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1 Maternal and infant factors that shape 2 neonatal gut colonisation by bacteria

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

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

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1 Abstract

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

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

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

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

20

1 Introduction

2 The human gut is home to a vast and diverse community of bacteria, fungi, archaea and
3 viruses collectively known as the gut microbiota [1]. This gut microbiota has profound effects
4 on our health and well-being throughout life, for example by contributing metabolic functions
5 such as digestion of fermentable dietary fibers and production of vitamins such as vitamin K
6 and B group vitamins [2]. Furthermore, the microbiota shapes and provides guidance to the
7 immune system during a critical developmental window in early life (extensively reviewed in
8 [3–5]). Notably, multiple studies of mother-infant pairs have indicated the existence of
9 vertical transmission of microbes from mother to infant that can contribute to this colonizing
10 microbiota [6–9]. Indeed, the composition of the infant microbiota is dynamic and dependent
11 on multiple factors, including maternal microbiota composition, delivery mode, infant feeding
12 mode, antibiotic usage and environmental factors, such as the presence of pets and siblings.
13 Disturbance of the microbiota composition during infancy is also associated with atopic
14 disease, asthma and obesity in later life [10]. This review will focus on recent developments
15 in understanding the factors in infant microbial colonization and the impact of these factors
16 on infant development and risk of later life disease. It is noted that the majority of studies
17 relate the relative abundance of microbes in fecal samples to the composition of the gut
18 microbiota, thus any changes or differences in microbial content discussed herein are based
19 upon changes/differences in relative abundance of microbes unless otherwise stated.

20

21

1 2 Microbiota colonization *in utero*

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

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

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

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

14 Whether or not the placenta harbors a microbiome is still under debate [12,13,16–
15 18]. Some studies have shown by either cultivation or DNA sequencing that the placenta is
16 dominated by Proteobacteria and contains species such as *Escherichia* and *Enterococcus*
17 *faecalis* [12,13,17,26]. More recently, however, a very thorough examination of the placental
18 microbiome which incorporated multiple controls for contamination showed that the
19 bacteria detected were contaminants or species acquired during labor [18]. One exception
20 was Group B *Streptococci*, a common infection that can lead to pregnancy complications.

21 A recent study reported on the presence of bacteria in human fetal intestines *in utero*
22 [28]. These bacteria were identified by scanning electron microscopy and 16S rRNA
23 sequencing in a small number of fetal meconium samples obtained from terminated
24 pregnancies. Despite the very low number of bacteria in the meconium, *Lactobacillus* and

1 *Micrococcus* were identified as the most abundant taxa in some of the samples and
2 *Bacteroides*, *Bifidobacterium* and *Prevotella* in others. In addition, *Micrococcus luteus* was
3 successfully cultivated from meconium [28]. *Micrococcus* spp. is often found in the
4 cervicovaginal microbiome, however, in this study bacteria were only isolated from
5 meconium so it is unclear if this exact strain originated from the mother's vagina [28]. These
6 findings in a small cohort suggest that colonization of the meconium *in utero* is highly
7 restricted both taxonomically and spatially. Detection of microbes in the meconium soon
8 after birth has been used as an indication that microbes colonize the infant gut before birth
9 [13,17]. Longer labor and time after labor before the meconium is passed impact on whether
10 bacteria can be detected in the meconium suggesting that bacteria detected in the meconium
11 are acquired after birth rather than *in utero* [11].

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

15 3 Maternal factors that contribute to the infant gut microbiota

16 3.1 Maternal diet and health status before and during pregnancy.

17

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

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

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

9 Maternal weight can also impact the microbes present in the infant gut. Infants born
10 to obese or overweight mothers are three times more likely to be overweight by one year of
11 age, which increases to five times in infants born by caesarean section [34]. An increase in
12 infant associated *Lachnospiraceae* was associated with overweight mothers but the genus of
13 *Lachnospiraceae* was different between vaginally born and caesarean section born infants.
14 This suggests that it is the maternal microbiome rather than delivery mode that is a factor for
15 obesity in early life. Maternal obesity impacts on the alpha diversity (diversity within a given
16 environment i.e. the diversity of microbes within a given infant fecal sample) of vaginally born
17 but not caesarean born infants at 6 weeks of age [38]. Furthermore, changes in beta diversity
18 associated with maternal obesity were only seen in vaginally and not caesarean born infants
19 [29]. Vaginally born infants with overweight mothers have been shown to possess a gut
20 microbiota with a higher relative abundance of *Escherichia*, *Enterococcus*, *Klebsiella* and
21 *Ruminococcus*, while infants born to obese mothers carry a gut microbiota with a higher
22 relative abundance of *Parabacteroides* and *Staphylococcus* [38]. These observed differences
23 have been shown to vary between studies as another investigation found that *Enterococcus*
24 was depleted in infants born to obese or overweight mothers [29], while *Bacteroides*

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

7 Weight gain during pregnancy is a normal physiological response, however, excessive
8 gestational weight gain (defined as 16 kg or above for women with BMI 19.8-25 or above 11.5
9 for women with BMI > 25 [40]) has been shown to result in increased relative abundance of
10 *Escherichia* and *Dorea* in vaginally born infants [38]. Metagenomics analysis has shown that
11 gestational weight gain impacts on the function of the infant microbiome [39]. Enrichment of
12 bacterial glucose pathways and phenylalanine, cysteine/serine, folate, thiamine, biotin, and
13 pyridoxine synthesis pathways in infants were shown to be associated with maternal
14 gestational weight gain [39]. These effects are seen up to 8 months post-birth, highlighting a
15 longer-term impact on microbiota function. The relative abundance of *Bifidobacterium*, a key
16 genus in the healthy early infant gut, was decreased in infants of mothers with excessive
17 weight gain [30] and those with insufficient weight gain (< 11.5 kg for women with BMI 19.8-
18 25 and < 7.0 kg for women with BMI > 25) [38], suggesting that this genus is sensitive to
19 maternal factors in pregnancy.

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

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

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

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

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

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

7

8 3.2 The vaginal microbiome

9

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

16 The composition and characterization of the vaginal microbiome has been studied
17 using culture-dependent [51,52] and culture-independent methods [53]. The diversity of the
18 cervicovaginal microbiota is generally low, dominated by one or more species of the genus
19 *Lactobacillus* [54]. Several studies reported that the most frequently identified species are
20 *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners* and *Lactobacillus jensenii*.
21 [55–58]. Genera that are present in lower relative abundance are *Enterococcus*, *Prevotella*,
22 *Bifidobacterium*, *Dialister*, *Atopobium*, *Gardnerella*, *Megasphaera*, *Peptoniphilus*, *Sneathia*,
23 *Eggerthella*, *Aerococcus*, *Fingoldia*, and *Mobiluncus* [47,59]. Because the vaginal microbiome
24 profile is very variable amongst individuals and over time, some researchers have suggested

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

5 The cervicovaginal microbiome is a very dynamic ecosystem, influenced by many
6 factors and physiological changes such as sexual development, sexual intercourse, personal
7 hygiene, menstruation cycle, pregnancy, menopause and hormonal changes [62]. It is worth
8 noting that one of the factors that has a major influence is racial background, where large
9 differences are observed in terms of diversity and abundance. Several studies have shown
10 that African women and women of African descent have a lower *Lactobacillus* abundance and
11 higher ecological diversity than Caucasian women [63–67], associating this fact with higher
12 risk of HIV infection [68,69]. Similar studies were carried out in Japanese and Chinese
13 populations, showing high similarity with those previously described for Caucasian women
14 [56,70].

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

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

6 The impact of the vaginal microbiome on the infant before birth remains unknown,
7 however gestational changes that happen during the pregnancy could be part of an adaptive
8 response to promote the correct development and health of the fetus [72]. The composition
9 of the vaginal and gut microbiota are related to each other, as studies have shown that many
10 bacteria are shared between rectum and vagina, including *Lactobacillus* and *Bifidobacterium*
11 [76,77]. Moreover, some of the earliest colonizers of the infant gut are species present in the
12 vagina [78]. The most dominant genus in the vagina, *Lactobacillus*, has been shown to persist
13 in the infant gut long after birth [8,79,80], although one study suggests that specific species
14 of *Lactobacillus* from the maternal vagina cannot be detected in the infant gut by one month,
15 while other vaginal species such as *Atopobium vaginae* and *Gardnerella vaginalis* were still
16 detected, although whether these strains are inherited from the mother is unknown [6].

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

23

1 4 Microbial colonization of the neonatal gut following birth

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

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

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

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

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

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

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

8

9 4.1 Factors that affect microbial composition after birth

10

11 4.1.1 Delivery mode

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

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

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

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

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

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

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

23

1 4.1.2 Breast milk microbiota and infant diet

2 4.1.2.1 Breast milk composition and microbial content

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

11 The composition of the milk varies and depends on different factors: stage of lactation
12 period; degree of breast fullness; infant feeding; mother-infant health status; maternal diet
13 and maternal genetics [106,107]. Breast milk is composed of carbohydrates, fats,
14 proteins/amino acids, iron/lactoferrin and antibodies that are important for infant nutrition
15 and health. Lactose is the primary carbohydrate and provides an efficient energy source for
16 the growing infant. In addition, breast milk also contains human milk oligosaccharides (HMOs)
17 that can only be digested by certain members of the gut microbiota. HMOs can prevent
18 infection by pathogenic bacteria by promoting growth of competitive commensal bacteria
19 [108,109]. The presence of certain HMOs in human milk is correlated with increased relative
20 abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. in the infant gut contributing to the
21 development of a bifidobacteria-rich gut microbiota that is believed to support health and
22 well-being of the infant [110,111].

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

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

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

1 Several studies have shown that not only the nutritional composition, but the
2 microbiota composition differs between colostrum and regular breast milk. Species like
3 *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus* and *Lactococcus* have predominantly
4 been detected in colostrum samples, while typical inhabitants of the oral cavity such as
5 *Veillonella* and *Prevotella* were shown to be significantly increased in later stage milk samples
6 [117]. To date, the origin of the human milk-associated bacteria remains unknown, leading
7 some authors to suggest that bacteria in human milk are contaminants from the skin or are
8 being regurgitated by the infant during feeding. A recent study showed that the microbiota
9 in pre-colostrum samples that had been collected before delivery and that of the infant oral
10 cavity are similar, indicating that there is no contamination from the infant, and suggesting
11 that an infant receives certain oral bacteria through breastfeeding [123]. Furthermore, two
12 studies have shown that bacteria consumed orally by lactating women or mice can later be
13 isolated from the milk [124,125] suggesting that bacteria reach the mammary gland through
14 an internal, systemic route. Two mechanisms have been proposed for how bacteria are
15 seeding into the breast milk, the Entero-mammary pathway and Retrograde translocation
16 [116,126]. The Entero-mammary pathway proposes that immune cells mediate translocation
17 of bacterial cells from the mother's gastrointestinal tract into the mammary gland [127]. This
18 hypothesis is supported by the fact that colostrum contains bacteria even before the newborn
19 has been breastfed [123]. Retrograde translocation consists of a reverse flow of milk from the
20 infant mouth back into the breast that occurs during breastfeeding, where bacterial origin
21 would be the areola area and the infant oral cavity, supported by the similarity of infant oral
22 microbiota to breastmilk microbiota [127–129].

23

1 4.1.2.2 *Impact of breast feeding on infant microbiota composition*

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

10 Gut microbial diversity, as determined from fecal samples, is increased in infants who
11 are FF compared to those who are predominantly or exclusively BF [87,92,94,130].
12 Furthermore, infants who are exclusively BF have a less diverse gut microbiota compared to
13 those who had received both formula and breastmilk [94]. The age of the microbiota in a
14 sample can be predicted by assessment of specific species found in BF infants and some
15 studies have used this prediction to show FF infants generally possess an 'older' microbiota
16 when compared to partially or exclusively BF infants [9,87,94]. This may have a negative
17 impact on immune development which is correlated with microbiota composition in early life
18 [131].

19 Taxonomic differences in microbes between BF and FF individuals are relatively
20 consistent across multiple studies from different geographical locations highlighting that
21 components of breast milk are promoting the enrichment of similar microbial communities
22 [94]. BF infants generally have higher relative abundance of *Bifidobacterium*, *Lactobacillus*,
23 *Streptococcus*, *Staphylococcus* and *Prevotella* in their stool [9,80,87,93,94]. Infants who
24 receive formula either exclusively or in combination with breastmilk have higher relative

1 abundance of Firmicutes, Bacteroidetes and Proteobacteria such as *Clostridium difficile*,
2 *Granulicatella adiacens*, *Enterobacter cloacae*, *Bilophila wadsworthia*, *Klebsiella oxytoca*,
3 *Enterococcus faecalis*, *Lachnospiraceae* and *Bacteroides* spp. [9,80,87,93,94]. Many of the
4 taxa in higher abundance in FF infants contain opportunistic pathogens (e.g *C. difficile* and
5 *Klebsiella* spp.) that encode virulence factors and antibiotic resistance genes. Thus, these taxa
6 have a higher pathogenic potential which is important as FF infants are at a higher risk of
7 infection compared to breastfed infants [100,132]. Moreover, infants with higher abundance
8 of Bacteroidaceae and Clostridiaceae in early life (as seen in FF infants) are associated with
9 increased occurrence of allergies, eczema and asthma compared to infants with higher
10 relative abundance of Bifidobacteriaceae and Lactobacillaceae seen in BF infants [133,134].
11 This may account for why breastfed infants have lower risk of developing allergic diseases
12 [100]

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

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

3

4 4.1.2.3 *Impact of weaning on microbiota composition*

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

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

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

3 4.1.3 Antibiotic usage

4 Antibiotics are an essential component of modern medicine and are often prescribed to
5 pregnant women either during pregnancy or labor [139]. Antibiotic administration has a
6 substantial effect on the microbiome by reducing microbial load and altering composition
7 [139], the effects of which are observed long after administration [140]. Thus, a number of
8 studies have attempted to identify the effect that perinatal and neonatal antibiotic
9 administration has on the infant fecal microbiome in the short and medium term. Caesarean
10 section birth alters the early infant microbiome composition substantially (see section on
11 delivery mode above) and some of these effects are likely due to prophylactic administration
12 of antibiotics to mothers to prevent surgical site infection [141,142]. *Bacteroides* species are
13 particularly sensitive to antibiotics as they are decreased in both neonates born by caesarean
14 section or vaginally where mothers have received perinatal antibiotics [80]. Transmission of
15 key microbes, such as *Bifidobacterium* and *Bacteroides*, from mother to infant is also reduced
16 in caesarean section born infants [80,143], which may be a result of perinatal antibiotic
17 exposure.

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

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

5

6 4.2 Impact of microbiota on preterm infant health

7

8 Preterm birth is the second most common cause of neonatal death in the world,
9 presenting an incidence above 10 % worldwide. Defined as an anticipated birth before 37
10 weeks of gestation, it depends mainly on maternal and fetal genetics. However, it has been
11 suggested that between 40-50 % of preterm births can be associated with microbial etiologies
12 [66]. Preterm birth has been associated with low levels of *Lactobacillus* and overgrowth of
13 strict anaerobic bacteria, such as *Gardenella vaginalis*, *Mobiluncus*, *Streptococcus* and
14 *Prevotella*, among other anaerobic vaginosis-associated genera [62,66,145].

15 Preterm infants are commonly prescribed antibiotics due to their underdeveloped
16 immune system and to prevent early onset sepsis [146]. This can markedly affect the gut
17 microbiome development and composition as reflected in reduced alpha diversity in preterm
18 infants who had a brief or intensive course of antibiotics compared to no antibiotic treatment
19 [146]. Prolonged use of antibiotics in preterm infants results in reduced relative abundance
20 of *Bifidobacterium* and *Lactobacillus* when compared to full term infants [147], however, the
21 relative abundance of *Bifidobacterium* increases with age [148]. Preterm infants have a higher
22 relative abundance of *Escherichia*, *Klebsiella* and *Enterobacter*, all of which are part of the
23 ESKAPE pathogen group and many of which are multidrug resistant [139,146]. Metagenomic
24 analysis has shown that members of the ESKAPE family encode the highest number of
25 antibiotic resistance genes in the preterm infant microbiome [139]. Knowledge of which

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

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

16

17 4.3 Expert opinion

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

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

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

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

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

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

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

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

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

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

9

10 4.4 Article highlights

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

24

1 Figures:

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

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

5

6 4.5 Bibliography

7

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