, systemic route. Two mechanisms have been proposed for how bacteria are seeding into the breast milk, the Entero-mammary pathway and Retrograde translocation [116,126]. The Entero-mammary pathway proposes that immune cells mediate translocation of bacterial cells from the mother's gastrointestinal tract into the mammary gland [127]. This hypothesis is supported by the fact that colostrum contains bacteria even before the newborn has been breastfed [123]. Retrograde translocation consists of a reverse flow of milk from the infant mouth back into the breast that occurs during breastfeeding, where bacterial origin would be the areola area and the infant oral cavity, supported by the similarity of infant oral microbiota to breastmilk microbiota [127–129].

4.1.2.2 Impact of breast feeding on infant microbiota composition

As described above, breast milk contains all nutrients for optimal infant growth and health. Formula milk is designed to be very similar to breastmilk in order to provide all required nutrients to the infant and represents an ideal option to mothers for whom breast feeding is not possible, or to infants who do not breast feed well. Most formula milk products contain prebiotics such as Galactooligosaccharides (GOS), Fructooligosaccharides (FOS) and, more recently, certain biosynthetic HMOs in order to provide bioactive compounds and nutrients for the microbiota [39]. Despite these similarities, the microbiota of formula-fed (FF) and BF infants are distinct.

Gut microbial diversity, as determined from fecal samples, is increased in infants who are FF compared to those who are predominantly or exclusively BF [87,92,94,130]. Furthermore, infants who are exclusively BF have a less diverse gut microbiota compared to those who had received both formula and breastmilk [94]. The age of the microbiota in a sample can be predicted by assessment of specific species found in BF infants and some studies have used this prediction to show FF infants generally possess an 'older' microbiota when compared to partially or exclusively BF infants [9,87,94]. This may have a negative impact on immune development which is correlated with microbiota composition in early life [131].

Taxonomic differences in microbes between BF and FF individuals are relatively consistent across multiple studies from different geographical locations highlighting that components of breast milk are promoting the enrichment of similar microbial communities [94]. BF infants generally have higher relative abundance of *Bifidobacterium*, *Lactobacillus, Streptococcus*, *Staphylococcus* and *Prevotella* in their stool [9,80,87,93,94]. Infants who receive formula either exclusively or in combination with breastmilk have higher relative

abundance of Firmicutes, Bacteroidetes and Proteobacteria such as *Clostridium difficile*, *Granulicatella adiacens*, *Enterobacter cloacae*, *Bilophila wadsworthia*, *Klebsiella oxytoca*, *Enterococcus faecalis*, *Lachnospiraceae* and *Bacteroides* spp. [9,80,87,93,94]. Many of the taxa in higher abundance in FF infants contain opportunistic pathogens (e.g *C. difficile* and *Klebsiella* spp.) that encode virulence factors and antibiotic resistance genes. Thus, these taxa have a higher pathogenic potential which is important as FF infants are at a higher risk of infection compared to breastfed infants [100,132]. Moreover, infants with higher abundance of Bacteroidaceae and Clostridiaceae in early life (as seen in FF infants) are associated with increased occurrence of allergies, eczema and asthma compared to infants with higher relative abundance of Bifidobacteriaceae and Lactobacillaceae seen in BF infants [133,134]. This may account for why breastfed infants have lower risk of developing allergic diseases [100]

Metagenomic sequencing of fecal samples has provided insights into why certain species thrive in the BF infant and others in FF infants, which corresponds to the compositional changes between these milk types. The gut microbiome of BF infants is enriched in pathways involved in the synthesis of amino acids such as methionine, cysteine/serine, arginine and branched-chain amino acids that are less concentrated in BM compared to FM [39]. Correspondingly, pathways involved in the biosynthesis of histidine, purines, pyrimidines and the tryptophan-precursor chorismate were lower in BF infants compared to FF, presumably because these nutrients are in high abundance in BM compared to FM. Many of the pathways enriched in BF infants could be mapped to *Bifidobacterium* spp. Certain members of the genus *Bifidobacterium* such as *B. breve*, *B. longum* subsp*. infantis* and *B. bifidum* are commonly found in very high prevalence and abundance in breastfed infants due to their ability to digest both lactose and HMOs found in BM [135–137]. These apparently

infant-adapted *Bifidobacterium* species have specific genomic clusters that allow for the intra- or extra-cellular digestion of HMOs.

4.1.2.3 Impact of weaning on microbiota composition

The impact of breastfeeding on the microbiome after weaning from milk to solid food is correlated with the duration of exclusive breastfeeding rather than the age at which weaning occurred [86,87]. The duration of exclusive breastfeeding was shown to be positively correlated with *Bifidobacterium*, *Veillonella*, *Megaspheara*, *Haemophilus*, *Lactobacillus*, *Enterococcus* and *Streptococcus* at an age of 9 months [86]. The effects of breastfeeding on the post-weaning microbiota reduces with time; *Bifidobacterium* spp. are replaced as the dominant species with a concurrent increased relative abundance of members of the phyla Bacteroidetes and Firmicutes such as *Bacteroides*, *Bilophilia*, *Roseburia*, *Ruminococcus* and *Clostridium* [9,86,87,94]. Changes in microbiota composition are delayed in infants that still receive breastmilk whilst at the same time are on a diet that includes solid foods [9,86,87]. The types of food that infants are weaned onto also have an effect on the microbiota composition and diversity [86]. Increased intake of protein and fiber in foods like meat, cheese and rye bread at 9 months is associated with an increase in alpha diversity. By the age of 18 months the effects of breastfeeding on the microbiota are no longer observed, most likely due to the cessation of breastmilk and transition to an exclusive diet of solid and more varied foods [86,87].

Breastfeeding is correlated with improved health outcomes for infants and is associated with reduced levels of sudden infant death syndrome, asthma and necrotizing enterocolitis (NEC) [138]. It is clear that breastfeeding also has a profound impact on the

infant gut microbiota though it is still obscure how these changes in microbial composition precisely impact on early life health outcomes.

4.1.3 Antibiotic usage

Antibiotics are an essential component of modern medicine and are often prescribed to pregnant women either during pregnancy or labor [139]. Antibiotic administration has a substantial effect on the microbiome by reducing microbial load and altering composition [139], the effects of which are observed long after administration [140]. Thus, a number of studies have attempted to identify the effect that perinatal and neonatal antibiotic administration has on the infant fecal microbiome in the short and medium term. Caesarean section birth alters the early infant microbiome composition substantially (see section on delivery mode above) and some of these effects are likely due to prophylactic administration of antibiotics to mothers to prevent surgical site infection [141,142]. *Bacteroides* species are particularly sensitive to antibiotics as they are decreased in both neonates born by caesarean section or vaginally where mothers have received perinatal antibiotics [80]. Transmission of key microbes, such as *Bifidobacterium* and *Bacteroides*, from mother to infant is also reduced in caesarean section born infants [80,143], which may be a result of perinatal antibiotic exposure.

Antibiotic usage in full term infants is common and has been linked to increased incidence of asthma, allergy, eczema, obesity and IBD development [100]. Antibiotic exposure within 1 month of birth has a significant effect on phylogenetic diversity and species observed but diversity recovered within the first year of life [92]. Reduced diversity may be explained by the finding that infants who received antibiotics in the first year of life have more species dominated by a single strain rather than multiple strains [144]. This may be due to the presence of antibiotic resistance genes in those strains giving them a competitive advantage.

Major changes in microbiota composition are not observed in infants who do not received antibiotics until weaning, a time that coincides with an increased immune response to new bacterial taxa [97]. It is possible that antibiotic usage and associated taxonomic changes could result in a premature immune response at the incorrect development stage i.e. pre-weaning.

4.2 Impact of microbiota on preterm infant health

Preterm birth is the second most common cause of neonatal death in the world, presenting an incidence above 10 % worldwide. Defined as an anticipated birth before 37 weeks of gestation, it depends mainly on maternal and fetal genetics. However, it has been suggested that between 40-50 % of preterm births can be associated with microbial etiologies [66]. Preterm birth has been associated with low levels of *Lactobacillus* and overgrowth of strict anaerobic bacteria, such as *Gardenella vaginalis*, *Mobiluncus, Streptococcus* and *Prevotella*, among other anaerobic vaginosis-associated genera [62,66,145].

Preterm infants are commonly prescribed antibiotics due to their underdeveloped immune system and to prevent early onset sepsis [146]. This can markedly affect the gut microbiome development and composition as reflected in reduced alpha diversity in preterm infants who had a brief or intensive course of antibiotics compared to no antibiotic treatment [146]. Prolonged use of antibiotics in preterm infants results in reduced relative abundance of *Bifidobacterium* and *Lactobacillus* when compared to full term infants [147], however, the relative abundance of *Bifidobacterium* increases with age [148]. Preterm infants have a higher relative abundance of *Escherichia*, *Klebsiella* and *Enterobacter*, all of which are part of the ESKAPE pathogen group and many of which are multidrug resistant [139,146]. Metagenomic analysis has shown that members of the ESKAPE family encode the highest number of antibiotic resistance genes in the preterm infant microbiome [139]. Knowledge of which

species encode these genes could lead to tailored antibiotic treatments in the future [149]. Necrotizing enterocolitis is particularly prevalent in pre-term infants and has a complex etiology, though development of abnormal gut microbial composition is important in progression of the disease [108] and can be exacerbated by the use of antibiotics which promote overgrowth of antibiotic resistant taxa such as those listed above. There is clear evidence the microbial supplementation of preterm infants can prevent NEC [150] thus a change in clinical practice should be considered. A recent single center study in the United Kingdom showed that supplementing pre-term infants with *Lactobacillus* and *Bifidobacterium*, species commonly associated with breastfed infants, reduced the incidence of NEC [151] suggesting that specific microbes are important in prevention of this disease.

The effect of antibiotic use on the microbiome is reported as being of a short-term in preterms [146,148], with one study identifying no effect on the microbiota 1-3 years after discharge from neonatal intensive care unit [152]. Despite this, early life is a critical window for immune development and disruption of the microbiota during this period may have longer-term impacts on health [4,5].

4.3 Expert opinion

The early life microbiota is a complex and changeable ecosystem that is affected by multiple factors. There is compelling evidence that delivery mode, infant diet and antibiotic usage are the most important factors in early life microbiota development, but we still do not understand exactly why compositional changes may impact infant health. The evidence is less compelling for the impact of maternal factors before and during pregnancy on infant microbiota development and health. It may be that maternal factors have a limited impact and targeting microbial therapies at the early life window would be more effective. Like all

areas of interest in the field of human microbiota, studies to late have been limited, for the most part, to describing differences in taxa under different conditions. While this information is informative, the field is lacking in knowledge of the underlying mechanisms behind these taxonomic differences and their impact on health.

To date studies have been limited by cost and technology, resulting in many studies being underpowered or using 16S rRNA gene sequencing to determine the changes in the microbial composition in different situations. These studies have reported important but limited findings as 16S sequencing is only reliable to genus level and does not tell anything about the functional capacity of the microbiome. Over the past decade there has been a considerable reduction in cost of DNA sequencing, meaning many more studies will employ more powerful sequencing technologies that provide much greater insight, not only into what microbes are present but also what metabolic functionalities these microbes possess.

Sequencing results can now be obtained and analyzed more quickly due to technological advances. In pre-term infants where gastrointestinal infection by multidrug resistant bacteria can result in poor outcomes or death, rapid diagnostics using sequencing can identify what antimicrobial resistance genes are present in the infant's microbiome thus helping clinicians quickly choose the correct antibiotics for treatment [149]. These results are far quicker, with results in hours, than traditional microbial cultivation techniques where results can take days. Microbial therapies also help prevent infection in pre-term infants and a change in practice is strongly suggested [150,153], however, there is limited information regarding which strains and species are the most effective. More clinical trials of are needed to address this issue and to provide better guidance to clinicians.

Large cohort studies using metagenomic, and sometimes metatranscriptomic, sequencing have provided greater insight into the functionalities of the microbes that are

present in the infant gut and show that some species, such as *Bifidobacterium*, are genomically adapted to specifically prosper in the infant gut. Given the advances and reduction in cost of sequencing and other "omics" technologies, it is expected that many more large cohort studies over the next five years will further add to the field

Another limitation of these studies is their reliance on the use of relative abundance of microbes as this incorrectly presumes that all individuals have the same microbial load and can exaggerate the impact of single species and underplay the role of low abundance microbes. Differences in microbial load have been linked to disease in adults and so it is important to establish any differences that may occur in infants.

While DNA and RNA sequencing provide a relatively insightful view of the functional capacity of the microbiome, there are many complex metabolic pathways that can only be inferred by this type of data. Detailed metabolomic, lipidomic, glycomic and proteomic efforts are expected to provide a greater insight into types of compounds produced by the microbiota that may interact with the host and impact on host physiology. Some of these types of studies have been published and give an even greater insight into the function of bacteria in the infant gut but need to be expanded to build a more complete picture of this complex ecosystem. Recent improvements in cultivation of culturable and so-called "unculturable" microbes will allow for the isolation and detailed examination of these bacteria, aimed at identifying key roles for specific microbes in the gut. Furthermore, cultivation of microbes from both mother and infant will lead to further understanding of the traits that are required for transmission to occur.

Linking the taxonomic changes to long term health outcomes is difficult due to many confounding variables. However, there is a clear impact of the microbiota on the development of the infant. In order to obtain a better understanding of the mechanisms at

play, more complex, carefully designed, longitudinal studies that focus on more than taxonomy are required. There are many questions left to answer including: is there an immune reactions to weaning in humans and how is this altered by disturbances to “normal” microbiota development; how do bacteria reach breastmilk?; and can microbial supplementation during pregnancy result in transmission to infant and improve clinical outcomes? Ultimately the goal of the field should be to improve infant and maternal health and only more detailed knowledge of mechanisms of action of microbiota-host interactions can get us to that point.

4.4 Article highlights

- Early life microbiota composition is influenced by maternal and infant factors
- Microbial colonization primarily occurs after birth but there may be some colonization *in utero*, although this remains highly controversial.
- Maternal factors during pregnancy that can affect the infant microbiota include maternal diet, weight, gestational weight gain and antibiotic usage but overall their impact is low in comparison to that of birth mode and infant diet.
- Microbes are passed from mother to infant during and after birth. The major contributor to the infant microbiota appears to be the maternal gut microbiota and environmental microbes whereas vaginal microbes are present in lower abundance.
- Delivery mode and breastfeeding have the largest effects on microbial composition in early life.
- Altered microbiota composition may lead to increased risk or asthma, eczema and allergies.

Figures:

Figure 1 title: Factors that affect the composition of the infant microbiota:

Figure 1 legend: A schematic outlining the factors the contribute to the infant microbiota composition in early. Further detail of all factors is outline in the text.

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