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DEVELOPMENT OF INTESTINAL DYSBIOSIS AND NECROTIZING ENTEROCOLITIS IN INFANTS AND THE TREATMENT OF ENTEROCOLITIS

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DEVELOPMENT OF INTESTINAL DYSBIOSIS AND NECROTIZING ENTEROCOLITIS IN INFANTS AND THE TREATMENT OF ENTEROCOLITIS

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ABSTRACT

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The human gut microbiota is a complex ecosystem of trillions of microorganisms that provide health benefits to the host. Gut colonization begins at birth and is affected by five primary factors – mode of delivery, antibiotic exposure, hygiene, mode of feeding, and prematurity. These colonization factors are also associated with gut microbiota dysbiosis and the development of necrotizing enterocolitis (NEC). NEC is an inflammatory gastrointestinal disease, mainly affecting premature infants. Development of NEC is associated with a distinct bacterial population, an immature immune system and contributes to an exaggerated immune response. This altered microbiota, or dysbiosis, is thought to lead to the chronic inflammation associated with NEC. Breast milk reduces the risk of NEC in preterm infants; however, not all infants will have access to breast milk. The protective effects of break milk are elicited by various components that promote colonization of the infant gut with protective bacteria. These effects may be mimicked by supplementing feedings with pre-, pro-, or synbiotics, promoting the establishment of a healthy microbiota in formula-fed infants. This review summarizes our understanding of the functions and development of the gut microbiota, and its association with dysbiosis and related diseases, with emphasis on NEC and its treatment.

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INTRODUCTION

The human body is colonized by trillions of microbes, with the highest concentration residing in the colon. These resident gut microbes are known as the gut microbiota and include bacteria, viruses, fungi, and protozoa (1). In healthy individuals, the dominant bacterial phyla in the microbiota include Proteobacteria, Bacteroidetes, and Firmicutes, amassing to 500-1000 microbial species per gram of colonic content (2, 3). These resident commensal bacteria interact with each other and their host to acquire and provide nutrients, shape the immune response, and protect the gut from colonization by pathogens (2). Dysbiosis, or alterations in the gut microbiota away from what is regarded as healthy, is associated with adverse health effects (4). Factors that cause dysbiosis include cesarean section, formula feeding, prematurity, antibiotic use, and an overly hygienic lifestyle (5–9). Exposure to any of the factors related to dysbiosis increases the risk of many illnesses such as allergies, asthma, inflammatory bowel disease, irritable bowel syndrome, colic, and necrotizing enterocolitis (NEC) (4, 10).

NEC is the most common gastrointestinal disease acquired by preterm infants, affecting 10-20% of very low birth weight (VLBW) infants with a mortality rate of 30% (11, 12). The annual estimated cost of NEC in the U.S. is between $500 million and $1 billion due to extended hospital stays and the need for surgery, posing a substantial

financial burden to families (13). Several risk factors for NEC have been identified, which include prematurity, antibiotic use, formula feeding, and others that are related to microbial dysbiosis of the infant gut (12, 14). NEC has never been observed in germfree animals, further implicating the association of gut microbiota with the development of NEC (11, 12). Because the etiology of NEC is not fully understood, preventing the disease is difficult. However, research has been started to elucidate potential preventative treatments.

Human breast milk has been known to protect against NEC, with pasteurized donor breast milk being just as protective (15). Since breast milk is incredibly complex, elucidating which components elicit protective effects has been challenging. However, studies have identified several protective components, which include epidermal growth factor (EGF), lactoferrin, lysozyme, and human milk oligosaccharides (HMOs), among others (16, 145-153). Unfortunately, a vast majority of premature infants are not able to be breast-fed or cannot tolerate breast milk, and therefore require formula feeding, which results in a lack of exposure to these protective components of breast milk. Therefore, supplementation of feedings for premature infants with pre-, pro-, and synbiotics as prophylaxis is being investigated as a therapeutic option for the prevention of NEC.

This review serves to give a brief overview of the functions and development of the gut microbiota, focusing on its association with the development of necrotizing enterocolitis and potential preventative therapies.

FUNCTIONS OF THE GUT MICROBIOTA

The gut microbiota is incredibly complex and has an extensive metabolic repertoire that is different from, but complements, the mammalian enzymes in the liver and gut mucosa (17). The gene set of the gut microbiota, the microbiome, is estimated to contain over 3 million genes, 150 times larger than that of the human genome (17). Comparative studies in germ-free, conventional microbiota or human microbiota-associated animals have identified three main functions of the gut microbiota – bioconversion, immune system development, and pathogen colonization resistance, each of which is discussed below (Table 1).

TABLE 1 Three functions of the gut microbiota.

Bioconversion	Convert indigestible food stuffs to short chain fatty acids – acetate, butyrate, propionate - Acetate: enters circulation; improves insulin response in pancreatic β cells; reduces lipolysis; increases production of leptin; protective against diet-induced obesity and insulin resistance - Butyrate: primary energy source for colonocytes; induces gut hormones; reduces food intake; protective against diet-induced obesity and insulin resistance; anti-inflammatory and anti-carcinogenic - Propionate: used by the liver for gluconeogenesis; implicated in reduced stress behaviors and increased reward pathway activity; anti-carcinogenic and anti-inflammatory; protective against diet-induced obesity and insulin resistance
Immune System Development	Tolerance to colonizing bacteria and common environmental antigens Maturation and proliferation of immune cells Maintenance of a "controlled physiological inflammation" Training for immunoglobulin class switching Promote production of anti-inflammatory cytokines and reduction of pro-inflammatory cytokines
Pathogen Colonization Resistance	Production of bacteriocins Alteration of the pH Consumption of nutrients required for pathogen growth Production of metabolites that alter pathogen gene expression Competition for space

Bioconversion

The digestive tract offers a nutrient rich, relatively favorable environmental niche for anaerobic and fermentative bacteria. These bacteria aid the host in bioconversion of indigestible foodstuffs to short-chain fatty acids (SCFAs) that act as energy sources for a variety of host cells (18, 19). Microbial metabolites, as well as digested dietary compounds, are metabolized by host enzymes in the liver and returned to the colon via bile for further metabolism or excretion (4). Non-digestible dietary carbohydrates including polysaccharides, oligosaccharides, lignin, and other plant materials, are metabolized to SCFAs – acetate, propionate, and butyrate namely – by our gut microbiota (20, 21). These SCFAs protect the host against pathogen translocation across the epithelial barrier, provide energy for intestinal cells, and have anti-carcinogenic and anti-inflammatory properties. The gut microbiota also produces vitamins for the host and mediates maturation of the immune response. Therefore, changes in diet can alter the composition of the gut microbiota and their metabolic activity significantly, impacting nutrient availability and host metabolism (22).

Acetate is the only SCFA that enters the circulation and reaches peripheral tissues (9). Elevated plasma acetate levels have been linked inversely to plasma insulin levels, improving the insulin response in pancreatic β cells (23). The presence of acetate has also been linked to a reduction of adipocyte lipolysis and production of leptin, preventing fatty liver disease, and regulating appetite (24, 25). Acetate, as well as butyrate and propionate, can protect against diet-induced obesity and insulin resistance (9). Butyrate is the primary energy source for colonocytes. The energy provided by butyrate improves the intestinal barrier by strengthening tight cell junctions, regulating the mucus layer, and inducing

proliferation of healthy cells while inducing apoptosis in unhealthy cells (20, 26). Further, butyrate induces gut hormones, and reduces food intake (27, 28). Propionate is primarily used by the liver for gluconeogenesis (29). Propionate has also been linked to the gut-brain axis in reduced stress behaviors and increased reward pathway activity (30). Both butyrate and propionate have anti-carcinogenic and anti-inflammatory effects (31–33) .

Immune System Development

Our immune system must be "primed" to tolerate the environment, while reacting to pathogens, and avoiding autoimmunity and allergy. Studies with germ-free animals have demonstrated that the gut microbiota plays an essential role in the development of a healthy immune system. Germ-free animals have underdeveloped mucosal-associated lymphoid tissue (MALT) and defective cell-mediated immunity due to the lack of a microbiota. Further, the germ-free animals exhibited a reduction in cytokine production, mucosal cell turnover, and production of antimicrobial peptides (34–38). The healthy gut microbiota and luminal contents aid in priming of the mucosal immune response and maintenance of a "controlled physiological inflammation" (39). The interactions of the gut microbiota and early immune cells promoted the proliferation of B and T cells in Peyer's patches and mesenteric lymph nodes (40). For example, *Bacteroides fragilis* has been shown to direct development of Foxp3(+) regulatory T cells (Tregs) and in turn increase the production of anti-inflammatory cytokines (3, 41). The gut microbiota has also been linked to increased production of interleukin (IL)-10, IL-12, and interferon (IFN)-gamma, and decreased production of IL-4, leading to the expansion of T_h1 cell populations (40). The gut microbiota also assists in training the immune system for

immunoglobulin class switching (42). Whether these immuno-stimulatory effects are attributed to specific commensal microbes remains to be elucidated; however, it has become clear that without proper immune stimulation, the host is at a higher risk for autoimmune and other immune-related diseases.

Pathogen Colonization Resistance

Commensal gut bacteria can prevent pathogen colonization via direct mechanisms that allow them to outcompete pathogens. These mechanisms include the production of bacteriocins, altering the pH of the environment, consumption of nutrients required for pathogen growth, production of metabolites that alter pathogen gene expression, and creating competition for space. Bacteriocins are proteinaceous toxins produced by commensal bacteria that inhibit the growth of members of related species (43). Production of SCFAs by commensal bacteria alters the pH of the gut environment, inhibiting the growth of specific enteric pathogens (44–47). Further, SCFAs can modulate the expression of virulence gene loci in intestinal pathogens (48, 49). Finally, preferential consumption of required nutrients by commensal microbes causes the starvation of pathogens and aids the preferential proliferation of commensal microbes to take up space, limiting potential adhesion sites for pathogens (50–53).

GUT MICROBIOTA DEVELOPMENT

Colonization of the gastrointestinal tract begins at birth and changes drastically until the age of three years old when the gut microbiota begins to resemble that of an adult (54–56). Infants are first colonized by facultative anaerobes that use up the oxygen present in the gastrointestinal tract and allow for colonization of the gut by strict anaerobes including *Bacteroides, Clostridium,* and *Bifidobacterium* spp. Characterized by low diversity and dominance by the phyla Proteobacteria and Actinobacteria, the infant gut microbiota becomes more diverse over time, with Firmicutes and Bacteroidetes becoming the dominant phyla (57, 58). Changes in the community composition of the infant gut microbiota are associated with life events and result in elevated expression of metabolic genes that are characteristic of the adult microbiome (55). However, community composition can also be altered by exogenous factors that lead to the development of an altered, or dysbiotic microbiota.

Factors Affecting Colonization

There are five factors which play essential roles in the establishment of a healthy infant gut microbiota – mode of delivery (5), exposure to antibiotics pre- and post-birth (6), hygiene (8), mode of feeding (9), and prematurity (59). Each of these factors exposes the infant to different microbial communities, leading to variations in colonization that increase the risk of various diseases throughout life.

Mode of Delivery

Our first exposure to microbes is at birth and infants will be colonized by different bacteria depending on if they are born vaginally or via cesarean section (C-section) (Table 2). Infants born vaginally are exposed to and become colonized by maternal vaginal and fecal microbes, specifically *Lactobacillus* and *Prevotella* (5, 60). Conversely, infants born via C-section are first exposed to microbes associated with the skin, such as *Staphylococcus* and *Peptostreptococcus* (5, 60). After four weeks, the gut of vaginally delivered infants is colonized by high numbers of *Bifidobacterium* and *Bacteroides,* while the gut of C-section infants is dominated by *Clostridium* (9). Further, infants born via C-section are more likely than those delivered vaginally to experience infections with methicillin-resistant *Staphylococcus aureus* (MRSA) and are more frequently colonized by *Clostridioides difficile* (5, 8, 9, 60–64). Infants born by C-section also show delayed colonization by *Bifidobacterium, Lactobacillus,* and *Bacteroides,* and increased incidences of allergies and asthma (65–67). The protective effects of natural birth have been associated with the microbiota of vaginally delivered infants; as C-section delivered infants have reduced cytokine levels and increased risk of immune disorders, type 1 diabetes, and obesity in infants delivered by C-section due to different microbiota (64, 68, 69).

TABLE 2 Comparison of the flora acquired from a cesarean vs vaginal birth.

Cesarean Section	**Vaginal Birth**
Skin normal flora - *Staphylococcus* - *Peptostreptococcus*	Vaginal normal flora - *Lactobacillus* - *Prevotella*
4 weeks: *Clostridium*	4 weeks: *Bifidobacterium* and *Bacteroides*

Antibiotic Exposure

Many infants born by C-section will also experience antibiotic treatment, further altering the infant microbiota. Most antibiotics in clinical use have a broad spectrum of activity, making them effective against not only pathogens, but also commensal gut bacteria. Because of the impact on commensals, short-term side effects of antibiotic use include antibiotic-associated diarrhea, gastrointestinal discomfort, gastritis, glossitis, and the development of a bacterial population in the gut that is antibiotic-resistant (8, 70–74). Studies suggest that long-term side effects of antibiotic use associated with gut dysbiosis include asthma, allergy, and an increased risk of obesity (75–77). In infants, antibiotic exposure before and after birth has been implicated in alterations of the microbiota. One study found that infants treated with ampicillin and gentamicin started within 48 hours of birth, had significantly higher proportions of Proteobacteria and significantly lower proportions of Actinobacteria, *Bifidobacterium,* and *Lactobacillus* compared to untreated infants 4 weeks after treatment. The same study found that after eight weeks, the levels of Proteobacteria remained significantly higher in treated infants than in controls, but the Actinobacteria, *Bifidobacterium,* and *Lactobacillus* levels returned to a similar level as the controls, although there was significantly less species diversity (78). Further, antibiotic use in infants has been associated with decreased numbers and diversity of *Bifidobacterium* and *Bacteroides* later in life, demonstrating the inability of the infant's gut to recover from antibiotic treatment (8, 79).

Hygiene/Family Lifestyle

The “Hygiene Hypothesis” stemmed from David Strachan’s observation that a large household size was associated with a decreased prevalence of hay fever and eczema. Therefore, he proposed that allergic diseases could be prevented by early infection in childhood via exposure to an unhygienic older sibling (7). He believed that the increased incidence of hay fever and allergies could be attributed to declining family size and improvements in personal hygiene and household amenities. A smaller family and improved hygiene would consequently limit children's exposure to environmental microbes, increasing their chance of immune reactivity, and in turn, atopic disease (7). Numerous epidemiological studies have been performed to confirm his theory and add additional factors that affect the risk of allergies, such as furred pets, C-section delivery, and antibiotic use (80–83). These studies suggest that altered microbial exposure, would consequently alter gut microbiota and affect the development of allergies and other atopic diseases.

In trying to elucidate which microbial exposures are vital for reducing or increasing the risk of these diseases, various studies using whole-genome sequencing have been performed to compare the gut microbiota of infants with various exposures. Studies found that the guts of older siblings had higher numbers of bifidobacteria and lower numbers of non-*E. coli* enterobacteria and clostridia (8, 63). Results from a Danish study suggested that having older siblings was associated with increased diversity and richness of bacterial species during early childhood, with pets having less of an effect on the gut microbiota (84). Here, richness refers to the total number of bacterial species present in the gut, while diversity is the amount of individual bacteria present from each

245 species. Interestingly, the opposite effect was observed in a small Canadian study, which
246 showed that infants with pets had more microbiota richness and diversity than those with
247 older siblings (85). Infants with pets also had less *Bifidobacteriaceae* and more
248 *Peptostreptococcaceae*, whereas infants with older siblings had less
249 *Peptostreptococcaceae* (86). Decreased microbiota richness and diversity, specifically
250 lacking members of the *Bifidobacteriaceae* and *Peptostreptococcaceae,* is associated
251 with increased risk of asthma, allergy, and other immunologic diseases (86).

252 **Mode of Feeding**

253 Mode of feeding, either breast milk or formula, plays a vital role in the
254 development of gut microbiota. Either method of feeding exposes the infant to different
255 microbial environments, leading to different colonization profiles. Human milk is
256 incredibly complex, with its composition changing constantly. Gestational age at birth,
257 lactation period, diet, and health of both the mother and infant, impact the composition of
258 breast milk. Breast milk provides further immune factors, hormones, and enzymes that
259 contribute to infant health, whereas formula is meant only to meet the nutritional needs of
260 the infant, (87–89). Further, breast milk contains other factors that enhance the
261 proliferation of beneficial bacteria and prohibit the growth of pathogens, leading to the
262 dominance of probiotic bacteria in breast-fed infants compared to formula-fed infants
263 (16).

264 Certain species of bacteria, such as *Bifidobacterium infantis*, are only found in
265 breast-fed infants, while other species, such as *Bacteroides fragilis*, are only found in
266 formula-fed infants. This indicates the presence of a growth factor only present in breast
267 milk that is required by *B. infantis* (8). Breastfeeding also exposes the infant to the

268 microbes of the skin, milk ducts, and breast milk, while formula feeding exposes the
269 infant to environmental microbes from the equipment used to process and package the
270 formula, as well as the water used to suspend the powder (8). Breast-fed infants have a
271 microbiota that is more stable, uniform, and less diverse than formula-fed infants. The
272 breast-fed microbiota is dominated by bifidobacteria and has lower levels of potential
273 pathogens (8, 59, 88, 90). Formula-fed infants are often colonized with *Escherichia coli, C. difficile, Bacteroides, Staphylococcus,* and enterococci (8). Because of the increased
275 species diversity in the formula-fed microbiota, the levels of SCFAs vary between the
276 two groups, with formula-fed infants having higher levels of propionate and butyrate in
277 their stool (91–93). Small amounts of formula supplementation have been shown to shift
278 the microbiota from breast-fed to a formula-fed pattern, with more diversity, and
279 increased numbers of *Clostridium* and *Streptococcus* (94). Cessation of breastfeeding was
280 found to be a requirement for conversion to an adult-like microbiota; therefore, formula-
281 fed infants make this switch earlier than their breast-fed counterparts (95).

282 Mode of feeding influences the colonization patterns of infants, and consequently
283 induces specific T-cell responses, playing a significant role in the development of
284 tolerance and a balanced immune response (96). Breastfeeding is protective against
285 various diseases, including allergic and autoimmune disorders, celiac disease, type I
286 diabetes, necrotizing enterocolitis, obesity, and inflammatory bowel disease (97).

287 **Prematurity**

288 According to the Centers for Disease Control and Prevention, 11.4% of all live
289 births were preterm in 2013 (98). An infant is considered premature when born before 37
290 weeks gestation, and with very low birth weight when born less than 1,500 grams (99). A

preterm infant often needs to overcome health challenges which may include an immature gut, immune, respiratory, or neurological issues, or exposure to extensive antibiotic and drug treatments. These challenges often involve long stays in hospitals, enduring ventilation, and artificial feeding (100). Several studies have identified differences in the fecal microbiota of pre- versus full-term infants. In preterm infants, richness is reduced, and colonization of *Bifidobacterium* and *Bacteroides* is delayed. Preterm infants also have significantly higher numbers of *Enterobacteriaceae,* *Enterococcus*, and other opportunistically pathogenic bacteria (9, 101–105). Higher numbers of Proteobacteria and *C. difficile* are found in preterm infant guts compared to their full-term counterparts (8, 9). The preterm infant microbiota also experiences abrupt population changes, with VLBW infants often becoming colonized by increased numbers of clostridia (106). The differences in colonization make the preterm microbiota more unstable than that of full-term infants and may contribute to a delay in the transition to and the establishment of an adult-like microbiota, and may affect short- and long-term health (106).

The immaturity of the infant gut and infant immune system paired with delayed "healthy" colonization patterns increases the risk of enteric and systemic infections in premature infants. Complications from preterm birth have become more common as more preterm infants are surviving and spending more time in neonatal intensive care units. Many of these complications, including ischemia, necrotizing enterocolitis, chronic lung disease, and retinopathy, lead to long-term developmental and health complications (107).

Dysbiosis

The factors discussed above - mode of delivery, antibiotic exposure, hygiene, mode of feeding, and gestational age - all contribute to the development of healthy gut microbiota. Any one of these factors can lead to changes in the colonizing microbiota that cause harmful health effects, named dysbiosis (5–9). Dysbiosis is generally thought to be associated with an over colonization of the gut with pathogenic or otherwise harmful bacteria, which increases the risk of infection, immune and metabolic disease, and overstimulates the immune system (108–110).

Associated Diseases

In developed countries, dysbiosis of the infant gut microbiota has been linked to the development of immune-mediated diseases like allergy, asthma, Crohn's disease, and type 1 diabetes (111). Dysbiosis has also been associated with obesity, and increased risk of colonization and infection by pathogenic bacteria (9, 61, 67, 69, 108, 112). Colonization with populations of antimicrobial resistant bacterial strains, diarrhea, and gastritis have also been associated with dysbiosis (70, 74–77). In infants, dysbiosis has been associated with higher risks of infectious morbidity in the first year of life, more frequent occurrences of otitis media, increased risk of lower respiratory tract and gastrointestinal infections, and metabolic diseases (16, 113–118). Further, dysbiosis in infants is associated with increased risk of nosocomial infections, growth reductions, developmental delays, and necrotizing enterocolitis, a severe inflammatory intestinal disorder (11, 119–121).

NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis is the most common gastrointestinal disease acquired by preterm infants, affecting 10-20% of VLBW infants (11). Symptoms of NEC include gastric retention of enteral feedings, abdominal distension and bloody stools caused by mucosal inflammation, intestinal epithelial cell apoptosis, sepsis, intestinal perforations, and multiple organ failure (122, 123). The disease takes more lives after the first week of life than any other pathology in preterm infants and has a case mortality rate of 30% (12). Necrotizing enterocolitis is also a substantial financial burden. The total annual cost of caring for infants with NEC in the United States is estimated to be between $500 million and $1 billion. One study aimed at determining the impact of NEC on hospital stay duration found that infants with NEC were hospitalized 60 days longer than healthy preterm infants if surgery was required, and 20 days if surgery was not required (13). The most common surgery required for infants diagnosed with NEC was a bowel resection and was the primary cause of short-gut syndrome in pediatric patients (124).

Identified risk factors for NEC include prematurity, mode of delivery, mode of feeding and timing of transitions to formula or soft foods, extended antibiotic use, and gut bacterial colonization. However, its etiology remains relatively unclear (12, 14). NEC has never been observed in germfree animals and is associated with high numbers of *Enterobacter, Escherichia, Enterococcus, Sphingomonas, Staphylococcus,* and *Klebsiella*

(11, 12, 125–130). Infants that develop NEC exhibit decreased microbial diversity relative to their non-NEC counterparts, which may reduce colonization resistance, allowing for colonization by more potential pathogens, and exacerbating the inflammatory response (131). Infants at risk of developing NEC also demonstrated far less microbial stability compared to healthy infants, which may act as a "trigger" to initiate an exaggerated inflammatory response causative of tissue damage (11).

Preterm infants have underdeveloped organs and therefore, immature enterocytes. These immature host cells could exhibit excessive signaling by toll-like receptor 4 (TLR4) in response to lipopolysaccharide (LPS) on gram-negative bacteria, which has also been suggested as a "trigger" for NEC development (12). Toll-like receptor 4 expression seems to be upregulated in fetal compared to adult enterocytes, accompanied by underexpression of IκB, an inhibitor of NF-κB responsible for inflammatory cytokine expression (132). Activation of TLR4 also triggered increased enterocyte apoptosis and reduced mucosal healing, and contributed to intestinal necrosis, which is a hallmark of NEC (133–135). Further, serum levels of several cytokines and chemokines increased in infants with NEC compared to their healthy counterparts. Specifically, interleukin (IL)-8, which is produced by epithelial cells and recruits neutrophils to the site of inflammation, can cause necrosis and increased production of acute-phase proteins (136). Activation of TLR4 seems to be required for NEC development, as mice deficient in TLR4 or given TLR4 inhibitors do not develop NEC (137, 138). The excessive inflammation associated with NEC has systemic effects, such as damaging organs like the brain and putting affected infants at increased risk for neurodevelopmental delays (139). In fact, an infant

recovering from NEC has a 25% chance of developing microcephaly or other severe neurodevelopmental delays (139).

Necrotizing enterocolitis does not develop until 8 – 10 days postpartum when anaerobic bacteria have colonized the gut. Pathogens including *E. coli*, *C. difficile*, and *Cronobacter sakazakii* have been implicated in single-institution outbreaks, but none have been consistently implicated in the disease (140). Abnormal gut colonization, however, is a hallmark of NEC (141). Specific characteristics of the microbiota associated with the development of NEC include reduced diversity and increased levels of potentially pathogenic microbes, compared to infants without NEC (129, 142). Using molecular techniques, scientists have been able to compare the gut microbiota of infants with and without NEC. Infants without NEC tend to have higher counts of bifidobacteria, suggesting a protective role of this genus against NEC. One study found that high species diversity and dominance by bifidobacteria was associated with healthy, preterm infants (11). Further, infants who do not develop NEC tend to have increased bacterial diversity, including an abundance of Firmicutes, *Veillonellaceae*, and bifidobacteria (11, 131, 143). In a study by Wang *et al*., infants with NEC had significantly decreased microbial diversity with an abundance of *Gammaproteobacteria* in their gut microbiota compared to their preterm counterparts without NEC (131). A further study implicated over-colonization with Enterobacteriaceae in the development of NEC (143). Further, researchers have repeatedly identified an increased abundance of Proteobacteria and *Clostridium perfringens* preceding NEC development (12, 14, 125, 129, 130) and high levels of Proteobacteria may increase the risk of bacterial translocation, bowel perforation, and subsequent sepsis development (144). The known colonization

differences between infants who develop NEC and those who do not, allow scientists to theorize prophylactic treatment options that benefit the infant by altering the gut microbiota.

Prophylactic Treatment Options

Due to the implication of the gut microbiota in the development of NEC, altering the gut microbiota may be a viable prophylactic option. Breast milk is regarded as the best prevention for the development of NEC. However, as breast milk is not an option for all infants, alternative options must be approached (15). Pre-, pro-, and synbiotics have been used to supplement foodstuffs and treat various ailments associated with gut dysbiosis for some time (145). Applying these supplements to formula or breast milk for premature infants may be a viable way to alter their microbiota and prevent NEC (Table 3).

TABLE 3 The benefits of breast milk, pre-, pro-, and synbiotics.

	Enhance immunity	Hormones	Enzymes	Enhance proliferation of beneficial bacteria	Bactericidal compounds	Protective against NEC?
Breast milk	✓	✓	✓	✓	✓	YES
Prebiotics	✗	✗	✗	✓	✗	POTENTIAL
Probiotics	✓	✗	✗	✓	✓	YES
Synbiotics	✓	✗	✗	✓	✓	POTENTIAL

Breast milk

Breast milk has long been recognized as protective against NEC. One study found that exclusively formula-fed infants were up to 20 times more likely to develop NEC than exclusively breast-fed infants, and three times more likely than infants who received formula and breast milk depending on their gestational age (15). The same study also found that pasteurized donor milk is as protective as raw breast milk, presenting a viable alternative to infant formula (15). Due to the complexity of breast milk, it has been challenging to elucidate which components of breast milk have protective effects against NEC. Components that potentially have protective effects against NEC include

412 epidermalgrowth factor (EGF), heparin-binding epidermal growth factor-like growth
413 factor (HB-EGF), lactoferrin, lysozyme, and human milk oligosaccharides (HMOs) (16,
414 146–154). Other anti-inflammatory molecules such as IL-10, TGFβ, antioxidants,
415 prostaglandins, and protease inhibitors may also protect against NEC by alleviating the
416 inflammatory activation of TLR4 (155).

417 Epidermal growth factor is abundant in breast milk, as well as in the amniotic
418 fluid, and is critical for intestinal development (146–149). Both breast milk and amniotic
419 fluid have been found to inhibit LPS-mediated TLR4 activation, rescue cell proliferation,
420 and reduce TLR4-mediated apoptosis via activation of the EGF receptor (16). In animal
421 models of NEC, experimental NEC is induced by hypoxia, a special formula, and LPS
422 exposure. In newborn mice with experimental NEC, breast milk reduced the severity of
423 experimental NEC, expression of pro-inflammatory nitric oxide synthase, enterocyte
424 apoptosis, and enhanced enterocyte proliferation (16). These protective effects were
425 dependent upon breast milk activation of EGFR and were reproduced by supplementing
426 formula with exogenous EGF (16). A different study associated low concentrations of
427 EGF in cord blood of extremely premature infants with development of NEC (156).
428 Further, HB-EGF accelerated enterocyte migration and proliferation, as well as increased
429 microvascular blood flow in the intestine (150, 151).

430 Human milk oligosaccharides (HMO) are the third-largest solid component of
431 human milk, behind lactose and lipids, yet are indigestible by the human gut (154). These
432 carbohydrates have no nutritional value to the infant but stimulate the growth of
433 beneficial bacteria and prevent colonization by pathogens. Once in the gut, these
434 carbohydrates are fermented and stimulate colonization of the gut by microbes that have

a positive health effect, and therefore, they are prebiotic (161). One bacterium in particular, *B. infantis*, is uniquely enriched by HMO's, evidenced by its absence in formula-fed infants, as well as culture studies employing HMOs (161). However, other species of *Bifidobacterium* and *Bacteroides* can utilize HMOs as well (162, 163). Human milk oligosaccharides can also bind to pathogenic bacteria, inhibiting their adherence to epithelial cell surface glycans, and also act as antimicrobials (164–167).

Human milk is highly complex and contains many components that potentially contribute to infant health. As the most preventative intervention available for NEC, it is essential to identify the factors that genuinely contribute to its protective effects. These protective factors are altogether missing from infant formula; therefore, it is crucial to identify ways in which formula can be supplemented to afford the infant the same protections.

Pre-, Pro-, and Synbiotics

While the composition of infant formula has been regulated and optimized to be as nutritionally similar to breast milk as possible, it still lacks many of the functional components that make breast milk protective (168). Therefore, supplementation of infant formula with pre-, pro-, and synbiotics has been attempted to prevent infant gut dysbiosis leading to the development of NEC.

Prebiotics. Prebiotics are nutrients that enhance the growth of potentially beneficial intestinal microbes (169). The intestinal microbiota is unstable and highly variable during infancy. Diet plays a vital role in the development of the microbiota during this period, making it an opportune time for manipulation with prebiotics. Prebiotic agents include non-digestible oligosaccharides like inulin, galacto-

oligosaccharides (GOS), lactulose, fructo-oligosaccharides (FOS) or combinations of these (169). Once prebiotics reach the gut, they work by enhancing the proliferation of endogenous microbes, mostly bifidobacteria. Shifting the microbiota towards dominance by bifidobacteria has a significant impact on the other bacteria that can colonize the gut. A study by Knol *et al.* found stimulation of bifidobacteria via prebiotic oligosaccharides significantly reduced the presence of pathogens, potentially protecting infants against intestinal infections (170). Further, bifidobacteria, specifically *B. infantis,* was able to counter the decreased expression of tight junction proteins that is associated with NEC, decreasing intestinal permiability (171). Therefore, by stimulating the growth of bifidobacteria with prebiotics, we may be able to prevent the development of NEC. However, this likely requires the presence of bifidobacteria in the infant's gut before treatment with prebiotics, and since premature infants often are lacking bifidobacteria, prebiotics may not be effective. Human milk oligosaccharides are natural prebiotics; however, various synthetic prebiotic oligosaccharides have been developed, as purification and isolation of HMOs from human milk is difficult and unfeasible (172).

Although HMOs have no nutritional value for the infant, they influence the gut microbiota through colonization resistance and proliferation of beneficial microbes. Because HMOs are native to the host, this makes them ideal prebiotics (162, 163, 173). There is more variability of HMOs found in milk from mothers with preterm infants, compared to those found in milk from mothers with full-term infants, which may explain the protective effect of human milk against NEC. However, the breast milk of mothers to infants developing NEC has less diverse HMOs than milk of mothers to infants that do not develop NEC (174). This is thought to be associated with the secretor and Lewis

status of the mother. Low levels of LNDH I, a neutral oligosaccharide produced by only secretor and Lewis-positive mothers, is associated with NEC development (174). Five monosaccharides – glucose, galactose, N-acetyl-glucosamine, fucose, and sialic acid – serve as the base of all HMOs (175). All HMOs contain lactose that can be further modified, leading to more than 150 variations of HMOs (175). Because these various structures were identified, structurally similar oligosaccharides for supplementation in preterm infants have been synthesized. Various mixtures of synthetic, non-human milk galacto-, fructo-, and acidic oligosaccharides have been extensively studied in preterm infants. It is believed that a combination of short- and long-chain oligosaccharides mimic HMOs the best. These synthetic oligosaccharides have been found to beneficially alter the gut microbiota, decrease fecal pH, feeding intolerance, colonization by potential pathogens, increase gastric motility and elevate fecal sIgA levels (169, 177).

Studies of single HMO structures are thus far limited to animal studies, but have demonstrated a protective effect against NEC. In a study by Cilieborg *et al*., c-section delivered preterm pigs were used to assess whether the most abundant oligosaccharide in human milk, α1,2-fucosyllactose (2'-FL), would prevent NEC development and benefit gut colonization. Piglets were given plain formula or formula supplemented with 2'-FL for five days. Half of the piglets receiving 2'-FL formula developed NEC while 71% of the piglets receiving plain formula developed NEC. Additionally, there was no difference in the gut microbiology of the two treatment groups, indicating a non-critical role of 2'-FL in gut colonization and intestinal immunity (178). Interestingly, a similar study by Good *et al*. in neonatal wild-type mice, found a protective effect of 2'-FL against NEC, decreased pro-inflammatory markers, and preservation of the integrity of the intestinal

mucosa (179). The observed effects were attributed to the up-regulation of endothelial nitric oxide synthase (eNOS), a vasodilator which maintains mesenteric perfusion, which was supported by the development of NEC in eNOS-deficient mice as well as in mice given eNOS inhibitors. When cultured endothelial cells were treated with 2'-FL, eNOS was again upregulated (179). The differences in these two studies were likely due to the difference in animal models. In the study by Cilieborg *et al*., the piglets were delivered by C-section, immediately treated with 2'-FL, and observed for the development of NEC. Meanwhile, in the study by Good *et al*., NEC was induced in 7-10 day old mice using a specific NEC formula and enteric bacteria collected from an infant with severe NEC. While the mice were highly representative of the dysbiosis seen in human NEC, the piglets may be better representatives for the development of NEC, as they developed the disease naturally. In order to determine which of these studies most accurately represents the function of 2'-FL, more animal studies should be conducted allowing the animals to develop NEC naturally, eventually moving forward to clinical trials.

A further study of pooled HMOs from breast milk found their addition to formula significantly reduced the risk of NEC in neonatal rats. The study further showed that GOS, which are added to infant formula to mimic HMOs but are structurally very different, did not affect the incidence of NEC, suggesting the benefits associated with HMOs are structure-specific. Finally, this study was able to identify a single oligosaccharide, disialyllacto-N-tetraose, from the pooled HMOs that independently reduced the incidence of NEC, suggesting a target HMO for purification and supplementation in formula (175).

Multiple studies have assessed the efficacy of combinations of galacto- and fructo-oligosaccharides in preventing NEC. In one study, preterm formula containing short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio used to supplement insufficient maternal milk had no significant impact on NEC development between the study and placebo groups; possibly because both groups of infants still received breast milk, and therefore still encountered various HMOs (180). Another study assessing preterm formula supplemented with the same 9:1 ratio of GOS to FOS found the study group had significantly increased bifidobacteria counts compared to the placebo group and changed the consistency of stools in the study group to be more similar to the stools of breast-fed pre-term infants (181). A third study used preterm formula supplemented with a 4:1 ratio of scGOS/lcFOS and acidic oligosaccharides (AOS) at increasing doses over 30 days, and again found no significant difference in the development of NEC (182). In a similar study, preterm formula was supplemented with either AOS alone or a 1:3 ratio of AOS and lcGOS/lcFOS. Fecal flora and pH were assessed for both groups and found that only formula supplemented with AOS/lcGOS/lcFOS had a bifidogenic effect and increased proliferation of lactobacilli (183). The same combination also had the most significant effect on stool consistency; however, the formula with AOS alone also significantly softened stool compared to the placebo group (183). Further, formula with the AOS/lcGOS/lcFOS combination led to a significant decrease in fecal pH, while the addition of AOS maintained the fecal pH, and the placebo cohort who received maltodextrin experienced an increase in their fecal pH (184). The same AOS/lcGOS/scFOS combination did not affect fecal IL-8 or fecal calprotectin (185). Other studies with GOS/FOS have shown a long-lasting bifidogenic

549 effect, decreased fecal pH, reduced stool viscosity, and GI transit. However, none of
550 these studies evaluated the impact on NEC (186–189).

551 Besides studying GOS/FOS, a pilot study assessed the effects of lactulose
552 supplementation to feeding in premature infants found the addition increased the number
553 of probiotic lactobacilli in the stool, compared to infants receiving dextrose as a control.
554 Infants receiving lactulose also had less intolerance to enteral feedings, fewer episodes of
555 late-onset sepsis or NEC, and improved calcium and protein absorption (190). Lactulose
556 has been used to prevent and treat portal-systemic encephalopathy and as a laxative since
557 the late 1960s, reducing intestinal ammonia production and absorption. Reduced
558 ammonia concentrations decrease the luminal pH, increasing the conversion of ammonia
559 to ammonium by gut bacteria, and killing urease-producing bacteria. Lactulose is also
560 used by *Bifidobacterium* species, promoting colonization by probiotic bifidobacteria, and
561 inhibiting colonization by *Salmonella* (191).

562 The studies above demonstrated the ability of prebiotics to alter the gut
563 microbiota towards that of a breast-fed infant. However, many prebiotic studies focus
564 only on alterations to the microbiota, fecal pH, and stool consistency; creating a
565 significant lack of evidence for their efficacy in the prevention of NEC. Further research
566 is required to identify synthetic oligosaccharides with prebiotic effects, as well as to
567 identify specific HMOs with protective effects against NEC. A comparison of
568 commercial products containing HMOs and their doses for preterm infants should also be
569 performed. Finally, research should be conducted comparing the gut microbiota of
570 exclusively breast-fed, formula-fed, and supplemented formula-fed infants to resolve if

571 an established population of *Bifidobacteria/Lactobacilli* is a requirement for prebiotic
572 efficacy.

Probiotics. Probiotics are a dietary supplement containing live microorganisms that are believed to confer health benefits (Figure 1) (145). Probiotics can be found in pill

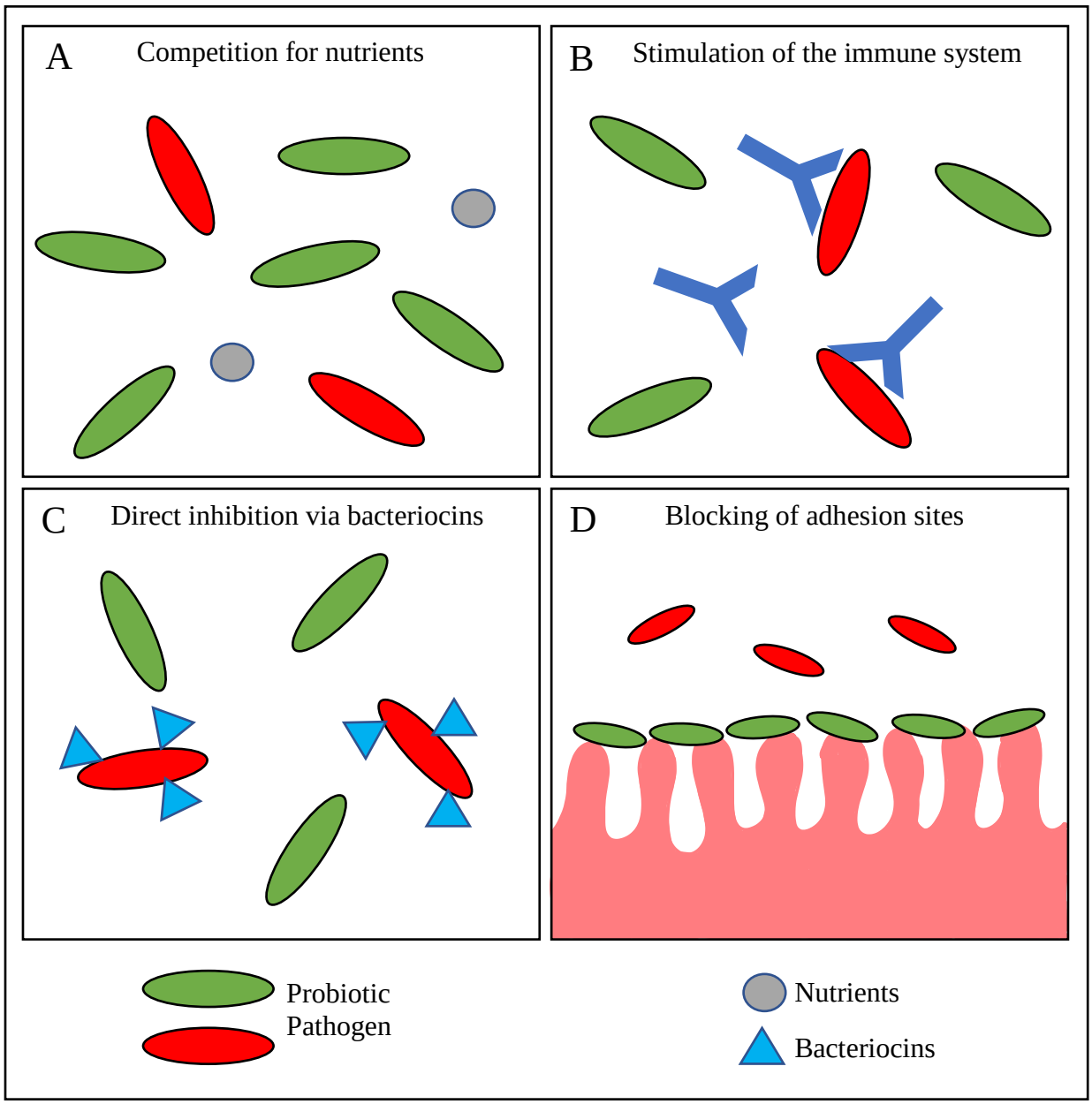

FIG 1 The benefits of probiotics. (A) Probiotic bacteria compete with pathogens and commensal bacteria for nutrients. (B) Probiotic bacteria stimulate the immune system against pathogens. (C) Some probiotic bacteria secrete bacteriocins, toxins which target and kill pathogenic bacteria. (D) Probiotic and commensal bacteria block the epithelial layer, preventing pathogen binding.

forms or mixed into foods, such as yogurt, and have exhibited beneficial effects that may resolve various diseases. In the treatment of NEC, results from studies using probiotics

are inconsistent, likely due to variation in dosing and strains used. However, probiotics given at an appropriate dose can decrease the risk of NEC. Routine administration of probiotics to all premature infants has become common practice in many countries (192).

Bifidobacterium infantis is a probiotic bacterium that is present in the gut of nearly all breast-fed infants, and absent from that of formula-fed infants (161). For this reason, *B. infantis* has been the subject of many probiotic studies and is included in many probiotic supplements. In combination with human milk, *B. infantis* increased colonization by *B. infantis*, decreased colonization by *Enterobacteriaceae,* and reduced the risk of NEC (161). The protective effect of *B. infantis* against NEC stems from its increased ability to bind and colonize the intestinal mucus layer, taking space from potentially pathogenic colonizers (193, 194). Further, *B. infantis* produces anti-inflammatory substances that suppressed the production of IL-6, IL-8, TNFα, and IL23, and the activation of TLR2 and TLR4, aiding in the development of a balanced innate immune response (195, 196). A bactericidal substance was also produced and secreted by *B. infantis* that attenuated *Cronobacter sakazakii*-induced colitis in mouse models (197). This is an essential function of *B. infantis* as *C. sakazakii* is a common contaminant of powdered infant formula and is associated with NEC and sepsis in premature infants (197). Finally, *B. infantis* decreased intestinal permeability, increased tight junction stabilization, and decreased the incidence of NEC (171). However, *in vitro* studies have shown the beneficial effects of *B. infantis* are dependent on the presence of HMOs, exhibiting decreased benefit when grown in the presence of lactose alone (193, 198, 199). Results from a dose-escalation trial comparing *B. infantis* and *B. lactis* in formula-fed infants, showed that *B. infantis* colonized the infant gut faster and longer than *B. lactis*,

and *B. infants* also increased bacterial diversity over time. The same study also compared the supplements in breast-fed infants, and found that they increased bifidobacteria and decreased γ-*Proteobacteria* more significantly following administration of *B. infantis* (200). Thus, confirming the observation that *B. infantis* is most effective in combination with human milk.

In a rat model of NEC, *B. bifidum* significantly reduced the incidence of NEC. This reduced incidence was attributed to a reduced inflammatory response in the gut, regulation of mucin production, and improved intestinal integrity (201). A subsequent study about the mechanisms of *B. bifidum* saw increased TLR-2 expression and significantly reduced apoptosis in the intestinal epithelium of rats with induced NEC (202). Further studies also found that *B. bifidum* decreased expression of antimicrobial peptides that are upregulated in NEC (203).

Another *Bifidobacterium* species, *Bifidobacterium breve,* reduced NEC and improved survivability in a rat model (204). It also downregulated molecules related to inflammation and barrier function which are implicated in NEC. Further, *B. breve* normalized TLR4 expression and increased TLR2 expression in comparison to rats not receiving *B. breve*. (204). A subsequent study found that *B. breve* had anti-inflammatory properties in a rat model of colitis, which could have contributed to its protective effects against NEC (205). However, in a multicenter study in England, infants born between 23- and 30-weeks gestation were given infant formula supplemented with *B. breve* within 48 hours. The authors found no difference in the incidence of NEC, sepsis, or death between the test and placebo groups, suggesting that *B. breve* does not protect against NEC development in humans (206). These contradictory results highlight the need for more

studies of *B. breve* supplementation in infants to determine the potential of *B. breve* in the prevention of NEC.

Besides *Bifidobacterium*, other genera of bacteria are used as probiotics. Lactic acid bacteria (LAB) produce antimicrobial factors and bacteriocins that prevent infection by enteric pathogens. In the human gut, *Lactobacillus* spp. are the most commonly found LAB, specifically *L. reuteri* (207). Reuterin is the primary antimicrobial produced by *L. reuteri*, exhibiting broad-spectrum activity against gram-positive and gram-negative bacteria, fungi, yeasts, and protozoa (208, 209). Reuterin has also been found to inhibit the growth of a different probiotic *Lactobacillus* spp., *L. casei* (207).

A study exploring *L. reuteri* supplemented formula in neonatal rats indicated that probiotic supplementation with *L. reuteri* significantly reduced the incidence and severity of NEC via downregulation of IL-6, TNF-α, TLR4, and NF-κB, as well as upregulation of IL-10 (210). A further study using *L. reuteri* supplemented cow milk-based formula in TLR2 $^{-/-}$ or wild-type (WT) mice, found a significantly reduced incidence of NEC in WT but not TLR2 $^{-/-}$ mice. Mice deficient in TLR2 also had significantly reduced activation of effector CD_4^+ T cells and increased activation of $Foxp3^+$ T cells, suggesting that the anti-inflammatory effects associated with *L. reuteri* require TLR2 (211). However, conflicting results have been found in human studies. One study showed that the probiotic *L. reuteri*, used as a supplement in milk or formula, did not protect against NEC or NEC-related death (212). A second group performed a similar study using *L. reuteri* and showed a similar, non-significant decrease in the incidence of NEC (213). Interestingly, in a retrospective cohort study, the introduction of prophylactic treatment with *L. reuteri* in infants born $\leq$1000 g was found to significantly decrease the incidence of NEC.

However, this study had a relatively small sample size, with the treatment group and the control group containing only 79 and 232 infants, respectively (214). In a study mixing *L. rhmanosus* in water and given via a feeding tube, the infants who received the probiotic had reduced incidence of NEC and NEC-related death (215). Finally, in a multicenter study in Italy, newborn infants < 33 weeks gestation or weighing < 1,500 g were given a standard milk feed supplemented with *Lactobacillus GG* from the first feeding to discharge. This study found no significant difference in the occurrence of NEC between the probiotic and placebo groups, although NEC did occur less in the probiotic group (216).

Multispecies probiotics, probiotics containing two or more bacterial species, have become prevalent, hoping for a synergistic effect of the combined species. A probiotic combination of *B. breve, B. bifidum, B. infantis*, *B. longum,* and *L. rhamnosus* was given to infants <32 weeks gestation from the first feeding until the infant reached 34 weeks and assessed for its ability to prevent NEC or death in very preterm infants (192). This combination significantly reduced the risk of NEC and death associated with NEC from 10% to 5% and 17% to 11%, respectively. (192). However, this study failed to measure if a difference in feeding had any effect on the clinical outcome, although most subjects were breast-fed. A multispecies study using a combination of *B. infantis, B. bifidum, B. longum,* and *L. acidophilus* with breast milk, found a significantly reduced incidence of NEC and death in infants exposed to probiotics (217). A study by Bin-Nun *et al*. using a multispecies probiotic of *B. infantis*, *B. bifidus,* and *Streptococcus thermophilus* found that the incidence of NEC in infants receiving the probiotic was significantly reduced from 16.4% in the control group, to 4% in the study group. Further, when NEC did occur

669 in infants receiving probiotics, the severity was also significantly reduced compared to
670 the control group, with only NEC related deaths occurring in the control group (218). In a
671 similar study by Jacobs *et al.*, the combination of *B. infantis, B. lactis,* and *S.*
672 *thermophilus* significantly decreased the risk of NEC development in infants receiving
673 the probiotic (219). Interestingly, an observational study using the same probiotic
674 combination found no difference in the incidence in NEC when probiotics were used.
675 However, the authors attributed this observation to small sample size, lacking the power
676 to differentiate any change in outcome (220).

677 Multispecies probiotic products containing *B. infantis* and *L. acidophilus* have
678 become common as well. A small study using a combination of only *B. infantis* and *L.*
679 *acidophilus* in newborns found a significantly decreased incidence of NEC and NEC-
680 related deaths (221). A similar study was done using the same probiotic combination
681 assessing prevention of NEC in two very low birth weight cohorts, 400-999 g, and 1000-
682 1500 g. The incidence of NEC was significantly decreased in both groups with the use of
683 the probiotic combination (222). A third study using the same combination of *B. infantis*
684 and *L. acidophilus* also found a significant protective effect attributed to treatment with
685 the probiotics (223). Due to the observed protective effects of *B. infantis* and *L.*
686 *acidophilus* on NEC, Ganguli *et al*. set out to determine if one or both species produced
687 secreted products that accelerate the maturation of the intestinal immune response.
688 Immature human enterocytes, intestinal xenografts, and primary enterocyte cultures of
689 NEC tissue were exposed to inflammatory stimuli and assayed for IL-8 and IL-6 after
690 treatment with either probiotic supernatant. The *B. infantis* supernatant had superior anti-
691 inflammatory properties compared to *L. acidophilus*, although *L. acidophilus* was anti-

692 inflammatory as well (195). A commercially available probiotic combination of *L.*
693 *acidophilus* and *B. bifidum* with breast milk was found to be protective, reducing the
694 incidence and severity of NEC (224). In a follow-up multicenter study, *L. acidophilus*
695 and *B. bifidum* were added to breast milk or a combination of breast milk and formula.
696 Probiotic supplementation again significantly decreased the risk of NEC (225). A further
697 study using the same probiotic product found it was only protective in infants receiving
698 breast milk, suggesting the effects of one or both bacterial species is dependent on breast
699 milk (226).

700 Overall, probiotics have been found to reduce the risk of NEC in preterm infants
701 significantly. However, further research is needed to determine the appropriate timing,
702 duration, dosage, and species/strains necessary to maximize these results. As
703 demonstrated above, not all probiotic species demonstrate protective effects against NEC,
704 so determining which species and respective strains elicit these effects is paramount. For
705 now, it appears that *Bifidobacterium* or multispecies supplements have the best effect,
706 with conflicting results from studies employing lactobacilli. Further, the probiotic
707 supplements should be monitored for contamination and maintenance of marketed CFUs,
708 as contamination could lead to increased infant infections and degradation would cause
709 the supplement to become ineffective. Larger study groups should also be used to
710 confirm the protective effects of probiotics. Finally, further studies should be done
711 assessing the efficacy of probiotics in formula-fed infants, especially because the effects
712 of some probiotic species are thought to be dependent on the presence of HMOs, which
713 are effectively missing from infant formulas.

Synbiotics. Synbiotics are dietary supplements that contain both pre- and probiotics, which beneficially affect the host by improving the survival, implantation, and colonization of health-promoting bacteria (145). These supplements are believed to act synergistically to increase gut health, providing a substrate for exogenously administered bacteria (probiotics) as well as for endogenous commensal bacteria (145).

In a study in India, preterm infants were given synbiotics containing *Lactobacillus, Bifidobacterium,* and fructo-oligosaccharides with breast milk. Infants that received the synbiotic had a 50% reduction in NEC incidence, with no effect on severity or mortality (227). Further, in a multicenter study in Turkey, VLBW infants were given a prebiotic (inulin), probiotic (*B. lactis*), synbiotic (*B. lactis* plus inulin), or placebo in breast milk or formula for up to 8 weeks and the rate of NEC was significantly reduced in the pro- and synbiotic groups, with the prebiotic group having no significant effect. However, all of the interventions resulted in shorter stays in the neonatal intensive care unit, lower rates of sepsis, and lower mortality rates (228).

Research on the use of synbiotics as prophylactic therapy for the prevention of NEC is lacking. The two available studies that have been performed demonstrated significant reductions in the incidence of NEC, highlighting the potential for synbiotics as a treatment option. With the many bifidogenic prebiotic factors that have been identified, finding a synbiotic combination to prevent NEC does not seem far off.

CONCLUSION

The human gut microbiota is incredibly complex and contributes to host health and disease. Its three main functions are the conversion of non-digestible foodstuffs to short-chain fatty acids, priming of the immune system and establishment of tolerance, and colonization resistance against pathogens (2). Dysbiosis of the gut microbiota is associated with infectious, metabolic, and inflammatory diseases, caused by several factors (4). The most important time to develop a healthy gut microbiota is during infancy, therefore, preventing or correcting dysbiosis at a young age is paramount. Dysbiosis in infancy is associated with gestational age, mode of delivery, mode of feeding, exposure to antibiotics, and family size/lifestyle, and can lead to the development of asthma, allergies, and necrotizing enterocolitis (4–10).

Necrotizing enterocolitis is the most common ailment of premature infants, with a mortality rate of up to 30% (12). It is a significant financial burden, costing up to $1 billion annually with a less understood etiology. Many of the risk factors associated with NEC are associated with dysbiosis of the infant gut (12, 14). Altered colonization of the gut and a chronic inflammatory intestinal response are hallmarks of NEC, and because it has never been observed in germ-free animals, the gut microbiota is easily implicated in the development of NEC (11, 12, 141).

Human milk is thought to be the best therapeutic option for neonates (15). Boasting many benefits, human milk is incredibly complex and difficult to duplicate. By providing nutrition as well as colonization factors, inflammatory mediators, and antimicrobial molecules to the infant, human milk has a dramatic impact on infant health. Because not all infants can tolerate breast milk, it is essential to find alternative treatment options that elicit the same preventative effects as breast milk.

Thus far, prebiotics have not shown great potential in the prevention of NEC. Prebiotics require the presence of the target bacterium to be efficacious. Due to the lack of bifidobacteria in the preterm gut, they may be more difficult to propagate by prebiotics. Synthetic oligosaccharides have shown variable results, while research of natural HMOs is thus far limited to animal studies. Synthesis of oligosaccharides that are both structurally and functionally similar to HMOs, could create prebiotics with significantly higher efficacy, but they may be most effective when given in combination with probiotics. Probiotics are efficacious in the prevention of NEC. However, more research needs to be done to determine which species of *Bifidobacterium* and *Lactobacillus*, or others are the most beneficial to the prevention of NEC. This must include the use of various species, strains, dosages, durations, and timings. Evaluation of efficacy should also be done in human milk and formula as breast milk seems to affect the efficacy of some strains. Further, elucidating which bacterial strains are protective will allow providers to treat preterm infants for the prevention of NEC more directly and effectively. Synbiotics need to be further studied, as the few studies that have been done show promise in the prevention of NEC. Further studies should also work to elucidate the full synergistic properties of synbiotics. Many bifidogenic prebiotic molecules have been

identified, and in combination with the species most readily able to use it, may be able to assist in alteration and colonization of the gut microbiota, thus preventing NEC.

In conclusion, understanding the etiology of NEC is vital to establishing practical guidelines for its prevention. The use of molecular methods will allow further insight into differences in the gut microbiota before the onset of NEC, as well as colonizers that may increase the risk of NEC. Optimizing infant formula with synthetic or natural HMOs may be an effective therapeutic option for prevention of NEC, although current data suggests that altering the microbiota via probiotics may be the best course of action.

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