Gut microbiota in preterm infants: assessment and relevance to health and disease

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BACKGROUND
In adults the microbial community of the gut (microbiota) influences a diverse range of health outcomes from obesity, diabetes, asthma and allergy to seemingly ‘remote’ diseases like Parkinson’s disease.1 In preterm infants, establishment of the gut microbiota is also of importance for key morbidities like late onset sepsis (LOS) and necrotising enterocolitis (NEC), both microbiota is also of importance for key morbidities like late onset sepsis (LOS) and necrotising enterocolitis (NEC), both conditions.4

PRETERM INFANTS’ MICROBIOTA
Establishment of the gut microbiota is key to developing barrier function, integrity, and mucosal and systemic immune function. They also ‘educate’ the gut associated lymphoid tissue, allowing the establishment of a ‘tolerant’ state between microbiota and the immune system, affecting intestinal function including tight junction structure and immune function.4 The initial colonisation affects host metabolic function including tight junction structure and immune function.4 The initial colonisation affects host metabolic function including tight junction structure and immune function.4–6 Patterns of initial colonisation affect host metabolic function: fat deposition, circulating leptin levels, and insulin resistance.5

In the preterm gut structural and immunological immaturity contribute to inflammatory necrosis and abnormal bacterial colonisation (dysbioses). This may result in decreased microbial diversity and increased inflammatory response exacerbated by an immature innate immune response that increases the risk of diseases like NEC or LOS. An improved understanding of the microbiota of infants cared for in neonatal intensive care, and how this is affected by current practices may allow clinicians to promote more ‘healthy’ gut microbiota patterns, and may be associated with reductions in mortality and improvements in long term outcomes.7

WHAT FACTORS ASSOCIATED WITH PRETERM BIRTH AFFECT THE GUT MICROBIOTA?
Early differences in delivery mode and care alter the development of the gut microbiota. Vaginally delivered term infants acquire their gut microbiota from maternal exposures (genital, stool, skin and breast milk flora) and the wider environment. In contrast, preterm infants are more commonly delivered by caesarean section, and have additional ‘risks and exposures’: antenatal and postnatal antibiotics, infection control measures (alcohol gel etc), minimal exposure to probiotics, infection control measures (alcohol gel etc), minimal exposure to breast milk (breast milk modified by pasteurisation, freezing, fortification, etc), and receipt of other medication such as H2 blockers that may directly or indirectly affect the microbiota (figure 1). Mechanistic ‘proof’ of an impact on the microbiota is incomplete for many of these factors even for term infants and important knowledge gaps remain.8 However, examples of postulated mechanisms exist: preterm infants who secrete less Fucosyltransferase 2 (FUT2) have an increased risk of NEC. FUT2 fucosylates luminal mucosal glycans without which many bacteria cannot adhere to bowel endothelium and colonise the gut. This suggests genotypic effects may be modulated by bacterial colonisation, with important clinical effects8 (mechanisms D and E figure 1). Altered colonisation also modulates the innate anti-inflammatory response: Enterococcus faecalis from 4-day-old children mediates the activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ correlating to IL10 expression (mechanism D).10

For preterm infants changes in microbiota appear pivotal to development of NEC and LOS, but the model is probably very complex. ‘NEC’ and ‘LOS’ are not single disease entities: LOS from ‘enteric organisms’ is more likely to be influenced by microbial manipulation. The disease described with the catch all term ‘NEC’ represents a spectrum of disease with differing presentations, and ranges from medically managed pneumatisis to devastating pan-enteric necrosis.

HOW IS THE GUT MICROBIOTA DESCRIBED BY NEW ‘MOLECULAR’ TECHNIQUES?
What are ‘molecular’ techniques?
Molecular techniques enable more detailed exploration of complex bacterial communities than standard culture,11 by utilising the ubiquitous 16S ribosomal RNA (16S rRNA) gene to differentiate prokaryotic taxa. Traditional Linnean bacterial classification (seven hierarchical taxa from kingdom to species) relies on pragmatic definitions of species, integrating phenotypic, biochemical and phylogenetic data, but for prokaryotes there is no accepted conceptual definition for a species.12

Bacterial DNA is extracted from biological samples and the 16S rRNA gene amplified by PCR. These amplified regions (amplions) then require differentiation into groups that share a pre-defined similarity to each other. These are known as ‘operational taxonomic units’ (OTUs). Separation methods differ: some depend on sequencing and comparison with known libraries (pyrosequencing), others (‘fingerprinting’) use electrophoresis to separate amplicons: temperature or denaturing gradient gel electrophoresis (T/DGGE). Different techniques offer different resolutions of the community, for example, metagenomic approaches (pyrosequencing) offer deep community sampling, with the derived OTU resolved to specific taxa (family/genus/species) depending on the length and quality of the sequence obtained. In contrast, fingerprinting methods usually identify the most abundant OTUs but require substantial downstream processing to obtain sequence information which is usually to order/family level.13

Molecular approaches are not perfect: sequencing techniques can overestimate diversity due to formation of amplicons

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derived from two OTUs (chimeras) and specific key taxa can still be missed: most widely used primers were recently shown to inadequately detect bifidobacteria. The technique can be extended to non-bacterial components using primers designed to amplify the ribosomal genes found in archaea and fungi. Table 1 compares common current molecular methodologies and standard culture approaches.

Analysis and presentation methods
Communities are described using several ‘measures’: diversity indices show relative contributions by different taxa, richness indicates the number of separate taxonomic units (dependent on the level of differentiation between OTUs by individual techniques), and evenness indicates relative abundance of different OTUs. Visualisation of differences in community structure can be made using pie charts, histograms or transitional plots, clustered heat maps (demonstrating the similarities and differences), or by the production of dendrograms, allowing visual linkage of the ‘most similar’ samples or populations. (figure 2).

Describing and analysing molecular data is complex because large numbers of OTUs are identified, and shifts in any, or their relative relationships, may be important. Identifying whether changes in the microbiota, between samples or through time are the cause or effect of the disease state is difficult because visualising the interplay in the preterm gut between many variables that may impact on the bacterial community structure and function both independently or via collective interactions (figure 1) is a significant computational and statistical challenge.

Ordination analyses, most commonly principal component analysis, help simplify data and identify the most significant variables that cause variation between samples. Moreover, bacterial communities from different samples can be compared to identify species and community response patterns to external variables. These can be further explored using techniques that combine the reduction of data complexity achieved by ordination with multiple regression analyses (constrained correspondence or redundancy analyses) to determine the variance attributed to specific variable(s). These approaches often facilitate the interpretation of community structure. Ultimately, as the effect of each explanatory variable on the bacterial community structure emerges, models can be developed that predict the impact of particular interventions (eg, antibiotic administration, feed change) on the microbiota. Such modelling might allow clinicians to manipulate infants’ microbiota towards a more ‘healthy’ pattern.

**CURRENT KNOWLEDGE OF THE PRETERM GUT MICROBIOTA**

**Molecular studies**
Molecular studies on preterm gut microbiota are currently limited by both methodological and population issues. Small cohorts are studied, samples are collected haphazardly, few informative (diseased) cases are recruited, or confounders are poorly controlled. Despite limitations, most studies show the preterm microbiota differs from term infants with more limited diversity, a more ‘unstable’ profile, and delayed acquisition of ‘adult’ profiles. For some organisms a gestational ‘threshold’ for colonisation of the bowel may exist: for bifidobacteria this appears to be ∼35 weeks, which is important as these organisms are key to healthy microbiota development. Recent data on colonisation with fungi, and viruses in 11 preterm infants revealed surprising eukaryotic and viral microbial diversity that merits further exploration.

**Relationship to LOS and NEC**
In animal models of NEC, conflicting findings exist: in rodents, *Enterobacteriaceae* predominate in NEC and *Firmicutes* in healthy animals. In pigs, *Firmicutes* were more prevalent in NEC. Similarly the literature in human infants with NEC is not consistent. Neither standard culture or molecular approaches have identified a single microbial ‘cause’ of NEC: in one study, standard culture identified a predominance of *Staphylococci* in NEC, although sample timing in relation to NEC was unknown, but this was not confirmed by molecular methods. An increase in the carriage of *Enterobacter, Klebsiella* and *Pseudomonas* spp. have also been reported. Molecular analysis of stool after development of NEC indicated that post-NEC infants microbiota were
lower in diversity with more *Proteobacteria*. Similarly, a pyrosequencing study sampling pre-NEC and within 72 h of symptoms, identified a bloom in *Proteobacteria* and a decrease in *Firmicutes* during the interval between sampling. Others have found no differences in community profiles in infants with NEC or controls. Potential difference were shown in the gut microbiota in those developing LOS compared with healthy infants, although in small numbers of infants, postulating the idea of a ‘healthy’ microbiota.

**INTERVENTIONS THAT AFFECT THE GUT MICROBIOTA**

**Mechanisms of effect**

Interventions that manipulate the microbiota may affect disease state (figure 1); effects are likely to be complex, multidimensional, and non-linear in causality. Some exposures or risks are not preventable, others are (e.g., ranitidine) or are deliberate manipulations. These are further discussed below and table 2 summarises key interventions and postulates the mechanisms via which they may act.

**Antenatal and delivery factors**

Maternal receipt of antibiotics affects maternal stool and vaginal microbiota. Whether and how this affects the infant microbiota remains unclear. Mode of delivery is usually dictated by obstetric factors, but ‘elective’ caesarean may disadvantage the development of a healthy microbiota.

**Postnatal factors**

**Feed type**

Nutritional, immune and metabolic functions are co-dependent in humans. Breast milk immuno-nutrients and growth factors exert a range of effects, with term formula and breast fed preterm infants demonstrating both metabolomic differences and distinct microbiota. In healthy breast fed term infants *Bifidobacterium* dominate by day seven, but not in preterms. The influence of feed type on the two-way cross-talk between the developing immune system and the microbiota is eloquently demonstrated by the presence of specific modulators within breast milk (prebiotic carbohydrates). These allow maternal milk to promote *bifidobacterial* dominance by exploiting specific catabolic pathways present in *Bifidobacterium longum* subsp. *infantis*, but absent in *bifidobacterial* species in adults, to utilise the otherwise indigestible oligosaccharides found in human milk.

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Figure 2  Examples of visual interpretation of pyrosequencing data derived from 3000 individual sequence reads from 11 individual patients. Numbers followed by letter represent longitudinal samples taken from those individuals. All data are analysed at a Class level taxonomic resolution. (A) Bar plot: showing the distribution and proportion of each unique operational taxonomic units within each sample. (B) Heatmap: comparing the phylogenetic similarity of each sample. (C) Cluster analysis showing a dendrogram illustrating the degree of similarity between each sample profile.
Antibiotic use

Oral vancomycin or gentamicin reduced rates of NEC\(^2^5\) but studies did not investigate the microbiota. Recent explorations of the preterm microbiota show a dominance of staphylococci within the preterm gut, and an increase in dominance associated with NEC providing a potential explanation for how these oral antibiotics modulate NEC.\(^2^1\)\(^2^6\)

Exploring the effects on the microbiota of specific antibiotics in relation to NEC development is difficult; attempts in the literature are limited, often confounded, and cause and effect difficult to separate.\(^2^3\)\(^2^7\) No effect of antibiotic receipt on colonisation with bifidobacteria has been demonstrated.\(^1^8\)\(^2^2\) Bjorkstrom\(^2^6\) however showed a reduction in lactic acid bacteria with vancomycin, and an effect of cefotaxime on Gram negatives. To further explore individual antibiotics effect on individual taxa or community changes, large, frequent sampling studies would be needed to distinguish effects of antibiotics from other management changes.

Deliberate interventions to manipulate the preterm gut microbiota

Antenatal manipulation of maternal microbial colonisation

Maternal colonisation is affected by environment and diet, and may be manipulated with probiotic containing food-stuffs for women receiving antibiotics or in or at risk of preterm labour, but this is untested.

Prebiotics and other nutrients

Prebiotics are nutrients that promote colonisation with probiotics and most often are short chain carbohydrates like oligosaccharides. They are now added to some formulas and appear to increase bifidobacterial counts in stool in preterm infants.\(^3^4\) In a rat model supplementation was shown to reduce the incidence of NEC by \(50\%\) with altered gut microbiota, supporting microbiotal manipulation as a mechanism for prebiotics.\(^3^5\) A serendipitous increase in \textit{Lactobacillus} spp. in preterm infants was associated with 1% lactulose added to feeds, potentially attributable to its prebiotic effects\(^3^6\) suggesting there is scope for further work in this area.

Probiotics reduce the incidence of NEC in many studies, but interestingly do not affect LOS.\(^3^7\) Few have explored associated changes in the microbiota as a result of probiotic administration. Work that has been reported indicates increased probiotic numbers (\textit{Bifidobacterium} and \textit{Lactobacillus} spp.) at the expense of Enterobacter, Enterococci and Clostridia—all organisms previously associated with NEC. Supplemetning \textit{Lactobacillus casei} increased the amount in infant stool and stabilised bowel flora.\(^3^8\) Some neonatal units now routinely use probiotics: they are reportedly well tolerated and appear microbiologically safe but for some concerns over safety persist. Further data on the optimal dose, species combinations and dose are needed before any beneficial effect might be maximised, but the studies required to obtain this data will necessarily involve very large numbers.

Lactoferrin

Bovine lactoferrin (BLF) supplementation has been demonstrated (in small numbers to date) to reduce LOS in very low birth weight infants.\(^3^9\) Lactoferrin is a key component of the innate response to infection and the major whey protein in human colostrum and breast milk. It enhances cell proliferation of enterocytes and aids tight endothelial cell junctions. At lower concentrations, lactoferrin stimulates differentiation of enterocytes and expression of intestinal digestive enzymes. Lactoferrin also suppresses free radical activity when iron is added to milk suggesting further anti-inflammatory actions that could modulate the pathogenesis of diseases linked with free radical generation: NEC, retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD). Studies on the mechanisms by which bovine lactoferrin (BLF) exerts its effect in vivo show it is both bacteriostatic, inhibits growth by sequestering iron, and binds to lipopolysaccharide on the cell surface of a number of enteropathogens inhibiting surface expressed virulence factors.\(^4^0\) It can also inhibit viral attachment and replication and exerts a fungicidal activity. Studies to date have not explored the impact on the microbiota.

Other potential modifiers not currently well explored

There are many other substances known to have a role in intestinal integrity or function currently not explored in terms of their microbiomic impact, some of which are used in NICUs. These include arginine and glutamine, oral immunoglobulins or protein supplementation and antacids. Modulation of microbial colonisation of milk mediated by feeding practices (continuous versus bolus feeding, freezing breast milk, addition of fortifier/other solute load) may all also affect the microbiota. In addition, infants with a stoma, whether from NEC or other pathology, may have specific differences in their microbiota. Dramatic shifts in flora have been seen with stoma formation, with more facultative anaerobes in those with stoma’s, reversing to obligate anaerobes on reversal. Modulation of the microbiota in infants with stomas may improve feeding issues.

**SUMMARY**

There are a number of studies that demonstrate the development and dynamics of the microbial community in the preterm infant gut and suggest that this process is significantly associated with diseases such as NEC and LOS. To date these studies report data from limited cohorts and do not explore underpinning mechanisms well. This limits their applicability and means that potential insights into the broader aspects between microbial community assembly and dynamics and disease remain elusive. Larger case series with detailed microbiomic exploration and exposure data are needed. Many maternal and early infant interventions that are common place in neonatal intensive care appear likely to affect the microbiota. Molecular studies of the gut microbiota as part of prospective controlled trials are now needed to translate basic scientific knowledge into improved care and outcomes.

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