Gut microbiota and the pathogenesis of necrotizing enterocolitis in preterm neonates

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Necrotizing enterocolitis (NEC) remains a devastating intestinal disease in preterm neonates. In this population, disruption of the gut microbiota development, mainly due to organ immaturity, antibiotic use and hospital microbial environment, plays a key role in the pathogenesis of NEC. This gut dysbiosis has been associated with opportunistic pathogens overgrowth, expression of virulence factors, altered metabolic functions and inflammatory dysregulated responses. In this review, we provide an updated summary of the host and gut microbiota interactions during the formative early life. We also explore the key determinants of gut dysbiosis in preterm neonates with NEC. Finally, we discuss the promising role of bacteriotherapy in the management of NEC, the aim being to shape or restore the beneficial gut bacterial communities.

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Human health is conditioned by an equilibrated beneficial gut microbiota that plays an essential role in nutrient digestion and metabolism, vitamin synthesis, immune tolerance and maturation of the intestinal mucosa [1]. The resident microbial community, referred to as the commensal microbiota, establishes a strong relationship with its human host through a dynamic process [2]. The resultant homeostasis plays an important role in resistance against colonization by pathogens and the overgrowth of opportunistic microorganisms [3]. In neonates, this relationship needs to develop and many factors including maternal microbiota, gestational age, mode of delivery, feeding, antibiotic exposure, gastric acidity and bile acids, will affect its delicate equilibrium, and thus its ability to promote health or to cause disease [4].

Alteration of the intestinal microbial composition, termed gut dysbiosis, has been associated with the occurrence of colorectal cancer [5], obesity [6], inflammatory bowel disease [7], Type 1 and Type 2 diabetes [8,9] and Clostridium difficile infection [10]. In preterm neonates, characterized by a functionally immature intestine [11], gut dysbiosis with a lower microbiota diversity has been linked to the occurrence of necrotizing enterocolitis (NEC) [12,13]. In this population, NEC is the most common and devastating gastrointestinal disease [14], with an estimated incidence of 1–3 per 1000 live births, and a prevalence between 4 and 11% in premature neonates with very low birth weight (<1500 g), the risk being inversely proportional to birth weight and gestational age [15]. Although this disease has been well described since the 1960s, it is still considered as emerging.
mainly due to the recent evolution of techniques in obstetrics and neonatology that have significantly improved the survival of increasingly premature newborns [14]. To date, mortality rate associated with NEC’s occurrence has reached 30%, and even higher in cases requiring surgery [16]. NEC, which is associated with extensive intestinal tissue necrosis and excessive inflammatory process in the context of a highly immunoreactive intestine, also extends its effects systemically, affecting distant organs such as the brain [17,18]. In fact, nearly 25% of the neonates recovering from NEC develop microcephaly and serious neurodevelopmental delays [19].

Relationships between the occurrence of NEC in preterm neonates and bacterial colonization were recognized by Sántulli et al. over three decades ago [20]. Additional observations noting outbreaks in neonatal intensive care units, finding *pneumatois intestinalis* in such cases, most likely representing submucosal gas produced by bacterial fermentation, and the fact that NEC could not be reproduced in germ-free animals, extended proofs of a microbial role in the pathogenesis of the disease [14]. Notably, NEC does not occur until at least 8–10 days postpartum in preterm neonates, the period when gut microbiota diversification and anaerobic bacteria colonization usually begin [14]. Moreover, the more premature one is at birth, the later the colonization process occurs with less diversity [21]. To date, multiple causative organisms have been definitively established, but none of them has been definitively established.

In this review we provide an updated summary of the host and gut microbiota interactions during the formative early life, highlighting the essential role of the gut microbiota in nutrient digestion and metabolism, vitamin synthesis, immune tolerance, as well as intestinal and brain development. Based on current research evidence, we also define the key determinants of gut dysbiosis in preterm neonates with NEC, in order to determine appropriate preventive measures. Finally, we discuss the promising role of bacteriotherapy in the management of NEC, the aim being to shape or restore the beneficial gut bacterial communities.

**Host & gut microbiota interactions in early life**

Human adult gut microbiota compounds tenfold more cells than the human body and 100-fold more genes than the human genome [22]. It is estimated to consist of up to 100 trillion microorganisms, comprising between 500 and 3000 different species [23]. Additionally, the microbial composition varies widely along the different regions of the gastrointestinal tract according to transit time, pH, exposure to oxygen, nutrient availability, host secretions (such as bile acids and digestive enzymes), mucosal surfaces and interactions with the immune system [24]. Current research provides evidence that commensal bacteria are involved in the regulation of multiple pathways, giving rise to interactive host–microbiota metabolic, signaling and immune-inflammatory processes connecting the intestine, liver, muscle and brain (Figure 1). The gut microbiota also synthesizes *de novo* molecules, such as vitamin K and constituents of vitamin B, that must be provided exogenously since the human host lacks the biosynthetic capacity for these [25].

- **Bacterial colonization**

  Establishing a core microbiota of diverse commensal species is critical and advantageous to the host as it provides competition with the pathogenic microbes [4].

  The early postnatal period, approximately the first week of life, is the most dynamic stage in the process of the intestinal microbiota establishment. Overall in this period, a variety of bacteria, mainly facultative anaerobes such as *Enterococcus spp.*, *Streptococcus spp.* and *Staphylococcus spp.*, transiently colonize the GI tract. This stage is considered essential for the establishment of reductive conditions favorable for colonization by strict anaerobes, including Bifidobacteria [28]. In a longitudinal study of a single preterm neonate born by cesarean delivery, nine stool samples taken during the third week of life showed a shift toward fermentation-based metabolism (obligate anaerobes), which overtook *Escherichia coli* as the most abundant microbial species [29]. After the first 3–5 years of life, a convergence to a relatively stable and common profile of bacterial communities has been described [30]. But studies investigating the colonization of the gastrointestinal tract showed high interindividual variability between neonates due to several factors that influence the bacterial composition, among which the geographical provenance and environment, making it difficult to define a ‘normal’ dynamic of colonization (Figure 2) [31,32]. In particular, dietary habits represent a major factor contributing to the diversity of the human gut microbiota [33],
Figure 1. Host and gut interactions in early life. (A) Commensal bacteria and metabolites like SCFAs play a role in intestinal mucosa (IM) integrity by facilitating the assembly of tight junctions and regeneration. Goblet cells produce mucin, a viscoelastic mucus gel layer that acts as a protective barrier against the harsh luminal environment. In response to bacterial antigens, Paneth cells release AMPs, such as REGIIIγ or α-defensins, into the lumen of intestinal crypts. Commensal bacteria induce IgA secretion from plasma cells within the gut lumen, protecting the mucosa from invasion by pathogens and reducing proinflammatory signals. Commensal bacteria may interact with the brain via cytokine release from mucosal immune cells, via the release of hormones, such as 5-HT, from enterochromaffin cells or via afferent neural pathways, including the vagus nerve (cholinergic anti-inflammatory system). (B) SCFAs are the result of fermentation of undigestible carbohydrates by commensal bacteria. A part of BAs escape the enterohepatic circulation and undergo microbial biotransformation in the large bowel to form the secondary BAs, DCA, and LCA. (C) Immune tolerance to commensal bacteria is in part mediated by TLRs. These detect MAMPs and promote accumulation of protective iTreg cells in the lamina propria and release of anti-inflammatory cytokines and regulating the adaptive immunity. Paneth cell-intrinsic MyD88 activation through TLR limits bacterial penetration of host tissues. Commensal bacteria can induce intestinal epithelial ROS and IK B production which both prevent NF-kB (proinflammatory transcription factor) activation. Secondary BA metabolites act as signaling molecules through the TGR5 and the FXR by inhibiting inflammation, preventing pathogen invasion and maintaining cell integrity. Macrophages include M1 forming part of the primary host defense, and M2 routinely repairing and maintaining tissue integrity [26]. (D) The gut–brain axis is a communication system that integrates neural, hormonal and immunological signaling between the gut microbiota and the brain [27]. BAs metabolism represent the link between gut and liver.

AMP: Antimicrobial protein; BA: Bile acid; DCA: Deoxycholic acid; FXR: Farnesoid X receptor; LCA: Lithocholic acid; MAMP: Microbial associated molecular pattern; ROS: Reactive oxygen species; SCFA: Short-chain fatty acids; TGR5: G-protein-coupled BA receptor.
explaining part of the variability associated with the geographical location [32]. Moreover, the nutrient requirement of individual commensals defines the composition and distribution of the microbiota throughout the gastrointestinal tract. The small intestine is rich in monosaccharides and disaccharides, as well as amino acids, which are used by the host and support the growth of certain bacteria, particularly proteobacteria and Lactobacillales. By contrast, the large intestine is enriched in Bacteroidetes and Clostridia, which can use, by fermentation, host-indigestible polysaccharides, including fibers and mucin, as energy sources [34]. For some specific species, this process allows to prevent damages by pathogens. For instance, *Bacteroides thetaiotaomicron* produces a mucin-derived fucose that modulates the virulence factor expression of a pathogenic *E. coli* [35].

### Intestinal mucosa

The commensal microbiota plays an important role in the development and function of the intestinal mucosa (IM), which in turn exert a control on the microbial composition. For instance, during the process of bacterial colonization there is an increased expression of genes that promote epithelial turnover and synthesis of mucus [36]. Commensal bacteria increase intestinal epithelial cell survival by inhibiting the activation of the epithelial cell proapoptotic pathway associated with pathogenic bacteria [37]. Commensal bacteria are also involved in maintenance of barrier function by enhancing intestinal epithelial integrity, through translocation of the tight junction proteins and upregulation of genes involved in desmosome maintenance (Figure 1) [38,39]. Fermentation products of commensal bacteria (i.e., butyrate) have been shown to enhance the intestinal barrier function by facilitating the assembly of tight junctions through the activation of AMP-activated protein kinase [40].

The host’s IM encourages colonization of commensal organisms by shifting its energy source in their favor, thus the commensals can gain control over the pathogenic species in the competitive intestinal ecosystem. For instance, fucosylation of intestinal epithelial glycans facilitates colonization by commensal species, for example, *B. thetaiotaomicron*, that use terminal fucose as an energy source [39]. Goblet cells in the IM produce mucin, a viscoelastic mucus gel layer that acts as a protective barrier against the harsh luminal environment by serving as a natural lubricant. This provides a mechanical protection and prevents epithelial damage by acidic secretions from the duodenum and stomach. It also

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**Figure 2. Factors influencing the gut microbiota development in early life.** The first 3 years of life represents the window of opportunity for interventions aimed at microbiota modulation to improve child health.
has a role in the binding of bacteria, enhancing the ability of commensal bacteria to colonize the intestinal tract while inhibiting the adherence of pathogenic ones (Figure 1) [41]. Mucin oligosaccharides are also a direct source of carbohydrates and peptides that can promote the growth of bacteria [42]. Germ-free animal experiments showed a series of anatomic, biochemical and physiologic perturbations (i.e., defect in villus capillary angiogenesis, dramatic enlargement of the cecum, mostly due to accumulation of degraded mucus, altered microvilli morphology and reduced rate of intestinal epithelial cells turnover) [39].

- Inflammatory-immune system

The single-cell epithelial layer of the IM confronts the largest antigenic microbial challenge of any other mucosal surface in the human body. Exposed to trillions of luminal microbes, the IM develops tolerance toward commensal bacteria by downregulating its innate immune system response [2,43]. Through a process of ‘crosstalk’ with the mucosal immune system, the microbiota negotiates mutual growth, survival and inflammatory control of the intestinal ecosystem [44]. One of the mechanisms of regulation of inflammatory signals is by activating IκB, the inhibitory component of NF-κB activation. Indeed, inflammatory or apoptotic responses to pathogenic bacteria, or stress signals, are controlled by NF-κB and caspase-dependent signaling [43].

The innate immune system, including dendritic cells as well as epithelial cells have mechanisms of recognition of bacterial products via a highly specialized ‘pattern recognition receptors’ called ‘Toll-like receptor’ (TLR), which have the ability to recognize specific microbial associated molecular patterns that include lipopolysaccharide, flagellin, peptidoglycans and formylated peptides [39]. This pathway causes differentially regulated responses to commensal or pathogenic bacteria. The recognition of commensal bacterial-derived molecules by TLRs represents a critical component of the homeostasis between the host and gut microbiota and is important for protection against intestinal injury (Figure 1) [42]. TLRs may also direct expression of the MyD88-dependent antimicrobial response, triggering expression of antimicrobial proteins that have been implicated in both establishing the barrier between the gut microbiota and the enterocytes, and in shaping the composition of the colonizing microbiota [45]. Paneth cell-intrinsic MyD88 activation also limits bacterial penetration of host tissues [46].

Moreover, continuous detection of commensal bacterial molecules promotes intestinal homeostasis by inducing the accumulation of microbe-induced iTregs in the lamina propria (Figure 1). These cells have an essential role in suppressing immune responses to environmental and food allergens, and restraining inflammation, by releasing anti-inflammatory cytokines (i.e., IL-7, TGF-β, IL-10) [47]. Additionally, it has been shown that commensal bacteria play a role in the upregulation of IL-25, which serves to repress expression of IL-23 and subsequent development of proinflammatory IL-17-producing Th17 CD4+ T cells [43].

Commensal bacteria can also induce intestinal epithelial reactive oxygen species production, which in turn can regulate homeostatic processes, preventing NF-κB activation through oxidative inactivation of its regulatory enzyme, Ubc12 [48]. A recent study has shown that symbiotic Lactobacillus spp. stimulate gut epithelial proliferation via NADPH oxidase 1 (Nox1)-mediated generation of reactive oxygen species [49].

Paneth cells release antimicrobial proteins (REGIIIγ, α-defensins) into the lumen of intestinal crypts [50]. In addition to exerting direct antimicrobial effects, defensins facilitate and amplify innate and adaptive immune responses, such as activation and degranulation of mast cells, cytokine production and secretion, maturation of dendritic cells and chemotaxis of immune cells [51].

Furthermore, commensal intestinal bacteria have also been shown to induce Immunoglobulins A secretion from plasma cells within the gut lumen, protecting the mucosa from invasion by pathogens and reducing proinflammatory signals [52]. Unlike other immunoglobulins, IgA does not participate in the inflammatory response and does not bind complement; instead, it serves as an immunologic barrier that inhibits uptake of antigens, without respect to antigenic specificity. This antibody forms complexes with antigens and bacteria in the gut lumen, thereby preventing their binding to the mucosal surface and subsequent absorption. Consequently, the presence of IgA within the gut lumen is important in maintaining gut barrier integrity [41].

The broad expression of recognition molecules by the innate immune system enables to promptly act after an invading pathogen or toxin.
Nutrient metabolism-like sequences were.

Bacterial composition & colonization

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such as SCFAs are primarily produced by Firmicutes. These resulting BA metabolites act as signaling molecules and regulate the intestinal homeostasis through the G-protein-coupled BA receptor (TGR5) and the farnesoid X receptor by inhibiting inflammation, preventing pathogen invasion and maintaining cell integrity [58].

Brain

The gut–brain axis is a bidirectional communication system. Enterochromaffin cells, neurons, immune cells are involved in this connection [59]. The brain is able to influence the gastrointestinal functions (such as motility, secretion and mucin production) as well as immune functions (including the modulation of cytokine production) [27]. Studies using behavioral tests of young germ-free animals have demonstrated the ability of the gut microbiota to influence brain development [60]. Epidemiological evidence links neurodevelopmental impairment in preterm neonates with NEC that is associated with gut dysbiosis [61]. Thereby, the notion of the gut–brain axis supports that intestinal microbiota can indirectly harm the brain of preterm infants (Figure 1).

Gut microbiota & the pathogenesis of necrotizing enterocolitis

Accumulating data suggest that disruption of the gut microbiota development and homeostasis plays a key role in the pathogenesis neonatal NEC in preterm neonates [14]. This gut dysbiosis is associated with a lack of beneficial commensal microbes, a low diversity of bacteria and an overgrowth of pathogenic bacteria [62].

Several factors influence gut dysbiosis and inappropriate inflammatory response leading to NEC; among them, prematurity and enteral nutrition are considered pivotal (Figure 3) [63].

Bacterial composition & colonization timing

The gut microbiota from preterm neonates with and without NEC both prior to and during the disease has recently been studied using high throughput culture-independent techniques. These studies suggested an association between gut dysbiosis and the occurrence of NEC. An overall reduction in bacterial diversity, especially when there has been a prolonged previous antibiotic therapy, was reported [64]. Changes in bacterial communities appear to occur 2–3 weeks prior to NEC [11]. In a comparative study, Citrobacter-like sequences were only detected in patients with NEC, whereas controls had an increased frequency of Klebsiella

Carbohydrates fermentation

The stomach and proximal small intestine are responsible for most nutrient digestion and absorption in humans. Between 10 and 30% of ingested dietary carbohydrates are resistant to small intestinal digestion and need to be digested by the colonic microbiota [54]. These nondigestible dietary carbohydrates are fermented by colonic bacteria to short-chain fatty acids (SCFAs), and gases such as CO₂, H₂ and CH₄ [54]. The major SCFAs produced as a result of carbohydrate and protein fermentation are acetate, propionate and butyrate. These SCFAs are primarily produced by Firmicutes such as Clostridium spp. and Bifidobacterium species [43]. Most of the absorbed SCFAs are then used as energy for the enterocytes [55]. Dihydrogen is excreted in the breath and/or oxidized by methanogenic archae, acetogens or sulfate reducing bacteria [56]. Additionally, SCFAs have immune-modulatory effects on colonic inflammation, facilitating intestinal mucosa tolerance to the presence of commensal microorganisms and controlling the overgrowth of pathogens [57].

Bile acids biotransformation

Bile acids (BAs) are essential for normal digestion and metabolism of lipids and cholesterol.

A part of BAs escapes the enterohepatic circulation and undergoes microbial biotransformation in the large bowel to form the secondary BAs, deoxycholic acid and lithocholic acid. These resulting BA metabolites act as signaling molecules and regulate the intestinal

is encountered and induces the adaptive immunity. Subtle differences exist between commensal bacteria, probiotic and pathogenic microorganisms with respect to the surface molecules that are present and their host interactions, determining the final inflammatory-immune response [53].

Compared with animals housed under conventional conditions, germ-free animals show extensive defects in the development of gut-associated lymphoid tissue and antibody production, fewer and less cellular lymphoid follicles (Peyer’s patches), a thinner and less cellular lamina propria and fewer plasma cells in germlinal centers of the mesenteric lymph nodes [52].

• Nutrient metabolism

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Figure 3. Perfect storms for developing necrotizing enterocolitis. A series of overlapping events contribute to the development and the pathogenesis of necrotizing enterocolitis.

NICU: Neonatal intensive care unit.

spp. sequences [65]. Mai et al. found that one of the bacterial signatures detected more frequently in NEC cases matched closest to \( \gamma \)-proteobacteria [12]. Norman et al. detected a higher Bacillales and Enterobacteriaceae relative abundance at early time points in stool samples from extremely preterm neonates who developed NEC, while healthy controls were more dominated by Enterococcus species [66]. Morrow et al. identified two distinct forms of dysbiosis (Firmicutes dominance during postnatal days 4–9 or Enterobacteriaceae dominance during postnatal days 10–16) associated with NEC together with a lack of Propionibacterium spp. and a lower diversity [13]. In this study, a metabolomic signature (i.e., high urinary alanine:histidine ratio) was predictive of NEC occurrence. While Millar et al. did not observe any difference [67], Stewart et al. found that the presence of Enterobacteriaceae and Staphylococcus spp. was associated with the development of NEC [68]. Sim et al. showed that two fecal microbiota signatures (Clostridium and Klebsiella) anticipated the onset of NEC in preterm neonates [69]. McMurtry et al. compared the gut microbiota of 21 preterm neonates with NEC categorized into three subgroups based on severity to 74 matched controls without NEC. They concluded that low bacterial diversity and the lack of Clostridia in lethal specimens were associated with NEC [70]. In another study using genome-resolved analysis, no strain was common to all infants who developed NEC [71]. Zhou et al. conducted a longitudinal analysis of 312 samples obtained from 12 NEC cases and 26 age-matched controls and found that the day of life was the major factor contributing to the colonization process. While in early onset the abundance of Clostridia was significantly higher, Escherichia/Shigella and Cronobacter among \( \gamma \)-proteobacteria showed an increasing and higher pattern prior to late onset NEC [72]. Finally, no single microbial pattern has been consistently identified as a risk factor predictive for NEC. Heterogeneity of results in these studies is likely due to the fact...
that molecular-methods identification induces several biases [33]. Moreover, the populations studied were heterogeneous. For instance, the wide range in age at the time of disease is a confounder in the microbiome comparison between NEC cases [72]. Further, variability in antibiotic therapy may be an important confounder in these studies. We found recently in a molecular and culture-based study that NEC was associated with cytotoxic Clostridium butyricum strains and dysbiosis with a poorly diversified gut microbiota [73]. Interestingly, the association between C. butyricum and NEC was confirmed in a larger population (n = 363) by testing stool samples with a specific quantitative real-time PCR. Promisingly, Claud et al. demonstrated a difference in microbial community structure by both taxonomy and function several weeks prior to NEC, suggesting a window of opportunity in which interventions to alter community structure could influence clinical outcome [21].

- Factors influencing the gut bacterial composition & NEC occurrence

In neonates, the gut microbiota fluctuates substantially and is more susceptible to environmental factors than the adult microbiota [31]. Composition of the neonate’s intestinal microbiota will depend on many factors. In preterm neonates, the neonatal intensive care unit environment, comorbid conditions, frequent indwelling catheters and invasive ventilation, extensive use of antibiotics and delayed oral feeding may induce a delayed colonization and reduced diversity of gut microbiota in preterm neonates [21]. The resultant gut dysbiosis leads to increased susceptibility to NEC.

Prenatal exposure

The presence of bacterial isolates and/or DNA, such as Enterobacteriaceae (including E. coli and Shigella spp.), Enterococcus spp., Staphylococcus spp. and Bifidobacterium spp., has been documented in meconium, amniotic fluid, fetal membranes, umbilical cord blood and placenta without signs of infection or inflammation [74]. Recently, DiGiulio et al. described from the amniotic cavity a greater diversity of microbes than previously suspected, including previously uncharacterized taxa [75]. Others showed that bacteria in the maternal mouth could reach the amniotic fluid via the bloodstream, particularly in the presence of gingivitis or periodontitis during pregnancy [76].

These findings suggest that fetuses are not sterile as previously thought and prenatal transmission of bacteria from mother to fetus exists in healthy pregnancies, influencing the gut microbiota composition.

Gestational age

Epidemiological evidence has established that prematurity is the most significant risk factor for development of NEC [65]. In preterm neonates, the dynamics of colonization are delayed, and the diversity is limited [21,77]. In this population, beneficial bacteria like Bifidobacterium spp. and Lactobacillus spp. are underrepresented during the first weeks of life [21]. Recently, Aujoulat et al. described bacterial colonization dynamics in consecutive stool samples of 30 very premature infants, by 16S rDNA-based PCR-Temperature Gel Electrophoresis. Gram-positive bacteria (Staphylococcus spp., Enterococcus spp. and Clostridium spp.) represented the quantitatively dominant microbiota established in the neonate gut during the first month of life, Staphylococcus species being the earliest colonizers found in a large majority of neonates. Gram-negative bacteria (Enterobacteriaceae and Veillonella spp.) established a delayed and inconstant colonization over the study period compared with Gram-positive bacteria. Preterm neonate’s microbiota has also been characterized by a lower presence of anaerobes [78]. Recently, La Rosa et al. demonstrated via 16S rRNA gene pyrosequencing of 922 samples from 58 preterm neonates residing in a microbiologically constrained ecosphere of a neonatal intensive care unit that their gut microbiota progresses through a well-synchronized succession of bacterial classes from Bacilli to γ-proteobacteria to Clostridia, interrupted by abrupt population changes. As neonates approach 33–36 weeks postconceptional age, the gut is well-colonized by anaerobes. Notably, external factors like antibiotics, mode of delivery, diet and age of the neonates when sampled did not influence the sequence of progression [79]. As NEC occurs after several weeks of age in the smallest preterm neonates compared with the first week for older preterm neonates, a role for a gestational age window of susceptibility to dysregulated inflammation in the timing of NEC has been suggested [80].

Intestinal immaturity & inflammation

In preterm neonates, the intestinal immaturity is associated with a delayed colonization by
beneficial commensal bacteria [21]. Functional immaturity is characterized by smaller amounts of gastric acid and mucus production, increasing intestinal barrier permeability and reducing peristaltic activity. In addition, the immune system immaturity is characterized by deficiency in local antibacterial products (i.e., IgA, defensins) and an alteration of immune cells [41]. All of these conditions promote intestinal inflammation that can in turn disrupt colonization resistance, alter microbiota composition and foster pathogen growth [3].

Delivery mode
Great differences have been shown between the microbial colonization of cesarean-delivered (CD) and vaginally-delivered (VD) neonates [81]. Dominguez-Bello et al. compared the fecal microbiota of neonates within 24 h of delivery with the microbiota of their mother’s skin, oral mucosa and vagina by 16S rDNA pyrosequencing [82]. Neonates born vaginally were colonized predominantly by Lactobacillus spp., Bifidobacterium spp. and Prevotella spp. after contact with mother’s vaginal and intestinal flora, whereas CD neonates were colonized by potentially pathogenic bacteria typically found on the skin and in the environment, such as Staphylococcus spp. and Acinetobacter species. Another study showed lower biodiversity in the stools from CD neonates at three days of age, harboring less Bifidobacterium spp. and Bacteroides spp. compared with neonates born vaginally [83]. Penders et al. showed that at one month of age, CD neonates had lower numbers of Bifidobacterium spp. and Bacteroides fragilis group, and were more often colonized with Clostridium difficile compared with VD infants [84]. However, delivery mode has little effect on the development of the premature neonate gut microbiota and has not been clearly associated with the occurrence of NEC [4].

Environment
The preterm neonate acquires its microbiota within the confines of the neonatal intensive care unit (NICU) where colonization is significantly influenced by iatrogenic manipulations including the hospital environment. In their study, Brooks et al. collected fecal samples from neonates together with environmental samples from around the NICU. The main species of bacteria they found in the neonates’ gut (Staphylococcus epidermidis, Klebsiella pneumoniae, Bacteroides fragilis and E. coli) were found throughout the NICU, suggesting the hospital environment may have been the source of these microbes [85]. Additionally, the perinatal determinant influencing Clostridia colonization appeared to be the NICU in the study by Ferraris et al. [86]. The fact that clostridial colonization incidence increased during the hospitalization suggested a colonization from the environment. This is consistent with the occurrence of NEC outbreaks and the effectiveness of implemented infection control measures [87]. Interestingly, Schmidt et al. showed that the establishment and development of the normal gut microbiota requires continuous microbial exposure during the early stages of life and this process is compromised under conditions of excessive hygiene [88].

Feeding
Feeding mode is one of the most important determinants of gut microbial diversity. Most NEC cases develop after enteral feeding has been initiated. This is consistent with the implicated role of an altered and proliferating gut microbiota that depends on luminal substrate. A recent review on this topic concluded that feeding with formula compared with donor breast milk results in a higher rate of short-term growth but also a higher risk of developing NEC [89]. It has been described that breast-fed infants have more diverse bacterial population than formula-fed infants with lower numbers of pathogenic microorganisms [84]. Such differences in microbial composition between breast-fed and formula-fed neonates may reflect the effects of the large quantity of structurally diverse human milk oligosaccharides, which are negligible in bovine milk and infant formula and stimulate the growth of specific gut bacteria, including Bifidobacterium spp. and Bacteroides species [90]. It has been showed that breast-fed neonates have predominance of Firmicutes mainly Lactobacillus spp., Bacteroides spp. and Bifidobacterium spp. compared with formula-fed that have predominance of proteobacteria and Firmicutes some with pathogenic characteristics, such as Clostridia and Staphylococci [84,91]. While human milk contains complex nutrients, it also harbors many immune components and a recently described microbiome (10^3–10^4 colony-forming units of select species per ml of human milk) [23]. For example, the glycoprotein lactoferrin, involved in innate immune host defenses, is found in significant concentrations
Antibiotic exposure

Perinatal exposure to antibiotics has been identified as independent risk factor for NEC occurrence, this risk being proportional to the duration of postnatal exposure [14].

Cotten et al. showed that prolonged duration of initial empirical antibiotic treatment is associated with increased rates of NEC and death for extremely low birth weight infants [94]. Using terminal restriction fragment length polymorphism (T-RFLP) analysis and qPCR, Tanaka et al. monitored the impact of broad-spectrum antibiotic exposure in the first four days of life on the development of intestinal microbiota [95]. Antibiotic-treated neonates had reduced overall diversity, with a specific reduction of Bifidobacterium spp. and overgrowth of Enterococcus spp. and Enterobacteriaceae in the first month of life compared with untreated neonates. Similar alterations were described in patients whose mother received the same antibiotic in the prenatal period [95]. Another recent study showed that early empiric ampicillin and gentamicin use in preterm neonates was associated with lower bacterial diversity and higher relative abundance of Enterobacter species. This dysbiosis was associated with a higher risk of NEC [96]. Schumann et al. showed in an animal model that neonatal antibiotic treatment alters the establishment of an efficient barrier to luminal antigens and bacterial colonization [97]. In this study, daily intragastric amoxicillin resulted in a significant reduction of Lactobacillus spp. associated with a dramatic reduction of colonic total aerobic and anaerobic bacteria, in particular Enterobacteria and Enterococci. This affected developmental genes expression including those involved in the innate immunity. Accordingly, the diminished colonization resistance allows pathogens overgrowth and expression of virulence factors [98]. Overall, specific antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric pathogens [99].

Oxidative stress

There is a critical balance between free radical generation and antioxidant defenses. Oxidative stress (OS) is caused by an imbalance between the production of free radicals (i.e., reactive oxygen species) and the ability of antioxidant system to detoxify them. Oxidative stress can occur early in pregnancy and continue in the postnatal period. Perrone et al. measured non-protein bound iron and total hydroperoxides in cord blood and showed that the determination of OS biomarkers was useful in identifying babies at high risk for NEC [102]. Recently, we showed that the occurrence of NEC was associated with an oxidized and acid gut microbiota when compared with age-matched controls [73].

Host genetics

Palmer et al. found that the gut microbiota was more similar in dizygotic twins than in unrelated children at any stage of development over the first year of life [30]. By contrast, Turnbaugh et al. reported that the similarity of fecal microbiota, in monozygotic twin pairs was not different from that in dizygotic twin pairs as assessed by next-generation16S rDNA pyrosequencing at a single point in time [6]. Another study provided clear evidence for the importance of host-genetic control in shaping individual microbiome diversity in a mouse model [103]. Further studies are needed to elucidate the role that host genetics may play in the selection and colonization of gut microbes in preterm neonates.

- Immunological & inflammatory dysregulated responses

Several aspects of immature intestinal function in preterm infants may contribute to NEC predisposition, including the inflammatory propensity of the immature gut, decreased intestinal barrier function and impaired intestinal immune defenses [62].

In a cultured human enterocyte model, commensal bacteria as well as pathogens have been
shown to evoke an excessive inflammatory response in preterm neonate’s enterocytes as compared with mature enterocytes [104]. This difference appears to be mediated by a developmental immaturity in the expression of IkB, the molecule that inhibits the activation of cytokines by transcription factor NF-κB.

In animal NEC models, the expression of known proinflammatory cytokines including IFN-γ, TNF-α, MIP-2, IL-1, IL-6, IL-8 and IL-12 have been shown to be increased [48]. Activation of the IL-10 pathway negatively regulates the expression of these cytokines [109]. Indeed, IL-10 deficient mice are more susceptible to NEC [106].

While at low levels, nitric oxid (NO) acts as a local vasodilator that improves mucosal blood flow, sustained upregulation of NO and its oxidative by-products have been shown to cause direct epithelial injury and to promote epithelial cell apoptosis [41].

Among the consequences of this inflammatory response are excessive apoptosis of epithelial cells, disruption of protein connections, increased permeability of the mucosa, bacterial translocation, changes in vascular tone and the microcirculation, as well as infiltration and accumulation of neutrophils [41].

- **Altered metabolic functions**

**Carbohydrates fermentation**

The association between enteral feeding and NEC occurrence has been related to insufficient digestive capacity of the premature intestine. Implication of bacteria is in part thought to be due to fermentation of nonhydrolyzed lactose, a consequence of the immaturity of the intestinal lactasic equipment in preterm neonates. There is increasing recognition that excessive production and accumulation of SCFAs due to bacterial fermentation of undigested polysaccharides contributes to the pathogenesis of NEC [107]. Indeed, *pneumatosis intestinalis* (gas in the bowel wall), which is pathognomonic of necrotizing enterocolitis may reflect the gas production (CO₂, CH₄, H₂) by the fermentation of carbohydrates like lactose [108]. The resulting gut distension compromises vascular perfusion of the tissue, leading to ischemia and hypoxia, which contribute to the pathological events leading to NEC [109]. Additionally, fermentation produces SCFAs such as acetate and butyrate that could directly cause damage to the intestinal mucosa [107,110]. Moreover, increased breath hydrogen excretion (an indicator of bacterial fermentation and an indirect measurement of SCFAs production) was found in NEC patients even prior to the onset of clinical symptoms [111].

Birds, unlike newborn mammals, do not have endogenous intestinal lactase, a situation that mimics the lactase deficiency of premature neonates. Szylit et al. used germ-free quails infected only with *C. butyricum* to develop a suitable experimental model to study the enteropathogenicity of this species. Lactose fermentation into butyric acid and hydrogen appeared to be a prerequisite to the caecal NEC-like lesions and removal of dietary lactose suppressed all mucosal damage [112]. However, most of these results should be considered cautiously because the translation of findings based on animal models to humans is hazardous [53].

**Bile acids biotransformation**

Abnormally increased concentrations of hydrophobic secondary BAs are cytotoxic, causing DNA damage and cell death through the likely mechanism of induction of oxidative stress [113]. Using a neonatal rat model of necrotizing enterocolitis, Halpern et al. recently showed that BAs accumulate in both the ileal lumen and enterocytes of neonatal rats with NEC and the increased BA levels were positively correlated with disease severity [114]. In addition, BA transporters are altered during experimental NEC development leading to a higher and more hydrophobic intraenterocyte BA levels [115]. This strongly suggests that the abnormal accumulation of BAs and of its metabolites, in part linked to gut dysbiosis, play a role in the development of ileal damage in experimental NEC.

- **Bacterial translocation**

Bacterial translocation has been suggested as one of the mechanisms contributing to the pathogenesis of NEC [116]. Bacterial translocation is not only restricted to the invasion of intestinal bacteria but can also include bacterial toxins or antigens that damage the intestinal epithelia and enter the circulation resulting in a systemic inflammatory response [116]. Kansagra et al. demonstrated that the intestinal barrier function was significantly less developed in full term newborn piglets receiving total parental nutrition compared with those receiving enteral nutrition [117]. During inflammation, production of NO alters the expression and localization of the tight junction. Disruption of the tight junctions...
leads to intestinal permeability and bacterial translocation [118].

**Bacterial overgrowth**

Bacterial overgrowth has been related to the occurrence of NEC [63]. It may result from factors such as intestinal dysmotility, reduced digestive function and immature immunological responses to microbial colonization, all of which are characteristics of the premature gut. Stasis of the intestinal contents and diet malabsorption triggers the overgrowth and fermentation by commensal and pathogenic bacteria, thereby overriding the weak protective factors of the immature mucosal epithelium [119,120].

**Bacterial virulence factors**

**Enterotoxins**

Evidence is available to support toxin-mediated injury in the pathogenesis of some cases of NEC. For instance, *C. perfringens* [69], *C. difficile* [121] and *C. butyricum* [122] have been implicated in several epidemics knowing that asymptomatic carriage of toxigenic strains is not uncommon. While knowledge about enterotoxins produced by *C. perfringens* and *C. difficile* is extended, it is less documented for *C. butyricum*. Genome sequencing allowed the identification of various toxin genes (i.e., enterotoxin [OA81_00270], hemolysins) present on *C. butyricum* strains [29,73]. Cushing et al. described two clusters of NEC associated with toxigenic *Escherichia coli* in which case patients had free heat-labile enterotoxin detected in stools by ELISA concomitantly with the bacteriae [123]. Additionally, Scheifele et al. described in their NICU an association between the presence of toxigenic Coagulase-Negative *Staphylococcus* in stool samples and the occurrence of NEC in preterm neonates [124]. The enterotoxin identified was a Delta-like toxin prone to damage a variety of cell types as a result of its detergent-like action on cell membranes. It was reported to cause dose-dependent damage to the bowels of guinea pigs, suggesting its enteropathogenicity [125]. Another clinical report identified delta toxin-producing methicillin-resistant *Staphylococcus aureus* in stool and blood samples of three patients with NEC [126]. A role for bacterial toxins in the pathogenesis of NEC is then plausible, but supporting evidence is still limited.

**TLR ligands**

TLRs play key roles in the induction of innate inflammatory cytokines secretion and also to regulate adaptive immune responses. TLR-4, a receptor on immune cells and epithelial cells, recognizes several virulence factors on pathogens (i.e., lipopolysaccharide present in Gram-negative bacteria; surface layer protein described on *C. difficile*) [127]. The important role of TLR-4 in the pathogenesis of NEC has been shown by Leaphart et al., who demonstrated that TLR-4 knockout mice were protected from the development of NEC [128]. By contrast, TLR-9 knockout intestinal epithelial cells show a reduced NF-κB activation threshold, and TLR-9 knockout mice are highly susceptible to colitis and NEC [129]. Therefore specific TLR ligands present in bacterial surface are potential virulence factors.

**Role of specific microorganisms**

A broad variety of bacterial, viral and fungal species have been implicated in both clinical and experimental NEC. Cases in preterm neonates most often occur sporadically, with no clear seasonal pattern, but outbreaks have also been described [87]. Moreover, several reports have pointed to the effectiveness of implemented infection control measures in reducing the incidence of NEC [87]. Here we summarize the microorganisms isolated in preterm neonates with NEC, highlighting those for which outbreaks were reported (Table 1). The fact that the same microorganisms could be frequently found in healthy preterm neonates lead to hypothesize an opportunistic role of suspected pathogens. Moreover, studies examining the role of some suspected NEC pathogens demonstrated that the ability to cause epithelial damage in NEC models was not a property of bacterial species as a whole, but rather a characteristic of specific strains. Strain-specific virulence may explain why the same microbial species could be symbiotic or pathogenic. In a recent study, we found that the presence of specific *C. butyricum* strains in an oxidized, acid and abnormally poorly diversified gut microbiota was linked to necrotizing enterocolitis [73]. Detailed knowledge about specific strains that can act as NEC opportunistic pathogens may lead to better diagnostics and pathogen-tailored antibiotic therapies.

**Prevention & therapeutic perspectives**

Although NEC is the most common and serious gastrointestinal disorder in preterm neonates, there is still no well-established prevention strategy or treatment for this disease [63]. While there is evidence that human milk prevents the...
Table 1. Microorganisms identified in preterm neonates with necrotizing enterocolitis.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Microorganisms</th>
<th>Outbreak</th>
<th>Positive patients; n (%)</th>
<th>Identification</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birenbaum (1997)</td>
<td>Echovirus type 22</td>
<td>Yes</td>
<td>19</td>
<td>Stool cultures, virus typing</td>
<td>[131]</td>
</tr>
<tr>
<td>Rotbart (1988)</td>
<td>Rotavirus</td>
<td>Yes</td>
<td>11 (73)</td>
<td>ELISA on stool samples</td>
<td>[132]</td>
</tr>
<tr>
<td>Herruzo (2009)</td>
<td>Rotavirus P4G2</td>
<td>Yes</td>
<td>11</td>
<td>Immunochromatography</td>
<td>[133]</td>
</tr>
<tr>
<td>Stuart (2010)</td>
<td>Norovirus Gil.3</td>
<td>Yes</td>
<td>4 (40)</td>
<td>PCR-RNA</td>
<td>[134]</td>
</tr>
<tr>
<td>Turchios-Ruiz (2008)</td>
<td>Norovirus</td>
<td>Yes</td>
<td>8</td>
<td>qPCR</td>
<td>[135]</td>
</tr>
<tr>
<td>Lodha (2005)</td>
<td>Torovirus</td>
<td>No</td>
<td>27 (61)</td>
<td>Stool cultures</td>
<td>[137]</td>
</tr>
<tr>
<td>Johnson (1977)</td>
<td>Coxsakie B2</td>
<td>No</td>
<td>1</td>
<td>Stool cultures</td>
<td>[138]</td>
</tr>
<tr>
<td><strong>Enterobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Speer (1976)</td>
<td><em>Escherichia coli</em></td>
<td>Yes</td>
<td>8</td>
<td>Stool cultures, blood cultures</td>
<td>[140]</td>
</tr>
<tr>
<td>Cushing (1983)</td>
<td><em>Escherichia coli</em> with heat-labile toxin</td>
<td>Yes</td>
<td>15 (71)</td>
<td>Stool cultures, ELISA (toxin)</td>
<td>[123]</td>
</tr>
<tr>
<td>Powell (1980)</td>
<td><em>Enterobacter cloacae</em> type 3305573</td>
<td>Yes</td>
<td>12</td>
<td>Stool cultures, blood cultures</td>
<td>[141]</td>
</tr>
<tr>
<td>Van Acker (2001)</td>
<td><em>Enterobacter sakazakii</em></td>
<td>Yes</td>
<td>6 (50)</td>
<td>Stomach aspirate, anal swab and/or blood sample</td>
<td>[142]</td>
</tr>
<tr>
<td>Gregersen (1999)</td>
<td><em>Klebsiella pneumoniae</em> with ESBL</td>
<td>Yes</td>
<td>6</td>
<td>Blood cultures</td>
<td>[143]</td>
</tr>
<tr>
<td>Mshvildadze (2010)</td>
<td><em>Citrobacter</em> spp.</td>
<td>No</td>
<td>3 (75)</td>
<td>Stool cultures, 16S rRNA PCR</td>
<td>[65]</td>
</tr>
<tr>
<td>Guner (2009)</td>
<td><em>E. coli</em> O157:H7</td>
<td>No</td>
<td>1</td>
<td>Blood cultures, stool cultures</td>
<td>[144]</td>
</tr>
<tr>
<td><strong>Clostridia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dittmar (2008)</td>
<td><em>Clostridium perfringens</em> (type A α-toxins)</td>
<td>No</td>
<td>9 (22)</td>
<td>Peritoneal culture, blood cultures, stool cultures, PCR, ELISA (α-toxin)</td>
<td>[146]</td>
</tr>
<tr>
<td>Kosloske (1985)</td>
<td><em>Clostridium perfringens</em></td>
<td>No</td>
<td>9</td>
<td>Blood cultures and peritoneal fluid</td>
<td>[147]</td>
</tr>
<tr>
<td>Sim (2014)</td>
<td><em>Clostridium perfringens</em> (type A β2-toxin gene)</td>
<td>No</td>
<td>4 (33)</td>
<td>Stool culture on selective media</td>
<td>[69]</td>
</tr>
<tr>
<td>Han (1983)</td>
<td><em>Clostridium difficile</em></td>
<td>Yes</td>
<td>13</td>
<td>Stool cytotoxin detection</td>
<td>[121]</td>
</tr>
<tr>
<td>Alfa (2002)</td>
<td><em>Clostridium neonatale</em></td>
<td>Yes</td>
<td>3 (37)</td>
<td>Blood cultures, stool cultures</td>
<td>[148]</td>
</tr>
<tr>
<td>Howard (1977)</td>
<td><em>Clostridium butyricum</em></td>
<td>Yes</td>
<td>9 (90)</td>
<td>Blood cultures, stool cultures, gaseous chromatography</td>
<td>[122]</td>
</tr>
<tr>
<td>Sturm (1980)</td>
<td><em>Clostridium butyricum</em></td>
<td>No</td>
<td>1</td>
<td>CSF, peritoneal fluid</td>
<td>[149]</td>
</tr>
<tr>
<td>Cassir (2015)</td>
<td><em>Clostridium butyricum</em></td>
<td>No</td>
<td>15 (100)</td>
<td>Stool cultures, 16S rRNA pyrosequencing, qPCR</td>
<td>[73]</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overturf (1990)</td>
<td><em>Staphylococcus aureus</em> (with δ-toxin)</td>
<td>Yes</td>
<td>3</td>
<td>Stool cultures, blood cultures, WB (δ-toxin)</td>
<td>[126]</td>
</tr>
<tr>
<td>Adler (2005)</td>
<td><em>Bacillus</em> spp.</td>
<td>Yes</td>
<td>4</td>
<td>Blood cultures</td>
<td>[150]</td>
</tr>
<tr>
<td>Scheifele (1987)</td>
<td>Coagulase-negative <em>Staphylococcus</em> (with δ-toxin)</td>
<td>No</td>
<td>10 (23)</td>
<td>Stool cultures, ELISA (δ-toxin)</td>
<td>[124]</td>
</tr>
</tbody>
</table>

We mention whether or not the isolation was part of an outbreak and the tool of identification.

CSF: Cerebrospinal fluid; ESBL: Extended-spectrum β-lactamases; qPCR: Real-time PCR; WB: Western-blot.

occurrence of NEC [151,152], the optimal way to feeding preterm neonates remains unclear. Recent meta-analyses have shown that delayed introduction or progressive enteral feeds did not prevent NEC [153,154]. Premji et al. reviewed data from seven trials in 571 infants on the effectiveness of continuous feedings in preterm neonates and concluded that there was no effect in the incidence of NEC [155]. However, promising recent studies suggest an opportunity for improvement in the management of NEC.

- **Lactoferrin**
  Lactoferrin, an iron-binding glycoprotein is suggested to be an important factor providing protective barrier and stimulating the innate
immune system [92]. In a recent double-blind placebo-controlled randomized trial conducted at 13 tertiary care NICUs involving 743 preterm neonates (<1500 g at birth) who were given bovine lactoferrin (BLF) and BLF plus Lactobacillus rehmannii GG (LGG) from birth to 30 days of life, Manzoni et al. concluded that compared with placebo, BLF supplementation alone or in combination with LGG reduced the incidence and death of NEC (Bell’s stage II or III) [156].

● **Bacteriotherapy**

A prebiotic is defined as a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota. It has been reported that supplementation of infant formula with prebiotics affected the composition of the gut microbiota. For example, the administration of infant formula milk containing short-chain galactooligosaccharides and long-chain fructooligosaccharides was associated with an increase in fecal Bifidobacteria and Lactobacilli loads [157]. However, a recent meta-analysis including both formula and breast-fed preterm neonates demonstrated that prebiotic supplementation had no effect on NEC [158]. Prebiotics may represent a complementary option in formula-fed preterm neonates when human milk is not available, contributing to restore a healthy balance of gut microbiota.

Probiotics are defined as living bacteria that confer a health benefit to the host. The two most commonly used probiotic agents are from the genera Bifidobacteria and Lactobacilli. It has been shown that specific species with probiotic properties can augment host intestinal defenses by promoting cyto-protective gene expression and anti-inflammatory signaling, blocking inflammatory signaling and improving gut barrier function [48]. A recent meta-analysis concluded that enteral probiotic supplementation to preterm neonates, slightly but significantly reduced the incidence of severe NEC and mortality [159]. However, clinical guidelines from the American Society for Parenteral and Enteral Nutrition among others did not support the use of probiotics in infants at risk for NEC [160].

The incomplete effectiveness of single strains or mixture of probiotics in preventing NEC may be partly explained by the persistence of gut dysbiosis. Following on the probiotics principle, but on a community rather than on strain level, fecal microbiota transplantation (FMT) is the process of transplanting fecal bacterial communities from a healthy individual to a recipient whose microbiota has been disrupted or altered. To date, FMT has most commonly been used to treat recurrent *Clostridium difficile* infection by replacing populations of commensal bacteria that have been wiped out by antibiotic therapy. Interestingly, recent reports demonstrated that FMT could successfully be applied for relapsing *Clostridium difficile* infection in young children [161,162]. However, the variability of donor commensal populations and the potential presence of hazardous microbes needs caution, especially in a fragile population such as preterm neonates. Thus, defined combinations of protective commensals that restrain the growth of gut pathogens should be evaluated in future trials.

**Conclusion**

Commensal bacteria foster a healthy intestinal microbiota; these resident bacteria are vital in protecting the host from pathogens. Preterm neonates have low bacterial diversity due to their immaturity, hospital environment and necessary medical care. This resultant gut dysbiosis favors the overgrowth of opportunistic pathogens and excessive inflammatory reaction. Bacteriotherapy represents a promising path for the prevention and/or treatment of NEC, as it may be able to restore beneficial bacteria that are essential for normal development and function of the digestive tract together with an enhancement of bacterial diversity. Such therapeutic intervention aimed to improve the adequate colonization of the neonatal gut microbiota, will certainly need to be associated with a reduction of factors leading to gut dysbiosis including formula-feeding, antibiotic exposure, H2-blockers administration.

**Future perspective**

Modern microbiology including culture-based methods is currently extending our knowledge on gut microbiota. Future studies would enable to refine the characteristics of the dysbiosis and its determinants associated with NEC; the aim being to identify the best window of opportunity in which interventions to shape the gut bacterial communities could influence clinical outcome. Biomarkers including microbial and metabolomics signatures represent a promising
tool for accurate and timely prediction of NEC and selection of appropriate therapy. Knowing that specific gut microbiota changes are associated with specific antibiotics [33] and that prolonged empirical antibiotherapy increases the risk of NEC, it would be critical to determine which antibiotics would be effective as part of the treatment of NEC. Further studies are needed to determine the most effective type(s) of probiotic(s), dosage, time and duration of administration to reduce the incidence of NEC. Although it should be cautiously applied, we hypothesize a future place for fecal bacteriotherapy in the management of severe NEC. An interesting development in the application of bacteriotherapy would be the use of synthetic microbial communities in place of undefined mixtures from donors.

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EXECUTIVE SUMMARY

Host & gut microbiota in early life
• Human health is conditioned by an equilibrated beneficial gut microbiota that plays an essential role in nutrient digestion and metabolism, vitamin synthesis, immune tolerance, maturation of the intestinal mucosa, brain development and resistance against colonization by pathogens.
• The first week of life is the most dynamic stage in the process of the intestinal microbiota establishment, after which reductive conditions are favorable for colonization by strict anaerobes.
• After the first 3 years of life, there is convergence of gut bacteria communities to a relatively stable common profile with increased diversity, found in the adult digestive tract.

Gut microbiota & the pathogenesis of necrotizing enterocolitis
• Necrotizing enterocolitis is the most common and devastating gastrointestinal disease in preterm neonates; it is an emerging disease with an estimated incidence of 1–3 per 1000 live births, and prevalence between 4 and 11% in premature neonates with very low birth weight (<1500 g).
• Gut dysbiosis plays an important role in the pathogenesis of necrotizing enterocolitis; it is associated with an overgrowth of pathogenic bacteria, expression of virulence factors, altered metabolic functions and inflammatory dysregulated responses.
• Factors leading to gut dysbiosis include prematurity, formula feeding, delayed oral feeding, antibiotic exposure and antimicrobial exposure.

Prevention of necrotizing enterocolitis & therapeutic perspectives
• Along with the reduction of factors leading to gut dysbiosis, bacteriotherapy represents a promising path for the management of necrotizing enterocolitis.

References
Papers of special note have been highlighted as: • of interest; ** of considerable interest
REVIEW


Study showing that biomarkers including microbial and metabolomics signatures would represent promising tools for accurate and timely prediction of necrotizing enterocolitis (NEC) and selection of appropriate therapy.


• Review exploring the gut–brain axis.


• Study revealing that chaotic shifts in the microbiome are associated with life events that shape the gut microbiota composition.


Gut microbiota & the pathogenesis of necrotizing enterocolitis in preterm neonates

**Review**


**Study providing the epidemiological link between NEC and neurodevelopmental impairments.**


**Study combining molecular and culture-based methods that reports an association between NEC, cytotoxic *C. butyricum* strains and dysbiosis with an oxidized, acid and poorly diversified gut microbiota.**


**Study showing evidence that human gut is not sterile at birth. Bacterial colonization begins in the prenatal period.**


80 Sharma R, Hudak MI, Tepas JJ et al. Impact of gestational age on the clinical presentation and surgical outcome of necrotizing
This study reports that vaginally delivered infants acquired bacterial communities resembling their own mother’s vaginal microbiota, dominated by Lactobacillus, Prevotella or Sneathia spp., and C-section infants harbored bacterial communities similar to those found on the skin surface, dominated by Staphylococcus, Corynebacterium and Propionibacterium spp.


Study showing the impact of the use of broad-spectrum antibiotics in neonatal intensive care units on the gut bacterial colonization that may impair the maturation of the gut barrier function via modulation of developmental gene expression.


Gut microbiota & the pathogenesis of necrotizing enterocolitis in preterm neonates

149 Sturm R, Staneck JL, Stauffer LR, Nebelt WW 3rd. Neonatal necrotizing...
enterocolitis associated with penicillin-resistant, toxigenic *Clostridium butyricum*. *Pediatrics* 66(6), 928–931 (1980).


