Decoding the bacterial microbiome of preterm babies – insights, unknowns and opportunities

This article reviews the importance of the gut bacteria (microbiome) to neonatal health, explaining recent advances in quantifying and analysing the complex community structures of the gut, the way in which the newborn preterm infant establishes its microbiome, and threats and challenges associated with being born prematurely. Data suggesting a microbiomic change in association with necrotising enterocolitis and late onset sepsis are reviewed and opportunities for enhanced understanding and future studies are described.

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Key points

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1. The preterm microbiome is a complex community of organisms with importance to short- and long-term health.
2. It is both modfiable and modified: sometimes unconsciously through NICU practices, but increasingly consciously, eg the use of probiotics and lactoferrin.
3. Mechanistic understanding of these interventions might reduce deaths, improve outcomes in survivors and have long-term health impacts.

It is increasingly recognised that the bacteria we carry influence both short- and long-term health in humans[1]. The gut bacteria appear uniquely important, affecting metabolic, immune and digestive function. In adults the range of diseases associated with alterations in the composition of the bacteria in the gut include diabetes, obesity, Parkinson's disease and other neurodegenerative disorders, inflammatory bowel disease, asthma and allergies. Newborn infants are faced with enormous challenges as they acquire their bacterial colonisers. They must move from a 'sterile' intrauterine environment to one where they exist in happy equilibrium with their gut microbes.

The neonatal period is thus of unique importance, and for preterm infants with relative gut and immune immaturity, this period is especially challenging. It is known that complex interactions occur between host cells of both the immune system and gastrointestinal tract and the microbes that they encounter and that host and bacteria operate synergistically to lead to a balanced state. This has been described as 'two-way cross talk'[2] meaning that both bacteria and the host interact to modulate host immune function and shape the gut bacteria that are carried. This allows carriage of bacteria within the gut in a way that is tolerated and becomes established as a community, referred to as the microbiome.

Our understanding of the composition of this community has increased with new molecular technologies. These methods circumvent the dependency of culturing an organism from stool, which is limited to around 20% of the overall gut diversity. Molecular techniques utilise small conserved fragments of DNA to identify the presence of an individual organism, for example the 16S ribosomal RNA in bacteria or the internal transcribed spacer (ITS) region in fungi. These molecular techniques have rapidly progressed since the 1990s and now next generation sequencing methodologies allow extremely detailed understanding of whole communities and how these change over time in individuals, or vary across diseased and non-diseased individuals. Molecular techniques offer new opportunities to understand disease processes that have so far eluded neonatology, particularly necrotising enterocolitis (NEC) and late onset sepsis (LOS), and may offer insights that suggest new diagnostic, prevention or treatment strategies[3].

Analysing the microbiome with molecular techniques

Over time the gut microbiota change in several key ways: what is present, how much is present, what genes are being expressed by the bacteria and, consequent upon this gene expression, the metabolic activity or function of the bacteria. Measuring, analysing and presenting such complex dynamic data sets is challenging. Instead of a report from a culture of stool identifying a single pathogen, studies have moved to complex identification, quantification and analysis

with increasing consideration for polymicrobial communities.

These molecular methodologies generate enormous datasets and handling them and performing appropriate statistical analysis is now the biggest challenge for many such studies[4]. Key terms that will be seen in papers describing microbiome communities are richness (their constituents), evenness (dominance by particular taxonomic units) and diversity (a combination of richness and evenness). When attempting to assess drivers of change, the data can be explored in such a way to generate and test hypotheses, using ordination analyses and constrained or redundancy analyses[5]. Such approaches allow the influence of factors like gestation, age or specific exposures to a treatment (like antibiotics) to be explored within a model.

The functions performed by bacteria in a complex niche like gut and microbe/host interactions are also key: metabolomic (measuring all metabolites) and proteomic (measuring protein products) techniques allow analyses that begin to examine functional microbiomic changes rather than just constituent changes, which may be associated with disease states, or promote health[6].

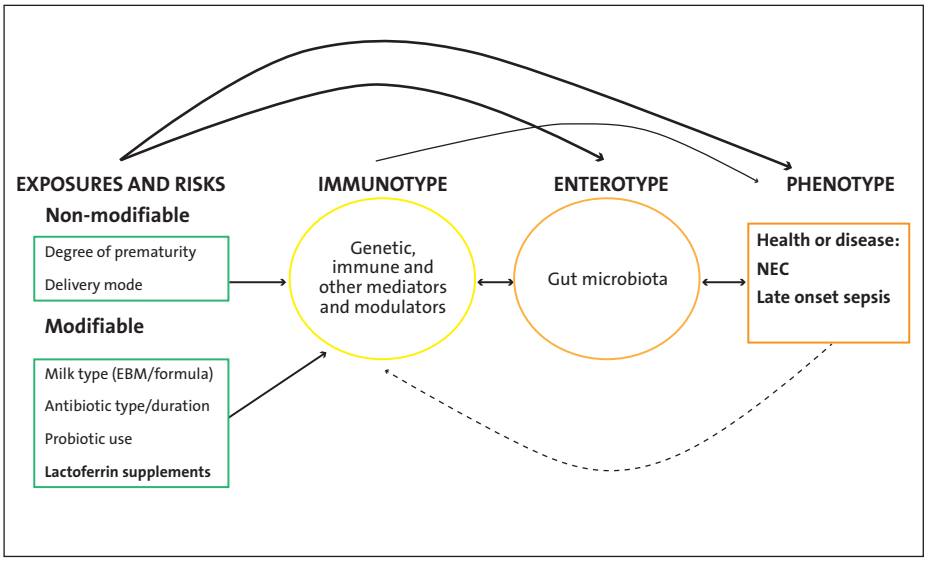

FIGURE 1 Factors affecting the preterm gut microbiota and mechanisms of effect, first published in Berrington et al, 2012[9]. Key: EBM = expressed breast milk, NEC = necrotising enterocolitis.

Meeting bacteria: the challenge for preterm infants

In healthy term newborn infants delivered vaginally and receiving breast milk, the gut receives its initial bacteria from mostly maternal sources – vaginal and bowel flora at delivery, skin contact during nursing, oral organisms from kissing – and later wider flora from environmental and family contact. This leads to a relatively preordained gradual acquisition of flora that progresses variably in individuals but eventually reaches a predictable adult-like pattern in the second year of life[7]. However events related to prematurity can interfere with this complex process.

Caesarian delivery, maternal antibiotics, neonatal antibiotics, lack of breast milk (or receipt of pasteurised or donor-expressed breast milk), hand gels and the sterile environment of the neonatal intensive care unit (NICU), all prevent the newborn preterm infant acquiring bowel flora in the expected way. This disruption of the normal developing microbiome is called dysbiosis. Dysbiosis is increasingly thought to contribute to diseases that are still important causes of mortality in preterm infants – LOS and NEC[8] – and adversely affect neurodevelopment in survivors.

While these inadvertent manipulations of preterm birth affect the microbiome, there is also an opportunity to potentially positively manipulate the microbiome with other therapuetic interventions, or wiser choices around existing interventions like antibiotics. **FIGURE 1** illustrates the dynamic and complex nature of factors that influence the microbiome and its role in health and disease states, with particular focus on the preterm perspective[9].

Host-microbiome interactions in preterm infants

Gut bacteria are separated by a single layer of epithelial cells from the blood and therefore careful regulation of pro- and anti-inflammatory effects of the presence of these bacteria are needed to maintain health, prevent bacterial translocation and not generate an inflammatory response[10]. This is achieved by a complex balance of host and bacterial elements, all of which need to be established after preterm birth, but in an immunologically immature host, with a fragile gut epithelium. In health, the host gut and immune cells respond to bacterial presence establishing a balanced T helper and T regulatory cell response, allowing establishment of a healthy microbiome[11].

In turn, commensal bacteria control more virulent bacteria by competition for evolutionary niches, for example through shared carbohydrate requirements[12] or by environmental manipulation ensuring the environment is hostile to more pathogenic species. For example, bifidobacteria, viewed as a 'healthy bacteria', use control of pH in the environment to limit growth of more pathogenic species such as *Escherichia coli*[13]. Some host molecules, like the antibacterial lectin Reg III-gamma, help control total bacteria numbers present by limiting the contact of these bacteria with host epithelium, affecting colonisation as opposed to transient carriage[14]. Some bacterial products, such as short chain fatty acids, produced by commensal microorganisms during starch fermentation also regulate host response to bacteria[15,16].

These processes and their interactions are complex, readily disrupted and may be programmed during gestation, such that preterm infants find establishing a healthy microbiome more challenging. Despite their apparent importance, the role of gestational age for the specifics of these complex processes is poorly studied.

The preterm microbiome

In health

A healthy gut microbiome has been defined as 'the intestinal microbial community that assists the host to maintain a healthy status under certain environmental conditions'[17].

In term neonates three phyla (Bacteroidetes, Proteobacteria and Firmicutes) seem to influence the community dynamics the most[18,19], but others have key roles. Some types of

Study group	Technique	Sampling strategy	Key findings	Citation
32 preterm infants 10 infants with NEC	DGGE	Weekly	No clear differences between NEC and non-NEC	Millar et al[37]
27 infants of <32 weeks 4 infants with NEC	Pyrosequencing	Weekly	Increased enterococci and *Klebsiella* in controls	Mshvildadze et al[38]
20 infants of <32 weeks 10 infants with NEC	Pyrosequencing	As soon as possible after NEC diagnosis	Increase in Gammaproteobacteria in NEC	Wang et al[39]
38 infants of <32 weeks 7 infants with NEC 13 infants with LOS	DGGE	Opportunistic surveillance study (n=99)	Enterococci associated with health Enterobacter associated with NEC Staphylococcus associated with LOS	Stewart et al[22]
18 infants of <32 weeks 9 infants with NEC	Pyrosequencing	3-10 days before NEC and within 72hrs of NEC onset	Increase in Proteobacteria and a reduction of Firmicutes in NEC Gammaproteobacteria family 'signature' for NEC	Mai et al[25]
68 infants of <27 weeks 12 infants with NEC	qPCR	Alternate days for four weeks	Increase in *E. coli* before NEC Associated increase in faecal s100A12 and hBD2	Jenke et al[26]
32 infants of <29 weeks 11 infants with NEC	Pyrosequencing	Day 4-9 and day 10-16 urine for metabolomics (NMR)	Two patterns of dysbiosis associated with NEC: 1) Firmicute dominant (Staphylococcus and Enterococcus) 2) Proteobacteria dominant (Enterobacteriaceae) Urinary alanine increased in Firmicutes dysbiosis	Morrow et al[27]
32 infants of <32 weeks 7 infants with NEC 13 infants with LOS	DGGE (16S rRNA and 28S rRNA)	First stool, then weekly	Sphingomonas associated with NEC Fungal data included	Stewart et al[28]
163 infants of <30 weeks 21 infants with NEC	DGGE	Three samples (day 0-5, 10 and 30)	No differences between NEC and non-NEC identified, but no data on sample timing relating to NEC	Smith et al[40]
27 infants of <32 weeks 5 infants with NEC 8 infants with LOS	Pyrosequencing	First stool then weekly	12 twin pairs and one triplet set A reduced diversity and increasing dominance of *E. coli* precedes NEC	Stewart [29]
6 preterm infants 2 infants with LOS 2 infants with no sepsis 2 infants with culture-negative systemic inflammation	Pyrosequencing	First stool, then weekly and at sepsis evaluation	Increased Firmicutes (Staphylococcus) in LOS. The authors postulate that a healthy microbiome may ameliorate risk of sepsis	Madan et al[31]
28 infants of <32 weeks 10 infants with LOS	Pyrosequencing	Weekly	Lower bifidobacteria in LOS cases	Mai et al[30]

TABLE 1 Studies of microbiomic analysis in preterm infants with NEC or sepsis. Adapted from Berrington et al[24].
Key: NEC = necrotising enterocolitis, DGGE = denaturing gradient gel electrophoresis, LOS = late onset sepsis, qPCR = quantitative polymerase chain reaction, NMR = nuclear magnetic resonance.

bifidobacterium that are found in breast milk may hold a pivotal role as they have some features attributed to probiotics[20]. Such naturally occurring probiotic bacteria – defined as being of health benefit to the host – could be the basis for a probiotic supplement designed for preterm infants using microbiomic knowledge[21]. This might be especially important as bifidobacterium abundance is low in the preterm microbiome[22], perhaps as a result of altered breast milk exposures.

Studies of the preterm microbiome have suggested that overall reduced diversity and greater instability are seen compared to term infants[23], even where the preterm infant remains healthy. Specific patterns of colonisation differ, with more facultative anaerobic organisms. Given the interaction between the gut microbiome and immune function, this altered progression may lead to delayed immune maturation, with implications for health. To date no large-scale studies of healthy preterm microbiomes have been undertaken. The focus of studies tends to be on disease states, possibly because the nature of preterm delivery, especially at the limits of viability, is such that no 'healthy' extremely preterm infant exists.

In NEC or infection

Both diseases appear to be influenced by the developing gut microbiome, but whether the association is causal or not remains unclear. If a specific microbiome pattern or a specific shift in the microbiome was known to predict NEC or LOS it might offer a diagnostic test, or allow insights into how to change this to prevent or reduce these problems.

Studies that have attempted to address this are shown in **TABLE 1** highlighting populations, molecular techniques employed and key findings. As can be seen, populations and techniques differ and importantly so do the timing of samples in relation to disease onset. Ideal sampling would be frequent enough that changes predicting NEC, happening many days before clinical illness, were detectable. One study found an increase in the numbers of Proteobacteria and a decrease in Firmicutes associated with NEC[25]. This same family was identified in a single preterm infant with lethal NEC where Proteobacteria, specifically *Klebsiella*, increased dramatically before NEC onset. Others have identified the Enterobacteriaceae family in association with NEC. One study[26] identified an increase in *E. coli* preceding NEC and showed functional and host changes associated with this. One group have suggested that NEC with different timings of presentation may have different associated microbiomic changes – early presentation being dominated by Firmicutes, later by Proteobacteria[27]. This would fit with the increasing recognition that all disease labelled NEC is not the same – rather a common clinical endpoint is seen from several causal pathways.

In infants <32 weeks' gestation, comparison of healthy infants and those with NEC revealed distinct microbiomic differences[22,28] and in twins studied over their NICU stay, where one developed NEC, lower diversity and more dominant *E. coli* was seen before the development of NEC[29]. The lack of normal exposure to environmental organisms that occurs in NICUs has led to the suggestion that preterm infants are less tolerant to gram negative organisms when they are exposed later, with a resultant excessive inflammatory response resulting in disease.

A similar imbalance of the gut microbiome with the same excess of Proteobacteria and Firmicutes has also been seen in association with LOS development in preterm infants. Changes in the permeability of the gut barrier, specifically related to function of the tight junctions, and impaired immune function with an imbalance between pro- and anti-inflammatory responses have also been implicated. Specific patterns predicting LOS were seen in the two weeks before diagnosis of LOS by two groups[30,31]. Interestingly there was dominance of Staphylococci, a common cause of LOS.

- Better understanding of gut communities in health and disease states
- Improved understanding of the effects of NICU practice
- Underpinning associated 'omics' (tests of function, not just presence)
- Deliberate manipulations to achieve a healthy microbiome based on this understanding:
  - lactoferrin
  - antibiotics formulated for neonatal use
  - new specific immunomodulatory products

FIGURE 2 Key steps and opportunities to help promote microbiome-related health in preterm infants.

Manipulating the microbiome

There are clear associations between NEC and LOS and interventions that affect the microbiome: NEC is more common after more antibiotic receipt[32], probiotics can reduce NEC[33], lactoferrin (a complex molecule evolutionarily conserved in mammalian milk, with multiple anti-inflammatory, anti-bacterial and immunological affects[34]) appears to reduce LOS and possibly NEC[35]. These interventions are either in common clinical use in NICUs, or have been the subject of clinical trials in neonates.

Despite this, there is currently a very poor understanding of microbial patterns associated with these interventions. If this was better understood, it may be possible to make more logical choices of products – reducing use of those that are associated with a microbiome less in keeping with health and avoiding emergence of drug-resistant pathogens.

Probiotics currently used in neonates were not formulated specifically for this purpose, and despite the many probiotic studies in neonates few examined the microbiome[36]. Where stool was examined the focus was on colonisation with the probiotic organism, but even this is poorly defined and understood. Is colonisation represented by finding the probiotic organism in the stool at all, or only in larger numbers than it was administered in (suggesting bacterial replication)? Indeed if mucosal adherence was necessary for colonisation might it be better not to find the organism in the stool? Should expression of adhesion molecules be looked for, and if so, how?

It will be of further importance to determine if any observed colonisation is sustained after the probiotic treatment is stopped. By not understanding these basic aspects of microbial manipulation, but undertaking clinical trials with these products, neonatology has in one sense run before it could walk.

If there was better understanding of how

these interventions interact with the microbiome both quantitatively and functionally, more logical steps to manipulating the microbiome could be taken and a gut microbiota more reflective of the healthy infant could be engineered (**FIGURE 2**). Manufacturing complex combination supplements is attractive but before this step a better understanding of the positive and negative effects of current interventions on the microbiome is needed.

Conclusions

Understanding the neonatal microbiome may hold the key to preventing LOS and NEC, improving growth and longer neurodevelopmental outcomes. Although patterns are emerging, the causal pathway is complex, likely to differ in individuals, and remains unclear. The focus of this article has been bacterial elements of the microbiome, but increasingly the potential role of viral, fungal and archaea elements has been recognised. Key steps to improving microbiome-related aspects of preterm health are given here.

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