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REVIEW

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Microbiome-targeting therapies in the neonatal intensive care unit: safety and efficacy

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ABSTRACT

Microbiome-targeting therapies have received great attention as approaches to prevent disease in infants born preterm, but their safety and efficacy remain uncertain. Here we summarize the existing literature, focusing on recent meta-analyses and systematic reviews that evaluate the performance of probiotics, prebiotics, and/or synbiotics in clinical trials and studies, emphasizing interventions for which the primary or secondary outcomes were prevention of necrotizing enterocolitis, late-onset sepsis, feeding intolerance, and/or reduction in hospitalization length or all-cause mortality. Current evidence suggests that probiotics and prebiotics are largely safe but conclusions regarding their effectiveness in the neonatal intensive care unit have been mixed. To address this ambiguity, we evaluated publications that collectively support benefits of probiotics with moderate to high certainty evidence in a recent comprehensive network meta-analysis, highlighting limitations in these trials that make it difficult to support with confidence the routine, universal administration of probiotics to preterm infants.

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Introduction

The gut microbiome is a dynamic community that is seeded during infancy, and which trains the immune system and supports healthy growth and development of its host.[1] Microbial assembly in the infant gut is influenced by numerous factors, including the maternal microbiota, delivery mode (cesarean vs. vaginal birth), diet (breastfeeding vs. formula feeding), and postmenstrual age at birth. Infants born preterm (gestational age (GA) <37 weeks) have an immature intestine characterized by deficient mucosal immunity and often experience perturbations that interfere with microbial community maturation.[2] Specifically, preterm infants are typically hospitalized for prolonged periods in the neonatal intensive care unit (NICU), experience variable durations and intensities of enteral feeding and maternal milk consumption, are exposed to frequent courses of antibiotics, and reside in microbially controlled environments.[3] Early-life hospitalization of preterm infants has been associated with persistent enrichment of antibiotic resistance genes in the gut and altered community profiles.[4] Compared to age-matched infants born after full-term gestations, infants born preterm have lower gut microbial richness and diversity,[4] which may impact intestinal development and increase the risk of immune and metabolic diseases later in life.[2] Gut microbial community composition and diversity are also associated with several pathologies and comorbidities afflicting preterm infants.[2] Gut microbiome modulation is, therefore, an appealing approach to preventing disease in infants born preterm and a strategy that is particularly timely given our expanding insights into human gut microbial communities.

Neonates have morbidity and mortality rates proportional to the degree of prematurity at birth.[5] Infants born after shorter gestations

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(i.e., <32 weeks) are especially vulnerable to necrotizing enterocolitis (NEC), a devastating necroinflammatory event that affects ~ 7% of very low birthweight (VLBW) infants (<1,500 g at birth) and has a mortality rate approximating 25%.[6] NEC often requires surgery to remove nonviable bowel, and survivors are prone to lifelong complications such as short-bowel syndrome and impaired neurodevelopment.[7] The precise etiology of NEC is unknown, but signatures of antecedent aberrant pre-NEC gut microbial communities prompt speculation that gut microbial populations could initiate organ injury.[8,9] Preterm birth is also a risk factor for late-onset sepsis (LOS), which develops after the first 72 hours of life and affects > 35% of extremely preterm infants (i.e., those born at <28 weeks GA).[10] Preterm infants have greater intestinal permeability than term infants,[11] which may facilitate translocation of enteric bacteria into the bloodstream. Indeed, data demonstrate that bloodstream pathogens can be cultured from stool prior to systemic invasion,[12] whereas the presence of *Bifidobacterium* in the preterm gut is associated with protection from LOS.[13] Given these findings, the infant gut microbiome has been proposed as a therapeutic target for the prevention of NEC and LOS. Finally, preterm infants commonly experience feeding intolerance (FI), defined as an inability to digest enteral feeds and which delays progression to full enteral feeding, prolonging hospitalization.[14] As gut bacteria affect intestinal homeostasis, microbiome-directed therapies may also present opportunities for improved nutritional support within NICUs.

While neonatal care has advanced significantly, convincing evidence in support of therapies that reduce morbidity and mortality by manipulating the gut microbiome composition or function in this vulnerable population remains elusive. Probiotics and prebiotics are microbiome-targeting therapeutics that have attracted intense research in neonatology in recent years.[15] However, altering the composition and function of the microbiome, while appealing, must be approached with caution. In this review, we provide a landscape analysis of the current state of the field, focusing on meta-analyses and systematic reviews published between 2017 and 2022 that have evaluated the performance of probiotics and prebiotics in clinical trials and studies. We study three different interventions to alter gut microbial content and/or function (probiotics, prebiotics, and synbiotics), and focus on studies in which the primary or secondary outcomes were prevention of NEC, LOS, FI, and/or reduction in hospitalization length or all-cause mortality.

Probiotics

Background

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer health benefit(s) on the host.[16] For vulnerable preterm infants, probiotics include bacteria and, to a lesser extent, yeast, that have been associated with healthy intestinal development and immune function. Bifidobacteria and lactobacilli have been the most studied, followed by *Streptococcus thermophilus* and the yeast *Saccharomyces boulardii*.[15]

Probiotic bacteria ferment indigestible carbohydrates such as human milk oligosaccharides (HMOs) abundant in human milk into short-chain fatty acids (SCFAs), which have beneficial effects on gut health.[17] Some bifidobacteria are especially robust producers of acetate, which enhances gut barrier function and lowers luminal pH, general attributes of a healthy gut.[18,19] Other commensals convert acetate and lactate produced by lactobacilli and bifidobacteria into butyrate, which fuels colonocytes and is considered anti-inflammatory.[20–22] *In vitro* studies demonstrate that some probiotics inhibit pathogen adherence to intestinal epithelial cells[23,24] and may have beneficial immunomodulatory (i.e., anti-inflammatory) properties.[25] Several trials have reported that preterm infants receiving probiotic supplementation have increased gut microbiome diversity and/or reduced abundance of disease-associated pathobionts, presumably via competitive exclusion.[19,26–29]

Given these theoretically beneficial properties, the concept of using probiotics to support healthy infant development and prevent disease is attractive. Indeed, many studies have been performed to determine if probiotics improve clinical outcomes in preterm infants, though considerable heterogeneity

exists between patient cohorts (e.g., average GA and birthweight (BW)) and probiotic formulations used in these trials, a challenge that has been emphasized in recent network meta-analyses.[30–32] In the following section, we analyze the existing evidence for the effect of probiotic administration on disease outcomes among preterm infants, highlighting consensus recommendations and safety considerations. In our review, we prioritize randomized controlled trials (RCTs).

Single- versus multiple-strain probiotics in infants born preterm – general considerations

It remains unclear how multi-strain probiotics may exhibit additive or synergistic effects that outperform single-strain probiotics.[33] Additionally, some mechanisms, including HMO utilization, are strain-specific, which has implications for the selection of probiotic strains for clinical use in the NICU.[34,35] *Bifidobacterium* species (spp.) have been the most common single-strain probiotic intervention tested, and combined *Bifidobacterium* spp. and *Lactobacillus* spp. comprise the most common multi-strain formulations.[36] Most single and combination probiotics have been studied few times in preterm infants, complicating comparisons of their efficacy between trials; indeed, a 2018 network meta-analysis found that a minority of the 25 studied probiotic strains or strain combinations showed any effect in reducing mortality or morbidity.[30] Although some studies report no difference between the effects of single- and multi-strain mixtures,[37,38] the consensus favors multi-strain formulations,[31,32,39–41] with specific outcomes discussed in further detail below.

Necrotizing enterocolitis (NEC)

The first cohort study investigating the effects of probiotics on NEC and NEC-related death in preterm infants was conducted in 1999 in Colombia by Hoyos et al.[42] In a pre-post analysis of 1,237 newborns, the administration of a combination of organisms (250 million live *L. acidophilus* and 250 million *B. longum* subspecies (subsp.) *infantis* (*B. infantis*) per dose) was associated with significantly decreased rates of NEC and NEC-related mortality compared to the prior year. Many subsequent RCTs, observational studies, and meta-analyses that combine these study results have tried to demonstrate whether probiotics prevent severe NEC (Bell stage ≥ II[43]) and associated mortality in preterm infants. A 2020 Cochrane Database systematic review by Sharif et al. reported results in favor of probiotic treatment in VLBW infants, calculating a reduced risk of NEC for trials at low risk of bias (16 trials; $N = 4{,}597$; risk ratio (RR): 0.70; 95% confidence interval (CI): 0.55, 0.89; study heterogeneity (I^2)) 25%).[36] Similar findings were reported in a 2017 meta-analysis, which concluded that probiotics resulted in a 45% reduction in NEC incidence in VLBW infants (25 RCTs; $N = 8{,}492$; 95% CI: 0.43, 0.70; $p < 0.001$).[44] A combination of *Lactobacillus* spp. and *Bifidobacterium* spp. showed the strongest evidence of effect against NEC (RR: 0.36; 95% CI: 0.23, 0.59) with no heterogeneity in meta-analysis ($I^2 = 0$)[36]. This finding was replicated in a 2020 meta-analysis conducted by Morgan et al. (odds ratio (OR): 0.35; 95% CI: 0.20, 0.59; $I^2 = 0$).[31] Despite such evidence of significantly reduced NEC incidence following probiotic administration, most benefit accrues to infants with BW > 1,000 g.[31,40,45–50] For instance, in a large, well-conducted RCT (ProPrem trial, $N = 1{,}099$) including VLBW infants with GA at birth <32 weeks, administration of a daily probiotic combination (*B. infantis BB–02* 300, *S. thermophilus TH–4* 350, and *B. lactis BB–12* 350, containing 1×10^9 total organisms) significantly reduced rates of NEC (RR: 0.46; 95% CI: 0.23, 0.93; $p = 0.03$).[45] However, a subgroup analysis of extremely low BW (ELBW) infants (<1,000 g) revealed that probiotics did not reduce NEC rates (RR: 0.73), with a non-significant interaction between probiotic treatment and GA or BW subgroup determined by logistic regression ($p = 0.08$).

However, probiotics have not uniformly reduced NEC rates in RCTs. The Probiotics for Preterm Infants (PiPS) trial, the largest probiotic RCT ($N = 1{,}315$) conducted to date to prevent NEC, allocated infants born between 23- and 30-weeks GA to receive *B. breve* BBG–001 (6.7×10^7 to 6.7×10^9 colony-forming units (CFUs)) or placebo. No difference in the primary outcomes of NEC or mortality was discerned.[51] Two observational studies even reported that probiotics were associated with

a higher incidence of NEC, though both studies used historical controls prior to routine probiotic use at a single center.[52,53] Furthermore, as mentioned above, there is a paucity of safety and efficacy data for probiotics in preventing NEC in ELBW infants, the subgroup at highest risk for NEC and other prematurity-related disorders.[47,54] Though some studies indicate significant reductions in NEC in probiotic-treated ELBW infants,[46,55] most conclude that probiotics do not prevent NEC in this lowest BW subgroup,[36,45,47,54,56,57] as in the rigorous ProPrem trial described above.[45] Sharif et al. amalgamated these studies after a subgroup analysis focusing on ELBW infants and arrived at the same conclusion ($N = 1{,}712$; RR: 0.90; 95% CI: 0.68, 1.21; low certainty of evidence).[36] The less developed gut physiology as well as higher rates and duration of antibiotic exposure in ELBW infants possibly contribute to differences in observed efficacy according to BW.[2,58]

Inconsistent case definitions of NEC among probiotic trials might have contributed to the non-uniform results.[59,60] Most papers specify the case definition as Bell stage ≥ II,[43] and do not include spontaneous intestinal perforation or Bell stage I cases. However, not all of them use the same Bell stage definition or apply additional clarification of Bell clinical diagnostic criteria such as the Vermont Oxford Network (VON) or the Centers for Disease Control and Prevention (CDC) definition.[59,60] To ensure comparability and generalizability of study results, there is a need for a more consistent NEC case definition. Case fatality rates may be one metric that can indirectly address sensitivity and specificity of definitions used by relating NEC severity between studies.

Late-onset sepsis (LOS)

Several studies and systematic reviews report that probiotics significantly reduce LOS rates in infants born preterm or with low BW (<2,500 g),[55,61] including VLBW infants.[46,53,54,62] For example, Sharif et al.[36] reported that probiotics probably reduce LOS ($N = 9{,}762$; RR: 0.89; 95% CI: 0.82, 0.97; moderate certainty of evidence) in VLBW infants. Most, however, report no significant reduction of LOS after probiotic supplementation in infants born preterm.[31,36,45,55,63–66] Specifically, the large ProPrem[45] and PiPS[51] studies reported no difference in rates of LOS between the probiotic and control groups.[67] Such findings in large and rigorous RCTs suggest that probiotic prophylaxis does not consistently prevent LOS in infants born preterm. Despite this lack of apparent efficacy, it is notable that probiotics do not considerably increase sepsis risk in the NICU, a safety concern given the increased intestinal permeability observed in preterm infants[11]; reports of probiotic-induced bloodstream infection are rare and respond well to antimicrobials,[68] though they highlight the need for active surveillance in probiotic trials.

Feeding intolerance (FI)

Probiotics have demonstrated mixed efficacy in improving feeding intolerance (FI) in preterm infants. A recent RCT found that a multi-strain probiotic supplementation of five *Lactobacillus* and *Bifidobacterium* spp. strains (*B. breve* HA −129 (1.2 billion CFUs), *B. bifidum* HA−132 (800 million CFUs), *B. longum* subsp. *infantis* HA−116 (600 million CFUs), *B. longum* subsp. *longum* HA−135 (400 million CFUs), and *L. rhamnosus* HA−111 (1.0 billion CFUs)) in 62 ELBW infants significantly decreased time to reach full enteral feeds compared to controls.[56] A recent meta-analysis of nine studies and over 1,200 infants born preterm reports that probiotics significantly reduced the incidence of FI and other related outcomes.[69] However, other studies,[44,49,66] including a 2021 meta-analysis,[55] found no such benefit.

The protean features of FI include abdominal distension, blood in stool, vomiting, bilious aspirate, time to reach full enteral feeds, and gastric residual volume. Significant variability in case definitions of FI makes it difficult to interpret the efficacy of interventions targeting this clinical entity.[70,71] A recent systematic review of current definitions used to diagnose FI identifies multiple different combinations and descriptions of these variables.[70] There is a clear need for a standardized definition of FI to gain clarity about the value of probiotics in RCTs that attempt to prevent or reduce the frequency of this clinical entity.

All-cause mortality

Lastly, we summarize the effects of probiotics on all-cause mortality, a categorical and important outcome. A 2017 meta-analysis reported results in favor of probiotic treatment in VLBW infants, calculating 22% reductions in mortality (21 RCTs; $N = 7{,}332$; 95% CI: 0.66%, 0.93%; $p = 0.01$).[44] At the species level, Morgan et al.[31] reported that a combination of 1 or more *Lactobacillus* spp. and 1 or more *Bifidobacterium* spp. reduced all-cause mortality (52 studies; $N = 14{,}003$; OR: 0.56; 95% CI: 0.39, 0.80; risk difference (RD): −2.2%; 95% CI: −3.1, −0.1; high certainty of evidence), as discussed in detail below. Consistent with these findings, at the strain level, van den Akker et al.[30] found that 3/25 of their tested strain combinations showed significant reductions in mortality rates: *B. bifidum* NCDO 1453 and *L. acidophilus* NCDO 1748 (two studies, $N = 494$); *B. bifidum*, *B. infantis*, *B. longum*, and *L. acidophilus* (one study, $N = 186$); and *B. infantis*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, and *S. thermophilus* (one study, $N = 150$). While these results are encouraging, they underscore the fact that not all probiotic formulations are equivalent, and robust evidence is needed at the strain-level to support evidence-based clinical recommendations of probiotic treatment for preterm infants.

Safety

Probiotics in the U.S. are considered dietary supplements and therefore are not subject to the degree of oversight that the Food and Drug Administration (FDA)[72] provides to drugs. In 2015, a premature infant died of a rare fungal infection after being administered a probiotic contaminated with *Rhizopus oryzae*,[73] raising concerns over possible contamination during manufacturing.[72] In another study, only one of 16 commercial probiotics matched its label organism for organism.[74] For this reason, it is advised that probiotics be evaluated for purity and viability before administration and again during a trial, but this practice has not been universally applied.[75] Also, there are few assessments of safety beyond the administration period, though this is a general limitation of many therapeutic interventions.

Ensuring product purity is especially important for preterm infants whose compromised immune systems render them susceptible to outgrowth of even commensal bacterial species.[76,77] In a recent systematic review of probiotic sepsis in preterm infants including reports published through January 2022, Kulkarni et al. found 16 studies with 32 total cases of probiotic-related sepsis, of which 25 cases were confirmed to be caused by the administered probiotic strain after full genomic analysis.[68] Further, they note that while there were no episodes of probiotic sepsis reported in the 2020 network meta-analysis by Morgan et al. (63 RCTs, $N = 15{,}712$),[31] only 12 RCTs conducted active and extended surveillance for such an event.[68] Many probiotics require anaerobic growth conditions, and that requires extra blood, incubated appropriately, to identify extra-intestinal dissemination accurately. Thus, although some studies have reported adverse event rates (most commonly bloodstream infections with the probiotic strain,[77–79]) uniform large-scale monitoring of such events and long-term effects are needed to evaluate the safety of the intervention more thoroughly.[64] Lastly, commercial probiotics can carry transferable antibiotic resistance genes (ARGs),[80] which was linked to a vancomycin-resistant Enterococcus outbreak among probiotic-treated VLBW infants in a Turkish NICU.[81] As preterm infants receive frequent courses of antibiotics that apply selective pressure to commensal microbes and are at particularly high risk of infection,[3] care should be taken to select probiotic strains that minimize the potential of ARG dissemination to the existing microbiome.

Limitations to published studies of probiotics in preterm

Infants

Marked heterogeneity among studies prompts caution when endorsing probiotics as standard of care for premature infants.[47,72,82,83] Studies often evaluate different probiotic species and strains that can

have quite different theoretical efficacy profiles, thereby lending bias to outcome measurements.[84] Differences in probiotic dose, length of treatment, and viability may further contribute to conflicting findings.[36,47] For example, in their network meta-analysis, Morgan et al. report substantial heterogeneity ($I^2 = 53.6\%$) among studies comparing the effect of *Bifidobacterium* spp. and *Streptococcus salivarius* subsp. *thermophilus* with placebo on NEC.[31] Substantial heterogeneity ($I^2 > 50\%$) was also reported for the comparisons of *L reuteri*; *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces boulardii*; *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp.; *Lactobacillus* spp. and *Bifidobacterium* spp.; and *Bifidobacterium* spp. and *S. salivarius* subsp. *thermophilus* vs. placebo on culture-proven LOS. It is worth noting, however, that the reported statistical heterogeneity was zero ($I^2 = 0$) for studies that evaluated the effect of the probiotic combination of *Lactobacillus* spp. and *Bifidobacterium* spp., which has shown the most evidence of having a protective effect against NEC based on GRADE certainty of evidence.[31,36] This suggests that at least for the combination of *Lactobacillus* spp. and *Bifidobacterium* spp. and NEC, there is low heterogeneity between trial results.

Several studies reported colonization of placebo infants which may have confounded negative results.[51,85,86] For example, the PiPS trial is one of the few RCTs that monitored rates of cross-contamination between the probiotic (*B. breve* BBG−001) and placebo arm. The authors report that *B. breve* BBG−001 was detected by culture in 49% of infants in the placebo group by 36 weeks postmenstrual age despite measures to minimize the possibility of cross-contamination such as cleaning all working surfaces after completing intervention preparations for each baby.[51] This underscores the need for more active surveillance for cross-colonization in probiotic RCTs.

Current recommendations

It is notable that professional societies have recently issued conflicting guidance regarding probiotic prophylaxis in preterm infants. While the American Gastroenterological Association (AGA)[87] and World Health Organization (WHO)[88] have given conditional recommendations for the use of probiotics in preterm infants based on moderate/high certainty evidence, the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) has taken an intermediate stance,[89] and the American Academy of Pediatrics (AAP) cautioned against the routine, universal use of probiotics in the NICU, especially for ELBW infants.[47]

The new 2022 WHO guidelines[88] broadly recommend probiotics for human-milk-fed very preterm infants based on the Cochrane review by Sharif et al.[36] These recommendations are conditional upon shared decision-making with parents,[88] emphasizing the need for clear evidence of benefits and safety that can be communicated to families when probiotics are offered. The AGA[87] recommend certain single- and multi-strain combinations of *Lactobacillus* spp. and *Bifidobacterium* spp. for preterm infants based on findings from the network meta-analysis by Morgan et al.[31] discussed in detail in a later section. The ESPGHAN[89] conditionally recommends specific strains to reduce NEC based on low certainty of evidence, while recommending against certain strains based on lack of efficacy and safety concerns. In all cases of conditional positive recommendations, the dosage, timing, and duration of treatment are not specified due to lack of data on optimal treatment course. In contrast, the AAP[47] supports a more cautionary approach based on the current lack of pharmaceutical-grade probiotics, variability in tested formulations, limited long-term safety information, and less convincing findings in ELBW infants. Unless compelling data – double-blind RCTs that are adequately powered to assess effects in ELBW infants, ensure purity and viability of the multi-strain probiotic compound (*Lactobacillus* spp. and *Bifidobacterium* spp.) prior to supplementation, and closely monitor stool samples for cross-contamination – demonstrate significant benefits in predeclared primary outcomes and long-term safety profiles,[90] many experts will likely continue to recommend against routine administration of probiotics to preterm infants. Considering these limitations, we believe there are insufficient data to currently endorse the routine, universal

administration of probiotics to preterm infants to prevent NEC, mortality, LOS, or FI.

Prebiotics

Background

Prebiotics, i.e., substrates used by host microbes to confer health benefit(s),[91] are another approach to targeting the preterm infant microbiome for disease prevention. Prebiotics are most commonly oligosaccharides that can be fermented by commensal bacteria to promote their colonization and growth in the intestine. Human milk provides an abundance of prebiotics in the form of HMOs.[92] These complex glycans resist degradation by host enzymes and arrive in the colon intact, where they serve as carbon sources for bifidobacteria (especially *B. infantis*), *Bacteroides*, and lactobacilli[93–96]. Bacterial fermentation of HMOs produces SCFAs, which promote epithelial barrier integrity and have anti-inflammatory activity *in vitro*.[97,98] Bacterial strains in the infant gut differ in their ability to ferment HMOs, and by-products generated by HMO metabolism can support the growth of other commensals through cross-feeding.[34,93] Besides promoting the growth of beneficial bacteria, HMOs have potentially immunomodulatory properties,[92] and some HMO-derived oligosaccharides can prevent Group B *Streptococcus* growth *in vitro*.[99,100] HMOs can also function as soluble decoy receptors, preventing adherence of pathogens to host cells *in vitro*.[101]

The most frequently studied prebiotic substrates include short-chain (sc) galacto-oligosaccharides (GOS), long-chain (lc) fructo-oligosaccharides (FOS), lactulose, and acidic oligosaccharides (AOS).[102] AOS comprise 12–14% of the total HMO fraction in human milk and pectin-derived AOS (pAOS) are included in some prebiotic mixtures.[103,104] As over 200 structurally unique HMOs have been described, commercially produced substrates cannot fully mimic the complexity of HMOs in human milk.[95] In turn, human milk-derived fortifiers containing donor-derived HMOs have been used to increase protein intake as part of an exclusive human milk diet.[95] However, these are not reviewed here as they also contain other bioactive human milk-derived compounds and evidence supporting their usage is currently limited. Plant-derived prebiotic oligosaccharides are thus low-cost alternatives to HMOs that approximate their beneficial effects. Preterm infants receiving prebiotics have increased bifidobacteria and reduced abundance of coliforms and potential pathogens in stool.[105–109] Considerably fewer trials have been conducted with preterm infants to evaluate the efficacy and safety of prebiotic supplements compared to probiotics, limiting the generalizability of results supporting their use. Nonetheless, prebiotics are appealing because they do not involve the administration of live bacteria, which carries a small risk of sepsis.[68] It is also probably easier to standardize prebiotic than probiotic product quality. In the following section, we review the findings from existing studies in preterm infants assessing the effects of prebiotics on the aforementioned outcomes.

NEC

Infants fed partially or exclusively with human milk are at decreased risk for NEC.[110] Among human milk's many bioactive compounds, HMOs are thought to be beneficial by modulating endogenous gut microbes and host defenses,[92] and commercial prebiotics may simulate these protective effects. Armanian et al. reported that infants receiving human milk supplemented with a 9:1 scGOS/lcGOS mixture had significantly less risk of suspected, but not proven, NEC in an RCT; however, providers were not blinded to an infant's treatment.[111] In contrast, four studies of preterm infants fed partially or exclusively with prebiotic-supplemented formula found no reduced NEC risk[112–115.] A 2019 meta-analysis concluded that based on pooled effects from 6 trials, prebiotics did not significantly reduce NEC morbidity (N = 737; RR: 0.79; 95% CI: 0.44, 1.44; $p = 0.44$).[116] Taken together, current data do not favor using prebiotics to prevent NEC. While these data are perhaps surprising given the presumed protection from NEC afforded by HMOs, they underscore the need to better understand the effects of prebiotic administration on bacterial function in the already perturbed preterm gut.

LOS

Prebiotics promote commensal bacteria growth and reduce pathogen adherence *in vitro*.[34,94,101] However, only one RCT, the ProPre-Save study, reported a lower frequency of LOS among preterm infants who received a prebiotic.[114] Other RCTs showed no benefit or only a statistically insignificant trend toward efficacy.[111–113,115,117] A 2019 meta-analysis by Chi et al. including 11 RCTs reported that LOS rates were significantly less in preterm infants treated with prebiotics (N = 1,106; RR: 0.64; 95% CI: 0.51, 0.78; $p < 0.001$).[116] A subgroup analysis showed that this effect was stronger for prebiotics added to human milk ($p < 0.001$) rather than distilled water ($p = 0.28$) and for those containing pAOS ($p < 0.001$), which have reported antibacterial properties,[99] compared to those without pAOS ($p = 0.13$). These conflicting results highlight the need for further research to understand if, and to what extent, prebiotic supplementation reduces LOS risk in infants born preterm.

FI

Several studies have reported that prebiotic supplementation softened stool and increased defecation frequency in preterm infants.[105,107,113,114,118] Additionally, one RCT of preterm infants given a 9:1 GOS/FOS mixture demonstrated that treated infants had decreased gastrointestinal transit time.[118] However, other studies found no significant changes in stool characteristics in infants born preterm.[109,112,117] The different prebiotic compositions, feeding modes, treatment dosages, and definitions of FI likely contribute to these mixed observations. When examining FI as a primary or secondary outcome, many studies report that prebiotics are well tolerated by preterm infants but do not necessarily improve or prevent this condition,[113,117,118] though a shortened timeframe to achieving full enteral feeds has been observed in some infants.[111,114] Interestingly, Modi et al. found a small but significant improvement in enteral tolerance among preterm infants given formula supplemented with 9:1 scGOS/lcFOS, an effect that was more pronounced at lower GA.[112] The meta-analysis by Chi et al. reported that prebiotics were overall associated with a shortened time to full enteral feeding (six trials; $N = 576$; mean difference (MD): −0.99; 95% CI: −1.15, 0.83; $p < 0.001$) but did not influence the degree of FI (four trials; N = 413; RR: 0.87; 95% CI: 0.52, 1.45; $p = 0.6$).[116] These conclusions warrant further study to determine if prebiotics enhance enteral tolerance, particularly in the most premature infants. However, as for probiotics, variable case definitions make it difficult to conclude that prebiotics can prevent or lessen the severity of FI.

Safety and efficacy

In adults, prebiotics can cause symptoms such as flatulence and abdominal bloating, especially at high doses.[119] However, prebiotics in preterm infants appear to be safe and well-tolerated, as adverse effects have not been documented. The long-term effects of prebiotic supplementation in preterm infants are also not well understood, but a follow-up study over the first year of life did not report adverse effects related to prebiotic treatment.[120]

Concerns have been raised that prebiotics could promote the growth of enteropathogens.[121] However, dietary prebiotics that resemble HMOs are preferentially metabolized by bifidobacteria and lactobacilli, a process that bolsters protection from pathogens.[18] Given preterm infants' especially vulnerable immune defenses, a better understanding of how prebiotics affect bacterial growth in the infant gut is needed to fully evaluate their safety within this population.

A 2019 meta-analysis suggested that prebiotics overall may benefit preterm infants by decreasing the rate of LOS and mortality and shortening the length of stay and time to reach full enteral feeds.[113] However, there was no significant effect on NEC incidence. Prebiotics may be low-cost interventions that benefit preterm infants and augment the effects of probiotics. Nonetheless, more trials are necessary to confirm or refute the postulated benefit of the most promising prebiotics on outcomes in infants born preterm. Until then, existing data suggest that while prebiotics are safe and well-

tolerated by preterm infants, their benefits are modest at best.

Synbiotics

Synbiotics combine probiotics and prebiotics in single administrations. Their combination enhances probiotic colonization and promotes therapeutic effects. Healthy term infants treated with a combination of *L. plantarum* and FOS for 7 days had high colonization rates of the probiotic for several months after treatment.[122] Most impressively, a large RCT of 4,556 term infants in rural India receiving this synbiotic reported reduced rates of LOS and mortality in the treatment group during the first 60 days of life.[123] Synbiotics have been evaluated in several RCTs in preterm infants. An RCT of exclusively breastfed preterm infants ($N = 220$) found that a consortium of *Lactobacillus* and *Bifidobacterium* spp. strains combined with FOS significantly reduced time to full enteral feeds, and there was a trend toward reduced NEC.[124] However, the intervention did not lower the risk of LOS. In comparison, the ProPre-Save study of 400 VLBW infants evaluated whether administration of a probiotic (*B. lactis*), prebiotic (inulin, a plant-derived lcFOS), or synbiotic (*B. lactis* plus inulin) reduced NEC risk compared to placebo.[114] Infants receiving the probiotic or synbiotic had a similarly reduced NEC risk, but the prebiotic alone did not have a significant effect. A 2022 Cochrane Database systematic review found low-certainty evidence that synbiotics reduce the risk of NEC (six trials; $N = 907$; RR: 0.18; 95% CI: 0.09, 0.40; $p < 0.0001$) and all-cause mortality (six trials; $N = 925$; RR: 0.53; 95% CI: 0.33, 0.85; $p = 0.008$), and very low-certainty evidence about their effect on LOS (five trials; $N = 707$; RR: 0.84; 95% CI: 0.58, 1.21; $p = 0.34$).[125] More specifically, a 2021 network meta-analysis including both probiotic and synbiotic intervention trials among preterm infants found that *Lactobacillus* spp. plus prebiotic was associated with a lower incidence of NEC (RR: 0.06; 95% CI: 0.01, 0.41) and LOS (RR: 0.18; 95% CI: 0.06, 0.44) compared to placebo, and *Bifidobacterium* spp. plus prebiotic was most strongly associated with lower mortality rates (surface under the cumulative ranking curve 83.94%) of the tested interventions.[32] Synbiotics may offer synergy between the protection afforded by probiotics and prebiotics individually. However, more RCTs that include larger numbers of preterm infants are necessary to rigorously determine the efficacy of synbiotic formulations on preterm neonatal health and their safety profile.

Probiotic efficacy: a closer look

Professional societies recently issued differing recommendations for using probiotics in infants born preterm to prevent NEC,[47,87–89] based on the same meta-analyses that we reviewed. Such variations in interpretations of the existing literature prompted us to review a subset of the primary literature cited on probiotics in preterm infants.

To do this, we used as a starting point the comprehensive and well-annotated network meta-analysis of Morgan et al.[31] These authors analyzed 63 publications reporting the use of probiotics in RCTs in which 15,712 preterm infants were enrolled. We focused on 21 of these publications (20 papers, one meeting abstract), because they were considered to provide, in the aggregate, moderate- to high-certainty evidence and statistically significant differences between the treatment and at least one other treatment and placebo in pooled analyses. These "extreme of effect" studies underlie the forest green cells representing four outcomes of interest (all-cause mortality, NEC occurrence, reduction in days to reach full feeds, and reduction in duration of hospitalization (DOH)) in Figure 2 in the meta-analysis by Morgan et al[31] as summarized in Table 1.

Morgan et al. concluded (1) *Lactobacillus* spp. and *Bifidobacterium* spp. prevented all-cause mortality and NEC, (2) *B. animalis* subsp. *lactis* prevented NEC and reduced DOH, (3) *L. reuteri* reduced DOH, and (4) a combination of *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces boulardii* reduced days to reach full feeds.[31] Two authors (AD, JR) examined the methodology and primary data in these primary papers, independently scored risk of bias in domains of sequence generation, allocation concealment, and blinding of data collectors/

Table 1. Probiotic interventions with efficacy against selected outcomes as supported by moderate to high certainty evidence and statistically significant differences between the treatment and at least one other treatment and placebo in pooled analyses (from Figure 2 in Morgan et al.[31]

Intervention	Outcome	References
Combination of *Lactobacillus* spp. and *Bifidobacterium* spp.	All-cause mortality	Lin et al.[126] Lin et al.[127] Samanta et al.[128] Rougé et al.[129] Braga et al.[130] Al-Hosni et al.[49] Roy et al.[131] Saengtawesin et al.[132] Van Niekerk et al.[133]
Bifidobacterium animalis subsp. *lactis*	NEC occurrence	Mihatsch et al.[134] Stratiki et al.[135] Hays et al.[136] Dilli et al.[114]
Combination of *Lactobacillus* spp. and *Bifidobacterium* spp.		Lin et al.[126] Lin et al.[127] Samanta et al.[128] Rougé et al.[129] Braga et al.[130] Al-Hosni et al.[49] Roy et al.[131] Saengtawesin et al.[132] Van Niekerk et al.[133]
Combination of *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces boulardii*	Days to reach full feeds	Shashidhar et al.[137] Hariharan et al.[138] Arora et al.[139]
Bifidobacterium animalis subsp. *lactis*	Duration of hospitalization	Mihatsch et al.[134] Stratiki et al.[135] Hays et al.[136] Dilli et al.[114]
Lactobacillus reuteri		Romeo et al.[140] Rojas et al.[141] Indrio et al.[142] Oncel et al.[143] Hernández-Enríquez et al.[144]

outcome assessors, noted if the study was registered at a public website such as ClinicalTrials.Gov or presented in a protocol paper, and assessed if the outcome referenced in Morgan et al.[31] was the primary goal of the study (Tables 2–5). Clinical content experts PIT and BBW were available to adjudicate discordant assessments, but agreement on all papers reviewed by AD and JR obviated the need for resolution.

Of the 21 publications, one (Hariharan et al.[138]) was an abstract that contained insufficient data and information to analyze. Only 12 studies relevant to the outcomes of interest were registered with clearly declared primary and secondary outcomes, so the reader can determine if the outcomes were pre-specified, or exploratory. While 17 of the 21 publications provided sufficient information to enable the reader to conclude that there was low risk of bias in treatment sequence generation and allocation concealment, only seven provided unambiguous information to conclude that there was low risk of bias by data collectors or outcome assessors. In 12 of the 21 studies, none of the outcomes of interest was a primary outcome of the study or designated either as a secondary outcome. Only six of the 21 studies met all criteria by being pre-registered and providing sufficient assurance that methods of sequence generation, allocation concealment, data collection, and outcome assessment had low risks of bias. Of these six studies, none showed a statistically significant benefit of probiotics for a primary outcome, and two showed a benefit for a secondary outcome. The papers cited in support of the value of probiotics in the five outcomes of interest enrolled their last participant a median of 11.5 years ago, and the most recent of the studies enrolled its last participant in 2016 (Table 6).

Even though there is little evidence in favor of probiotics improving the five outcomes of interest, it is possible that there was benefit to probiotics despite the non-significant differences between treatment and control groups. Specifically, two sources of type II error (failing to find a benefit to

Table 2. Effects of *Bifidobacterium animalis* subsp. *lactis* on NEC risk and/or duration of hospitalization (DOH).

Study	Country	NEC (Bell stage ≥ II) frequency (no. with event divided by no. in treatment arm)		Average duration of hospitalization (days)		Registered	Sequence generation & Allocation concealment	Data collectors/ outcome assessors blinded	Year last enrolled participant	Primary Outcome(s)	Comments
		Treatment	Control	Treatment	Control						
Stratiki et al.[135]	Greece	0/41	3/36	NA	NA	No	Low risk	Clinical staff were blinded as to treatment status, but blinding of outcome assessors not specified in text.	2005	Intestinal permeability	NEC stated as secondary outcome
Mihatsch et al.[134]	Germany	2/91	4/89	31.1	30.8	No	Low risk	Blinding of caregivers and investigators not specified in text.	2003	Nosocomial infections	NEC stated as secondary outcome
Dilli et al.[114]	Turkey	2/100*	18/100*	37*	50*	Yes (NCT01807858)‡	Low risk	The study is described as double blind in the methods, and the authors provide assurance that the staff and families were not aware of the treatment or control status of the infants. However, they also state that " . . . the only personnel who knew of the infants' group assignments were the investigators," but do not provide specific assurance that the treatment assignment was concealed to the outcome assessors.	2014	NEC	DOH stated as secondary outcome
Hays et al.[136]	France	2/50	3/52	"The mean duration of hospitalization was 50.4 ± 17.4 days, which was similar for both treatment groups."		Yes (NCT01379417)‡	Low risk	The investigators knew infants' group assignments.	2010	Short-term postnatal growth and body composition	Outcome category unclear but not primary; secondary outcome was safety of probiotic administration

NA = not available.
*Difference in outcome between treatment and control arms was significant.

Table 3. Effects of *Lactobacillus reuteri* on duration of hospitalization (DOH).

Study	Country	Sample size		Average duration of hospitalization (days)		Registered	Sequence generation & Allocation concealment	Data collectors/outcome assessors blinded	Year last enrolled participant	Primary Outcome(s)	Comments
		Treatment	Control	Treatment	Control						
Romeo et al.[140]	Italy	83	83	17.8	31.3	No	The three arms had different dosing regimens and were, therefore, not concealed to the staff or families.	UTD	2007	Incidence of enteric fungal colonization and fungal and bacterial infections	Outcome category unclear but not primary
Rojas et al.[141]	Colombia	372	378	20.0	20.0	Yes (NCT00727363)‡	Low risk	UTD	2011	Death or nosocomial infection	DOH stated as secondary outcome. Subgroup analysis demonstrated that this outcome was significant for infants with birth weight <1500 g.
Oncel et al.[143]	Turkey	200	200	38*	46*	Yes (NCT01531179)‡	Low risk	Low risk	2013	NEC or death	DOH stated as secondary outcome
Hernández-Enríquez et al.[144]	Mexico	24	20	39.3	50.6	No	Low risk	The neonatologists who diagnosed and classified necrotizing enterocolitis knew the group allocation.	2013	NEC	Outcome category unclear but not primary
Indrio et al.[142]	Italy	30	30	13.4*	22.4*	Yes (NCT00985816)‡	Low risk	Low risk	2012	Feeding tolerance	DOH stated as secondary outcome

NA = not available.
UTD = unable to be determined based on information provided.
*Difference in outcome between treatment and control arms was significant.

Table 4. Effects of a combination of *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces boulardii* on days to reach full feed.

Study	Country	Treatment	Sample size		Average days to reach full feed		Registered	Sequence generation & Allocation concealment	Data collectors/ outcome assessors blinded	Year last enrolled participant	Primary Outcome(s)	Comments
			Treatment	Control	Treatment	Control						
Hariharan et al.[138]	India	*L. acidophilus*, *B. bifidum*, *S. boulardii*	93	103	23.6*	32.4*	No	UTD randomization method	UTD	UTD	Time to achieve oral feeds	Abstract only
Arora et al.[139]	India	*L. rhamnosus*, *L. acidophilus*, *B. longum*, *S. boulardii*	75	75	8.53	10.7	No	UTD randomization method	UTD	2016	NEC	Days to reach full feed stated as secondary outcome
Shashidhar et al.[137]	India	*L. rhamnosus*, *L. acidophilus*, *B. longum*, *S. boulardii*	48	48	11.2	12.7	Yes (CTRI/ 2012/08/ 002853)¶	Low risk	Low risk	2013	Time to full enteral feeding	

NA = not available.
UTD = unable to be determined based on information provided.
*Difference in outcome between treatment and control arms was significant.

Table 5. Effects of a combination of *Lactobacillus* spp. and *Bifidobacterium* spp. on NEC occurrence and/or all-cause mortality.

Study	Country	Treatment	Mortality (no. with event divided by no. in treatment arm)		NEC (Bell stage ≥ II) frequency (no. with event divided by no. in treatment arm)		Registered	Sequence generation & Allocation concealment	Data collectors/outcome (NEC) assessors blinded	Year last enrolled participant	Primary Outcome(s)	Comments
			Treatment	Control	Treatment	Control						
Lin et al.[126]	Taiwan	*L. acidophilus*, *B. infantis*	7/180*	20/187*	2/180*	10/187*	No	Low risk	The investigators knew the infants' group assignments, but NEC was diagnosed by individuals who were not aware of the allocation of the participants.	2003	NEC or death	Efficacy not reported by birthweight of participants
Lin et al.[127]	Taiwan	*L. acidophilus*, *B. bifidum*	2/217†	9/217†	4/217	14/217	Yes (NCT00540033)‡	Low risk	The investigators knew the infants' group assignments, but NEC was diagnosed by individuals who were not aware of the allocation of the participants.	2007	NEC or death	Protective effect not apparent in children whose birth weights were ≤ 1,000 g
Samanta et al.[128]	India	*B. infantis*, *B. bifidum*, *B. longum*, *B. acidophilus*	4/91*	15/95*	5/91*	15/95*	No	Low risk	UTD	2008	NEC, sepsis, death, days to reach full feed, DOH were co-primary outcomes	P-values not corrected for multiple comparisons
Rougé et al.[129]	France	*B. longum* BB536, *L. rhamnosus* GG	2/45	4/49	1/45	2/49	Yes (NCT00290576)‡	Low risk	UTD	2007	Percent of infants receiving more than half of their nutritional needs enterally	Mortality but not NEC stated as secondary outcome
Braga et al.[130]	Brazil	*L. casei*, *B. breve*	26/119	27/112	0/119	4/112	Yes (67165178)§	Low risk	Low risk	2008	NEC	P-value for efficacy in preventing NEC was 0.054
Al-Hosni et al.[49]	United States	*L. rhamnosus* GG, *B. infantis*	3/50	4/51	3/50	4/51	Yes (NCT01164124)‡	UTD randomization method	UTD	2009	Growth velocity, weight gain, volume of feeding	Outcome category unclear but not primary
Roy et al.[131]	India	*L. acidophilus*, *B. longum*, *B. bifidum*, *B. lactis*	7/56	8/56	2/56	2/56	Yes (CTRI REF/2012/12/004378)¶	Low risk	UTD	2013	Incidence of enteric fungal colonization	NEC stated as secondary outcome
Saengtawesin et al.[132]	Thailand	*L. acidophilus*, *B. bifidum*	0/31	0/29	1/31	1/29	No	Low risk	UTD	2013	NEC	

(*Continued*)

Table 5. (Continued).

Study	Country	Treatment	Mortality (no. with event divided by no. in treatment arm)		NEC (Bell stage ≥ II) frequency (no. with event divided by no. in treatment arm)		Registered	Sequence generation & Allocation concealment	Data collectors/outcome (NEC) assessors blinded	Year last enrolled participant	Primary Outcome(s)	Comments
			Treatment	Control	Treatment	Control						
Van Niekerk et al.[133]	South Africa	*L. rhamnosus* GG, *B. infantis*	2/54 (HIV-unexposed) 3/37 (HIV-exposed)	5/56 (HIV-unexposed) 1/37 (HIV-exposed)	0/54 (HIV-unexposed) 0/37 (HIV-exposed)	2/56 (HIV-unexposed) 2/37 (HIV-exposed)	Yes (NCT01868737)‡	Low risk	Low risk	2012	NEC	

UTD = unable to be determined based on information provided.
* Difference in outcome between treatment and control arms was significant.
† Difference in incidence of death not attributable to NEC was significant.

Table 6. Year at which the last participant of the study was enrolled.

Intervention	Outcome	Year last participant enrolled
Bifidobacterium animalis subsp. *lactis*	NEC, duration of hospitalization	2014, 2010, 2005, 2003
Lactobacillus reuteri	Duration of hospitalization	2013, 2013, 2012, 2011, 2007
Lactobacillus spp., *Bifidobacterium* spp., and *Saccharomyces boulardii*	Days to reach full feed	2016, 2013
Lactobacillus spp. and *Bifidobacterium* spp.	NEC, all-cause mortality	2013, 2013, 2012, 2009, 2008, 2008, 2007, 2007, 2003
All studies		Median: 201.5 (11.5 years ago), IQR: 9–15 years; Mean: 2009.85 (12.15 years ago), SDL 3.73 years

**If the outcome cited in Morgan et al.[31] was categorized as primary or secondary in the original paper, we noted it as such. If the outcome was not specifically designated as secondary in a paper in which another outcome was primary, we described it as outcome category unclear, but not primary.
‡https://clinicaltrials.gov/. There should not be a "." after this url as it causes problems when pasting it into a browser.
¶ctri.nic.in. There should not be a "." after this url as it causes problems when pasting it into a browser.
§isrctn.com. There should not be a "." after this url as it causes problems when pasting it into a browser.

probiotics when one actually exists) may have been present in many trials. First, the propensity of probiotics to colonize controls in the same NICUs raises the possibility that the non-treated group might have been inadvertently treated by nosocomial spread in some studies, thereby reducing the apparent benefit of the intervention.[51,85,86] Second, the probiotics might have lost viability, and treatment groups were not administered a bioactive intervention. However, as many studies evaluated neither for colonization of controls nor for viability of the intervention, such exculpatory interpretations remain speculative. We also note that for the prevention of NEC, a subset of studies suggests that probiotics are effective, and the effects are reproduced in children with BW > 1,000 g, a commonality that lends some biological credence to the concept that microbial therapeutics might reduce NEC risk, though regrettably the benefit is demonstrated in those who are less likely to experience this outcome. We also wish to note that the primary studies were well-intentioned attempts to prevent profound consequences of preterm birth, and some shortcomings, such as non-registration of the trials, were more common in past decades than they are currently.

Meta-analyses attempt to amalgamate data from multiple studies to detect trends that can be employed in clinical settings, but which are not apparent in smaller studies. Morgan et al.[31] and the other five meta-analyses we used as sources of data[30,32,36,44,55] for our review were conducted to combine evidence into point estimates (with confidence intervals) of the effects of interventions combined across multiple studies. These reviews graded and reported the quality of evidence (chiefly risks of bias relative to observed magnitude of effect) according to standard criteria. However, while meta-analyses often identify limitations of primary papers, conclusions do not always convey circumspection warranted by the quality of the data. Hence, while the AGA's recommendation[87] of specific combinations of probiotics " … for prevention of NEC over no and other probiotics" seems commensurate with the review[31] on which it was based, most studies at the root of the meta-analyses, as demonstrated in Tables 2–5, rarely met criteria for trials that would qualify probiotics interventions as drugs. The presentation of data in a graded fashion in meta-analyses to illustrate the effects of risks of bias on assessment of the certainty of the evidence[145] might generate more circumspect recommendations.

Conclusions

It is understandable that gut bacterial communities be considered as targets for beneficial manipulation in infants born preterm. There is increasing evidence that variations in gut microbial makeup are associated with undesirable outcomes in these infants, and such outcomes can be devastating. In the case of probiotics, cogent arguments have been offered in support of these interventions to prevent NEC,[146] especially in view of the difficulties conducting large and convincing RCTs.[147] For centers choosing to use probiotics, the administration of a live biotherapeutic product that is not yet approved by a regulatory authority for this indication is a complex process.[148] However, if current

standards expected of drugs are employed, the interventions discussed in this review do not yet offer sufficient evidence to recommend their routine use in preterm infants.

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