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Intestinal colonization in premature and very low birth weight infants: Influencing factors and necrotizing enterocolitis (NEC)

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Contents

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Abstract

The intestinal microbiota has been recognized to influence metabolic, immune system and neurological development through host-microbe interactions. Preterm infants show aberrant intestinal colonization compared to infants born at term, implicated in development of gastrointestinal disorders, including necrotizing enterocolitis (NEC). Since the last decade interest for strategies to modulate the microbiota establishment in preterm infants has increased, in order to reduce the high morbidity and mortality among these infants. In order to modulate intestinal colonization, the regular colonization pattern and factors influencing this should be investigated in infants born preterm. The aim of this review is to describe associations between intestinal colonization and gestational age, delivery mode, antibiotic use, and type of nutrition. Compared to infants born at term, an increased proportion of preterm infants are born via caesarean section (CS) instead of vaginally, treated with broad-spectrum antibiotics, and formula-fed instead of human milk-fed. These factors negatively affect the establishment of a healthy intestinal microbiota in term infants and are believed to do the same in preterm infants. Indeed, this review shows that low gestational age and the use of broad-spectrum antibiotics induce an aberrant intestinal microbiota. Interestingly, intestinal microbiota does not seem significantly different between CS and vaginally delivered preterm infants and between formula-fed and human milk-fed preterm infants. However, preterm animal models demonstrate a clear difference due to delivery mode and type of nutrition in premature cases. Since above mentioned factors are involved in intestinal colonization, which is implicated in NEC development, they also have an effect on NEC. Several factors could possibly be modulated to decrease morbidity and mortality in preterm infants.

KEYWORDS: preterm infants, intestinal microbiota, premature, delivery mode, antibiotics, human milk, formula, necrotizing enterocolitis

Introduction

Importance of healthy microbiota development in early life

It is believed that before birth the fetal intestines are sterile and that colonization starts immediately from delivery[1]. Establishment of a healthy commensal microbiota, one that is relatively stable and aids in maintaining homeostasis in the intestines and immune system under common environmental changes, takes several years[1]. Intestinal bacteria play a role in our digestive system by degrading otherwise un-degradable food products[1]. Each bacterium uses specific substrates for growth, either from the host's diet or from intestinal origin such as mucus[1]. During degradation several products are formed among which vitamins and short chain fatty acids (SCFA)[1,2]. SCFA are beneficial to the host because they can be taken up by intestinal cells as energy source or as a signaling molecule. SCFA are not only involved in digestion, but also in host metabolism and functioning of the immune system, by affecting activity of receptors, histon deacetylases

or autophagy[2]. Another role for commensal bacteria is to provide colonization resistance by potentially pathogenic bacteria through secretion of antimicrobial products or occupying adhesion sites[3,4]. For example, Bifidobacteria have been shown to strongly inhibit several possible pathogenic Gram-negative bacteria ex vivo, such as *Escherichia coli* and *Salmonella* spp., by lowering the pH and producing organic acids[3]. Indeed, Bifidobacteria are considered beneficial for the human intestines, as well as Lactobacilli[1]. Other bacteria, including *Staphylococcus* spp. and several *Clostridium* spp. *(C. difficile, C. perfringens)* are considered to be potentially pathogenic for the human intestines, depending on the abundance and situation[5,6]. Overall, these studies outline a critical role for development of an intestinal microbiota that maintains homeostasis and promotes healthy intestinal, immune system and neurological development.

Factors that influence intestinal colonization

Various factors have been shown to have an influence on intestinal microbiota development in early life since these factors influence the infants' interactions with microbes[1]. Believed to be the most determinative are mode of delivery, antibiotic supplementation, type of nutrition and gestational age[1]. Infants born at term with a natural vaginal delivery (VD) are initially colonized by maternal vaginal and fecal bacteria, while CS-delivered infants are predominantly colonized by maternal skin or hospital-derived (nosocomial) bacteria[7]. When a newborn shows signs of infection they are administered with antibiotics[8], either narrow-spectrum antibiotics if the source of infection is known or broad-spectrum antibiotics if the source is unknown. Providing broad-spectrum antibiotics (i.e. ampicillin plus gentamicin) in order to reduce pathogenic bacteria may also limit colonization by (potential) beneficial bacteria such as *Bifidobacterium* spp. and *Bacteroides* spp., resulting in short- and long-term shifts in intestinal colonization[8,9]. Alteration in intestinal microbiota also occurs upon nutritional adjustment[1]. Infant nutrition starts with human milk or formula and is followed by solid food after several months, which all three contain a different set of substrates for bacteria that may promote growth of distinct bacteria[1]. Maintaining a healthy intestinal microbiota may be promoted by human milk-feeding. Human milk naturally contains pre- and probiotics such as HMOs, promoting specific bacterial growth, and potential beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. for intestinal colonization of the infant[10]. An alternative for human milk-feeding is formula, generally made from bovine milk and over the years research has been done to make adaptations in order for formula to induce similar beneficial effects as human milk[11]. However, there are significant compositional differences compared to human milk, changing the available substrates for intestinal bacteria, which may be the cause of intestinal microbiota differences found between breast-fed and formula-fed infants[1,12,13].

The above mentioned factors are all increasingly relevant in preterm infants (infants born before 37 weeks gestational age (GA)) and term infants born with a very low birth weight (VLBW, ≤1500g). In the Netherlands 5.7% of singleton births were delivered preterm in 2008, in other European countries this ranged between 4.3-8.7%[14]. Premature birth is the primary cause of VLBW, and because approximately half of the infants born by multiple live births are premature, these infants are at high risk of being VLBW[14,15]. These statistics show that there is quite a proportion of premature infants born each year. Of this proportion a quarter dies with death directly attributable to prematurity (including VLBW and NEC cases)[16]. Disturbances in intestinal microbiota are often seen in premature infants and associated with disease later in life[1]. Therefore it is

necessary to investigate intestinal colonization of premature infants and factors influencing this colonization.

The CS-rate of infants born preterm is approximately 3-fold higher than in term infants[17], suggesting these preterm infants are more likely to be colonized by skin and nosocomial bacteria instead of vaginal and fecal bacteria, as seen in infants born at term[7]. Once delivered, premature and VLBW infants are kept in a sterile environment in the hospital, further reducing the chances of encountering beneficial bacteria while they have prolonged exposure to nosocomial bacteria. Additionally, infants delivered by CS have on average a longer hospital stay compared to VD infants[18]. Because of fear for infections due to an underdeveloped immune- and digestive system, preterm infants are often supplied with antibiotics [19,20]. Although this may provide protection against several pathogens, it also may have the same effect as in term infants: a decrease of beneficial bacteria counts such as *Bifidobacterium* spp. and an increased colonization by potential pathogenic bacteria including *Enterococcus* spp. and *C. difficile*[8,9]. Additionally, due to an underdeveloped digestive system and/or delayed maternal milk production, premature infants are less breast-fed and often require paternal nutrition, enteral formula or donor milk-feeding[21,22]. Due to lower gestational age, higher CS-rates and antibiotic use and less human milk-feeding, premature infants are probably extra susceptible to disturbances in microbial composition[1].

Early microbiota detection methods were culture-based, limiting bacterial detection to culturable bacteria. New molecular techniques, generally based on 16S rDNA sequencing, gave new insights into intestinal microbiota development and exposed many species that were previously undetectable by culturing methods[1]. Therefore there are new studies elaborating more accurately on preterm intestinal colonization patterns and the influence of above mentioned factors. There are many observational and intervention studies done with preterms, investigating their microbiota development by looking at fecal samples. Others have done studies with preterm piglets in order to look more closely at colonization of specific sites of the intestines and the influence of delivery mode, diet and antibiotic treatment[23–25]. This thesis will describe recently gained knowledge on the influence of GA, delivery mode, antibiotics and type of nutrition on the microbiota development in preterm and VLBW infants and additionally discuss the impact on developing necrotising enterocolitis (NEC), which is the cause of high morbidity and mortality among preterm infants[16,26].

Current knowledge on microbiota development in preterm & VLBW infants

Intestinal colonization in infants born at term starts with facultative anaerobes such as *E. coli* and *Streptococcus* spp. in the first 48 hours, followed by *Staphylococcus* spp., *Enterococcus* spp. and *Lactobacillus* spp.[1]. Gradually, due to oxygen consumption by facultative anaerobes, obligate anaerobic bacteria start to colonize the intestine including *Bifidobacterium* spp., *Bacteroides* spp., and *Clostridium* spp., while Enterobacteriaceae levels decrease[1]. In premature infants this intestinal colonization pattern is aberrant[1]. In this chapter I will describe how GA, delivery mode, antibiotics, and type of nutrition influence intestinal microbiota development in premature and VLBW infants.

Influence of gestational age in early life on intestinal microbiota development

It's believed that the fetal intestines are sterile before birth and colonization starts during delivery[1]. However, data from several studies have demonstrated bacteria in meconium[24,27,28], the first stool of a newborn composed of ingested material during intrauterine maturation. This indicates that there are intrauterine bacteria ingested by the fetus before delivery and thus the fetal intestines are not sterile before birth[24]. A trend of increased proportion of non-sterile meconium in preterms has been observed[24] and its microbial composition differed from subsequent fecal samples, indicating the ingested intrauterine bacteria are not permanent intestinal colonizers[27]. Predominant bacteria in the meconium of preterm infants (≤32w GA) were *Staphylococcus* spp., *Lactobacillus* spp., *Streptococcus mitis* and Enterobacteriales[27,29]. This is supported by findings of Ardissone *et al* (2014) where GA was negatively correlated with *Lactobacillus* spp., *Enterococcus* spp. and Enterobacteriaceae *Enterobacter* spp.. Taken together, dissimilarities in bacterial composition of meconium between preterm and term infants suggests that the ingested bacteria found in meconium of preterm infants might induce a fetal immune response and thereby induce preterm labor[24].

Decades ago it was already reported that preterm and VLBW infants have an aberrant fecal colonization pattern compared to term infants. These differences might be due to distinct intrauterine bacterial composition, or as a result of underdeveloped intestines and immune system combined with different environmental factors[1]. High interindividual variability, particularly in the first week or month[30–32], make it complicated to illustrate an intestinal microbial colonization pattern that is specific for premature intestines. Preterm intestinal colonization during the first weeks after birth has been extensively studied (Table 1). A number of studies with preterm and VLBW infants have observed delayed colonization and reduced microbial diversity compared to infants born at term[20,32–38]. Microbial diversity is especially low in the first fecal samples of preterm infants born between 28-33 weeks GA[20,34,35]. Firmicutes are the most detected phylum in fecal samples from the first week, mainly from the Bacillales (*Staphylococcus* spp.) and Lactobacillales order (*Enterococcus* spp.) [27,30,32,39]. However, also Enterobacteriaceae are frequently detected, including several potentially pathogenic bacteria: *E. coli*, *Klebsiella pneumonia*, and *Serratia marcescens*[27,32,39]. In the following weeks *Enterococcus* spp. and species belonging to proteobacteria increase, including Gram negative bacteria *E. coli*, *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., and *Klebsiella* spp.[27,30–32,39]. In general, preterm infants show increased levels of facultative anaerobes Enterobacteriaceae, Streptococcaceae, Staphylococcaceae, and Enterococcaceae for a prolonged period compared to infants born at term, and reduced

or delayed colonization with obligate anaerobes Bifidobacteriaceae, Lactobacillaceae, and *Bacteroides* spp.[5,20,27,29,30,32–42].

Bifidobacterium spp. and *Lactobacillus* spp. are widely accepted as beneficial commensal bacteria, their growth being promoted by breastfeeding[13,43–45]. In the study of Björkstrom *et al* (2009) *Bifidobacterium* spp. and *Lactobacillus* spp. belonged to the most identified bacteria in the first three weeks after preterm birth, which might be explained by early pasteurized human milk feeding in these infants. Arboleya *et al* (2012) found higher levels of *Lactobacillus* spp. in preterm compared to term infants. These findings are in contrast with most studies, where compared to term infants delayed and reduced levels of *Bifidobacterium* spp. and *Lactobacillus* spp. are observed in premature infants, sometimes despite breastfeeding [20,27,31–33,37,38,42]. It has been suggested that colonization with *Bifidobacterium* spp. is impaired in infants born before 32.9 weeks GA, possibly due to the low degree of intestinal maturation[42] or use of broad-spectrum antibiotics[38]. When *Bifidobacterium* spp. appeared (with a median of 11 days after delivery), the most frequently detected species were *B. longum, B. breve*, and *B. bifidum*[27,42]. A lack of *Bifidobacterium* spp. could lead to loss of its antimicrobial products, thus possibly favoring colonization by potentially pathogenic bacteria such as *E. coli* and *C. perfringens* which are often detected in preterm infants[27,32,39].

In addition to the low counts of beneficial bacteria, increased occurrence and/or levels of (potential) pathogens have indeed been identified in preterm infants. Most frequently, and in some cases predominantly, detected are *Klebsiella* spp. (specifically *K. pneumonia*), *C. perfringens, C. difficile*, *Pseudomonas* spp., *E. coli*, and *Serratia* spp.[20,23,27,29–32,34,36–39,46,47]. Albeit at very low frequencies, Arboleya *et al* (2012) could detect *C. difficile* only in preterm infants while Ferraris *et al* (2012) found a trend of increased clostridial colonization in infants born before 29 weeks GA (including *C. perfringens* and *C. difficile*) in addition to increased clostridium diversity throughout hospitalization. The results presented thus far indicate that there is an imbalance of beneficial and potential pathogenic bacteria in the gastrointestinal tract of preterm infants, possibly disturbing healthy intestinal and immune development and making these infants prone to infections and associated diseases like necrotizing enterocolitis.

Influence of delivery mode in early life on intestinal microbiota development

During natural vaginal delivery at term the infant is initially exposed to and believed to be mainly colonized by bacteria of the birth canal and perineum, rich in *Lactobacillus* spp., *Prevotella* spp., and *Sneathia* spp.[7]. In CS-delivered term infants, maternal skin and nosocomial bacteria are the first colonizers, including *Staphylococcus* spp., *Corynebacterium* spp., and *Propionibacterium* spp.[7]. Additionally, term infants born by CS experience delayed colonization and reduced microbial diversity when compared to VD-infants[1,48]. Findings demonstrate lower prevalence of *Bacteroides* spp., specifically *B. fragilis* up to 6 months of age, delayed colonization by *Bifidobacterium* spp. and *Lactobacillus* spp., decreased diversity and counts of *Bifidobacterium* spp., and increased colonization levels of *C. perfringens* at one month of age(Grönlund et al. 1999; Mikami et al. 2009). However, in one of these studies the mothers of infants born by CS all received antibiotics before delivery, whereas the mothers of VD-delivered infants did not, which could possibly have influenced the delayed and reduced colonization by *Bifidobacterium* spp., *Lactobacillus* spp. and *Bacteroides* spp. that was observed in these CS-delivered infants[49].

Table 1 - Weekly intestinal colonization in early life of premature infants

Ref*[1]	Meconium/Week 0	Week 1	Week 2	Week 3	Week 4	Week 5+
[30] **n=10**		43% Enterococcus 29% Bacillales	22% Enterococcus 25% Enterobacteriaceae 48% Bacillales	28% Enterococcus 38% Enterobacteriaceae 25% Bacillales	65% Enterococcus 17% Enterobacteriaceae 13% Bacillales	52-25% Enterococcus 30-50% Enterobacteriaceae 27-7% Bacillales
[39] **n=44** ***2**	*Lactobacillus spp.(4)* *Bifidobacterium* spp.(4) *Staphylococcus* spp.(4) *Enterococcus* spp.(3.5) *Escherichia coli* (3) *Bacillus* spp.(5)	*Lactobacillus* spp.*(5)* *Bifidobacterium* spp.(5) *Staphylococcus* spp.(4) *Enterococcus* spp.(4) *Escherichia coli* (5) *Bacillus* spp.(6) *Pseudomonas* spp.(5)	*Lactobacillus* spp.(5) *Bifidobacterium* spp.(5) *Staphylococcus* spp.(2) *Enterococcus* spp.(4) *Escherichia coli* spp.(5) *Bacillus* spp.(6) *Pseudomonas* spp.(6) *Klebsiella* spp.(6)	*Lactobacillus* spp.(5) *Bifidobacterium* spp.(5) *Enterococcus* spp.(4) *Escherichia coli* (4) *Staphylococcus* spp.(2) *Klebsiella* spp.(6) *Proteus* spp.(6) *Pseudomonas* spp.(5)		
	Species mainly detected: *Lactobacillus: L plantarum, L paracei, L acidophilius;* Increasing proportion of Gram-negative bacteria (*E. coli, Klebsiella* spp., *Pseudomonas* spp., *Proteus* spp.)					
[27] **n=14** ***3**	*Staphylococcus* spp. (6.5) *Enterococcus* spp. (6) *Streptococcus* spp.(5) *Lactobacillus* spp.(5) *Klebsiella* spp. (7)	*Staphylococcus* spp. (8) *Enterococcus* spp.(9) *Klebsiella* spp.(9) *Escherichia coli* (9) *Serratia* spp. (9) *Streptococcus* spp.(8) *Lactobacillus* spp.(7.5)	*Enterococcus* spp.(8.5) *Escherichia coli* (9) *Klebsiella* spp.(9) *Staphylococcus* spp. (7) *Serratia* spp.(9) *Lactobacllus* spp.(7) *Bifidobacterium* spp.(9) *Streptococcus* spp.(6)	*Enterococcus* spp.(8) *Escherichia coli* (9) *Staphylococcus* spp.(7) *Klebsiella* spp.(9) *Serratia* spp.(9) *Lactobacillus* spp(6) *Bifidobacterium* spp.(9.5)		
	Species mainly detected: *Staphylococcus* spp.: S. *epidermidis, S. aureus*. *Enterococcus* spp.: *E. faecalis, E. faecum*. *Klebsiella* spp.: *K. pneumonia*. *Serratia* spp.: *S. marcescens*					
[32] **n=21**		*Klebsiella pneumonia* *Clostridium cluster IV* *Desulfovibrio* spp. *Streptococcus* spp. *Staphylococcus* spp.	*Klebsiella pneumonia* *Clostridium cluster IV* *Streptococcus* spp. *Desulfovibrio* spp. *Clostridium perfringens* *Staphylococcus* spp. *Clostridium cluster XIVa*	*Klebsiella pneumonia* *Clostridium perfringens* *Streptococcus* spp. *Clostridium cluster IV* *Staphylococcus* spp. *Clostridium cluster XIVa* *Desulfovibrio* spp. *Atopobium* spp. *Clostridium difficile*	*Klebsiella pneumonia* *Streptococcus* spp. *Clostridium cluster IV* *Clostridium cluster XIVa* *Clostridium perfringens* *Atopobium* spp. *Staphylococcus* spp. *Desulfovibrio* spp. *Clostridium difficile*	
[31] **n=10**	Gammaproteobacteria Bacilli Clostridia (C. *difficile, C. perfringens*) *Pseudomonas* spp. (0-10%) *Acinetobacter* spp. (0-23%)		Gammaproteobacteria Bacilli Clostridia (other than C. *difficile, C. perfringens*) *Acinetobacter* spp. (0-40%)		Gammaproteobacteria Clostridia (other than C. *difficile, C. perfringens*) Bacilli Bacteroidetes *Pseudomonas* spp. (0-1%) *Acinetobacter* spp. (0-12%) *Haemophilus* spp. (0-19%) *Veillonella* spp. (0-32%) *Roseburia* spp. (0-12%)	

*1 Gestational age 22-35 weeks; % Relative mean prevalence; *2 in order of decreasing occurrence, >10% (median $\log_{10}$ CFU/g for those samples in which growth was detected); *3 (mean bacterial counts as $\log_{10}$ CFU/ml)

Since the CS-rate among preterm infants is extremely high[17] they are expected to be more likely to experience abnormal microbial colonization subsequent to CS delivery. The microbial community structure in meconium of CS-delivered preterm infants was more diverse than in CS-delivered infants born at term, with higher abundances of Lactobacillales and Clostridiales species[24]. This raises the question whether these differences occur due to CS or due to intrauterine bacteria causing premature birth, which occurs more often via CS in preterm compared to term infants[17]. However, the differences found in bacterial content of meconium between infants born by CS and VD doesn't seem to be permanent. No associations were detected in preterm infants between delivery mode and intestinal microbiota composition or diversity of fecal samples[30,34]. Studies looking at a specific class, order or genus demonstrated similar results: Clostridia, *Bifidobacterium* spp., and Lactic acid bacteria (LAB, including *Bifidobacterium* spp. and *Lactobacillus* spp.) were not affected by delivery mode in infants ranging from 24 to 36 weeks GA[38,39,42,47]. Additionally, microbial diversity of maternal fecal samples right after delivery and the first fecal samples of the infant were distinct, suggesting that bacteria found in maternal feces are not all transferred to the infant during delivery[30]. Only few studies found an effect of delivery mode on intestinal colonization in preterm infants[20,51]. CS delivery was correlated to an accelerated increase in microbial diversity[20], suggesting microbial diversity may be decreased shortly after birth in CS-delivered preterm infants which disappears after accelerated increase. Others detected a significantly higher prevalence of *E. coli* at 7 but not at 14 days of age, while *Staphylococcus* spp. dominated in both CS and VD-delivered infants[51]. These results differ from findings in term infants by Grönlund *et al* (1999), where other bacteria dominated in VD-infants, possibly due to distinct culturing methods[51]. However, preterm piglets born 10% preterm, resembling preterm infants of 28-30 weeks gestation[52], have shown decreased bacterial levels and diversity when born by CS when compared to preterm piglets born by induced-VD[53]. This indicates that different delivery modes can have an impact on intestinal colonization in preterm piglets. Surprisingly, all the findings in preterm infants are in contrast with observations in term infants where differences in microbiota diversity and composition were observed between CS and VD-delivered infants[7,44,48,49]. This suggests that intestinal colonization in preterm infants is not differently affected by CS or VD, in contrast with findings in infants born at term and preterm piglets.

Influence of antibiotic use in early life on intestinal microbiota development

When newborns show signs of infection or there is increased susceptibility for infection, they are given antibiotics, either narrow-spectrum antibiotics for a specific target or broad-spectrum antibiotics if the source of infection is diverse or unknown[19,20]. Providing antibiotics may reduce pathogenic bacteria but it also limits colonization by beneficial bacteria[8]. Infants treated with ampicillin/gentamicin in the first week after birth had a lower prevalence and reduced diversity of *Bifidobacterium* spp. up to eight weeks of age[8]. This suggests that antibiotics disturb optimal natural intestinal colonization and function by inhibiting growth of beneficial bacteria and thereby allow proliferation of opportunistic pathogenic bacteria and appearance of resistant bacteria such as *Enterococcus* spp., *C. difficile* and Gram-negative Enterobacteriaceae[1,4,9]. Increased proportions of *C. difficile* are a known problem of antibiotic use, which leads to a higher production of toxins, and possibly infection and disease[9,54].

Table 2 - Influence of delivery mode on intestinal microbiota in preterm cases

Ref	n	GA*[1]	CS/VD delivery	Main findings
[30]	20	22-23	8/12	- No microbial difference between CS and VD-delivered infants
[42]	52	30-35	24/28	- Colonization by bifidobacteria colonization is not affected by mode of delivery
[39]	44	27	36/8	- No association between lactic acid bacteria colonization and mode of delivery
[34]	27	23-32	17/12	- Microbiota diversity is not affected by mode of delivery
[47]	76	24-36	35/41	- Incidence of clostridial colonization is not affected by mode of delivery
[24]	52	23-41	33/19	- Microbial community structure is more diverse in CS-delivered infants when compared to VD-delivered infants - Higher relative abundance in CS-delivered infants compared to VD-delivered infants of genera belonging to the Firmicutes: *Negativococcus* spp., *Leuconostoc* spp., *Vagococcus* spp., and *Butyrivibrio* spp.
[20]	29	≤30	24/5	- CS delivery was associated with an accelerated increase in diversity
[51]	63	<33	30/33	- CS-delivered infants had significantly higher prevalence of *E. coli* at 7 days of age - Both CS-delivered and VD infants were dominated by *Staphylococcus* spp. at 7 days of age
[53]	32 pigs	10% preterm	16/16	- CS delivery was associated with decreased microbial counts and diversity

*1 Gestational age in weeks

Vancomycin and metronidazole are antibiotics used against *C. difficile*, however these also often induce recurrent *C. difficile* infections and resistance of other genera including *Enterococcus* spp.[9]. A different antibiotic, clindamycin, was shown to affect *Bacteroides* spp. colonization in adults up to 2 years after treatment[9]. Treatment of *Helicobacter pylori* infection with clarithromycin and/or metronidazole also suppressed *Bacteroides* spp., as well as *Bifidobacterium* spp. and *Clostridium* spp. and allowed increase of *Enterococcus* spp.[9]. In conclusion, antibiotics frequently affect both the pathogenic bacteria and commensal bacteria, resulting in short and long-term shifts of normal intestinal colonization[9].

Infant antibiotic exposure is associated with GA[24]. Antibiotics are frequently provided to preterm infants or their mothers[19,20,24], since their gastrointestinal function and immune system are underdeveloped, allowing bacteria to more easily transfer the intestinal epithelial lining and cause dangerous infections[55]. Because of the fear for infections, broad spectrum antibiotics are frequently administered within days after preterm birth, with ampicillin and gentamicin being the most commonly prescribed combination[56](Table 3). Another possibility is that mothers receive antibiotics, either antenatal to treat infections or intrapartum to prevent infections in the mother and/or newborn. Antenatal, intrapartum and neonatal antibiotics may all reach the newborn and might disturb normal microbial colonization.

Preterm infants from mothers who received intrapartum antibiotics show a trend of decreased microbial diversity in the first week after birth and lower *Bifidobacterium* spp. levels at 10 days of age[32,34]. *Bifidobacterium* spp. counts in human milk of women receiving antibiotics intrapartum or after delivery was significantly decreased compared to women who did not receive antibiotics, suggesting that this might decrease intestinal *Bifidobacterium* spp. levels in premature infants. Neonatal antibiotics also caused reduced levels of *Bifidobacterium* spp. in preterm infants at 10 days of age[32]. These

findings are in contrast with Butel *et al* (2007) who show *Bifidobacterium* spp. colonization incidence was not affected by either intrapartum or neonatal antibiotics. GA, type of nutrition, and techniques were similar between these studies, suggesting that different antibiotics might have been used in the study of Butel *et al* (2007) (not registered for all studies) that might explain the lack of association between *Bifidobacterium* spp. and intrapartum and neonatal antibiotics in this study[32,34,42]. Intrapartum antibiotics also did not affect *Clostridium* spp. colonization or incidence levels, whereas antenatal and prolonged (>10d) neonatal antibiotics did decreased *Clostridium* spp. levels [47]. Although the overall *Clostridium* spp. levels decreased, the potential pathogenic *C. butyricum* significantly increased in preterm infants exposed to antibiotics as well as a slight increase of *C. difficile* and *C. paraputrificum* levels[47]. These increases of potential pathogenic species with antibiotic exposure could possibly be due to antibiotic resistance in a high proportion in these species[47].

Interestingly, infants of mothers who received intrapartum antibiotics are more likely to receive empirical antibiotics[56]. This is often the policy of a hospital, to prevent spread of potential pathogenic bacteria that might have transferred from maternal (vaginal) infections during delivery. Empiric, broad-spectrum antibiotic therapy in preterm cases, such as a combination of ampicillin and gentamicin or vancomycin and cefotaxime, reduces microbial diversity[29] and total counts of both anaerobes and aerobes[19,23]. In addition, in preterm infants treated with broad-spectrum antibiotics a predominance of *Staphylococcus* spp. and presence of *S. marcescens* was detected[27,29]. Another broad-spectrum antibiotic, vancomycin, active against Gram-positive bacteria, decreased LAB counts, while cefotaxime, active against both Gram-positive and Gram-negative bacteria, only decreased Gram-negative bacteria counts in preterm VLBW infants of 27 weeks GA[39]. Overall, these results indicate that intrapartum antibiotics and particularly neonatal broad-spectrum antibiotics cause an imbalance in the intestinal microbiota development of the preterm infant, and therefore they should be used sparsely and with caution.

Table 3 - Frequently used broad-spectrum neonatal antibiotics and their bacterial targets

Antibiotic	Bacteria targeted	Used in:
Ampicillin *mostly in combination with gentamicin	Beta-lactam ring: Gram-positives and several Gram-negatives	8,19,20,31,32,36,39,41,56
Gentamicin *mostly in combination with ampicillin	Gram-negatives (*Pseudomonas* spp., *Proteus* spp., *Serratia* spp.) *Staphylococcus* spp.	8,19,31,32,36,38–41,56,57
Vancomycin	Gram-positives (*Staphylococcus aureus* *Staphylococcus epidermidis*, *Clostridium difficile*)	20,36,38,39,41,57
Metronidazole	Anaerobes (*Clostridium difficile, Bacteroides fragilis, Fusobacterium* spp., *Clostridium* spp., *Helicobacter* spp.)	8,19,38,40
Cefotaxime	Gram-positives (*Streptococcus* spp., *Staphylococcus* spp., *Enterococcus* spp.) Gram-negatives (*Escherichia* spp.)	20,31,36,39–41,56

Table 4 - Antibiotic influence on intestinal colonization in preterm cases

Ref	n	GA*[1]	Antibiotics		- Neonatal antibiotics (% exposure) *[4] - Min - max duration	Main findings
			Intra*[2]	Neo*[3]		
[42]	52	30-35	48%	29%	- Antibiotics N.R. - 0-7 days	- *Bifidobacterium* spp. colonization incidence not affected by intrapartum or neonatal antibiotics
[39]	44	27	N.R.	61%	- Neonatal: Ampicillin (41%), gentamicin (39%), cefotaxime (17%), vancomycin (15%) - Duration N.R.	- Vancomycin decreased LAB levels - Cefotaxime decreased Gram-negative bacteria levels
[34]	27	23-32	26%	70%	N.R.	- Trend for decreased diversity with intrapartum antibiotics in the first week
[27]	14	≤32 or ≤1200 g	N.R.	93%	- Antibiotics N.R. - 3-15 days	- Presence of *Serratia* spp. (mainly *S. marcescens)* associated with neonatal antibiotics
[29]	6	24-27	67%	100%	- Neonatal: ampicillin+gentamicin followed by vancomycin+cefotaxime - 2-12 days	- Empiric, prolonged antibiotics decreased microbial diversity and increased *Staphylococcus* spp. levels
[47]	76	24-36	42%	54%	- antenatal: amoxicillin (82.7%), gentamicin (27.6%), metronidazole (10.3%) - intrapartum: amoxicillin (87.5%), gentamicin (56.2%) - neonatal: amoxicillin (80.5%), cefotaxime (85.4%), aminoglycoside (gentamicin or amikacin) (80.5%), vancomycin (29.3%) - 2-11.5 days	- No association between incidence of clostridial colonization and antibiotics (antenatal, intrapartum, neonatal) - Decrease in clostridial levels with antenatal antibiotic treatment and prolonged (>10d) neonatal antibiotic therapy, not with intrapartum antibiotic therapy. - Higher *Clostridium butyricum* frequency in preterm infants exposed to antibiotics
[56]	4039	26	68%	100%	- Neonatal: ampicillin+gentamicin (83%) - 1-36 days	- Infants of mothers that received intrapartum antibiotics were more likely to receive empirical antibiotic treatment
[32]	41	30-35 38-41	T: 0% P: 29%	T: 0% P: 29%	- Intrapartum: ampicillin, ampicillin+ erythromycin, ampicillin+gentamicin, clindamycin - Neonatal: ampicillin + gentamicin - 0-8 days	- Decreased *Bifidobacterium* spp. levels at 10d of age due to intrapartum or neonatal antibiotics
[20]	29	≤30	72%	86%	- Neonatal: penicillin G (14%), amoxicillin (14%), cefotaxime (50%), amikacin (82%), vancomycin (68%), fluconazol (11%) - 1-16 days	- Early neonatal antibiotherapy was associated with an accelerated increase in microbial diversity
[23]	57 pigs	T: 13 P: 44	-	T: 0% P: 46%	- Neonatal: ampicillin+gentamicin+ metronidazole (100%) - dayly	- Neonatal antibiotics in pigs decreased total bacterial counts (anaerobes & aerobes)
[24]	52 pigs	23-41	46%	29%	N.R.	- Antibiotic exposures was associated with low GA

*1 Gestational age in weeks
*2 Intrapartum antibiotics
*3 Neonatal antibiotics
*4 neonatal antibiotics with >10% exposure

T Term
P Preterm
N.R. Not registered

Influence of type of nutrition in early life on intestinal microbiota development

Human milk naturally contains pre- and probiotics, including HMOs and a wide diversity of bacteria (<10^3 cfu/ml), such as *Bifidobacterium* spp. and *Lactobacillus* spp.[1,13,43,44,58]. HMOs may block pathogen adhesion[59] and are a growth substrate for several *Bacteroides* and *Bifidobacterium* species that are frequently found in infant intestines[43]. Human milk composition changes over time and, besides HMOs and bacteria, contains antioxidants, lipids, carrier proteins (lactoferrin), and anti-inflammatory markers (TGF-β, IL-10) that together modulate the intestinal microbiota and immune function[10,60,61]. Besides *Lactobacillus* spp. and *Bifidobacterium* spp., genera most frequently cultured from human milk are: *Staphylococcus* spp., *Streptococcus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Weissella* spp., *Enterococcus* spp., and *Propionibacterium* spp.[58]. These human milk bacteria are a potential source of commensal intestinal bacteria for infants. Presence of the same *Lactobacillus* spp. in maternal milk and infant feces has been shown[62,63], indicating transfer of bacteria via human milk to promote healthy intestinal colonization of the infant. In contrast, Hunt *et al* (2011) did not find the beneficial *Lactobacillus* spp. and *Bifidobacterium* spp. in human milk, possibly due to technical limitations of the different methods to study microbial compositions[11]. The genera mentioned above are found by culturing methods in a number of studies[58], while Hunt *et al* (2011) used pyrosequencing of the 16S rRNA gene, which is suggested to not properly detect *Bifidobacterium* spp.[11]. However, other studies have identified *Lactobacillus* spp. and *Bifidobacterium* spp. in human milk by molecular techniques [58,64], suggesting that the distinct results might be due to study cohorts from different areas with diverse backgrounds, diets, habits and antibiotic use[58]. Hunt *et al* (2011) found nine genera detectable in all maternal samples of which *Staphylococcus* spp., *Corynebacteria* spp., and *Propionibacteria* spp. are typical skin microbes, suggesting that maternal breast skin is the source of these bacteria in human milk[64]. Human milk can be derived either from the mother or a donor, and it can be administered directly or after it has been frozen, pasteurized and/or fortified with minerals and proteins to accommodate to the needs of the growing infant. However, these processes may alter human milk components and reduce its health-promoting capacity.

The alternative for human milk-feeding when the mother is unable or decides not to is formula feeding. Infant formula is often bovine milk-based but can also be goat milk-based or fortified human milk[13]. Bovine milk-formula contains minimal amounts of HMOs compared to human milk[1]. Although (clinical) research is done to create formula that resembles the beneficial effects of human milk, there are still significant compositional differences, probably the reason for differences found in microbiota diversity and composition between human milk-fed and formula-fed infants[1,11]. In a recent clinical study it was demonstrated that levels of several *Bifidobacterium* spp. and *Lactobacillus* spp. increased when specific prebiotic oligosaccharides were added to formula, resembling the species found in intestinal microbiota of human milk-fed infants[11]. Formula without those prebiotics induced an intestinal microbiota with more *Bifidobacterium* spp. that are also found in adult microbiota[11]. In addition, intestinal microbiota of human milk-fed infants contained less potential pathogenic *Clostridium* spp. when compared to regular formula-fed infants, while adding specific prebiotics to formula caused a decrease in detection of several pathogens[11]. Collectively, these findings suggest that human milk induces beneficial effects which, with additional extensive research, may possibly be mimicked by formula in the future via the additiona of pre- and probiotics.

Several differences in intestinal colonization have been observed between human milk-fed and formula-fed term infants. First, bacterial diversity is higher in formula-fed infants[12,13]. Second, compared to human milk-fed infants, formula-fed infants have lower amounts of *Lactobacillus* spp.*,* Bifidobacteriaceae, and Bacteroidaceae[11–13]. In addition, studies showed that the lack of *Bifidobacterium* spp. in formula-fed infants leaves space for growth of *Bacteroides* spp., and also *Clostridium* spp. and Enterobacteriaceae[11]. Thus the third difference: formula-fed infants have an increased prevalence of potentially pathogenic *E. coli*, *Staphylococcus* spp., and Clostridia[65]. Taken together, aside from delivery mode and antibiotic administration, the type of nutrition has also been proven to be a very important factor in microbiota development. Human milk-feeding seems to promote intestinal colonization of several beneficial bacteria, while this promotion by formula-feeding is limited, allowing growth of several potentially pathogenic bacteria. Therefore human milk-feeding might be recommended over formula-feeding in term infants.

In preterm infants, breastfeeding is not always possible. Sometimes the mother has not started lactation yet, the production is insufficient due to preterm delivery, or the premature infant is unable to obtain or swallow the milk safely, necessitating the use of maternal milk, donor milk or formula. This nutrition can be administered by either bottle feeding or enteral feeding, which can also be used when supplementation of nutrients is needed. Additionally, premature infants are frequently fed through parenteral feeding, later followed by or supplemented with enteral feeding. Parenteral feeding is necessary if the intestines are too immature to process food and thus breastfeeding or enteral feeding could be harmful or insufficient. Despite of the many benefits of human milk-feeding identified in infants born at term, these are hardly found in preterm infants. A common finding was decreased microbial diversity in human milk-fed infants compared to formula-fed infants, but only in the first week of life[34]. *Bifidobacterium* spp. colonization was not affected by human milk or formula-feeding[38,42]. Another study found an influence, but only due to volume and not type of enteral feeding, which increased LAB and Gram-negative bacteria levels[39]. When comparing microbiota of infant feces and maternal milk, abundance of bacteria from the Bacillales order was the only similarity[30], indicating that intestinal colonization of the preterm infant is not much influenced by human milk bacteria. These findings suggests that intestinal colonization in preterm infants is hardly differently affected by human milk-feeding and formula-feeding, in contrast with findings in infants born at term.

Interestingly, research on preterm piglets born either by CS or induced-VD and fed with formula demonstrated increased *Clostridium* spp. colonization, particularly *C. perfringens*, increased *Streptococcus* spp., impaired intestinal function and a higher incidence of necrotizing enterocolitis when compared to colostrums-fed piglets[53]. Other findings due to formula-feeding compared to colostrums-feeding in preterm piglets showed increased diversity, lower counts of total anaerobes, LAB and *Lactobacillus* spp., higher *C. perfringens* prevalence, and higher *Enterococcus* spp. levels in proximal parts of the intestines[66]. These findings are identical from findings in term infants, indicating that human milk and formula might induce different intestinal microbiota development in preterm infants as well, although it is not measurable possibly due to abundant confounding factors.

In summary, the results discussed so far show that GA, antibiotic use, type of nutrition, and possibly delivery mode have either a positive, negative or intermediate influence on intestinal microbiota development in preterm infants. These factors, combined with

immaturity of the preterm infant and type of feeding, might contribute to development of NEC[52,66,67]. In the next chapter I will discuss some of these factors in association with NEC, since NEC is one of the leading causes of morbidity and mortality in preterm infants[16,26,67].

Table 5 - Influence of feeding on intestinal microbiota development in preterm cases

Ref	n	GA*[1]	Type of nutrition	Main findings
[30]	20	22-23	H: 100% B F: 5+days 100% M: 100%	- The only similarity between infant feces and breast milk is the Bacillales abundance - breast milk contained: Bacillales, Streptococcaceae
[42]	52	30-35	H: 35% F: 100% M: 35%	- *Bifidobacterium* spp. colonization not affected by type of nutrition
[39]	44	27	H: 76% F: 10% M: N.R.	- Volume of enteral feeding positively correlated with lactic acid bacteria and Gram-negative bacteria levels
[34]	27	23-32	H: 56% F: 30% M: N.R.	- Decreased microbial diversity in human-milk fed infants, but in samples from the 1st week of life
[38]	15	26-30.5 37-42	P: enteral T: H: 66% F: 33% M: 27%	- *Bifidobacterium* spp. colonization not affected by type of nutrition

*1	Gestational age in weeks	H	human milk
T	Term infants	F	formula
P	Preterm infants	M	mixed: H+F
N.R.	Not registered		

Preterm birth, microbiota development and relation to NEC

Role of intestinal colonization in NEC development

Intestinal microbiota development of preterm infants is distinct from term infants partially because of GA, delivery mode, antibiotic use and type of feeding. Due to a low GA both the gastrointestinal tract (GIT) and the immune system are underdeveloped, exposing the neonate to a higher chance of abnormal intestinal colonization and infection. Increased intestinal permeability in the premature underdeveloped GIT may lead to bacterial translocation in the neonate. In combination with an immature immune system this may reach systemic organs and tissues and cause disease[68]. Necrotizing enterocolitis (NEC) is a life-threatening disease affecting the GIT of infants mostly between 7-27 days of age[26], presenting with intestinal lesions, uncontrolled inflammation and necrosis of intestinal tissue[23,52]. It is frequently characterized by low microbial diversity, bacterial overgrowth, and presence of pathogenic bacteria (e.g. *Clostridium* spp., *E. coli*, *K. pneumonia*)[52]. NEC has an incidence of 0.3 to 2.4 infants/1000 live births, affecting 2-5% of premature infants[68]. Insight in this disease has been obtained from both premature infants and animal models of prematurity. Here I will discuss how intestinal colonization in preterm infants and piglets affects the development of necrotizing enterocolitis in the light of the factors discussed in this review.

NEC incidence is correlated with low GA and low birth weight[68]. Study cohorts for research on intestinal microbiota development of preterm infants frequently include infants that develop NEC. Findings show that microbiota diversity was either lower in NEC infants[69] or equal[34,41,57] when compared to healthy preterm infants. The microbiota composition differed one week before NEC diagnosis, with a higher variability within the NEC group compared to variability within the control group[57]. Additionally, several bacteria identified in the control group could not be identified in NEC cases and vice versa[41], indicating that not all NEC infants have the same microbiota structure prior to NEC. At early time points in patients developing NEC, bacteria belonging to the Firmicutes phylum (Bacillales, *Enterococcus* spp.) were relatively high or had a higher prevalence [30,57,68]. However, shortly before NEC diagnosis, Firmicutes, Actinobacteria, and Bacteroidetes proportions were decreased and Proteobacteria proportions were increased[41,57]. In contrast, others found an increase in Actinobacteria proportion prior to and during NEC, mainly consisting of *Propionibacterium* spp.[70]. *Propionibacterium* spp. is also regularly found in human milk[58,64] and since the proportion of human milk-fed infants in the study of Stewart *et al* (2012) was higher compared to the other two studies[41,57], this suggests that this might be the reason for the increase in Actinobacteria in these NEC patients.

Many potential pathogens are identified with increased frequency or levels prior to NEC development, suggesting that these may contribute to disease development or may be a consequence of disease onset. Increased frequency of Proteobacteria detection in preterm NEC infants compared to healthy preterm infants comes predominantly from the Gammaproteobacteria; Enterobacteriaceae family[30,57,69]. The Enterobacteriaceae genera identified are frequently Gram-negative potential pathogens: *Klebsiella* spp., *Enterobacter* spp., *E. coli*, *Cronobacter* spp., *Pseudomonas* spp., and *Proteus* spp.[39,41,57,69,70]. From these potential pathogens, presence of *Klebsiella* spp. in early fecal samples and presence of *Enterobacter* spp. have been associated with NEC development[41,70]. In addition, Mshvildadze *et al* (2010) suggested association of *Citrobacter* spp. with NEC, since this genus was only found in 3/4 NEC cases compared to none of the 17 controls. However, this was refuted by Morowitz *et al* (2011) who also

detected this genus in a preterm infant without NEC, suggesting that *Citrobacter* spp. might be involved in some cases of NEC but can also be found in healthy infants[34,46]. Potential pathogens from the Firmicutes phylum were identified with increased frequency or levels prior to NEC development, including *Staphylococcus epidermidis*, *C. perfringens*, and *Enterococcus* spp. [34,41,57,70]. In contrast, a decrease of *Enterococcus* spp., specifically *E. faecalis*, has also been seen in NEC cases when compared to healthy controls[30,70]. Together, these results provide important insight in microbial colonization of preterm infants developing NEC. However, despite of all these potential pathogens regularly detected in NEC cases, there is no common pathogen found that could be the initiator of NEC development.

In order to discover basic mechanisms, colonization patterns and the influence of environmental factors, multiple animal studies have been conducted, many in pigs born 10% preterm[52]. Preterm pigs with NEC showed lower microbial diversity and higher *C. perfringens* levels when compared to healthy preterm pigs[23]. Increased abundance of *C. perfringens* is probably a consequence of NEC since inoculation with the strain did not induce and immunization with the strain did not prevent NEC[23]. Ileal mucosa of preterm pigs had an increased abundance of Firmicutes and Actinobacteria[25]. Additionally, preterm piglets with NEC had a higher prevalence of *Clostridium* spp. *C. butyricum*, *C. neonatale*, *C. proteolyticum*, and *Streptomyces* spp. and *Leptolyngbya* spp.[25]. These results mainly confirm results found in preterm infants with NEC. Additionally, results from piglet models demonstrate that increase of *C. perfringens* is a consequence rather than a causative agent of NEC.

Factors influencing NEC development

Delivery by CS in infants born at term induces higher levels of potential pathogens[7,44,49]. An increase of potential pathogens was not as obvious in CS-delivered premature infants compared to infants born by CS at term[51]. Nonetheless, higher abundance of potential pathogens might increase the chance of bacterial transfer by a potential pathogen in the premature infant that could possibly cause infection and induce NEC[68]. However, NEC incidence was not higher among CS-delivered preterm infants[68,69] or preterm pigs when compared to VD controls[53]. These findings indicate that delivery mode is not involved in NEC development[52,53].

Broad-spectrum antibiotics are regularly used in preterm infants, increasingly with shorter gestation, because the immature intestines are sensitive to bacterial transfer of potential pathogens and this increases the fear of infection[19,20,24]. One could suspect that broad-spectrum antibiotics prevent infections and thereby decrease NEC incidence. However, there are studies in preterm infants that have not detected differences in proportion, duration and effects of intrapartum and neonatal antibiotics between NEC infants and controls[41,68]. Others detected the opposite of suspected, namely a 7% increased odds of NEC with each extra day of neonatal broad-spectrum antibiotic administration[56]. This is in line with results from Wang *et al* (2009), who show duration of antibiotic treatment prior to onset of disease is significantly longer in infants who develop NEC. Additionally, broad-spectrum antibiotic therapy has been shown to reduce *Bifidobacterium* spp. and LAB levels in preterm infants[32,39]. A decrease in these bacterial levels could possibly also be involved in NEC development, since commensal bacteria are involved in maturation and maintenance of the epithelial barrier function and stimulation of the immune system[71]. Findings in animal studies have shown the opposite. Broad-spectrum antibiotics protect against infection and NEC in preterm piglets[19,23]. The combination of broad-spectrum antibiotics used was gentamicin, ampicillin, and metronidazole for 5 days, which decreased bacterial counts and

prevented NEC at least until day 10, while in the control group that did not receive antibiotics, 85% developed NEC by day 5[19]. Bacteria that survived after antibiotic treatment were *Clostridium paraputrificum, Clostridium perfringens, Bacillus cereus, Cronobacter sakazakii, Enterococcus faecium/hirae*, and *Staphylococcus pasteuri*, albeit at lower levels than in controls[19]. These conflicting results in infants and animal models demand further research on effects of antibiotic treatment on NEC development.

Since type of nutrition and mode of feeding have been shown to influence intestinal colonization in term infants and preterm piglets[11–13,53], it could also be of influence on NEC development. Evidence revealed that fewer preterm infants with NEC were exclusively breastfed when compared to healthy preterm infants[41]. Additionally, healthy controls started earlier with breastfeeding, reached full dose of human milk about two weeks earlier than infants developing NEC, and were younger when feeding with human-milk fortifier was initiated[68]. This suggests that breastfeeding may be beneficial to the premature infant compared to formula feeding, and that breastfeeding promotes healthy GIT and immune development, thereby protecting against NEC development[67,72]. LAB, including *Bifidobacterium* spp. and *Lactobacillus* spp., are present in human milk and promote healthy microbiota development and immune function in infants born at term[10], and also have been suggested to play a role in protecting against NEC[42]. Indeed, NEC cases had lower *Bifidobacterium* spp. counts one and two weeks before diagnosis, although there was no difference within 3 days before diagnosis[41]. Again, these findings indicate that human milk-feeding might protect against development of NEC when compared to formula-feeding. One study detected LAB at high levels prior to disease onset[39], indicating that high LAB levels do not always protect against NEC. They also reported a positive correlation between volume of enteral feeding and LAB levels, suggesting that the increase prior to disease onset might be due to enteral feeding[39]. In addition, bacterial composition was not distinct between human milk of mothers of healthy infants and infants with NEC [30], indicating that components in human milk probably do not influence NEC development.

Animal experiments demonstrated the protective function of breast/colostrum-feeding[53,72]. NEC incidence in preterm piglets was higher with formula-feeding compared to colostrums-feeding, with increased *Clostridium* spp., increased proinflammatory cytokine levels, and impaired intestinal function[53]. In addition, it was shown that 52% of CS-delivered preterm piglets, formula-fed through enteral feeding, developed NEC[25]. Considering delivery mode is not involved in NEC development[52,53], the high NEC incidence in this group might be due to either formula or enteral feeding or both. It has been reported that enteral feeding at young GA may damage the underdeveloped preterm GIT, thereby further enhancing changes of bacterial transfer and subsequent infection and onset of NEC[52]. Preterm pigs show NEC-like lesions after initiation of enteral feeding, especially with formula-feeding[52,73]. NEC was only diagnosed after enteral feeding, in higher levels with formula-fed preterm piglets, and incidence increased when this was preceded by parenteral feeding[73]. Taken together, above mentioned results from animal and human studies show that compared to formula-feeding, colostrum/human milk-feeding protects against NEC and that enteral feeding may induce damage to the immature intestines, leading to NEC.

In conclusion, infants born preterm are prone to developing NEC due to an immature gastrointestinal tract and immune system, facilitating bacterial transfer. Transfer of opportunistic or potentially pathogenic bacteria may induce infection and an uncontrolled inflammatory reaction. Human milk-feeding, minimal enteral feeding and possibly a short duration of antibiotic supplementation could possibly lead to reduced chances of NEC development in premature infants.

Discussion

This review has described the many factors influencing intestinal colonization in infants born preterm. Intestinal microbiota development in early life is extremely important since host-microbe interactions contribute to gastrointestinal, immune system and neurological development[1]. Aberrant intestinal colonization has been implicated in increased risk for gastrointestinal diseases such as necrotizing enterocolitis[1]. Preterm infants have an underdeveloped gastrointestinal tract and immune system and additionally frequently spend their first days to weeks of life in the neonatal intensive care unit (NICU), making them prone to atypical intestinal colonization. This review has shown that the results of studies on preterm intestinal colonization are often contradicting. Contrasting results in human studies can arise from many confounding factors such as distinct gestational age, birth weight, nutrition and hospital environment. In addition, studies use different sampling times and vary in techniques for the detection and identification of bacteria. These factors and high variability in colonization patterns between individuals make it complicated to draw uniform conclusions. Animal studies on piglets have been done in order to minimize confounding factors and acquire basic information about prematurity, intestinal microbiota in early life, and the impact of one or two specific factors on intestinal colonization. However, animal physiology is not always compatible with human physiology; therefore translation of findings in animal models to human cases should be done with caution

In most studies described in this review fecal samples are used to detect bacteria that are present in the GIT. However, animal experiments have shown that colonization incidence and levels are distinct in different parts of the GIT, indicating that each bacterial species has their own niche[25]. Fecal microbiota might thus not be representative of the intestinal microbiota. However, unless a preterm infant has an ileostomy or colostomy, using fecal samples is the only ethical option to study intestinal microbiota development in infants. Additionally, ileal and colonic fluid of preterm infants are not fully representative either since an ileostomy and colostomy might allow oxygen passage to these parts of the GIT that may induce growth of facultative anaerobes[40]. It should be kept in mind that fecal microbiota is possibly different from intestinal microbiota. Again, animal studies are a good alternative to study colonization of specific sites in the GIT and to study host-microbe interaction mechanisms, although results are not always representative for humans.

Intestinal microbiota development in infants born at term starts with facultative anaerobes during delivery and proceeds to obligate anaerobes after approximately one week[1]. Preterm infants tend to have delayed colonization, reduced diversity and aberrant microbial composition with an increased abundance of facultative anaerobes including potentially pathogenic bacteria[1]. Interestingly, intestinal microbiota development in preterm infants seems less affected by different delivery modes (VD vs CS) and types of nutrition (human milk vs formula) than has been observed in infants born at term. A possible explanation could be that the many confounding factors in premature infants (prematurity, delivery mode, antibiotic use, type of nutrition, hospital environment) or the smaller patient groups investigated, result in non-significant differences. Additionally, the immature GIT and immune system of a preterm infant react differently to bacterial colonization[52], possibly affecting microbiota development in a way that it conceals the effect of vaginal delivery versus C-section and human milk versus formula feeding. With animal models these confounding factors could be diminished. Indeed, studies in preterm pigs have shown that delivery mode and type of nutrition do affect intestinal microbiota development despite prematurity of the GIT[23,53,67]. However,

despite these associations in preterm piglets, translating these results to humans should be done with caution, and only in the same context as in which it is interpret in the animal model.

The immature GIT of an infant born preterm has increased permeability, partly due to reduced numbers and function of tight junctions between epithelial cells[68]. Commensal bacteria are involved in maturation and maintenance of the epithelial barrier function and stimulation of the immune system[71]. Preterm infants have an aberrant intestinal colonization and an immature immune system, resulting in insufficient elimination of intestinal infections, transfer of bacteria past the epithelial barrier, and an abnormal inflammatory reaction, possibly leading to NEC development[74]. Humans can generally not considerably influence GA and delivery mode. Therefore it is important to influence antibiotic use and nutrition in the most beneficial way in order to induce healthy intestinal microbiota development in preterm infants. Since delivery by CS is often necessary with preterm birth, intestinal microbiota could be modulated by bringing preterm infants born by CS in contact with vaginal microbiota. The vaginal microbiota contains the beneficial *Lactobacillus* spp.[7]. This bacterium could possibly decrease colonization by less beneficial facultative anaerobic skin bacteria, which the infant is exposed to during CS[7], and reduce epithelial permeability by binding to intestinal mucins[68].

It has been shown that intrapartum and neonatal broad-spectrum antibiotics cause an imbalance in the intestinal microbiota of preterm infants. Preterm infants might benefit from narrow-spectrum antibiotics compared to broad-spectrum antibiotics. Narrow-spectrum antibiotics could minimize the short and long-term shift of intestinal microbiota development, maintain levels of beneficial bacteria and limit the opportunity for potential pathogenic bacteria to overgrow[9]. Therefore I would recommend screening for bacterial infections in the mother and infant, to be able to use specific narrow-spectrum antibiotics if possible. Additionally, colonization incidence of potential pathogenic bacteria such as several *Clostridium* spp. differs per NICU[47], suggesting that the hospital should be aware of bacteria that are present in their NICU, to pay attention to possible infections by those bacteria. Increased duration of antibiotic use has been associated with increased incidence of NEC in preterm infants[69], thus antibiotics should be used as short as possible. In contrast to findings in infants, Jensen *et al* (2013) demonstrated a decrease in bacterial load and prevention of NEC at least until day 10 after 5 days of broad-spectrum antibiotic therapy in preterm piglets. However, NEC in infants is regularly developed between 7-27 days of age[26], suggesting that the preterm pigs may have not been followed long enough to detect onset of NEC. The bacteria that survived after antibiotic treatment included potential pathogenic bacteria[19]. Decreased bacterial load and diversity after antibiotic treatment[9] may allow these potential pathogenic bacteria to proliferate extensively, induce an infection and/or transfer the intestinal epithelial lining. Thus, broad-spectrum antibiotics might prevent infection at time of treatment and shortly after, but later contribute to onset of NEC. This indicates that broad-spectrum antibiotic therapy possibly only causes delay of NEC development in cases prone to this disease, as was also suggested by a study in infants[69]. On the other hand, intestines of preterm pigs treated with broad-spectrum antibiotics showed no atrophy, dysfunction, or inflammation, and had increased expression of genes involved in intestinal metabolism and immunity in contrast to the control piglets[19]. Hence, it seems that further research is needed to establish the exact effects of antibiotics on the intestinal colonization and the GIT.

Another factor that could be modulated is nutrition. Findings in term infants show that human milk has beneficial effects on the intestinal microbiota although this was not

detected in preterm infants. Human milk contains many immunological factors that protect the premature infant against inadequate epithelial maturation and intestinal infections[74]. The beneficial bacteria found in human milk, *Bifidobacterium* spp. and *Lactobacillus* spp. have been shown to promote survival of epithelial cells[71] and create an unfavorable environment for pathogenic bacteria by lowering the pH of the intestinal lumen[68]. However, milk from mothers who delivered preterm has been shown to have a different composition, possibly less beneficial to the preterm infant than milk from mothers who delivered at term[74]. Therefore feeding human milk of mothers who delivered at term to preterm infants might promote a healthier intestinal development and better protection against infection compared to feeding their own mothers' milk. Another option to promote healthy intestinal colonization in preterm infants might be to add health-promoting components to formula in order to induce similar beneficial effects as promoted by human milk-feeding. Extensive research has been done in the last decade to search for pro- and prebiotics that resemble those in human milk[11,71]. Probiotics, such as the beneficial bacteria found in human milk[71] and prebiotics, such reviewed by Oozeer *et al* (2013) could be added to human milk. Formula supplemented with short-chain galacto-oligosaccharides (scGOSs) and long-chaing fructo-oligosaccharides (scGOS+lcFOS) affects the intestinal microbiota in early life in a way that it resembles that of human milk-fed term infants[11]. Compared to regular formula, proportions of *Lactobacillus* spp. and *Bifidobacterium* spp. increased, with higher levels of *B. breve* and *B. longum*, and lower levels of *B. catenulatum*[11]. *B. catenulatum* is mainly found in adult microbiota, while the first two are predominantly found in early life, because they are able to degrade specific HMOs[11,43]. *Bifidobacterium longum biovar infantis* preferentially degrades small chain glycans that are predominantly present in human milk of the first month after delivery[61]. Thus, HMOs specifically promote growth of several bacterial species. By adding these specific oligosaccharides to formula, formula induces similar effects on infants' microbiota as is seen with human milk and thus it better promotes a healthy GIT development that could possibly reduce incidence of NEC in preterm infants[11].

This review has examined the role of GA, delivery mode, antibiotic use and nutrition on intestinal microbiota development in preterm infants. Overall, preterm infants have an aberrant intestinal colonization and their GIT and immune system react abnormal to intestinal bacteria compared to infants born at term. This is partially due to prematurity and the higher proportion of broad-spectrum antibiotic therapy in preterm infants compared to term infants. Interestingly, CS and formula feeding seem to influence intestinal microbiota less negatively than in term infants, although negative influences are found in preterm animal models. Prematurity, antibiotic use and type of nutrition also influence development of NEC. Extra research is needed to investigate how nutrition and antibiotic use can be modulated to reduce morbidity and mortality among preterm infants.

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Lekensamenvatting

Het is bekend dat bacteriën in de darm invloed hebben op ontwikkeling van de darmen, het immuunsysteem en het zenuwstelsel via interacties met de gastheer. Babies die te vroeg geboren worden (prematuren) vertonen, vergeleken met babies die op tijd geboren worden (voldragen kinderen), vanaf de geboorte een afwijkende bacterie samenstelling in de darmen (darmflora). Er zijn verbanden gelegd tussen een afwijkende darmflora en ontwikkeling van darmziektes, waaronder necrotiserende enterocolitis (NEC). NEC is een aandoening waarbij een hevige ontsteking van darmweefsel leidt tot afsterving van dit weefsel, mogelijk met de dood tot gevolg. De laatste decennia is belangstelling onstaan voor strategieën om de ontwikkeling van de darmflora te beïnvloeden en zo een gezonde ontwikkeling van prematuren teweeg te brengen. Om de ontwikkeling van de darmflora te kunnen beïnvloeden moet het afwijkende ontwikkelingspatroon en factoren die hier effect op hebben in prematuren onderzocht worden. Het doel van dit rapport is om de verbanden te beschrijven tussen ontwikkeling van de darmflora en zwangerschapsduur, manier van bevalling, gebruik van antibiotica en type voeding. Prematuren worden vaker via keizersnede geboren, behandeld met antibiotica die ook invloed hebben op de darmbacteriën, en gevoed met poedermelk in plaats van moedermelk. Deze factoren hebben een negatieve invloed op de ontwikkeling van een gezonde darmflora in voldragen kinderen en worden verondersteld hetzelfde effect te hebben in prematuren. Dit rapport laat zien dat korte zwangerschapsduur en gebruik van antibiotica inderdaad voor een afwijkende darmflora zorgen in prematuren. Interessant is dat de darmflora van prematuren niet beïnvloed lijkt te worden door verschillende manieren van bevalling (natuurlijk of via keizersnede) en type voeding (poedermelk of moedermelk). Echter, diermodellen hebben wel verschillen aangetoond. Aangezien bovenstaande factoren een afwijkende darmflora kunnen veroorzaken, en dit betrokken is bij ontwikkeling van NEC, zouden deze factoren ook invloed kunnen hebben op NEC. NEC is een van de oorzaken van hoge ziektecijfers en sterfgevallen bij prematuren en kennis van de factoren die de afwijkende darmflora beïnvloeden is daarom van groot belang.