

# Intestinal microbiota and its relationship with necrotizing enterocolitis

Ravi Mangal Patel<sup>1,2</sup> and Patricia W. Denning<sup>1,2</sup>

Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in infants born prematurely. After birth, the neonatal gut must acquire a healthy complement of commensal bacteria. Disruption or delay of this critical process, leading to deficient or abnormal microbial colonization of the gut, has been implicated as key risk factor in the pathogenesis of NEC. Conversely, a beneficial complement of commensal intestinal microbiota may protect the immature gut from inflammation and injury. Interventions aimed at providing or restoring a healthy complement of commensal bacteria, such as probiotic therapy, are currently the most promising treatment to prevent NEC. Shifting the balance of intestinal microbiota from a pathogenic to protective complement of bacteria can protect the gut from inflammation and subsequent injury that leads to NEC. Herein, we review the relationship of intestinal microbiota and NEC in preterm infants.

**N**ecrotizing enterocolitis (NEC) is a major cause of mortality and morbidity in infants born prematurely (1,2). Approximately 7% of very-low-birth-weight ( $\leq 1,500$  g at birth) infants will develop NEC. More worrisome, 20–30% of the very-low-birth-weight infants with NEC will die (3) and the remaining survivors will be at substantial risk for long-term complications, including impaired neurodevelopment and short bowel syndrome (4). Although the underlying pathogenesis of NEC is not completely understood, current evidence suggests a multifactorial pathophysiology that can be categorized into four areas: premature birth, abnormal intestinal microbiota, enteral feeding, and other potential factors (Figure 1).

## RISK FACTORS FOR NEC

Premature birth is the major determinant of NEC, as this disease almost exclusively occurs in infants born prematurely and the incidence of NEC is inversely proportional to the gestational age at birth (5,6). Several aspects of immature intestinal function in preterm infants may contribute to NEC predisposition, including the inflammatory propensity of the immature gut (7,8), decreased intestinal barrier function (9,10), and impaired intestinal immune defenses (11). Additional

potential risk factors that are controversial and based on observational data but may confer an increased risk of NEC include impaired intestinal oxygen delivery from anemia (12), aberrant gut vascular regulation (13), and/or red blood cell transfusion (14), which may lead to gut reperfusion injury. Further, the use of formula instead of breastmilk increases the risk of NEC (15,16). Although older studies suggested that rapid and aggressive advancement of enteral feedings were associated with an increased risk of NEC (17,18), recent meta-analyses have not shown an effect of aggressive feeding or delayed initiation of enteral feeding on NEC (19,20). Finally, abnormal intestinal microbiota, either from a lack of beneficial commensal microbes, a low diversity of bacteria or a preponderance of pathogenic bacteria, are likely to contribute to the risk of NEC. In this review article, we discuss the role of the intestinal microbiota in the pathogenesis of NEC.

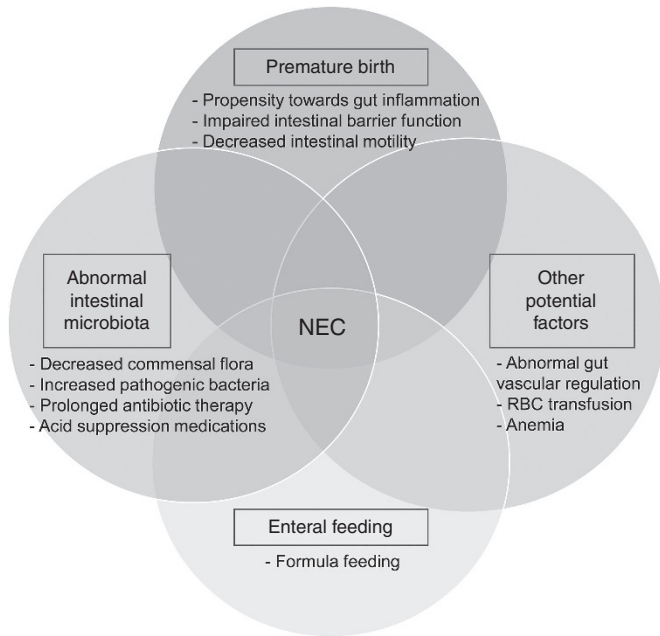
## ROLE OF MICROBIOTA IN INTESTINAL HEALTH AND HOMEOSTASIS

The human intestine is the largest immune organ in the body and a major interface to the external environment, where the gut must protect against harmful antigens, such as toxins and pathogens, while housing and tolerating beneficial commensal bacteria. This is a difficult task, as alterations in the balance between bacterial pathogens and commensal bacteria shift a gut from healthy intestinal homeostasis (21) to uncontrolled inflammation that can lead to injury and, potentially, the development of NEC (2). Commensal bacteria provide a number of benefits to the human host. These benefits include maintenance of intestinal homeostasis and protection from injury (22,23), support of digestion (24,25), and regulation of intestinal immune function (26,27). After birth, the immature neonate must acquire and sustain these beneficial commensal microbes as they begin to enter the intestinal lumen from the environment. Eventually, the number of bacteria will outgrow the host by a factor of ten to one (28,29).

Although the newborn infant's gut has previously been thought to be a sterile environment, emerging evidence suggests the potential that gut microbial acquisition may begin *in utero* under nonsterile intrauterine conditions. This is suggested by correlation of microbiota in meconium with those

<sup>1</sup>Department of Pediatrics, Division of Neonatal-Perinatal Medicine, Emory University School of Medicine, Atlanta, Georgia; <sup>2</sup>Children's Healthcare of Atlanta, Atlanta, Georgia. Correspondence: Patricia W. Denning (pdenning@emory.edu)

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**Figure 1.** Risk factors for NEC. Figure depicts the multiple risk factors that influence the development of NEC in premature infants. Abbreviations: NEC, necrotizing enterocolitis; RBC, red blood cell.

found in amniotic fluid (30) as well as detection of acinetobacter in the airways of preterm infants at birth (31). However, until these findings are replicated, they should be viewed with some caution given the concerns for effects of bacterial contamination in nonculture-based sequencing methods (32) and sampling under conditions where the amnion may not be intact.

### MAJOR EVENTS IN THE DEVELOPMENT OF THE INTESTINAL MICROBIOME

The fetal gut under physiologic conditions rapidly begins to acquire commensal microbiota following birth, and this process may potentially even occur *in utero* as mentioned previously. The initial composition of microbiota is derived from maternal colonic and vaginal flora that is acquired, in vaginally delivered infants, by passage through the birth canal. These microbiota include *Enterobacteriaceae*, *Enterococci*, and *Staphylococci* (33). Emerging evidence also suggests that placental transfer of microbes may also influence the development of the gut microbiome (34). The acquisition of gut microbiota appears to be an orderly process, with successive colonization of various bacterial classes beginning with Bacilli and then Gammaproteobacteria and Clostridia species (35). The intestinal microbiota is further developed and altered by feeding, particularly by breastfeeding, which contains prebiotics. These enterally delivered prebiotics promote the growth of commensal bacteria, which are beneficial to the infant (36). Term infants who are exclusively breastfed and born by vaginal delivery show a favorable composition of gut microbiota, with higher amounts of *Bifidobacteria* and lower amounts of *Clostridium difficile* and *Escherichia coli* compared to infants born by cesarean section and/or formula fed (37). Delivery in a

hospital environment, compared to home, also leads to greater intestinal colonization by *Clostridium difficile* (38,39).

In term infants, further expansion and alteration of the gut microbiota occurs with weaning from milk and the introduction of solid foods, with complete adult colonization by 2 y of age (33). Additional factors such as exposure to family members and local environments also impact the development of the intestinal microbiome (40). However, once an infant reaches adulthood, the composition of gut commensal microbiota remains relatively stable (41). As we discuss further, a number of factors disrupt the acquisition of gut microbiota in preterm infants and these are likely to contribute to the development of NEC.

### INTERACTION OF MICROBIOTA WITH TOLL-LIKE RECEPTORS: MEDIATORS OF BOTH INTESTINAL HEALTH AND NEC

The human intestine possesses “Pattern recognition receptors” (PRRs) that are able to specifically recognize “Microbial associated molecule patterns” or MAMPs. These PRRs then send signals that lead to cellular responses important in a number of homeostatic and host defense pathways. Common MAMPs include microbial cell-wall products such as lipopolysaccharide and peptidoglycan. Both commensal intestinal microbes and pathogens contain MAMPs, and normal flora can be proinflammatory under abnormal host conditions (42). Toll-like receptors (TLRs) are the most well-characterized PRR in the human intestine. These receptors must walk a tightrope between bacterial homeostatic vs. inflammatory effects in the preterm neonatal gut, as high-level activation by commensal microbiota can result in gut inflammation and injury. Activation of TLRs, while previously thought to be exclusively proinflammatory due to activation from pathogenic bacteria, is now known to also support important functions of intestinal health by activation from commensal bacteria (22). These functions include maintenance of intestinal homeostasis by supporting cell growth and proliferation, cytoprotection, regulation of barrier function, and antimicrobial peptide secretion (22,27,43). Mechanisms of gut tolerance to MAMPs are discussed in the next section.

Soon after birth, the preterm neonate receives a challenge of MAMPs through the acquisition of gut microbiota. Further, activation of TLR-4 occurs as part of the innate response to lipopolysaccharides from Gram-negative bacteria and has been implicated in the pathogenesis of NEC (44–46). In addition, the immature intestine, compared to a mature gut, is more likely to mount increased cytokine-mediated inflammatory responses by interleukin-8, which increases neutrophil chemotaxis and inflammation that can lead to tissue injury and NEC (10,47). Some studies suggest that immature intestines may have a propensity for exaggerated inflammatory responses, due to deficient expression of inhibitors of the NF- $\kappa$ B pathway, a major proinflammatory pathway (7,8). In addition, the gut inflammatory response in preterm infants may be developmentally regulated (48), as mice demonstrate a peak in inflammatory response to colonization at a postnatal maturational

window that is similar, in epithelial characteristics, to the gut of extremely preterm infants (48,49). These findings have an obvious implication for NEC, as there is a developmental window that centers on a common postmenstrual age when most infants develop disease (50). Further, immature intestines may develop abnormal inflammatory responses as the gut becomes colonized due to downregulation of interleukin 1 receptor-associated kinase 1 in immature intestine, which is an essential component of TLR type 4 (TLR-4) signaling (51). This suggests that tolerance to MAMPs from gut microbes may require early exposure to microbial ligands to promote tolerance to the continued acquisition of commensal microbiota.

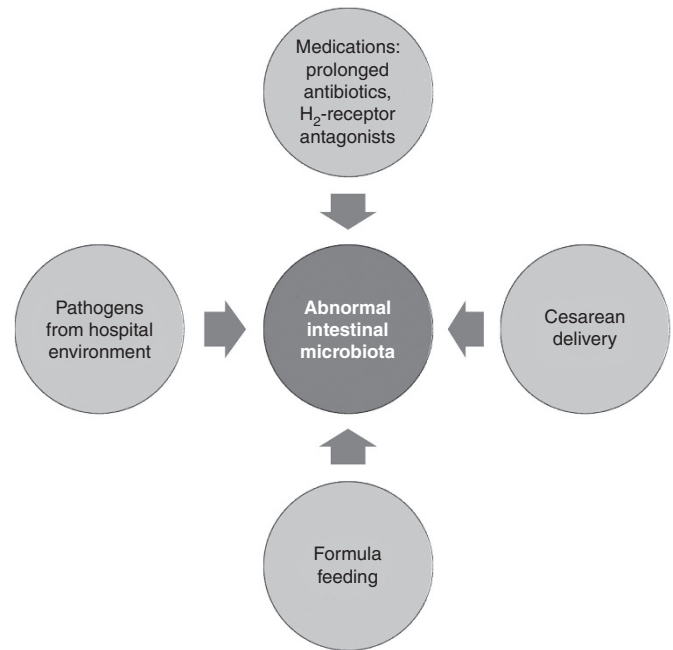
In addition to TLRs, other PRRs that mediate host-commensal and host-pathogen interaction include formyl-peptide receptors, G-protein coupled transmembrane receptors which are stimulated by microbial peptides (52,53), and the NOD family of PRR, which detect intracytoplasmic MAMPs to mediate inflammation (54). Bacterial production of short chain fatty acids such as butyrate, which supports enterocytes growth and differentiation (55), can also decrease inflammation (56).

### TOLERANCE OF GUT MICROBIOTA

The human gut must be able to house the beneficial microbiota that colonize it, without causing inflammation and injury. This is a remarkable feat, as the gut has numerous TLRs on its surface that are constantly sampling microbes and ready to mount an inflammatory response to protect the gut from pathogens. Commensal microbiota help the gut tolerate these microbes by preventing their recognition by the TLRs or by downregulating the proinflammatory NF- $\kappa$ B pathway (57). This is accomplished by blocking degradation of I $\kappa$ B, an inhibitor of the proinflammatory NF- $\kappa$ B pathway (58). Probiotic bacteria, similar to commensal microbiota, can attenuate inflammation by regulating TLR-4 dependent NF- $\kappa$ B activation through the same mechanism (59,60). In addition, low-level activation of the TLR receptors by commensal microbiota play an important role in gut homeostasis, as previously mentioned.

### DISRUPTED ACQUISITION OF COMMENSAL MICROBIOTA

Several factors may disrupt commensal colonization, by preventing or delaying the acquisition of commensal bacterial and lead to an abnormal composition of intestinal microbiota in premature infants (Figure 2). Prolonged antibiotic therapy can eliminate commensal bacteria and increase the risk of NEC, potentially by promoting colonization of more resistant pathogenic organisms which thrive in the hospital environment (61–64). Infants who receive antibiotic therapy soon after birth show lower amounts of *Bifidobacteria* (37). In addition, several studies have shown that prolonged empiric antibiotics therapy is associated with an increased risk of both NEC and late-onset sepsis (61,63,64). As antibiotic therapy depletes the gut of commensal bacteria, innate and adaptive immune defense are also potentially altered and weakened (65). Similarly, the use of acid-suppression medications such as ranitidine are associated with an increased risk of NEC (66,67), and a



**Figure 2.** Factors disrupting the acquisition of commensal microbiota. Multiple factors promote abnormal gut colonization in preterm infants by delaying or disrupting the acquisition of beneficial commensal bacterial from the infant's mother and external environment.

lower fecal microbial diversity with a higher proportion of Enterobacteriaceae and fewer Firmicutes, which includes bacteria such as *Lactobacillus* (68).

Further, delivery by cesarean section instead of vaginal delivery, where the opportunity to acquire maternal flora from passage through the birth canal is lost, may lead to decreased colonization of beneficial commensal bacteria such as *Bifidobacteria*, *Lactobacillus*, and *Bacteroides* (37,69). In addition, the lack of human milk feeding, which contains important prebiotics that promote the growth of commensal bacteria, may further disrupt the acquisition and growth of commensal bacteria.

### DIFFERENCES IN THE INTESTINAL MICROBIOTA OF INFANTS WITH AND WITHOUT NEC

Infants who develop NEC have altered gut microbiota compared to those infants without NEC. Culture-based studies, conducted over two decades ago, demonstrated differences in fecal bacteria up to 72 h before the onset of NEC (70). These changes in fecal bacteria included increases in *Enterobacter cloacae* and *E. coli*, with concomitant decreases in *Streptococcus faecalis* and *Staphylococcal* species.

More recent advances in nonculture-based methods have allowed for a molecular analysis of the gut microbiome of premature infants. These molecular-based studies have shown changes in the prevalence of *Clostridium perfringens*, Firmicutes, Proteobacteria, and *Enterobacter* prior to the onset of NEC (71–75). Further, decreases in *Bifidobacteria* and potential changes in yet unidentified strains similar to *Klebsiella* have also been described (76). These changes in the patterns of the developing gut microbiome occur rapidly after birth, within

the first few weeks of postnatal age (77). Further, alterations in the microbiome in infants who later develop NEC occur as distant as 3 wk prior to diagnosis (78). These studies suggest that the development of the early postnatal microbiome may be as important, if not more important, than the changes immediately prior to NEC onset. Further, infants with NEC tend to have a lower diversity of bacteria prior to the onset of NEC (72,75). It is likely that known factors associated with NEC, such as formula feeding and extended empirical antibiotic use, may cause some of these changes observed in the gut microbiome of infants with NEC. This emerging literature has yet to show a consistent pattern with many different species purported to play a role in NEC pathogenesis. However, analysis of the intestinal microbial population is a complicated process which has yielded variable results from study to study. Results may differ due to platform used for data acquisition and/or sophistication of bioinformatics analysis. Further, variability in antibiotic therapy may be an important confounder in these studies.

#### MICROBIAL PATHOGENS RECOVERED FROM INFANTS WITH NEC

Although bloodstream infection develops in up to one-third of infants who develop NEC (79), it is unclear whether this is an effect of NEC, resulting from increased bacterial translocation from a disrupted intestinal barrier, or if bloodstream infection is involved in the pathogenesis of NEC. In these instances of co-occurrence of bacteremia with NEC, most bloodstream infections are caused by Gram-negative bacteria, predominantly by *E. coli* and *Klebsiella* (80). Further, the presence of late-bacteremia with NEC may increase the risk of adverse neurodevelopmental outcome, particularly for infants who require surgical treatment for NEC (81).

#### PROMOTING, PRESERVING, AND RESTORING HEALTHY GUT BACTERIA

##### Human milk feeding: promoting a beneficial gut microbiome

Breastfeeding is associated with a lower risk of NEC. Part of the protective effect of breast milk is likely mediated by prebiotics within breast milk as well as the presence of beneficial *Lactobacilli* and *Bifidobacteria* (82). The presence of anti-inflammatory cytokines such as interleukin-10 may also confer protection against NEC (83). Importantly, pasteurization of donor human milk may reduce these beneficial components in breast milk that influence the microbiota, although studies suggest that the use of donor human milk, when maternal breast milk is not available, may also lower the risk of NEC (84). Further, a single randomized trial demonstrates that the use of an exclusive human milk based diet with human milk derived fortifiers decreases the risk of surgical NEC (85), although additional confirmatory trials are necessary.

##### Limiting prolonged empiric antibiotic therapy: preserving a beneficial gut microbiome

Limiting the duration of prolonged empiric antibiotic therapy may help preserve a healthy quantity and balance of beneficial

commensal bacteria. In a national network of academic centers in the United States, the duration of empiric antibiotic therapy for culture-negative early onset sepsis varied widely with the median duration of empiric treatment ranging from 3 to 9.5 d at any particular center (86). Further, data from this same study demonstrated that the odds of NEC was 40% higher for infants receiving 7 vs. 2 d of empiric antibiotic therapy for culture-negative sepsis. This association between early empiric antibiotic use and NEC has been validated in additional observational cohort studies (63,64,75).

Given the relatively low incidence of culture-proven early-onset sepsis, which is estimated to occur in ~1% of infants with a birthweight of 401–1,500 g (87), reducing early antimicrobial exposure or duration of exposure may help reduce the risk of NEC. However, further studies are needed to determine better identification of neonates with a low probability of early-onset sepsis and develop tailored approaches to narrow the spectrum or duration of antibiotic exposure when treatment is necessary. Newer informatics approaches, including the use of clinical decision support (88), may be useful in reducing antibiotic exposure in the preterm population (89).

##### Therapeutic modification of intestinal microbiota with probiotics and prebiotics

Since abnormal gut microbiota is implicated in the pathogenesis of NEC, therapeutic modification of the gut microbiota is a logical approach to prevent NEC. Both prebiotics and probiotic bacteria can accomplish this goal. Therapeutic administration of probiotics can supply beneficial commensal strains that help protect the gut against intestinal inflammation and injury that may lead to NEC. Some of the mechanisms by which probiotics exert their beneficial effects include: (i) anti-inflammatory effects by attenuation of NF- $\kappa$ B activation (58); (ii) increases in cytoprotective gene responses (23,90); (iii) promotion of barrier function by induction of tight junction protein expression (9,91); (iv) generation of low levels of reactive oxygen species important in cell signaling (48,92); (v) prevention of apoptosis and cell death (90,93). Many different probiotic agents have been used to treat preterm infants (94), with clear efficacy in preventing NEC as demonstrated in a recent meta-analysis of 20 out of 24 randomized controlled trials enrolling ~5,500 neonates that demonstrate probiotics reduce both severe NEC (pooled relative risk (RR) 0.43; 95% CI 0.33–0.56) and all-cause mortality (RR 0.65; 95% CI 0.52–0.81) (95). The two most commonly used probiotic agents are from the genera *Bifidobacteria* and *Lactobacillus*. Both of these types of probiotics show similar efficacy in the prevention of NEC (96). Further, the aforementioned meta-analysis did not demonstrate any differences in effect by various subgroups of probiotic types, including *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, or combinations thereof, ( $P = 0.48$  for test of subgroup differences).

Centers that have implemented the use of probiotic therapy as standard-of-care have demonstrated similar reductions in NEC, as reported in recent observational cohort studies from Canada (97) and Germany (98). These studies support

the external validity of the results from the randomized trials. However, concerns regarding probiotic-associated sepsis and non-FDA regulated preparations have limited widespread use, although FDA-approved probiotic preparations are unlikely to be available in the near future (99). Importantly, a recent meta-analysis found no effect of probiotics on the risk of sepsis (pooled RR 0.92; 95% CI 0.81–1.04), although there was significant heterogeneity among included studies (95). Of note, these studies have varied in species of probiotic used, dose (colony forming units per day), duration of therapy, selection criteria, and study population, all of which may influence the generalizability of the findings. Although insufficiently studied in the prevention of NEC, prebiotics such as GOS and/or FOS have been shown to increase beneficial commensal bacteria, such as *Bifidobacteria*, and may be another option in restoring a healthy balance of gut microbiota (100). However, a recent meta-analysis demonstrated prebiotic supplementation had no effect on NEC (101). In addition, prebiotic supplementation may have limited effects for infants already receiving mother's milk that contains prebiotics. This is highlighted by a recent study demonstrating a lack of significant increases in beneficial *Bifidobacteria* among prebiotic supplemented infants who were receiving maternal breast milk (102). In addition, prebiotics differ substantially between donor human milk and maternal breast milk, with some oligosaccharide concentrations higher and others lower in maternal breast milk compared to donor human milk (103).

## CONCLUSION

In conclusion, intestinal microbiota can have both a protective and pathologic role in NEC. Achieving a healthy complement of commensal bacteria, potentially through interventions such as probiotic therapy, can promote and maintain a healthy composition of intestinal microbiota or shift the balance of intestinal microbiota from a pathogenic to protective complement. This in turn, may protect the preterm infant from gut inflammation and injury that can lead to the development of NEC.

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