Review

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The neonatal microbiome *in utero* and beyond: perinatal influences and long-term impacts

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Abstract: The neonatal microbiome offers a valuable model for studying the origins of human health and disease. As the field of metagenomics expands, we also increase our understanding of early life influences on its development. In this review we will describe common techniques used to define and measure the microbiome. We will review *in utero* influences, normal perinatal development, and known risk factors for abnormal neonatal microbiome development. Finally, we will summarize current evidence that links early life microbial impacts on the development of chronic inflammatory diseases, obesity, and atopy.

Keywords: antenatal; dysbiosis; microbiome; microbiota; neonate; preterm.

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Introduction

The neonatal microbiome offers a valuable model to explore the developmental origins of health and disease. Multiple studies have described the importance of the first 1,000 days of life, during which key physiologic exposures can have profound impacts on future health outcomes [1]. The establishment of a healthy microbiome, encompassing trillions of microbial cells and their respective genomes, is critical during this time [2]. The neonatal microbiome begins as early as the antenatal period, where multiple studies suggest that maternal microbiota colonize the infant via the *in utero* environment [3–5]. From here, mode of delivery, breastfeeding, diet and environment lead to a rapid expansion in bacterial complexity as the microbiome becomes established. This steady trajectory of progressive bacterial colonization becomes critical for immunologic priming, physiologic function across multiple organ systems, and lays the foundations for future health and disease [6].

The study of the human microbiome goes back to the late 1800s in Europe, when German paediatrician Theodor Escherich first described the role of the “gut flora” through his discovery of *Bacterium coli commune* (posthumously named *Escherichia coli*) [7]. His research culminated in an 1885 publication of two articles: *The Intestinal Bacteria of the Neonate and Breast-Fed Infant*, an article in which he described the presence of *E. coli* in the stool of healthy babies, and *The Intestinal Bacteria of the Infant and Their Relation to the Physiology of Digestion*, which comprehensively described the microbiology of infant stool [7, 8]. This work was continued by French scientist Henry Tissier, who administered early forms of probiotic bacteria to children and adults to improve gastrointestinal ailments. Tissier used *Bacillus acidipara-lactici*, a bacteria that he knew to be resistant to acid degradation and had strong “fermentative power,” and administered this to breastfed infants [9, 10]. The use of stool from various species of animals has been used as remedies across Europe since the 18th century [11]. During World War I, Alfred Nissle patented gelatin capsules of *E. coli* Nissle 1917 to treat bacterial dysentery, after discovering that some *E. coli* strains impeded the growth of Salmonella [12]. His capsules of *E. coli* Nissle 1917 were

cultured from the stool of a soldier who appeared immune to the epidemic of dysentery in Dobrudja, in the Balkans [13].

This rich history of early microbiome work re-emerged over the past two decades, as national funding initiatives such as The Human Microbiome Project (HMP), launched in 2007 by the United States National Institutes of Health, was the first national funding initiative to explore the role of the microbiome on human health [14]. Since 2007, additional funding from federal research funds and private enterprise have improved tools used to measure, and describe the human microbiome. Advances in “-omic” and systems biology have increased our knowledge of the composition and function of the microbiome. Affordability, access, and rapid results have contributed to the explosion in microbiome research, enabling investigators worldwide to study and describe its many roles.

The human microbiome challenges traditional concepts of bacteria and other microorganisms as pathogens. Microbes are overwhelmingly non-pathogenic and generally exist in a state of symbiosis with the host [15]. In 1989, David Strachan first reported how within household, younger children often had lower rates of hay fever and asthma [16]. Strachan hypothesized that this was due to early-life exposures to their older siblings who created a more “unhygienic” environment in the home. This sentinel work became the basis for the hygiene hypothesis, which proposes that exposure to microorganisms is critically important for regulating immune response and that the absence of early life exposure to bacteria and other microbes may increase the risk of atopy and allergic disease. In 2012, Blumberg et al. demonstrated this using mice raised under sterile, germ free conditions [17]. These animals were more likely to develop inflammatory colitis and a form of allergic asthma. Importantly, reconstituting their gut with commensal bacteria obtain from standard (non germ free) colony mice prevented these intestinal and respiratory symptoms, but only if administered to very young germ-free mice [6, 17, 18]. A similar framework may be applied to the development of the neonatal microbiome [19]. Thus, while the neonatal immune system is relatively immature and susceptible to pathogenic infections, it is also an important period for the establishment of microbial communities that are critical for future immune maturation.

In this review, we will describe the development of the neonatal gut microbiome, key influences, and the impact of early life microbial stressors on long-term health outcomes.

Definitions and measurement: the vocabulary of microbiome research

The field of microbiome research is rapidly evolving and its accompanying terminology is frequently misused [20].

An initial understanding of several common terms is first necessary.

The *microbiota* refers to the totality of microorganisms (bacteria, fungi, viruses) that exist in a particular environment [21]. These include commensal, symbiotic, and pathogenic organisms. The vast majority of human microbiome research focuses on describing the microbiota in the human gastrointestinal tract, generally inferred based on the composition of microbiota detected in stool. While previous research once estimated higher ratios of microbial cells to human cells by an overwhelming ratio of 10:1, more recent estimates approximate a ratio closer to 1.3:1. Despite these more modest numbers, the biological importance of microbiota remains unchallenged.

The *microbiome* refers to the totality of *microbiota* (bacteria, fungi, viruses), along with their collection of genes and genomes [21].

Dysbiosis refers to a change or imbalance in the composition of microbiota found in a community, relative to what would be found in otherwise healthy individuals [22]. These changes may involve an abnormal distribution and/or abundance of microorganisms. While the term, dysbiosis, is used frequently in the literature there remains no clear gold-standard for what constitutes a “normal” microbiome. Composition varies widely across different geographies, households, and diets. Nevertheless, specific, commonly seen differences in distribution and/or abundance of particular bacterial taxa have been described across particular diseases and conditions. Dysbiosis can contribute to the onset of diseases in three main ways. First, pathogenic bacteria can colonize and overgrow, and their respective functional metabolic output can cause infectious diseases or systemic inflammation. Second, protective commensal bacteria and their functional metabolic outputs can be lost, or suppressed, permitting the onset of indolent disease. Finally, a combination of pathogenic overgrowth; and reduction in protective commensal bacteria can occur, ultimately leading to disease [23].

Tools for microbiome analysis

Several approaches have been developed to measure the composition and function of microbial communities. Culture-based methods were once the primary method for profiling bacterial taxa. This labour-intensive process was limited by the ability to grow certain organisms. As many as 60–85% of bacteria became nonviable during processing and would not grow in standard culture media [24, 25].

The field of *metagenomics* was largely developed as a culture-independent, high-throughput approach to assessing bacterial deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). 16S-based metagenomics uses the variable regions of the 16S ribosomal RNA (*rRNA*) gene to classify different bacteria. 16S *rRNA* genes are highly conserved across phylogeny, and measurement of these sequence variants (known as operational taxonomic units, or OTUs) can allow for rapid classification of bacterial species. Drawbacks to this technique include the inability to accurately differentiate closely related species due to shared sequence homology of the 16S *rRNA* hypervariable regions. Genus-level taxonomy is generally the limit of bacterial classification through 16S *rRNA* sequencing techniques [26]. Other drawbacks include differences in bacterial detection depending on which hypervariable region is employed (or amplified) by a particular sequencing technology, and being unable to differentiate between culturable (viable) and non-viable taxa [27].

Whole genome sequencing (WGS), also called “functional metagenomics” offers information about the microbiome, reporting the total gene content and its metabolic capacity [28], based on the presence of genes which encode for specific metabolic pathways. A significant amount of data is generated from this technique, which provides detailed taxonomic information (including classification to species, and strain levels) and functional information (by inferring functional output based on the relative abundance of known genes). Drawbacks of this technique are high cost and bioinformatics demands of processing large amounts of raw data [29].

Finally, metabolomics describes the array of metabolites generated by one’s microbiota. Techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are often used. This analysis can offer valuable data about the types of downstream, biologically active metabolites produced by the bacteria which could potentially interact with the host.

Ultimately, a fulsome understanding of the microbiome requires combining data obtained through multiple techniques and analyses. Both WGS and 16S *rRNA* sequencing describe the composition of the bacterial community, and its *potential* function [30]. Additional tools, such as metabolomics, metatranscriptomics, and metaproteomics can offer further detail on *actual* function [31]. These topics are beyond the scope of our review but have been described in greater detail [20]. Integrating data presents significant bioinformatics challenges, but together offer the most comprehensive view of the contribution of the microbiome.

The *in utero* microbiome

The traditional belief was that all fetal development occurs within a sterile, *in utero* environment. Microbial colonization was thought to begin at the time of birth via vertical (maternal) transmission and horizontal (environmental) transmission [32, 33]. In 2010, Mshvildadze et al. described the presence of microbial DNA in the meconium of neonates, first suggesting an intrauterine origin of neonatal microbiota [34]. Since then, additional research continues to challenge the concept of a sterile *in utero* environment. Multiple studies have detected the presence of microbiota within the first meconium samples taken from infants, including *Staphylococcus*, *Enterobacteriaceae*, *Enterococcus*, *Lactobacillus*, and *Bifidobacterium* [3]. In addition to amniotic fluid and meconium, additional evidence has suggested bacteria present within the umbilical cord blood, and possibly within the placenta itself [4, 5].

The extent of *in utero* colonization and the mode of bacterial passage continue to be the subject of disagreement. Mesa et al. have described how commensal and pathogenic bacteria may ascend from the vagina and seed *in utero* [35]. However, due to the physiologic decline in bacterial diversity within the vaginal microbiome during pregnancy, this has been challenged as the sole origin for the neonatal microbiome [36]. Fardini et al. suggested that maternal oral microbiota, including *Fusobacterium nucleatum* may affect the permeability of the placental vascular endothelium, allowing for hematogenous seeding and translocation of maternal microbes from the oropharynx and intestinal lumen [37]. Finally, Versalovic et al. suggested there may be a distinct placental microbiome, based on the detection of distinct bacterial taxa from 16S *rRNA* sequencing of placental tissue [5]. These findings have been challenged. The presence of bacterial DNA on the placenta does not confirm that these taxa were viable [38], and contamination remains an ongoing issue in microbiome studies, particularly with tissues that are not handled under sterile conditions [39].

Physiologic influences on the neonatal microbiome

Several common perinatal exposures can affect the development of the neonatal microbiome (Figure 1). Prenatal factors including maternal obesity, maternal diet, smoking and the use of antibiotics have all been described to affect the development of the neonatal microbiome. Animal studies have shown that a high-fat diet in pregnancy can lead to decreased microbial diversity within the offspring for up to one year [40]. These changes may persist longer [41, 42]. Additional evidence suggests that maternal

Figure 1: Factors influencing the development of the infant microbiome and immune system.

A variety of factors exert important influences on the neonatal microbiome including mode of delivery, mode of feeding, perinatal exposure to antibiotics, maternal genitourinary and intestinal microbiota, maternal skin flora, maternal comorbidities, and genetics. Created with BioRender.com.

obesity and extent of weight gain during pregnancy may also alter the neonatal microbiome compared to mothers without obesity [43]. This has been proposed as a potential factor in the development of childhood obesity [44].

Mode of delivery is widely regarded as the first, and most significant contributor to early life microbial colonization; however, the long-term effects of this remain unclear. *Bacteroides fragilis* appears to be predominant in vaginal deliveries; this species has been associated with greater intestinal microbial diversity and faster maturation. Conversely, infants born via elective caesarean section tend to have decreased bacterial diversity [45]. This may be a consequence of intrapartum antibiotics, lack of exposure to vaginal microbes, and increased exposure to skin microbes [45]. Of note, while mode of delivery appears to influence bacterial profiles immediately after birth, this does not appear to persist over time [46]. It is possible that the indication for C-section, rather than the mode of delivery itself, exerts a greater impact on the neonatal microbiome in the long term. It can be challenging to tease apart the many confounders to assess this further [42].

Duration of gestation may impart short and long-term consequences for the neonatal microbiome [42]. The preterm neonatal microbiome has decreased bacterial diversity, and overall abundance compared to term infants [47]. Premature infants appear to have a delay in microbial colonization, as well as higher relative abundance of potentially pathogenic bacteria like *Klebsiella* species and *Clostridium* species [48]. This may be due to increased pathogenic bacteria within the hospital/neonatal intensive care unit (NICU) environment, and decreased microbial diversity in the preterm gut. Ultimately, these differences may result in increased susceptibility to opportunistic infection [49]. Several studies have described similar intestinal microbial profiles in term and preterm infants by age 1–3 years [42]. However, while this may illustrate how early-life differences in gut microbial colonization converge over time, the neonatal period in a preterm's life has high morbidity with frequent neonatal infections and complications. These initial differences may have major impacts on neonatal outcomes.

Normal perinatal microbiome development

Standard trajectory of gut bacterial colonization

While questions remain about the extent of microbial colonization *in-utero*, the greatest expansion of bacterial colonization and diversification occurs at birth (Figure 2). The neonatal microbiome is suddenly exposed to potential maternal antibiotic use, vaginal vs. caesarean section delivery, breastmilk, formula, solids, and multiple environmental exposures [42, 50]. It is estimated that only 9% of the intestinal microbiome is affected by the host's genetic background; the majority of bacterial colonization is therefore derived from external sources [32, 41].

Initial colonizers of the infant microbiome vary based on mode of delivery and gestational age. In term infants born

via spontaneous vaginal delivery, neonatal gut colonization is dominated by genera such as *Lactobacillus* and *Prevotella*, *Bifidobacterium* and *Bacteroides*. In contrast, infants born by caesarean section (C-section) are more likely to become colonized by environmental microorganisms from maternal skin and the hospital environment, including *Proteobacteria* and *Firmicutes* species, and *Clostridium sensu stricto* (cluster I) and *Clostridium difficile* [51, 52]. Subsequent maturation of the microbiome is characterized by increased density and diversity of obligate anaerobes, such as *Bacteroidetes*, *Firmicutes*, *Clostridia*, *Lactobacillaceae* and *Bifidobacteria* [50]. Breast milk contains a significant amount of *Bifidobacteriaceae*, which use human milk-derived oligosaccharides (HMOs) as their primary substrates [53]. HMOs play an important role in neonatal growth and nutrition by increasing intestinal mucin production, thereby decreasing mucosal stimulation by gut bacteria [50]. As infants transition from human milk to formula, *Bifidobacteriaceae* levels decrease, along with a commensurate rise in *Clostridia*,

Figure 2: Microbial changes from gestation to adulthood.

A healthy infant microbiome is characterized by increased concentrations of *Bifidobacterium* and *Lactobacilli*, and decreased concentrations of *E. coli*, Enterobacteriaceae, and Bacteroidetes. The mucous layer also increases in density from infancy to adulthood. Imbalances in microbial communities alters immune priming and increases the risk of atopy and inflammatory disease. Created with BioRender.com.

Ruminococcus, and *Bacteroides* [42]. It is suspected that translocation of maternal microbiota into breastmilk occurs via enteromammary pathways, given the presence of milk microbes in infants who have never latched [54]. Further studies will continue to support whether bacterial contamination from infant secretions, or bacterial translocation from the maternal microbiome, constitute the source of *Bifidobacteria* in breastmilk.

Impact on neurodevelopment

Early life microbial dysbiosis may influence neurodevelopment, including neurodevelopmental and neuropsychiatric disorders [55]. Developmental windows for the gut microbiota and nervous system parallel in early life. Several studies have linked alterations in the gut microbiome to neuropathologies such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), and Rett syndrome, and disruptions in the microbiome have been linked to neuropsychiatric disorders, such as schizophrenia, mood disorders, and dementia [56]. Microbiota can inhibit histone deacetylases (HDAC), affect microglia, produce neurotransmitters, translocate across the blood brain barrier, and interact with SCFA free fatty acid receptors (FFAR) in the brain [57–60]. These effects can all play a critical role in brain development, particularly in preterm infants [61]. A case control study by Wan et al. compared 17 children with ADHD to 17 healthy, age-matched controls and found the ADHD group had significantly lower *Faecalibacterium prausnitzii*, *Lachnospiraceae* bacterium, and *Ruminococcus gnavus* compared to healthy controls, while *Bacteroides caccae*, *Odoribacter splanchnicus*, *Paraprevotella xylaniphila*, and *Veillonella parvula* were significantly increased in children with ADHD. Interestingly, (inferred) functional analysis of these taxa revealed significant between-group differences in the metabolic pathways of neurotransmitters (serotonin and dopamine) [62]. Bojović et al. showed that children with neurodevelopmental disorders had less butyrate-producing taxa and an increase in *Clostridia* when compared to health age-matched controls [63].

Further studies are required to elucidate the relationship between the gut microbiota composition, and the neurodevelopmental and neuropsychiatric disorders, yet this data has clear implications for the neonatal microbiome. A 2021 study by Carlson et al. showed that lower gut microbiota alpha-diversity at 1-month of life, and increased abundance of *Veillonella*, *Dialister*, and *Clostridiales* at 1-year was positively associated with increased fear, and anxiety-behaviors among infants [64].

Immune priming

The microbiome of an infant in the first three years of life broadly differs from an adult microbiome. Neonatal, and infant intestinal microbiota have lower diversity, reflected by fewer OTUs (alpha diversity), and higher interindividual variability (beta diversity) [65]. Increased phylogenetic composition and smaller interpersonal variation evolves over the first three years of life, and then mostly remains constant through adulthood hereafter. Significant differences persist between individuals living in different countries and consuming different diets, suggesting that age, geography, and cultural traditions primarily explain intestinal microbiome variation [66].

During pregnancy, fetal immune development is supported by microbial metabolites originating from the maternal microbiota and maternal dietary. Innate immune cell populations, such as monocytes, innate lymphoid cells (ILC), and neutrophils are intrinsically immature at this stage. At birth, the emerging immune system of the newborn first encounters live bacteria and remains dependent on maternal antibody protection, which is provided through breastmilk. Along with passive immunization via breast milk, neonatal invariant natural killer T (iNKT) cells, natural killer (NK) cells, ILCs, and the gastrointestinal epithelial barrier guard against invading pathogens and foster the beneficial interplay with the neonatal microbiota.

The “window of opportunity” for commensal bacteria to support the proper development of the immune system is unclear in humans. In mouse models, this window appears to close around the time of weaning, after which further exposure to healthy microbiota does not appear to reverse immune dysfunction, as described in germ-free mouse models. Infants appear to develop relatively stable microbial composition by approximately 2–3 years old. Nevertheless, further changes may still occur later into early childhood (Figure 2).

Growth and nutrition

The gut microbiome plays an important role in affecting growth. Turnbaugh et al., led by the pioneering microbiome investigator Jeffrey Gordon, described the effects of transferring stool from obese to lean mice, in which recipient mice gained greater adiposity following transfer [67]. Adipocyte production is initiated when gut microbiota ferment polysaccharides into short chain fatty acids (SCFA) and monosaccharides absorbed by the intestine, thereby stimulating lipid conversion. Studies also described the influence of the gut microbiome on malnutrition. A study

by Smith et al., also through the Gordon lab, described Malawian twins discordant for kwashiorkor and differences in their respective intestinal microbiomes. Although no specific taxa were found to be specifically related to kwashiorkor, when stool from the affected twins were transferred into gnotobiotic mice, the mice subsequently developed weight loss and changes in metabolism, in keeping with their human donor [68].

Early exposure to antibiotics has been shown to alter the composition and metabolic activity of intestinal microbiota. This has been associated with changes in the production of SCFA. SCFAs interact with G-protein-coupled receptors (GPCRs), which regulate adiposity, insulin secretion, and appetite and satiety signaling [69].

Neonatal antibiotic exposure has been shown to impact growth in children up to age 6 years, through impacts on the colonization of the intestinal microbiome. A 2021 study by Uzan-Yulzari et al. assessed the long-term impact of antibiotic use in a cohort of 12,422 full term children. Decreased abundance and diversity of faecal *Bifidobacteria* was seen until age 2 years, and decreased weight and height gain until age 6 years, in antibiotic-exposed children compared to unexposed controls. Further, faecal transfer from antibiotic-exposed children to germ-free mice also resulted in significant growth impairment in mice [70].

Insults and perturbations to normal microbiome development

Maternal factors influencing microbiome development

Maternal microbiome

The development of the neonatal microbiome has been associated with several maternal factors [71]. Maternal breast milk contains IgA antibodies, which bind to intestinal bacteria in the infant and may have a protective role against the development of necrotizing enterocolitis (NEC) [72].

Maternal BMI

Body composition and dietary habits of the mother have been shown to have several effects on neonatal development, including neonatal microbial colonization, immune function, and the development of obesity and metabolic syndrome [73]. Collado et al. found that mothers who were overweight or obese prior to pregnancy had significant differences in their gut microbiome

compared to normal weight mothers in their first and third trimesters of pregnancy [73–75]. Mothers who gained excessive weight during gestation, regardless of pre-pregnancy weight, were also found to have significant differences in their gut microbial profiles than normal weight mothers [43, 74, 75]. Infants of overweight and obese mothers were found to share similar microbial compositions, suggesting that maternal weight can direct neonatal microbial composition [73, 75].

Maternal diet

Maternal diet has been implicated in the development of the neonatal microbiome. Chu et al. demonstrated that infants of mothers who consumed a high-fat diet exhibited persistent depletion of *Bacteroides* and enrichment of *Enterococcus* [76]. This has been supported by animal models, where non-human primates who habitually consumed a high-fat diet during gestation and lactation caused significant alterations in the microbial composition of their offspring. More importantly, this dysbiosis in their offspring remained relatively fixed, and did not completely correct even after offspring switched to a healthier control diet after weaning [77]. As such, the maternal diet may be implicated in early bacterial colonisation and may have long-lasting effects on infant development, including increased risk of metabolic and immunological disease patterning [73].

Maternal antibiotic usage

Antibiotics have known impacts on the intestinal microbiome, and are frequently prescribed to mothers during pregnancy and intrapartum (Table 1) [78]. Maternal antibiotics have a significant effect on reducing maternal microbial load and changing microbial composition [79, 80]. Prenatal maternal antibiotic exposure has been shown to alter both maternal and neonatal microbial composition [81–84]. Specifically, *Streptococcaceae*, *Gemellaceae* and *Lactobacillales* were significantly decreased in infants of mothers who received intrapartum antibiotics, while *Proteobacteria* were significantly enriched [85]. This suggests that maternal antibiotic usage may directly affect the infant microbiome. While these studies have attempted to control for confounders, the administration of maternal antibiotics are generally reserved for settings where suspected chorioamnionitis, *Group B Streptococcus* infection, or other fetal or maternal distress has occurred. Thus, it is challenging to tease apart cause and effect when assessing the impacts of maternal antibiotic exposure on the infant microbiome.

Table 1: Early life exposures and impact on microbiome.

Early life exposure	Associated alterations in intestinal microbiota
Intrapartum antibiotics [86, 87]	↓ <i>Bacteroides</i> (g) ↓ <i>Bifidobacterium</i> (g) ↓ <i>Blautia</i> (g) ↓ <i>Roseburia</i> (g) ↓ <i>Ruminococcus</i> (g) ↑ <i>Oscillospira</i> (g) ↑ <i>Pseudobacter</i> (g) ↑ <i>Veillonella dispar</i> (s) ↓ Actinobacteria (p) ↑ Proteobacteria (p) ↑ <i>Enterococcus</i> (g) ↑ <i>Clostridium</i> (g)
Postpartum antibiotics [88]	↓ <i>Bifidobacterium</i> (g) ↑ <i>Enterococcus</i> (g) ↑ <i>Enterobacteriaceae</i> (f)
Vaginal delivery vs. Caesarean delivery [89]	↓ <i>Bifidobacterium</i> (g) ↑ <i>Bacteroides</i> (g) ↑ <i>Lachnospiraceae</i> (f) ↑ <i>Streptococcus</i> (g) ↑ <i>Staphylococcus</i> (g) ↑ <i>Lactobacillus</i> (g) ↓ <i>Enterobacteriaceae</i> (f)
Breast milk vs. formula milk [41]	↑ <i>Bifidobacterium</i> (g) ↑ <i>Bacteroides</i> (g) ↓ <i>Streptococcus</i> (g) ↓ <i>Enterococcus</i> (g) ↓ <i>Lachnospiraceae</i> (f) ↓ <i>Veillonella</i> (g) ↓ <i>Clostridioides</i> (g) ↓ α-Diversity
Maternal smoking [90]	↑ <i>Bacteroides</i> (g) ↑ <i>Escherichia</i> (g) ↑ <i>Shigella</i> (g) ↑ <i>Ruminococcus</i> (g) ↑ <i>Akkermansia</i> (g) ↑ <i>Ruminococcaceae</i> (f) ↑ <i>Lachnospiraceae</i> (f) ↑ <i>Staphylococcus</i> (g) ↑ α-diversity
Maternal high fat diet [76]	↑ <i>Enterococcus</i> (g) ↓ <i>Bacteroides</i> (g)

p, phylum; f, family; g, genus.

Maternal stress

Perinatal adverse events, including maternal stress have been implicated in microbial changes in offspring and may influence fetal and infant development [91]. Psychological stress generates a systemic inflammatory response involving the release of cytokines, chemokines, and inflammatory mediator molecules [91]. An association has been described between the intestinal microbiome and chronic exposure to these inflammatory

mediators, and changes in the gut microbiome of offspring have been described among mothers who experienced significant stress exposures during pregnancy [92]. Gur et al. described significant differences in the microbial composition of infants born to mothers with high-vs. low-stress exposures during pregnancy [91]. Prenatal stress in animal models has been linked to reduced *Lactobacilli* in the maternal vaginal microbiome, reductions in *Bifidobacteria* and *Lactobacilli* in offspring microbiome, and alterations in key hormonal mediators of energy metabolism in infants [93, 94].

Mode of delivery

The neonatal microbiome experiences significant transitions during delivery and is strongly influenced by mode of delivery [71, 95, 96]. *In utero* the fetus is suspended in amniotic fluid. This rapidly changes after birth as the neonate is exposed to a microbe-rich environment [86]. Bokulich et al. assessed 43 mother and infant dyads, and performed longitudinal 16S rRNA sequencing of stool samples collected immediately after birth and up to age 2 years. Birth mode (caesarean delivery vs. vaginal delivery) was shown to result in profound, prolonged gut microbial dysbiosis [97]. Infants delivered via caesarean delivery had greater bacterial diversity immediately after birth, but this diversity declined below infants delivered via spontaneous vaginal delivery by one month of life [97]. This has been challenged by Chu et al., who found that mode of delivery impacted the external neonatal microbiome (skin, nares, oral cavity) but not the meconium microbial composition [76]. Additional research is needed to determine how mode of delivery impacts infant microbial composition and risk of microbial dysbiosis.

Gestational age

There are significant differences in the intestinal microbiome of term infants (≥37 weeks gestational age) compared to preterm infants (<37 weeks gestational age). In particular, preterm infants have decreased levels of *Lactobacillus* and *Bifidobacteria* and relatively greater abundance of *Escherichia*, *Klebsiella*, and *Enterobacter* [41, 97]. Very premature infants (<32 weeks) have increased levels of Gram positive bacteria, including *Staphylococcus*, *Clostridia* and *Enterococcus* during their first month of life [98, 99]. In combination with an immature immune system, this pre-term microbial composition increases the risk for impaired immune priming, inflammation, and infection in very

premature infants and has been postulated to be associated with the development of necrotizing enterocolitis [98].

Preterm infants experience multiple insults to their intestinal microbiome; gestational age alone does not contribute to all these differences. Preterm infants admitted to NICUs have differences in feeding (including human donor milk, formula feeding), higher antibiotic exposure, and often significant multiorgan dysfunction secondary to prematurity compared to term infants. In a study by Chernikova et al. [100] assessing the effect of gestational age on the microbiome, even after accounting for various exposures, alpha diversity varied between extremely preterm infants (<28 weeks gestational age) and very preterm infants (<32 weeks gestational age), as well as between extremely preterm infants and moderate-to-late preterm infants (≥ 32 weeks to <37 weeks gestational age).

Breastfeeding vs. formula feeding

Food intake can significantly impact the microbiome even from infancy. Breastmilk contains HMOs and serves as primary nutrition, a source of important metabolites, bioactive factors (IgA, lactoferrin, growth factors), and maternal microbiota [101]. *Bifidobacterium* species (*Bifidobacterium breve*, *Bifidobacterium bifidum*), *Lactobacillus* species, and particularly *Bifidobacterium longum* subspecies *infantis* metabolize HMOs [102]. These taxa are prevalent in the breastfed infant gut [86, 103]. HMOs have a unique role in the neonatal gut, inhibiting the binding of pathogenic bacteria to intestinal mucosa and preventing their proliferation [103]. As HMOs can only be digested by certain taxa in the distal gastrointestinal tract, this results in a decreased alpha diversity, but presumably healthier microbial composition in the neonatal gut [103]. Conversely, formula fed infants have a more diverse microbiome, which is more similar to a child or adult [53]. This suggests that the use of bacterial diversity as a proxy for microbial health may be an incomplete, simplistic characterization.

In addition to *Bifidobacteria* and *Lactobacilli*, breastfed babies also have a higher relative abundance of *Streptococcus*, *Staphylococcus* and *Prevotella*. Human milk has its own microbial composition, leading to a transfer of these genera to the infant gut. While mothers share similarities in their milk composition, there are a number of maternal factors (body mass index, maternal diet, maternal genetics, delivery mode) that also contribute to differences in the maternal milk microbiome [86, 103, 104]. It continues to be unclear, however, how the breast milk microbiome develops [103, 104]. Formula-fed infants tend to have a higher relative

abundance of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* [103, 105]. However, this has not been consistently shown across all studies [103].

As our understanding of HMOs has grown, formulas have been fortified with prebiotics such as galactooligosaccharides (GOS), fructooligosaccharides (FOS) and biosynthetic HMOs. In one study of formula fortified with GOS and FOS, the percentage of *Bifidobacteria* rose to 73.4% among formula fed infants, compared to 90% in breastfed infants [106]. In a recent study of infants receiving formula fortified with HMOs, the microbiome also shifted in diversity towards that of breastfed infants [107]. However, while these additions are helpful, the microbiome of a formula fed infant will likely always be somewhat different from an infant who has been breastfed [86]. The impact of these differences on short- and long-term health outcomes is unclear.

Antibiotic exposure

Antibiotics have been shown to cause changes to an infant's microbiome [108]. There are three time points at which antibiotics are typically administered to the neonate: (a) perinatally, to mothers at the time of delivery, (b) at birth or within the first few days after birth (usually within the first 48 h), and (c) within the first few weeks of life (particularly if a neonate is admitted to the NICU). Even after accounting for prematurity, mode of delivery and type of feeding, Bender et al. found that antibiotics given at birth significantly altered the infant's microbiome with a relative decrease in species richness [108]. Further studies have shown that early antibiotics led to decreases in *Bifidobacteria* and *Lactobacilli*, which have been described as being important for a healthy intestinal microbiota [83, 109]. In functional analyses, pathways involving folate synthesis, glycerolipid metabolism, fatty acid biosynthesis and glycolysis were most affected. Changes to these pathways were driven by decreases in *B. breve* and *E. coli*, and increases in *Klebsiella* and *Bacteroides* species [108]. *Bacteroides* abundance, however, has been shown to decrease in response to antibiotics across numerous studies [110, 111]. Further data may help reconcile heterogeneity in this area.

While antibiotics given within the first few days of life have been shown to lead to an overall decrease in diversity, the microbiome was found to recover by the first year of life among infants exposed to antibiotics [97]. These findings were also true for a population of 91 premature neonates in the NICU, who were randomized to receive antibiotics or no antibiotics within the first 48 h of life [112].

Long-term outcomes associated with perinatal microbial dysbiosis

Early life impacts on the gut microbiota have been associated with multiple long-term sequelae (Table 2).

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is an acute inflammatory disease of the intestine which primarily affects preterm infants. It is a leading cause of morbidity and mortality in the NICU. Attempts to identify microbial signatures that are predictive for the development of NEC have been inconsistent. Longitudinal stool studies of very low-birth weight preterm infants have described increases in *Enterobacter* (phylum: Proteobacteria), *Klebsiella* (phylum: Proteobacteria), and *Pseudomonas* (phylum: Proteobacteria) in the first stool samples of infants who would later develop NEC [121, 122, 125]. A 2016 prospective trial by Heida et al. assessed twice-weekly stool samples in 11 gestational-age/birthweight matched infants who developed NEC and matched healthy controls. The abundance of *Clostridium perfringens* and *Bacteroides dorei* in meconium correlated with the development of NEC compared with controls (0.1 and 0.2%; both species, $p < 0.001$). The abundance of staphylococci was also

found to be negatively associated with NEC development ($p = 0.1$ and $p = 0.01$ for consecutive samples) [125]. Other studies have described decreased bacterial diversity and greater abundance of Proteobacteria in affected infants. A cross-sectional study of faecal microbiota in nine, early-preterm (< 32 weeks gestational age) infants who developed NEC, and nine age-matched healthy controls, neonates who developed NEC had an increase in Proteobacteria and decrease in Firmicutes within 72 h of symptoms [123, 124]. Other studies have failed to identify differences in the microbiota of neonates who developed NEC from healthy controls [126, 127]. The study of microbial influences in the development of NEC, a surgical emergency that continues to have a 24–35% rate of mortality and long-term morbidity, remains challenging [128]. Whether these described microbial changes are the cause or result of changes at the intestinal epithelium remain unclear.

Atopic dermatitis

Perturbations of the gut microbiome in early life have been associated with the development of atopic dermatitis (AD) in infants and children. AD has been associated with a high proportion of *F. prausnitzii* subspecies in the intestinal microbiome [113, 114]. Symptoms of AD were found to improve as the abundance of fecal *Coprococcus eutactus*, a butyrate-producing bacterium, increased [129]. *F. prausnitzii* is a member of the Firmicutes phylum that has classically been associated with butyrate production; however faecal samples from patients with AD have a higher proportion of a compositionally different strain of *F. prausnitzii* (A2-165) that reduces the number of butyrate and propionate producers [114]. SCFA like butyrate and propionate are preferred substrates of colonocytes. It has been hypothesized that decreases in taxa which support colonocyte integrity may alter the intestinal epithelial barrier, allowing greater passage of bacterial antigens and promoting an aberrant Th2-type immune response to cause AD [130]. A 2017 study by Kennedy et al. described how the density of colonization of skin microbiome with commensal staphylococci at two months of life was associated with lower rates of AD among infants at one year of life.

Gut microbiome and food allergy

Food sensitization and the development of food allergy have strong relationships with the intestinal microbiota. Children with food sensitization in early life had increased number of Firmicutes and decreased Bacteroidetes [131]. Antenatal antibiotic use, before and during pregnancy has also been

Table 2: Diseases associated with early life dysbiosis.

Disease	Microbial alteration
Eczema	↑ <i>Escherichia coli</i> (s)
Atopic dermatitis	↑ <i>Faecalibacterium prausnitzii</i> [113, 114] (s)
Asthma	↑ <i>Clostridium difficile</i> (s) ↓ <i>Faecalibacterium</i> (g) ↓ <i>Akkermansia</i> (g) ↓ <i>Lachnospira</i> (g)
Increased BMI	↑ <i>Bacteroides fragilis</i> (s) ↑ <i>Staphylococcus aureus</i> (s)
Type 1 diabetes mellitus	↑ <i>Ruminococcus</i> [115] (g) ↑ <i>Streptococcus</i> [115] (g) ↓ Reduced Firmicutes:Bacteroides ratio [116] (p) ↓ α-Diversity [117]
Inflammatory bowel disease	↑ <i>Gammaproteobacteria</i> [118] (c) ↑ <i>Enterobacteriaceae</i> [119] (f) ↓ <i>Bifidobacteria</i> [118] (g) ↓ <i>Faecalibacterium prausnitzii</i> [120] (s)
Necrotizing enterocolitis	↑ <i>Enterobacter</i> (g) ↑ <i>Klebsiella</i> (g) ↑ <i>Pseudomonas</i> [121, 122] (g) ↓ Firmicutes [123, 124] (p)

p, phylum; f, family; g, genus; c, class; s, species.

associated with risk of food allergy in neonates [132]. Antibiotic use during the first months of life are associated with increased risks of cow's milk allergy. Savage et al. quantified the burden of antibacterial exposures by measuring urinary levels of triclosan, a common antibacterial and antifungal found in consumer products (toothpastes, soaps, detergents, toys). A significantly higher odds ratio (OR 3.9; $p=0.02$) was found of food sensitization with increased urinary levels of triclosan among 860 participating children [133].

Gut microbiome and asthma

Antimicrobial diversity has been correlated with the development of asthma in neonates and infants. *Clostridia* species have been shown to affect CD4+ T-cell proliferation and function, which is associated with the development of asthma. A large systematic review demonstrated that infants breastfed for the first 2 years of life had a significantly lower risk of developing asthma than those who were not [134, 135]. Unfortunately, while intriguing, large epidemiological studies remain affected by multiple confounders. Further, protective taxa, or interventions (i.e., probiotic supplementation, prebiotic enrichment) to enrich these taxa remain unclear [136].

Body mass index (BMI)

Obesity has been associated with microbial dysbiosis. Multiple studies have described an “obesogenic” microbiota, which participates in greater energy-harvesting from food sources and alters key regulatory hormones involved in energy metabolism [137].

Multiple studies have suggested that early life influences on the intestinal microbiota, from as early as age 6 months, may impact the colonization of gut microbiota and predispose to the development of obesity [138–142]. Lower abundance of *Bifidobacteria* and higher abundance of *Staphylococcus aureus* was described in stool samples of children age 7 years, who were initially classified as overweight at ages 6 and 12 months [138]. In a cohort of 138 full term infants, higher *B. fragilis* and lower *Staphylococcus* was found in stool at ages 3–26 weeks, which correlated with a higher BMI z-score in children between ages 1–3 years [140]. A prospective cohort study of 218 full term infants found *Bacteroides* spp in stool samples detected at age 1 month was associated with a reduced growth trajectory over the first 6 months of age [142]. Two large birth cohorts, the Danish National Birth Cohort and the United Kingdom Avon Longitudinal Study of Parents and Children, both found increased risk for overweight at

ages 3 and 7 years among infants with antibiotic exposures before age 6 months [143, 144]. This was further corroborated by a large United States study of 65,000 children, showing that frequent courses of antibiotic treatment within the first 2 years of age correlated with a risk of developing obesity between ages 1–5 years [145], and a Canadian study by Azad et al., reporting greater odds of childhood overweight at ages 9 and 12 years among infants with antibiotic exposure during their first year of life [146].

Whether observed changes in the microbiome are obesity-promoting, or merely observed differences in the intestinal microbiome resulting from broader influences on energy regulation remain unclear. Nevertheless, it is highly plausible that neonatal microbial dysbiosis is a common pathway for the development of overweight and obesity. Future interventions will need to target these early life determinants.

Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder involving destruction of the insulin-producing beta cells in the pancreas. The cause of T1DM remains unknown, but microbiota-mediated immune dysfunction is implicated. Two broad mechanisms have been proposed. Gut dysbiosis may lead to dysregulation of the innate and adaptive immune system, resulting in beta cell destruction. Alternately, disassembly of tight junctions and disruption of the mucosal barrier may enhance intestinal permeability, allowing microbial antigens to translocate across the intestinal epithelia, stimulate reactive T-cells, and promote the destruction of pancreatic beta cell tissues.

Several independent studies have suggested that early life impacts on the gut microbiota could affect the incidence of T1DM. Large epidemiological studies have demonstrated that breastfed infants have decreased incidence of T1DM [147]. Studies of probiotic and prebiotic supplementation have described protective effects of early life supplementation. Uusitalo et al. studied 7,473 infants with genetic susceptibility to T1DM who were supplemented with probiotics (mixtures of *Lactobacillus* and *Bifidobacterium* species) during the first 28 days of life. Probiotic supplemented infants had a reduced risk of beta cell autoimmunity (HR 0.66; 95% CI, 0.46–0.94) compared to unsupplemented controls [148]. A 2021 double-blind, randomized, placebo-controlled trial by Groele et al. to determine whether probiotics can improve beta-cell function in children ages 8–17 years with *newly diagnosed* T1DM failed to show benefit [149]. Whether this reflects intervention occurring outside a

critical window for gut microbiota adaptation is unclear. Further randomized-controlled trials in larger cohorts are necessary. Interventions aimed at potential early life determinants may hold greatest potential.

Inflammatory bowel disease

Numerous studies have described the intestinal microbiota of patients with inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). This chronic autoimmune disorder of the gastrointestinal tract is thought to be strongly influenced by early life bacterial colonization. Several studies have described relationships between perinatal microbial influences and the development of IBD. Prenatal exposure to antibiotics (OR 1.8; 95% CI 1.2–2.5), early life otitis media (OR 2.1; 95% CI 1.2–3.6) and tobacco smoke (OR 1.5; 95% CI 1.2–1.9) were associated with CD and UC [150]. Population-based studies have also described an association between exposure to antibiotics in infancy and IBD (OR: 1.7, 95% CI 0.97, 2.9) [151].

A recent 2020 study by Torres et al. described the effects of maternal IBD on maternal, and offspring microbiome. Infants born to mothers with IBD had enrichment in *Gammaproteobacteria*, depletion in Bifidobacteria, and decreased overall bacterial diversity [118]. Follow-on studies involving transfer of third trimester maternal stool, and infant stool at 90 days of life to germ-free mice showed significantly reduced microbial diversity and fewer memory B-cells, and regulatory T-cells in the colon [118]. These immune disturbances are mimicked in patients with known IBD [152]. How this data may be used to recommend early-life preventative strategies is the focus of ongoing research.

Conclusions, future directions

The role of the microbiome has been studied in every field of medicine. As we develop tools to integrate large data sets certain themes arise, including the importance of early life influences and their long-term impacts on the gut microbiome. Our review of neonatal microbiome development, and the many maternal, and environmental factors that affect it, underscores the importance of being attentive to these factors. Maternal health has significant, lifelong importance on offspring. Perinatal exposures, including single-dose intrapartum antibiotics, can exert health impacts throughout childhood.

Preventing early life dysbiosis may impact future rates of obesity, atopic disease, and neuropsychiatric illness. Identifying effective preventative strategies has been challenged by the prevailing difficulties of clearly defining

dysbiosis and having a suitable comparator of a “healthy microbiome.” Nonetheless, good maternal health, term gestation, spontaneous vaginal delivery, breastfeeding, and avoidance of intrapartum and early life antibiotics correlate best with microbial diversity, and positive neonatal outcomes. We can look forward to a future that involves innovative microbial therapeutics to correct early-life dysbiosis. As this field matures, we should instead focus on promoting standard and least-invasive neonatal care.

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