Role of the microbiome in pathophysiology of necrotising enterocolitis in preterm neonates

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ABSTRACT
Although necrotising enterocolitis (NEC) is a serious, life-threatening disease, improved neonatal care is increasing the number of survivors with NEC among extremely preterm neonates. Therapy is nevertheless mostly symptomatic and the mortality rate remains high, especially among neonates requiring surgery. Therefore, it is important to focus on preventing the disease and modifiable risk factors. NEC’s pathophysiology is multifaceted, with key factors being immaturity of the immune and barrier protective mechanisms of the premature gut and exaggerated proinflammatory reaction to insults like gut hypoxia, enteral nutrition or microbial dysbiosis. The role of the intestinal microbiome in the pathophysiology of NEC has been a subject of research for many years, but to date no specific pathogen or type of dysbiosis has been connected with NEC development. This review assesses current knowledge as to the role of the intestinal microbiota in the pathophysiology of NEC and the possibilities for positively influencing it.

INTRODUCTION
Necrotising enterocolitis (NEC) is a serious disease and is the leading cause of mortality in preterm neonates surviving beyond the first week of life.1 Although improvements in neonatal care and increased numbers of survivors from the category of extremely preterm neonates are leading to decreased respiratory mortality, the number of patients with NEC is nevertheless increasing.2 Each year worldwide, 1.5 million preterm infants are born before the 32nd week of gestation, making NEC not just one of the most serious but also one of the most expensive diseases in neonatology.3 The diagnosis and therapy of NEC remain non-specific and research is currently focused on understanding its multifactorial pathophysiology, which is primarily the consequence of an interaction between an immature immune system of the preterm gut and proinflammatory reaction to diverse stimuli such as hypoxia, enteral nutrition and gut dysbiosis.4 This review evaluates current knowledge as to the role of microbiota in the pathophysiology of NEC and the possibilities for influencing them positively.

Necrotising enterocolitis
NEC is a severe, life-threatening disease characterised by acute gut ischaemia that can progress to necrosis and destruction of the entire gut wall. The typical histological finding comprises gut epithelium disruption, coagulation necrosis, inflammatory cells infiltration and bacterial overgrowth, especially in the terminal ileum and colon.5 The incidence of NEC is about 1–3 per 1000 live-born infants and it affects approximately 7% of neonates born before the 32nd week of gestational age and/or with birth weight under 1500 g.6 The mortality rates are as high as 30% and increase with lower gestational age. Moreover, NEC is the leading cause of mortality in neonates requiring surgical intervention.7 NEC is also associated with a high rate of late morbidity, among the most prominent of which morbidities are delay in neurodevelopment and chronic lung disease.8

The diagnosis of NEC is based on the clinical picture, laboratory findings and some radiological features; recently, ultrasonography is being used frequently.4 Inasmuch as clinical and laboratory findings are non-specific, the diagnosis of NEC is definitive only after a surgical intervention or postmortem. Bell defined diagnostic criteria (which were subsequently modified in 1987), but this

KEY MESSAGES
⇒ Prevention and therapy of necrotizing enterocolitis (NEC) remain unmet needs in neonatology, and better understanding of NEC’s pathophysiology is needed.
⇒ In preterm neonates, exaggerated inflammatory reaction to gut dysbiosis may lead to NEC’s development.
⇒ Modifying the intestinal microbiome may play a role in NEC’s prevention.
staging system has never been externally validated and, for example, spontaneous intestinal perforation was not separated from NEC. Juhl determined in their retrospective study that in one-third of neonates who were given an NEC code at discharge, a different diagnosis was given by an expert panel. It seems that various diseases with different phenotype presentations may be hidden under the diagnosis of NEC, all of which are characterised by disorder of the intestinal wall integrity. This is important to bear in mind when analysing the quality of studies in NEC patients, as close attention must be given to how the cohort of NEC patients has been defined. Therapy of NEC is mostly symptomatic and supportive. It comprises withdrawing enteral nutrition, abdominal decompression, antibiotics, as well as ventilatory and circulatory support. Surgical procedures are necessary in cases of gut necrosis and perforation.

**NEC pathophysiology**

The pathophysiology of NEC is multifactorial, with the key factors being immaturity of the immune and barrier protective mechanisms of the premature gut and exaggerated proinflammatory reaction to insults such as gut hypoxia, enteral nutrition or microbial dysbiosis (figure 1). The immature gut is shorter in length and characterised by fewer villi, inadequately developed mucosa and the junction of epithelial cells, lower amounts of Goblet’s and Paneth cells, lower acidity, and intestinal dysmotility.

In addition to its role in the digestion and absorption of nutrients, the gut has a very important immunological function. Dysregulation of the immune system, especially through Toll-like receptor 4 (TLR 4), seems to be crucial in the pathogenesis of NEC. TLR4 belongs to a family of pattern recognition receptors expressed by the immune cells and which play a role in recognising distinct molecules typical for pathogens or damaged cells of their own organism. As a part of the innate immune system, their activation is primarily induced by contact with lipopolysaccharides of Gram-negative microorganisms. Subsequent activation of inflammatory reaction may lead to NEC’s development with irreversible damage to the intestinal mucosa. TLR 4 activation also is associated with endothelial damage leading to vasoconstriction and intestinal ischaemia, and it is involved in a loss of glia resulting in gut dysmotility. Activation of TLR 4 in the lung leads to lowering of the endothelial nitric oxide synthase production that causes decreased perfusion and lung injury associated with NEC.

Figure 1  A model of the pathophysiology of necrotising enterocolitis. TLR 4, Toll-like receptor 4.
Another very important field of research in the pathophysiology of NEC is the impact of inflammation on splanchnic vasoregulation in preterm neonates. Before the 32nd week of gestation, the intestinal vasculature is underdeveloped, as more pronounced expression of vascular endothelial growth factor and its VEGFR2 receptor are not seen until later phases of gestation. This natural maturation of vasculature can be disrupted by inflammation.\textsuperscript{15}

**Intestinal microbiome**

The composition of the intestinal microbiome (herein-after just the ‘microbiome’) has an essential influence on our health. The microbiome is involved in the metabolism and digestion of nutrients and in producing amino acids and vitamins; it promotes growth and gut maturation; and it plays an important role in the development of the immune system.\textsuperscript{16} Favourable microbiome composition is characterised by rich diversity, which prevents the overgrowth of pathogens. By contrast, dysbiosis with a dominance of pathogenic bacteria is associated with many diseases (eg, atopic dermatitis, diabetes, obesity, colorectal carcinoma, inflammatory bowel disease and neurological diseases).\textsuperscript{17} Microbiome development in full-term neonates has been well described. The authors of a study including 900 neonates from 6 centres in Europe and the USA described three phases of the microbiome’s development.\textsuperscript{18} The first phase, lasting about 2 weeks, is characterised by a dominance of facultative anaerobes (Esherichia coli, streptococci, enterococci, Propionibacterium spp), which generate an anaerobic environment suitable for the obligate anaerobes (Bifidobacterium spp, Bacteroides spp). In the final phase, the diversity of Bacteroides spp and anaerobic G+/coccii (peptococci, peptostreptococci) increases.\textsuperscript{19} Many studies describe the influences of several factors on microbiome evolution, such as mode of delivery, use of antibiotics and type of enteral nutrition.

**Microbiome of preterm neonates**

The intestinal microbiome of the preterm neonate is generally characterised by lower diversity.\textsuperscript{20} Bacterial settlement of the gut begins already in utero—the microbiome of the meconium is about a 50% match with that in the amniotic fluid.\textsuperscript{19} Prelabour rupture of the membranes, chorioamnionitis and delivery by caesarean section are often connected to prematurity and negatively affect the microbiome of the neonate.\textsuperscript{21} The influence of antibiotics on modulation of the microbiome is also well described. Antibiotics are among the drugs most frequently used in neonatal intensive care units (NICUs) and their use leads to a greater abundance of Proteobacteria.\textsuperscript{22} La Rossa et al showed that NICU-related factors, such as diagnostic and therapeutic interventions, delayed enteral feeding and antibiotics, are associated with greater abundance of potential pathogens (E. coli, Klebsiella spp, staphylococci) and smaller abundance of favourable Bifidobacterium spp and lactobacilli.\textsuperscript{23} Those authors pointed out, however, that the main factor influencing the microbiome is the prematurity itself.

**Intestinal microbiome and NEC**

The hypothesis that the onset of NEC is preceded by dysbiosis, a change in bacterial colonisation of the gut, was first declared by Claud et al.\textsuperscript{24} Dysbiosis is characterised by low microbiome diversity and change in its composition, and especially by overgrowth of some pathogens, as has been well described by many studies in preterm neonates.\textsuperscript{25-27} The overgrowth of pathogens and lower number of anaerobes (above all lactobacilli and Bifidobacterium spp) present a higher risk for NEC’s development.\textsuperscript{26}

Given that with the use of culture methods we are able to detect only a minimal fraction of microorganisms, it was not until the development of sequencing methods (16S rRNA, whole-genome sequencing, metagenomics) that we were able to achieve deeper understanding of the gut microbiome’s composition. Published studies assessing the relationship between the microbiome and NEC have focused on cross-sectional description of the gut microbiome and its longitudinal changes before the onset of NEC. This approach has been applied to cohorts of different gestational age and using various analytical methodologies. We searched the relevant literature published from January 2009, when the first study described the use of 16S RNA sequencing in the gut microbiome of neonates, to March 2023. We searched the terms ‘microbiome’, ‘microbiota’, ‘necrotizing enterocolitis’ and ‘preterm dysbiosis’ in PubMed. We excluded non-English-language publications and studies focused on NEC in children born after the 32nd week of gestational age. The remaining 18 studies are summarised in table 1 and further discussed here.

The first study to describe gut microbiome composition in preterm neonates at time of NEC using 16S rRNA was a monocentric study by Wang et al.\textsuperscript{29} They compared the microbiome in 10 neonates with NEC to a control group of 10 preterm neonates. The authors confirmed the generally lower diversity of the preterm microbiome and that it was even lower in the NEC group. In the control group, four phyla were present (Firmicutes, Proteobacteria, Bacteroides and Fusobacteria), while only two phyla, Firmicutes and Proteobacteria, were detected in the NEC group. They showed a significantly higher relative abundance of Proteobacteria at the time of NEC onset, these composing as much as 90% of the microbiome. From the same group, the authors later confirmed in a longitudinal study that significant changes in microbiome composition occurred already 3 weeks before the onset of NEC. At that time, the relative abundance of Proteobacteria started to increase while on the other hand Firmicutes decreased.\textsuperscript{24} Mai et al investigated the microbiome in two time intervals and described very similar changes of microbiome with the dominance of Proteobacteria 1 week before the onset of NEC. At the level of operative taxonomic units, the bacteria closest to Enterobacteriaceae were represented (E. coli, Enterobacter
Table 1  
Studies comparing gut microbiome composition in preterm infants between NEC patients and controls

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>No of participants NEC/controls</th>
<th>GA (weeks) and/or BW (g)</th>
<th>Stool samples collection</th>
<th>Methods of microbiome evaluation</th>
<th>Results: microbiome profiles in NEC vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang et al, 2009 [29]</td>
<td></td>
<td>10/10</td>
<td>25–32</td>
<td>Onset of NEC</td>
<td>16S rRNA</td>
<td>Lower absolute richness and diversity</td>
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<td></td>
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<td></td>
<td></td>
<td>Higher abundance of Proteobacteria</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Only 2 phyla in NEC (Proteobacteria, Firmicutes)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>4 phyla in control group (Proteobacteria, Firmicutes, Bacteroides, Fusobacteria)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>570–1269</td>
<td></td>
<td></td>
<td>Decrease of Firmicutes (Proteobacteria, Firmicutes, Bacteroides, Fusobacteria)</td>
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<td>Torrazza et al, 2013 [31]</td>
<td></td>
<td>18/35</td>
<td>23–30</td>
<td>2 weeks prior to NEC 1 week prior to NEC Onset of NEC</td>
<td>16S rRNA (V6–V8 region)</td>
<td>Increase in Proteobacteria 2 weeks before NEC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>570–1269</td>
<td></td>
<td></td>
<td>Increase in Actinobacteria 1 week before NEC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>650–980</td>
<td></td>
<td></td>
<td>Early onset (7–21 days)—Firmicutes dominate in the 1st sample</td>
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<td>Later onset (19–39 days)—Proteobacteria dominate</td>
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<td>(Enterobacteriaceae – Escherichia coli, Enterobacter spp)</td>
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<td></td>
<td>Operative taxonomic units close to Klebsiella pneumoniae strongly associated with NEC</td>
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<td></td>
<td>487–965</td>
<td></td>
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<td>Control—higher relative abundance of Enterococcus spp</td>
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<td></td>
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<td></td>
<td>Shotgun metagenomics</td>
<td>Increase of Proteobacteria 2–3 weeks before NEC</td>
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<td>Increase of Firmicutes in the control group</td>
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<td>Shotgun—increase of Enterobacteriaceae and decrease of Veillonella spp (Firmicutes) before NEC</td>
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<td>Zhou et al, 2015 [36]</td>
<td></td>
<td>12/26</td>
<td>24–31</td>
<td>Weekly</td>
<td>16S rRNA (V3–V5 region)</td>
<td>Early-onset NEC—higher abundance of Clostridium spp Late-onset NEC—higher relative abundance of Escherichia spp/Shigella spp from 6 days prior to NEC and of Cronobacter spp 1–3 days prior to NEC</td>
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<td></td>
<td></td>
<td></td>
<td>940–1860</td>
<td></td>
<td></td>
<td>Clostridia decrease with increasing severity of NEC</td>
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<td>McMurtry et al, 2015 [37]</td>
<td></td>
<td>21/74</td>
<td>24–31</td>
<td>1–5 days prior to NEC</td>
<td>16S rRNA (V3–V5 region)</td>
<td>NEC—lower relative abundance of Actinobacteria and Clostridium Clostridia decrease with increasing severity of NEC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>940–1800</td>
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<tr>
<td>Cassir et al, 2015 [38]</td>
<td></td>
<td>15/15</td>
<td>Preterm</td>
<td>Onset of NEC</td>
<td>16S rRNA (V6 region) qPCR Clostridium butyricum</td>
<td>Lower diversity</td>
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<td></td>
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<td></td>
<td></td>
<td>Higher abundance of Clostridium ssp, especially Clostridium butyricum</td>
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</tbody>
</table>

Continued
30 Other studies also describe the dysbiosis defined by an increase of Proteobacteria before the onset of NEC.31–33 Warner et al found these changes especially among the most immature neonates born before the 27th week of gestational age.25 On the other hand, another group of authors investigated only neonates born before the 26th week of gestational age and did not confirm this observation.34 The opposing results may be a consequence of the different methodologies being used (ie, different variable sections of 16S rRNA).28

Morrow et al described two types of dysbiosis preceding NEC.35 The first, already mentioned, was the predominance of Proteobacteria (especially Enterobacter spp, E. coli). Their abundance was as great as 99.6% in a group of neonates with late-onset NEC after the 19th day of life. The second type of dysbiosis was observed among neonates with early onset of NEC, and it was characterised by significantly greater representation of Firmicutes (up to 99%).

Stewart et al in their study succeeded in dividing the microbiome based on dominant bacteria into six clusters, known as preterm gut community types or PGCTs.36 The second and fifth cluster types, characterised by dominance of Klebsiella spp, Enterococcus spp and Escherichia spp, were observed most frequently in neonates with NEC.
and their microbiomes were generally very unstable. The sixth cluster had much greater diversity; showed higher relative abundance of Bifidobacteria, lactobacilli, Clostridia and streptococci; and was found only in the control group.

In some studies, the presence of Clostridia at the time of NEC was described. In two studies, the authors found a relationship between cytotoxic Clostridium butyricum and NEC, but both studies described the microbiome only at the time of NEC diagnosis. At that time point, the results could be influenced by the course of the disease and its therapy. Heida et al found a significantly higher abundance of Clostridium perfringens and Bacteroides dorei in the meconium of preterm neonates. McMurtry et al grouped neonates with NEC according to severity of the disease. They found that at the time of diagnosis the abundance of Clostridia decreased with severity of the disease and in lethal cases Clostridia were absent altogether.

Several studies have pointed to a greater abundance of Klebsiella spp before the onset of NEC. Torrazza et al found significant association between the presence of Klebsiella pneumoniae during the first week and subsequent NEC development.

Recently, increased availability and lower price of whole-genome sequencing have enabled its common utilisation, and this has resulted in deeper and more accurate resolution of the microbiome. Olm et al confirmed on a relatively robust cohort (34 NEC, 126 controls) the dominance of Proteobacteria in all neonates. Before the onset of NEC, at species level, K. pneumoniae was present in 52% of samples. Considering that the same pathogen was present also in 23% of control samples, the authors warn that this could reflect an effect of their NICU colonisation.

Original in its approach was a study by Stewart et al, who examined gut microbiomes in paraffine blocks from samples of intestinal resections in neonates with NEC. They chose samples of spontaneous intestinal perforation as a control group. The authors found significantly lower diversity in the NEC samples and large interindividual differences testifying to an unstable microbiome. They also described a significantly higher relative abundance of Proteobacteria. While the stool offers the possibility to evaluate microbiome of the colon, it may not reflect the state of the most frequently affected part, the terminal ileum, the microbiome of which may be better assessed by examination of resected intestine. The authors point out the fact that, optimally, we should compare the microbiome of the stool and the tissue from the same neonates, ideally right after resection of the intestine.

The utilisation of machine learning seems to be an attractive option in evaluating microbiome composition as an early predictor of NEC. Hooven et al developed a computer analysis to predict the onset of NEC based on assessment of clinical parameters and a once weekly analysis of stool microbiome, but their method has not yet been validated.

MODIFIABLE RISK FACTORS OF NEC RELATED TO MICROBIOME
Regulation of inflammation
Recent research in NEC prevention has focused on the suppression of exaggerated inflammatory reactions through influencing the TLR 4 signal pathway and gut dysbiosis. The results of animal studies have shown that in mice without TLR4 expression no NEC would develop. Various ways to inhibit this pathway are being investigated, for example, by activation of nucleotide-binding oligomerisation domain 2 receptor or using the C34 molecule (2-acetamido pyranoside), which inhibits lipopolysaccharide-stimulated inflammatory response via downregulation of TLR4 signalling. This leads to a decrease of proinflammatory factors and chemokines, such as nitric oxide, tumour necrosis factor α or interleukin-6. Lately, an integral role of Paneth cells in maintaining intestinal homeostasis, regulation of microbiota and level of inflammation has been studied. Paneth cells contain many antimicrobial peptides and immunomodulating proteins, but they do not become fully immune competent until closer to the full term of gestation. Disruption of their normal role in the host–microbial axis in immature gut may facilitate NEC development.

Enteral nutrition
The role of enteral nutrition in the aetiopathogenesis of NEC has been investigated for many years. A prolonged period of fasting after birth increases the risk of NEC and a protective approach may be to standardise feeding. Use of mother’s breast milk lowers the risk of NEC compared with donor breast milk or formula, dependent also on the amount of milk. The highest number of proteins, growth factors and immunomodulatory factors (secretory IgA, lactoferrin, lactadherin, oligosaccharides) are contained in colostrum and preterm breast milk. Research is ongoing as to the effect of bovine colostrum. A recent study in 12 preterm neonates did not prove it to have positive effect. Potentially, that may be because the bovine colostrum is administered enterally in the studies, meaning that the protective factors contained therein do not come into contact with oropharyngeal-associated lymphoid tissue and therefore do not exert their immunomodulating effects. Pasteurised milk is less beneficial, as pasteurisation at 62.5°C for 30 min destroys enzymatic activity (eg, of lipase), which results in poorer growth, and it also may destroy many positive immunomodulatory factors with proven protective effects against NEC. Some growth factors contained in breast milk and also in the amniotic fluid are being investigated, including, for example, heparin-binding epidermal growth factor and epidermal growth factor, which inhibit the TLR4 signal pathway.

Antibiotic use
Antibiotics constitute another important factor with strong influence on microbiome composition. These are among the drugs most frequently used in NICUs. During the first 3 days of life, as many as 87% of newborns with
extremely low birth weight receive antibiotic treatment. In addition, almost 60% of them continue the therapy beyond the 4th day even if laboratory results are negative.⁵⁵ After even a short course of antibiotics there is a drop in microbiome richness and diversity together with overgrowth of some resistant pathogenic bacteria. Several weeks are needed for restitution of the microbiome and so prolonged antibiotic therapy increases the risk of NEC.⁵⁶ Therefore, it is important to consider carefully every indication of antibiotic treatment and also its early cessation.

**Probiotics use**

An important topic in NEC prevention is the use of probiotics. Probiotics are living organisms which, through their effects in the gut, improve the gut’s barrier function, mucosa IgA response and tolerance of enteral nutrition.⁵⁷ Probiotics also reduce the growth of pathogens and produce various anti-inflammatory cytokines. Through downregulation of inflammatory pathway, they contribute to reducing the incidence of late-onset sepsis and NEC.⁵⁸ The results of various studies in this area are not unequivocal, however. Most of them describe a greater abundance of Bifidobacteria in the neonate gut as a result of administering probiotics.⁵⁹ Several problems are inherent in these studies, such as inconsistencies in methodology (differing study designs, infant diets and/or gestational age) and the various types of probiotics being evaluated at different dosages. Nevertheless, the use of probiotics is a promising way to influence gut microbiome development in preterm infants and prevent NEC.

**CONCLUSION**

NEC is a serious disease that has a strong negative influence on the prognosis of preterm neonates, and its treatment places considerable financial demands on health systems worldwide. At present, it seems necessary to re-evaluate the diagnostic criteria for NEC and to focus research on reaching a better understanding of NEC’s pathophysiology. This not only could improve its diagnostics but also point to new possibilities for how to prevent NEC’s development. That dysbiosis plays a role in the pathophysiology of NEC seems indisputable, but a better comprehension of its relationship to NEC and the surrounding context is important. Improved understanding is needed of the microbiome’s metabolic functions and its interaction with the neonate’s innate immune system. In order to optimise the outcomes of new studies, their reproducibility and comparability, it is important to standardise the methodology for evaluating the microbiome. Furthermore, preventive measures to reduce the risk of NEC’s development, such as the use of probiotics or suppression of proinflammatory factors, warrant further investigations.

**Acknowledgements**

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