Pediatric Gastroenterology, Hepatology & Nutrition

pghn

Review Article

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Probiotics in the Prevention and Treatment of Necrotizing Enterocolitis

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ABSTRACT

Necrotizing enterocolitis (NEC) is a disease with high morbidity and mortality that occurs mainly in premature born infants. The pathophysiologic mechanisms indicate that gastrointestinal dysbiosis is a major risk factor. We searched for relevant articles published in PubMed and Google Scholar in the English language up to October 2020. Articles were extracted using subject headings and keywords of interest to the topic. Interesting references in included articles were also considered. Network meta-analysis suggests the preventive efficacy of *Bifidobacterium* and *Lactobacillus* spp., but even more for mixtures of *Bifidobacterium*, *Streptococcus*, and *Bifidobacterium*, and *Streptococcus* spp. However, studies comparing face-to-face different strains are lacking. Moreover, differences in inclusion criteria, dosage strains, and primary outcomes in most trials are major obstacles to providing evidence-based conclusions. Although adverse effects have not been reported in clinical trials, case series of adverse outcomes, mainly septicemia, have been published. Consequently, systematic administration of probiotic bacteria to prevent NEC is still debated in literature. The risk-benefit ratio depends on the incidence of NEC in a neonatal intensive care unit, and evidence has shown that preventive measures excluding probiotic administration can result in a decrease in NEC.

Keywords: Microbiota; Enterocolitis, necrotizing; Probiotics

INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe and potentially lethal intestinal disease that occurs almost exclusively in preterm infants. It is characterized by mucosal inflammation, epithelial cell death, and transmural perforation of the intestinal wall with leakage of intestinal fluids. In severe cases, it leads to sepsis and multiorgan failure, and surgical removal of the necrotized intestine is the only treatment option. The symptoms of NEC are often nonspecific, varying from temperature instability and changes in vital parameters to feeding intolerance, distention of the abdominal wall, and bloody stools.

The prevalence is around 7% in preterm babies with a weight <1,500 g and has a mortality rate of 20–30% [1]. NEC is predominantly seen in infants born at a gestational age younger than 32 weeks, and its incidence is inversely proportional to the gestational age [2]. NEC

usually develops between the second week and second month of life and rarely occurs in utero or prior to the first feeding [3]. Many risk factors have been identified, including small for gestational age, premature rupture of membranes, assisted ventilation, sepsis, and hypotension [4]. Other risk factors include formula feeding, exposure to acid suppression medication, and use of antibiotics [5,6]. The latter category of modifiable risk factors alters the intestinal microbiome, which supports the hypothesis that dysbiosis is an important determinant factor leading to NEC. Consequently, probiotics are frequently used in neonatal intensive care units (NICUs). In the United States of America, out of 78,076 infants, 3,626 (4.6%) received probiotics. Probiotic use increased over the study period, from 1997 to 2016, and varied among NICUs [7].

The effect of evidence-based strategies to decrease NEC, including (1) a standardized feeding protocol; (2) early initiation of enteral feeding using human milk; (3) optimizing the osmolality of preterm milk feeds using standardized dilution guidelines for additives; and (4) promotion of healthy microbiome using probiotics, early oral care with colostrum, and restriction of high-risk medications, and prolonged use of empirical antibiotics, was tested for four consecutive years in one center. Baseline patient characteristics, including sex, gestational age, and birth weight, were similar during the study period. The incidence of NEC in very-low-birth-weight infants was 7% in 2014 and dropped to 0% ($p<0.001$) in 2018. The duration of parenteral nutrition, use of central line, and days to full feeding were also reduced significantly ($p<0.05$) [8].

METHODS

We searched for relevant articles published in English up to October 2020 using PubMed and Google Scholar. Articles were extracted using subject headings and keywords of interest to the topic. A second selection was made by reading the abstract. Articles that answered different questions were used. Interesting references in included articles were also considered.

RESULTS

The microbiome, dysbiosis, and necrotizing enterocolitis

The number of species growing in the gut increased significantly with gestational age. The abundance of species and their diversity is, other than by gestational age, also influenced the use of (intrapartum) antibiotics, method of feeding, and mode of delivery. The presence and abundance of *Clostridium perfringens*, which produces alpha toxin, and *Bacteroides dorei* in the meconium were associated with NEC, suggesting that factors during pregnancy, delivery, and the first moments of life may contribute to the formation of an NEC-associated microbiota [9].

Studies found that preterm babies being formula fed had an increased abundance of Proteobacteria, including *Klebsiella* and *Enterobacter*, and a decreased abundance of Firmicutes. Moreover, *Bifidobacterium* species, which are beneficial commensal bacteria abundant in breastfed term infants, seem to be less common and especially less abundant in preterm infants who go on to develop NEC [10,11].

The use of antibiotics increased the abundance of Proteobacteria and decreased the relative abundance of Firmicutes and Actinobacteria. Infants not receiving antibiotics showed an

increased abundance of the genus *Clostridium* and unclassified Clostridiaceae. According to a meta-analysis [12], infants who developed NEC had an increased abundance of Proteobacteria and a decreased abundance of Firmicutes and Bacteroidetes compared to healthy controls. Although no association was found between the mode of delivery and the development of NEC, a meta-analysis found that children born by cesarean section showed an increased abundance of Firmicutes, while an increased abundance of Bacteroidetes was found in infants born through vaginal delivery [12].

Interestingly, the absence of Clostridia, particularly *Clostridium difficile*, is associated with the development of NEC [13]. Although this seems in contrast with the fact that infants with an abundant amount of *C. perfringens* in the meconium are associated with NEC, it was hypothesized that the non-toxinogenic *C. difficile* provides effective protection against enterotoxin-mediated diseases caused by *C. perfringens* [14]. Similarly, newborns are frequently colonized with *C. difficile* without suffering from any illness, while they become more prone to *Clostridium*-related diseases when they are no longer *Clostridium* carriers [15].

As mentioned, NEC occurs almost exclusively in preterm infants, a group in which cesarean section and perinatal antibiotic administration are extremely frequent compared to term infants. Many preterm infants are formula fed, and if mother's milk can be given, it has to be acknowledged that mother's milk that has been banked has lost some of its benefits [16]. In mice, maternal administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* (during pregnancy and lactation) promotes intestinal development, improves small intestinal barrier function, and decreases inflammatory responses in preweaned pups [17].

Most relevant mechanisms of probiotic action in the preterm infant

Probiotics have different sites of action that enable them to protect the premature intestine for the development of NEC. One of the most important mechanisms is the modulation of toll-like receptors (TLRs), whose essential function is to recognize components of pathogenic microbes and trigger a specific inflammatory response [11]. The immature gut has a propensity to inflame, whereby TLR4 is thought to play a crucial role in the regulation of injury and repair balance in the intestinal epithelium [18,19]. Animal studies have shown increased TLR4 expression in the immature gut compared to that in the full-term gut. This is probably due to the fact that TLR4 is also essential for the activation of the Notch signaling pathway, leading to activation of the intestinal stem cells, which is required for normal proliferation and differentiation [18]. In utero, TLR4 expression is downregulated by epithelial growth factor (EGF), which is present in the amniotic fluid that the fetus constantly swallows; however, extrauterine EGF is present in breast milk [20-25]. The activation of TLR4 in the premature gut leads to death of the intestinal epithelium, leading to NEC. Probiotics stimulate the production of TLR9, which prevents TLR4 signaling [26]. For example, Proteobacteria are known to be activators of TLR4 [27].

Furthermore, the immature intestine is constantly exposed to newly colonizing commensals and pathogens. When the intestines of preterm infants are colonized with pathogenic bacteria, probiotics compete and may limit the overgrowth of such pathogens [28]. Lactate-producing bacilli, including *Staphylococci* and *Streptococci*, lower the pH via the production of lactate, impairing the overgrowth of pathogenic Enterobacteriaceae [29,30]. In addition, probiotics are known to support barrier maturation and function of the intestinal wall [31,32].

Probiotics and prevention of necrotizing enterocolitis

As described earlier, probiotic bacteria are present in mother's milk, and maternal milk has a protective effect against NEC. However, breast milk is not always available, especially in mothers of preterm infants; hence, maternal production is poor due to premature delivery. Therefore, the administration of probiotics seems to be a logical step in the prevention of NEC.

Hoyos [33], a neonatologist in Bogota Colombia, was the first to investigate the role of probiotics in the prevention of NEC. Twenty years ago, she decided to treat preterm neonates in the NICU with the probiotic Infloran® (https://www.infloran.com.au), containing *B. infantis* and *L. acidophilus*, for the duration of their hospital stay and saw a highly significant decrease in both NEC and NEC-related death ($p<0.0002$ and $p<0.005$, respectively) (**Table 1**). Since then, many investigators have started clinical trials, including more than 40,000 infants [11].

A wide variety of probiotic preparations have been studied, including *Bacillus*, *Lactobacillus*, and *Saccharomyces* spp. and probiotic combinations. However, the most commonly used preparation was *Lactobacillus* spp., *Bacillus* spp., or a combination of both. Most randomized controlled trials (RCTs) compared the supplementation of a probiotic with placebo or no supplementation. In addition, most trials started supplementation within the first week of birth, usually with the first enteral feed. The supplementation was usually continued until 28 days postpartum or until discharge from the hospital [34,35].

Jacobs et al. [36] reported in 2013 that the combination of *B. infantis*, *Streptococcus thermophilus*, and *Bifidobacterium lactis* was effective in the reduction of NEC. A large trial in the United Kingdom on probiotic supplementation investigating the effect of the single strain *Bifidobacterium* breve found no effect on the reduction of NEC [37]. In the study by Costeloe et al. [37], the probiotics were given in a formula matrix, whereas in other studies, the probiotic was administered in separate drops. The matrix in which the probiotic is administered may contribute to differences in outcomes. According to data from Spain, between 2005 and 2017, the incidence of NEC remained stable at 8.8% among the 25,821 included infants during the

Table 1. Probiotics and prevention of necrotizing enterocolitis

Study	Country	No. of patient	Probiotic dose	Duration of administration	Effect
Hoyos, 1999 [33]	Columbia	1,237	250×10^6 *Bifidobacterium infantis*, 250×10^6 *Lactobacillus acidophilus*	1 year	↓ NEC and NEC-related death ($p<0.0002$ and $p<0.005$, respectively)
Jacobs et al., 2013 [36]	Australia, New Zealand	1,099	10^6 *B. infantis*, 10^6 *Streptococcus thermophilus*, 10^6 *Bifidobacterium lactis*	Discharge	↓ NEC of Bell ≥stage 2 (2.0% vs. 4.4%; RR, 0.46; 95% CI, 0.23 to 0.93; p=0.03; NNT, 43; 95% CI, 23–333)
Costeloe et al., 2016 [37]	United Kingdom	1,315	1 mL *Bifidobacterium breve* BBG-001 (6.7×10^7–10^9 cfu)	Birth to 36 wk GA	Evidence of benefit but does not support routine use of probiotics in preterm infants (cross colonization?)
Zozaya et al., 2020 [38]	Spain	25,821	Several	NA	NEC incidence not changed
Gray et al., 2020 [7]	United States of America	2,178	Several	NA	Population: 23–29 weeks old ↓ NEC (OR, 0.62; 95% CI, 0.48–0.80) and death (OR, 0.52; 95% CI, 0.39–0.70), ↑ Candida infection (OR, 2.23; 95% CI, 1.29–3.85), no change in bloodstream infection (OR, 0.86; 95% CI, 0.70–1.05) or meningitis (OR, 1.18; 95% CI, 0.40–3.46)

cfu: colony-forming unit, GA: gestational age, NA: not available, NEC: necrotizing enterocolitis, RR: relative risk, CI: confidence interval, NNT: number needed to treat.

whole study period and remained stable when comparing 4-year subperiods. Prophylactic probiotics were implemented during the 12-year study period in some units, reaching 18.6% of the patients in 2015–2017. However, when all trials with different protocols and different strains were grouped together, the incidence of NEC remained stable despite the increase in protective factors [38].

The most recent Cochrane review [9] performed a meta-analysis comparing different probiotic preparations (**Table 2**). The reviewed population included 10,812 preterm infants distributed over 56 trials with an average gestational age of 28-32 weeks and an average birth weight of 1,000–1,200 g. The overall conclusion was that supplementation with probiotics reduced the risk of NEC (relative risk [RR], 0.54; 95% confidence interval [CI], 0.54–0.65) with the combination of *Bacillus* and *Lactobacillus* spp. being the most effective (RR, 0.36; 95% CI, 0.23–0.59). Other effective combinations were *Bacillus* spp. and *Streptococcus* spp. (RR, 0.35; 95% CI, 0.19–0.68) and *Bacillus* spp., *Lactobacillus* spp., and *Streptococcus* spp. (RR, 0.42; 95% CI, 0.22–0.77). The single genus *Bacillus* spp. was also found to be effective (RR, 0.72; 95% CI, 0.54–0.96) like *Lactobacillus* spp. (RR, 0.45; 95% CI, 0.28–0.71), but less effective than their combination. Few data (from seven of the trials) were available for extremely preterm or extremely-low-birth-weight infants. Meta-analyses did not show their effects on NEC, death, or infection (low-certainty evidence). Sensitivity meta-analyses of 16 trials (4,597 infants) at low risk of bias did not show any effect on mortality or infection. Evidence was assessed as low certainty because of the limitations in the trial design and the presence of funnel plot asymmetry consistent with publication bias [9].

Studies comparing the efficacy of different strains with each other are lacking, and as a consequence, conclusions should be interpreted cautiously. Therefore, different network meta-analyses on probiotics and NECs have been published in 2020. Network analysis allows the comparison of the efficacy of different strains tested in different studies. According to van den Akker et al. [39], all safety issues are met, and there is currently a conditional recommendation (with low certainty of evidence) to provide either *Lactobacillus rhamnosus* GG ATCC 53103 or the combination of *B. infantis* Bb-02, *B. lactis* Bb-12, and *S. thermophilus* TH-4 to

Table 2. Meta-analyses on probiotics and prevention of necrotizing enterocolitis

Study	No. of patient	Outcome
Sharif et al., 2020 [9]	10,812	All: ↓ NEC: RR, 0.54; 95% CI, 0.45–0.65; NNT, 33; 95% CI, 25–50
		Trials at low risk of bias: ↓ NEC: RR, 0.70; 95% CI, 0.55–0.89; NNT, 50; 95% CI, 33–100
		All: ↓ mortality (RR, 0.76; 95% CI, 0.65–0.89; NNT, 50; 95% CI, 50–100) and late-onset invasive infection (RR, 0.89; 95% CI, 0.82–0.97; NNT, 50; 95% CI, 33–100)
van den Akker et al., 2020 [39]	?	Conditional recommendation (with low certainty of evidence) to provide either *Lactobacillus rhamnosus* GG ATCC 53103 or the combination of *Bifidobacterium infantis* Bb-02, *Bifidobacterium lactis* Bb-12, and *Streptococcus thermophilus* TH-4
Chi et al., 2021 [40]	12,320	B+L: ↓ mortality (risk ratio, 0.56; 95% CI, 0.34–0.84) and NEC morbidity (risk ratio, 0.47; 95% CI, 0.27–0.79)
		L+prebiotic: ↓ NEC morbidity (risk ratio, 0.06; 95% credible interval, 0.01–0.41)
		B+prebiotic: lowest mortality
		L+prebiotic: lowest NEC
Morgan et al., 2020 [41]	15,712	↓ All-cause mortality: combination of ≥1 L spp. and ≥1 B spp. (OR, 0.56; 95% CI, 0.39–0.80)
		↓ Severe NEC: *Bifidobacterium animalis* subspecies *lactis*, *Lactobacillus reuteri*, or *Lactobacillus rhamnosus* (OR, 0.35; 95% CI, 0.20–0.59; OR, 0.31; 95% CI, 0.13–0.74; OR, 0.55; 95% CI, 0.34–0.91; and OR, 0.44; 95% CI, 0.21–0.90, respectively)
		↓ Number of days to reach full feeding (mean ↓ of 3.30 days): ≥1 L spp. and ≥1 B spp. and *Saccharomyces boulardii* (95% CI, reduction of 5.91–0.69 days)
		↓ Duration of hospitalization: *B. animalis* subsp. *lactis* or *L. reuteri*: mean duration of 13 days (95% CI, reduction of 22.71–3.29 days) and mean reduction of 7.89 days (95% CI, reduction of 11.60–4.17 days), respectively

NEC: necrotizing enterocolitis, RR: relative risk, CI: confidence interval, NNT: number needed to treat, B: *Bifidobacterium*, L: *Lactobacillus*, ≥1: one or more, OR: odds ratio.

reduce NEC rates. Chi et al. [40] included 45 trials with 12,320 participants. The combination of *Bacillus* and *Lactobacillus* spp. was associated with lower rates of mortality (risk ratio, 0.56; 95% credible interval, 0.34–0.84) and NEC morbidity (risk ratio, 0.47; 95% credible interval, 0.27–0.79) in comparison to placebo; *Lactobacillus* spp. in combination with prebiotics was associated with lower rates of NEC morbidity (risk ratio, 0.06; 95% credible interval, 0.01–0.41) in comparison to placebo; *Bacillus* spp. in combination with prebiotics had the highest probability of having the lowest rate of mortality (surface under the cumulative ranking curve 83.94%); and *Lactobacillus* spp. in combination with prebiotics had the highest probability of having the lowest rate of NEC (surface under the cumulative ranking curve 95.62%) [40]. An important limitation is that only a few studies have reported the data of infants with a lower birth weight or gestational age [40]. According to a recent matched cohort study in 23- to 29-week-old infants, probiotic administration was associated with a decrease in NEC (odds ratio [OR], 0.62; 95% CI, 0.48–0.80) and death (OR, 0.52; 95% CI, 0.39–0.70) and an increase in Candida infection (OR, 2.23; 95% CI, 1.29–3.85), but no increase in bloodstream infection (OR, 0.86; 95% CI, 0.70-1.05) or meningitis (OR, 1.18; 95% CI, 0.40–3.46) [7]. To achieve optimal effects on premature infant health, the combined use of prebiotics and probiotics, especially *Lactobacillus* or *Bacillus*, is recommended [40]. In a systematic review and network meta-analysis performed by Morgan et al. [41] of the McMaster Probiotic, Prebiotic, and Synbiotic Work Group to determine the effects of single-strain and multi-strain probiotic formulations on outcomes of preterm, low-birth-weight neonates, a moderate to high evidence for the superiority of combinations of one or more *Lactobacillus* spp. and one or more *Bacillus* spp. vs. single- and other multiple-strain probiotic treatments was reported [41]. The combinations of *Bacillus* and *Enterococcus* spp. and one or more *Bacillus* spp. and *Streptococcus salivarius* subsp. *thermophilus* might produce the largest reduction in NEC development [41].

Safety

Since preterm infants are a fragile and immunocompromised population and probiotics are live bacteria supplements, it is extremely important to be aware of the possible risks. The most feared side effect is probiotic sepsis, whereby it is important to realize that it may be difficult to diagnose because the traditional pediatric culture bottle impairs the growth of anaerobic strains. Probiotic sepsis may be the result of not only intestinal translocation, but also contamination of the central lines after the preparation of the probiotic [39].

Probiotic sepsis, mostly associated with *B. infantis* and *L. rhamnosus* GG, has been described in several single or multiple case studies. However, other probiotic strains have also been cultured, including *Lactobacillus reuteri*, *Saccharomyces boulardii*, *B. breve*, and *Escherichia coli Nissle*. Two recent papers described *L. rhamnosus* bacteremia in infants with a central line that did not receive a probiotic but were just sharing the same room with the infant receiving a probiotic [42,43].

However, even though the case reports should be taken seriously, it is also important to note that in all 56 trials included in the most recent Cochrane review, no probiotic-related sepsis was found [9].

Lactic acidosis is another potential adverse effect, specifically relevant for this age group, as preterm infants tend to be acidotic and are more prone to suffer from conditions that make them more acidotic, such as sepsis and renal insufficiency. Lactate may be produced by *Lactobacilli* strains in two different isoforms: D-lactate and/or L-lactate. Some strains, such as *L. rhamnosus* GG ATCC 53103, produce mainly L-lactate, but *L. reuteri* DSM 17938 or *L. acidophilus* NCDO 1748 produces larger proportions of D-lactate. In particular, the

production of D-lactate could be problematic in preterm infants because it is difficult to dispose of after enteral uptake, therefore possibly leading to acidosis [44]. Moreover, in contrast to L-lactate, D-lactate cannot routinely be measured in blood gases, and as a consequence, it may be difficult to identify the cause of metabolic acidosis. Although *L. reuteri* DSM 17938 has been approved for use in term infant formula by the Food and Drug Administration, and despite the fact that in term born infants, an elevated urinary D-lactate concentration after being fed an *L. reuteri* DSM 17938-containing formula was not associated with blood acidosis [45], it may be wise not to take risks in the premature population because of the aforementioned reasons. Moreover, several case reports have described lactate acidosis in infants with short bowel syndrome [46,47]. Therefore, it may be wise to follow the statement of the Codex Alimentarius, stating that preterm infants should only receive probiotics that mainly produce L-lactate until more research on this topic is available [48].

Another important safety issue is related to the quality control of probiotic supplementation, whereby differences have been found between the label and the actual content. A recent report found that only 1 out of 16 tested commercial probiotic products matched exactly the label, including probiotics marketed specifically for infants [49]. Since probiotics are usually marked as food supplements instead of drugs, they fall under the regulatory framework of food, and consequently, manufacturers may change the product content or production process without the obligation to properly address those issues [50].

In addition, a limited number of follow-up studies are available to assess the long-term efficacy or safety of probiotics used in preterm infants. A randomized trial of 400 very low birth weight infants with follow-up of 18-24 months showed that the use of *L. reuteri* did not increase or decrease the risk of adverse neurocognitive outcomes [51]. A large trial called ProPrems is currently ongoing and will hopefully provide additional data regarding the long-term benefits of probiotics [52].

CONCLUSION

NEC is a devastating disease that is responsible for the morbidity and mortality of premature infants. Since early dysbiosis has been associated with the development of NEC, many studies have been conducted in the past decades to study the role of probiotics in the prevention of this disease. Different (network) meta-analyses have been performed reviewing clinical trials involving more than 10,000 infants, leading to the overall conclusion that probiotics could play a role in the prevention of NEC.

The most recent Cochrane review [9], dated October 2020, including 56 trials, found an overall beneficial effect of probiotics in the prevention of NEC, whereby most effects were observed for *Bifidobacterium* and *Lactobacillus* spp., but even more for mixtures of *Bifidobacterium* and *Streptococcus* spp. and *Bifidobacterium* and *Streptococcus* spp.

Besides the fact that the effect of probiotics is strongly species-specific, the effect of a single strain in NEC might also differ from that of combined strains [53].

Despite the promising results of the Cochrane review and other (network) meta-analyses, its implementation in clinical practice has been difficult because of concerns about the efficacy and safety of probiotics [54].

Since the efficacy of probiotics is highly strain-specific, more studies should be performed comparing individual strains face-to-face. So far, only network analyses have been used to compare the different strains, but it is important to realize that as indirect comparisons are not randomized, the effect size may be confounded by factors other than purely the difference between the strains [9].

Moreover, there is a lack of studies investigating the optimal dose and duration of therapy. Within RCTs, there is variability in the doses, age at initiation, and duration of the therapy. It has been suggested that at least $1×10^9$ colony-forming units are required to guarantee the passage through the gastrointestinal tract and gut colonization to exert a measurable beneficial effect [55]. However, most trials vary in dose from 1 to $6×10^9$ per day, showing mixed results, leading to the conclusion that the dose-dependent effects are in fact strain-dependent. Therefore, studies should be performed to determine the optimal dose for specific probiotic strains.

In addition, studies systemically investigating possible adverse effects, such as D-lactate acidosis and probiotic sepsis, would be of great value. Studies should preferably be performed with probiotic products to guarantee that the actual content matches its label.

A model demonstrated that prophylactic probiotics are a cost-effective strategy for NEC reduction. Sensitivity analysis confirmed that the model is customizable to various clinical settings and, thus, can aid in understanding the economic impact of this intervention [56]. Uncertainty about the therapeutic role of probiotics in preventing NEC is partly due to the wide range of bacterial strains with no previous evidence of efficacy used in clinical trials [57].

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