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## The pathophysiology of necrotizing enterocolitis in preterm infants

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2016

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Heida, F. H. (2016). *The pathophysiology of necrotizing enterocolitis in preterm infants: New insights in the interaction between the gut and its microbiota*. University of Groningen.

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A NECROTIZING  
ENTEROCOLITIS-  
ASSOCIATED GUT  
MICROBIOTA IS  
ALREADY PRESENT  
IN THE MECONIUM:  
RESULTS OF A  
PROSPECTIVE STUDY

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Clinical Infectious Diseases 2016; 62(7): 863-870. Doi: 10.1093/cid/ciw016.

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## Abstract

**Background:** Anomalous intestinal microbiota development is supposedly associated with the development of necrotizing enterocolitis (NEC). This study aimed to identify the intestinal microbiota of patients at risk for NEC.

**Methods:** In a prospective trial, investigating prognostic factors for development of NEC in high-risk neonates (NTR4153), 11 NEC cases were gestational-age/birth-weight matched with controls (ratio of 1:2). Feces were collected twice a week. For the present analysis we used the first feces sample of the study of each patient (meconium), as well as the last two feces samples prior to NEC. DNA was extracted and the bacterial 16S rRNA genes were analyzed on a MiSeq sequencer.

**Results:** The presence and abundance of *Clostridium perfringens* (8.4%) and *Bacteroides dorei* (0.9%) in meconium were increased in neonates who developed NEC development compared to controls (0.1% and 0.2%; both species  $p < 0.001$ ). In post-meconium samples, the abundance of staphylococci became negatively associated with NEC development ( $p = 0.1$  and  $0.01$  for consecutive samples) while *Clostridium perfringens* continued to be higher in NEC cases. Early enteral feeding, and in particular breast milk was correlated with an increase of lactate producing bacteria in post-meconium samples ( $\rho = -0.45$ ;  $p = 0.004$ ).

**Conclusions:** A NEC-associated gut microbiota can already be identified in meconium samples; *Clostridium perfringens* continues to be associated with NEC from start to end. Contrariwise, in post-meconium, increased numbers of staphylococci were negatively associated with NEC. These findings suggest causality but this should be verified in induced infection trials in animals, targeted antibiotics and/or probiotics trials.

## Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammatory disorder found mostly in preterm neonates, and goes along with high mortality rates (20-30%).<sup>1</sup> Anomalous intestinal microbiota are supposedly associated with NEC development, but the details of this relationship remain poorly understood.<sup>2,3</sup> Associations between bacterial diversity and the presence of microorganisms, such as *Clostridium spp*, *Klebsiella pneumoniae*, and *Escherichia coli*, have been related with subsequent increased risk for NEC development.<sup>3-7</sup> Unfortunately, the results of these studies vary and only few analyzed the microbiota during the whole interval between birth and NEC development.

Advances in 16S rRNA based sequencing technologies nowadays allow for a rapid and detailed analysis of the bacterial composition of feces, the so-called microbiota.<sup>3,8-10</sup> Sequencing assisted exploration of the alterations in the microbiota of neonates at risk for NEC is important to gain more insight into the association between microbiota and NEC development. This technique allows us to identify not only the presence, but also the (relative) abundances of bacteria and also to follow the development of the microbiota over time. Identification of bacterial groups that are strongly associated with NEC might enable us to develop new preventative options. Therefore, the aim of this study was 1) to determine the diversity and the composition of the intestinal microbiota in preterm neonates at risk for NEC and its relation to NEC development, and 2) to determine associations between maternal- and/or neonatal factors and the intestinal microbiota, which could lead to NEC development.

## Material & Methods

### Patients

This study was part of a prospective cohort trial in our tertiary referral NICU between October 2012 and February 2014 (NTR4153 in the Dutch Trial Registry), in which we included 100 neonates with a high-risk for developing NEC. All of the following neonates were eligible for recruitment into this study: 1. neonates born at a gestational age (GA)  $\leq$  30 weeks, and a birth-weight (BW)  $\leq$  1000 gram, 2. neonates born at a GA  $\leq$  32 weeks and categorized as small for gestational age (SGA;  $\leq$  1200 gram), 3. neonates born with cardiovascular disease resulting in a possible reduced splanchnic blood flow, and 4. neonates who were antenatally exposed to maternal tocolysis using indomethacine. Patients with congenital intestinal disorders were excluded. The local medical ethics committee approved the study. We obtained written informed consent from both parents in all cases.

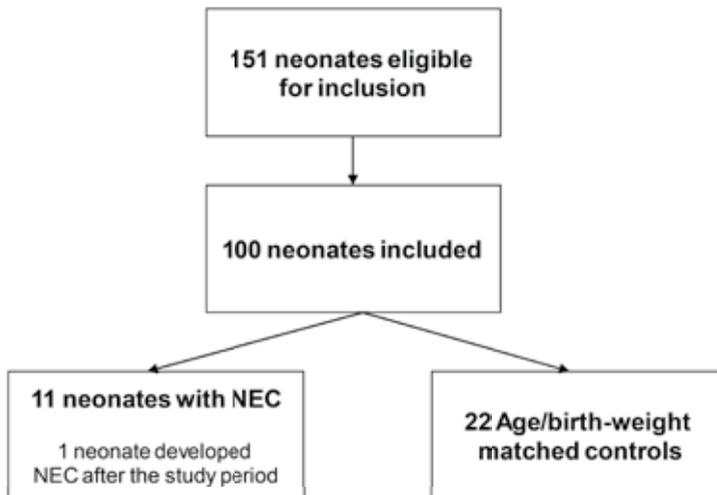
NEC was diagnosed when pneumatosis intestinalis, portal venous gas, or both were present on abdominal X-ray (Bell's stage  $\geq$  2).<sup>11</sup> Neonates remained in the study for

five weeks, until discharge from the NICU, or until NEC development, whichever came first.

We performed a case-control study out of this cohort. We matched every NEC case with 2 controls without NEC based on GA, BW and the availability of samples of the same postnatal age as their matched counterpart (Figure 1).

### FIGURE 1:

#### Flow chart of the study



\*11 neonates (one NEC) participated in the double-blinded early nutrition study (ENS). In these patients data concerning the feeding regime could not be obtained

#### Demographic and clinical variables

We extracted data on maternal and neonatal characteristics from patient files. Maternal factors included: Group B Hemolytic Streptococci (GBS+) carriership, chorioamnionitis as assessed by a pathologist, premature prolonged rupture of membranes (PPROM), use of antenatal antibiotics, and mode of delivery. Neonatal factors included: GA, BW, gender, positive blood cultures, antibiotics, the need for mechanical ventilation, the treatment of circulatory failure (i.e. volume expansion, vasoactive drugs), time of first meconium, delayed meconium (>48h), all available dietary information (i.e. time of first enteral feeding, type and amount of feeding), and length of NICU stay.

## Feces sampling

We collected fecal samples starting with the first feces after birth. Samples were thereafter collected twice a week. Samples were stored at -80 °C until batch analyses. Ideally we used three fecal samples per patient: the first sample of the study (meconium) and the two samples collected in the week prior to onset of NEC (when available). The samples of the controls were collected at the same postnatal day as their matched NEC cases. We classified feces samples as meconium and non-meconium, based on consistency and color.

## DNA extraction and MiSeq preparation

Total DNA was extracted from a 0.25 g faecal sample in exactly the same manner as described by de Goffau et al.<sup>12,13</sup> The V3-V4 region of the 16S rRNA gene was amplified from the bacterial DNA by PCR using modified 341F and 806R primers (Supplementary Table 1) with a 6 nucleotide barcode on the 806R primer as described elsewhere.<sup>14,15</sup> A detailed description of the PCR, DNA cleanup and MiSeq library preparation using a 2x300 cartridge are found in Online Supplemental Data file 1.

## Analyses of sequence reads

The software used to analyze the data received from Illumina paired-end sequencing included PANDAseq,<sup>16</sup> QIIME and ARB.<sup>17</sup> Readouts with a quality score lower than 0.9 were discarded by PANDAseq. QIIME identified sequences down to the family and genus level while ARB was used to identify sequences to the species level. To find aberrant microbiota compositions indicative of NEC development, we compared the intestinal microbiota of neonates with NEC development with the microbiota of those who did not develop NEC per sampling point. Both the presence and abundance of microbial groups in the fecal samples were analyzed in relation to NEC development.

## Statistics

Principal component analysis (PCA) was performed to find clusters of similar groups of samples or species.<sup>18</sup> Thereby, PCA was used to find bacterial patterns in time and to relate this data with clinical variables.<sup>18</sup> The Simpson index on the family level was used for analyses concerning the bacterial diversity. We performed statistical analyses using the Statistical Package for the Social Sciences (IBM SPSS Statistics 22, IBM Corp., Armonk, New York, USA). Two sided p-values <0.05 were considered statistically significant. Non-parametric tests were used, as microbial abundances are rarely normally distributed. For two categorical variables the chi-square test was used, for two continuous variables the Spearman rho-test and for the combination of a categorical and a continuous variable we used the Mann-Whitney *U* test.

## Results

Eleven patients developed NEC at a median of 12.5 days after birth (range: 4-43). One patient developed NEC after the study period. One patient developed NEC after 4 days after birth. For this patient we used sample A and the second sample (which we considered the sample prior to NEC (C)). We included 22 controls matched for GA, BW and sample frequency (maximum deviation GA = 8% and BW = 26%). Table 1 presents the patient characteristics.

**TABLE 1:**  
**Patient characteristics**

Characteristics	NEC n=11	Controls n=22	P value
Sex (male)	6 (55%)	10 (45%)	0.64
Median gestational age, weeks (range)	27 (24-29)	26 (24-29)	0.32
Median birth weight, grams	970 (560-1630)	995 (615-1735)	0.81
Maternal age (years)	31 (19 – 41)	28 (23 – 35)	0.38
Number of previous gravidities	1 (0 – 10)	0 (0 – 5)	0.96
Number of previous parities	1 (0 -7)	0 (0 – 7)	0.79
Mother GBS+	3 (27%)	2 (9%)	0.50
Choriomnionitis	5 (45%)	7 (32%)	0.47
PPROM	5 (45%)	5 (23%)	0.26
Maternal antibiotic exposure during labor	3 (27%)	5 (23%)	0.32
Delivery mode (CS)	4 (36%)	13 (59%)	0.93
Interval birth – first meconium, days	1 (0-3)	1 (0-3)	0.89
Start enteral feeds after birth, hours	6.5 (2-10)	3 (2-8)	0.02
Breast-feeding <24h postpartum (%of total enteral feeding)	6 (27%)	11 (12%)	0.99
Formula feeding <24h postpartum (%of total enteral feeding)	9 (73%)	22 (88%)	0.10
Parenteral feeding <24h postpartum (%of total feeding)	11 (87%)	22 (87%)	-
Need for mechanical ventilation	7 (64%)	9 (41%)	0.23
Need for inotropics	3 (23%)	8 (36%)	0.42
Positive blood cultures during study period	1 (9%)	4 (18%)	0.51
Antibiotic exposure within 48hours postpartum	9 (82%)	17 (77%)	0.42
Antibiotic exposure after 48hours postpartum	11 (100%)	14 (64%)	0.09
Age of onset NEC symptoms, days (range)	12.5 (4-43)	-	-
Mortality	4 (36%)	2 (9%)	-
Mean survival days	30 (6 – 49)	17.5 (10 – 25)	-
Cause of death			
- NEC	3	1	-
- Intracranial hemorrhage		1	-
- Sepsis	1		-

Data are expressed as median (range) or as numbers unless specified otherwise.

Abbreviations: GBS: Group B hemolytic streptococci; PPRM: preterm prolonged rupture of membranes; CS: Caesarian section; NEC: necrotizing enterocolitis

The first fecal samples were collected at a median of 1 day (range: 0-4) after birth. All of these samples consisted of meconium. The last two samples prior to NEC development were collected at a median of 5 days (range: 2-7) and 2 days (range: 0-4), respectively.

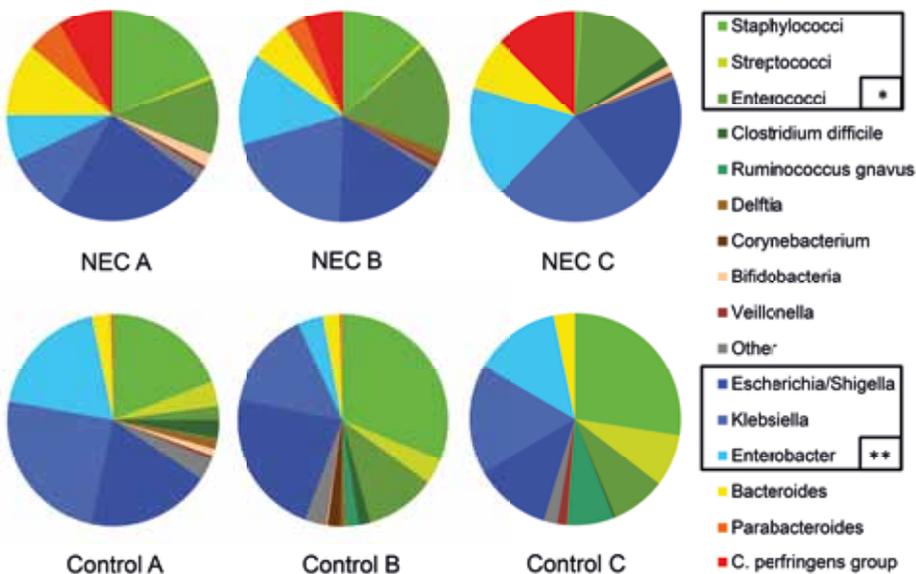
### Intestinal microbiota in the neonate at risk for NEC

The development of the intestinal microbiota over time in both NEC and control cases is summarized in Figure 2 and in Supplemental Figure 2. In the meconium samples, both the presence and abundance of *C. perfringens* and *B. dorei* were significantly higher in patients who developed NEC compared to those who did not ( $p < 0.001$ ). On the other hand, abundances of *C. difficile* were lower in meconium samples from patients who developed NEC compared to controls (Table 2). The enterobacteria family accounted for 50% of the reads on average in feces samples and thus represented the most abundant bacterial group in this study yet was not significantly correlated with NEC development (Table 2).

#### FIGURE 2:

### Changes in microbial composition over time between necrotizing enterocolitis cases and controls.

Proportions of the microbial species at the three time points (from the first sample of the study (A) until the last two samples before NEC development (B and C)) are visualized for NEC cases versus control samples. \* lactate producing bacilli, \*\* *Enterobacteriaceae*.



In the last two samples before the onset of NEC both the presence and abundance of *C. perfringens* and *B. dorei* remained higher in neonates who developed NEC compared to those who did not (percentages are depicted in Table 2). We observed a shift – also revealed via PCA analysis - from a more *Enterobacteriaceae* dominated microbiota into one with higher abundances of staphylococci (19%) and other lactate producing bacilli in controls compared to NEC cases (Figure 2, Table 2 and Figure 3). NEC cases either remained dominated by *Enterobacteriaceae* or developed an aberrant microbiota composition that forms a distinct cluster in Figure 3 (consisting of *C. perfringens* and *B. dorei*).

**TABLE 2:****The relation between necrotizing enterocolitis associated bacteria over time\***

	Time point A		Time point B		Time point C	
	p-value	NEC vs. controls (%)	p-value	NEC vs. controls (%)	p-value	NEC vs. controls (%)
<i>C. perfringens</i> -like bacteria						
- Presence	< 0.001(↑)	90% vs. 24%	0.002(↑)	80% vs. 23%	0.019(↑)	60% vs. 14%
- Abundance	< 0.001(↑)	8.4% vs.0.1%	0.006(↑)	6.0% vs. 0.003%	0.012(↑)	12.6% vs.< 0.001%
<i>C. difficile</i>						
- Presence	0.014(↓)	30% vs 76%	0.07(↓)	20% vs. 55%	0.13(↓)	20% vs. 50%
- Abundance	.012(↓)	0.02% vs 2.7%	.08(↓)	0.02%vs.1.8%	0.18(↓)	1.4% vs.0.34%
<i>B. dorei</i>						
- Presence	<0.001(↑)	80% vs. 14%	0.01(↑)	40% vs. 5%	0.35	20% vs. 7%
- Abundance	0.001(↑)	0.9% vs. 0.2%	0.006(↑)	0.7% vs.0.005%	0.36	3.7% vs.< 0.001%
Bacteroidaceae						
- Abundance	0.003(↑)	11% vs. 2.9%	0.7	6.0% vs. 2.5%	0.31	7.9% vs.3.2%
<i>Enterobacteriaceae</i>						
- Abundance	0.08(↓)	38% vs. 64%	0.5	52% vs. 42%	0.6	60% vs. 43%
Staphylococci						
- Abundance	0.5	17% vs. 17%	0.095(↓)	12% vs.30%	0.013(↓)	0.5% vs. 23%
Streptococci						
- Abundance	0.5	0.8% vs. 3.8%	0.27(↓)	0.7% vs.3.9%	0.16(↓)	0.1% vs.7.8%
Enterococci						
- Abundance	0.18(↑)	11% vs. 2.1%	0.33	17% vs. 11%	0.36	14% vs.8.3%
Lactate producing bacilli (all)						
- Abundance	0.9	30% vs. 25%	0.44	31% vs. 47%	0.16(↓)	16% vs. 44%

\*For exact distributions of the microbial groups over time in each sample see Supplemental Figure 2. An overview of the associations between NEC development and the most relevant bacterial groups found in this study. Timepoints A, B and C represent the meconium sample (A) and the last two samples collected in the week prior to onset of NEC (B and C). The arrows represent the direction of association with NEC.

## The association between maternal- and neonatal factors and the intestinal microbiota

In Table 3 the relation between neonatal- and maternal factors and NEC are presented. An earlier start of enteral feeding after birth is associated with a higher amount of lactate producing bacteria in post-meconium samples ( $\rho=0.31$ ;  $p=0.05$ ). In addition, in particular the amount of breast milk as a percentage of the total amount of feeding is correlated with an increase in the abundance of lactate producing bacteria ( $\rho=0.45$ ;  $p=0.004$ ) and a decrease in the number of Gram-negative species, which include the enterobacteria and bacteroidetes families ( $\rho=-0.35$ ;  $p=0.028$ ). The amount of breast milk just before NEC onset is negatively associated with NEC ( $p=0.04$ ).

**TABLE 3:**

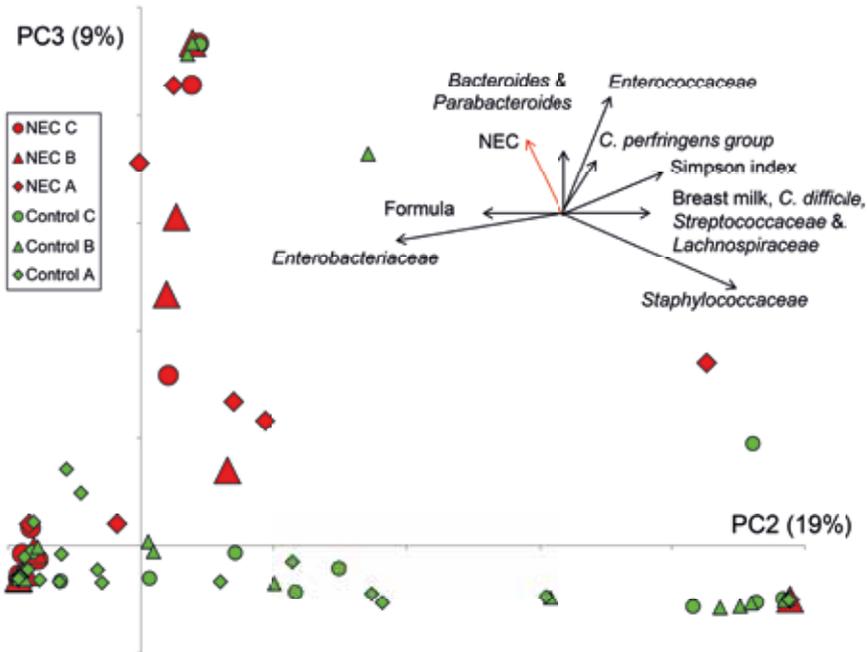
### The relation between neonatal- and maternal factors and necrotizing enterocolitis

Maternal factors	P value	Neonatal factors	P value	Neonatal factors	P value	Neonatal factors	P value
Chorionionitis		Antibiotics <2 days		Amount of breast feeding at moment A		Amount of formula feeding at moment B	
NEC	0.6	NEC	0.9	NEC	0.7	NEC	0.4
						Lactate producing bacteria	$\rho=-0.44$ 0.044(↓)
						<i>Enterobacteriaceae</i>	$\rho=0.40$ 0.076(↑)
PPROM		Delayed meconium (>2 days)		Amount of formula feeding at moment A		Amount of parenteral feeding at moment C	
Vaginal birth	0.03 (↑)	NEC	0.4	NEC	0.6	NEC	0.3
NEC	0.6						
Staphylococci	0.016(↓)						
C-section		Duration between birth and enteral feeding (hours)		Amount of parenteral feeding at moment B		Amount of breast feeding at moment C	
NEC	0.3	NEC	0.02 (↑)	NEC	0.9	NEC	0.04 (↓)
Staphylococci	0.004(↑)			Streptococci	$\rho=-0.51$ 0.018(↓)	<i>Bacteroidaceae</i>	$\rho=-0.41$ 0.093(↓)
Antibiotics antenatal		Amount of parenteral feeding at moment A		Amount of breast feeding at moment B		Amount of formula feeding at moment C	
NEC	0.8	NEC	0.09(↓)	NEC	0.3	NEC	0.5
		<i>C. perfringens</i>	$\rho=-0.54$ 0.014(↓)	Lactate producing bacteria	$\rho=0.40$ 0.073(↑)	Lactate producing bacteria	$\rho=0.50$ 0.03(↓)
				<i>Enterobacteriaceae</i>	$\rho=-0.41$ 0.069(↓)		

Abbreviations: GBS: Group B hemolytic streptococci; PPROM: preterm prolonged rupture of membranes; CS: Caesarian section; NEC: necrotizing enterocolitis

## Bacterial diversity

In general the microbial diversity (Simpson index) was negatively associated with the abundance of *Enterobacteriaceae* and a shift to the (upper) right in Figure 3. However, the microbial diversity was not associated with NEC development and only weakly with diet as 1) abundances of staphylococci were also frequently found (Supplemental Figure 2) and 2) *C. perfringens* and *Bacteroidaceae* both contributed to the diversity score.



**FIGURE 3:**

### Principal component analysis (PCA) on the family level.

PC2 (19%) and PC3 (9%), represented on the x- and y-axis respectively, highlight some of the main differences between those samples (expressed in time points A, B, and C) from neonates who developed NEC and those who did not (Figure 3). Meconium samples (A) from neonates without NEC development are more often found in the lower left corner of the graph and can be characterized as being mainly dominated by *Enterobacteriaceae*. From the lower left a series of samples from children without NEC development can be seen heading to the lower right of the graph. These samples are populated increasingly with staphylococci, streptococci (3.3%), *C. difficile* and/or *Lachnospiraceae* (1.9%). This shift to the right in sample B and C is associated with enteral feeding, specifically breast milk. A series of samples from neonates who developed NEC, whose microbiota does not mainly consist out of *Enterobacteriaceae*,

are found increasingly higher on PC3, representing an increase of *C. perfringens*, (*Para*) *bacteroides* and/or *Enterococcaceae*. The few non-NEC samples also scoring high on PC3 are invariably samples that have high *Enterococcaceae* abundances. Principal component 1 (PC1) described most of the variation in the data (58%), however was not directly associated with NEC development as it basically described the variation in the abundance of *Enterobacteriaceae* versus the rest.

### Relation between microbiota and mortality

The abundance of bacteroidaceae in meconium is positively associated with mortality ( $p=0.02$ ) while the abundance of lactate producing bacteria is negatively associated with mortality in the sample just prior to NEC development ( $p=0.04$ ).

### Discussion

This study demonstrates the presence of a NEC-associated gut microbiota, with *C. perfringens* and *B. dorei*, already in the meconium. Therefore, this study suggests that factors during the first days of life, during delivery or even in utero, might affect the formation of a NEC-associated microbiota. In post-meconium samples, a higher number of staphylococci later in life seemed protective against NEC. The abundance of lactate-producing bacteria correlated with an early commencement of enteral nutrition and moreover with the amount of breast milk.

One of the most prominent findings of our study is the fact that a NEC-associated gut microbiota was already present within days after birth. *C. perfringens* strains, that produce alpha-toxin, have been associated with NEC development before.<sup>7,19–22</sup> Almost all studies on the subject studied feces samples that were obtained around NEC diagnosis. Only de La Cochetière, et al.<sup>23</sup> suggested a significant relationship between early colonization by *Clostridium* spp and development of NEC. *B. dorei* was also associated already in the first days of life with the development of NEC. To our knowledge, this is the first study that describes an association between *B. dorei* in meconium and subsequent NEC development and mortality. Only Brower-Sinning et al.<sup>24</sup> previously suggested a relationship between *Bacteroides* and NEC based on findings derived from resection specimens, while Blakely et al.<sup>20</sup> stated, after analyzing feces samples of neonates around NEC onset, that *Bacteroides* were less frequently isolated from neonates with NEC. These findings might not be inconsistent with each other as we only found *Bacteroides* to be associated with NEC and mortality in meconium samples. *Bacteroides* generally behave as normal anaerobic (beneficial) commensal bacteria in the human gut but they are also known to cause severe pathology. This, combined with its resistance to many antibiotics and its ability to influence the immune system, makes *Bacteroides* a classic example of a pathosymbiont.<sup>25</sup> Its exact role in regards to NEC development hence remains elusive.

In post-meconium fecal samples, lactate-producing bacteria (including staphylococci and streptococci) seemed protective against the development of NEC. Thereby, the abundance of lactate-producing bacteria were associated with lower mortality. Cassir et al.,<sup>26</sup> similarly found Staphylococci to be more prevalent in controls than in NEC cases. La Rosa et al.,<sup>5</sup> observed that lactate-producing bacteria are best represented in the earliest weeks of life. Lactate-producing bacteria may have a similar protective role as bifidobacteria and lactobacilli.<sup>27</sup> They can do so by lowering the pH via the production of lactate and thereby hampering the growth of opportunistic pathogens, such as *C. perfringens*.<sup>28-30</sup> Lactate-producing bacteria were in this study in addition stimulated by breast milk. Whereas bifidobacteria and lactobacilli are stimulated by breast milk and are often thought to provide protection against NEC development,<sup>28-30</sup> this was not the case in this study, as they were only present in low numbers. Bifidobacteria and lactobacilli are usually only seen in fecal sampling after a gestational age greater than 33 weeks, and their presence increases with gestational age.<sup>29-31</sup> Our cohort was significantly younger than that. Based on the present data we might therefore speculate that in neonates with a gestational age below 33 weeks staphylococci in particular are capable of taking over the (protective) role and the bacterial niche of the still largely absent bifidobacteria.

*C. difficile* was negatively associated with NEC in the present study. *C. difficile* is considered a common nosocomial pathogen.<sup>32-34</sup> However, colonization with non-toxigenic *C. difficile* strains provides effective protection against toxigenic *C. difficile* strains.<sup>32-34</sup> In addition, healthy newborns are very frequently colonized with *C. difficile* (60-70%), yet they do not suffer any ill effects.<sup>34</sup> In fact, children become more prone to *C. difficile* related diseases as they become older when they are no longer *C. difficile* carriers.<sup>34</sup> The protective effects of being a *C. difficile* carrier might also shield children against *C. perfringens* enterotoxin mediated diseases.

The level of microbial diversity in NEC is controversial.<sup>4</sup> Multiple studies observed low bacterial diversity associated with NEC.<sup>4,6,35</sup> In our cohort, similar to multiple previously performed studies, there was a low diversity of microbiota in feces samples of premature neonates, but this was not associated with NEC development.<sup>35-38</sup> We hypothesize that a low diversity of microbiota makes the premature neonate vulnerable for the development of NEC because it provides less protection against pathogens, but does not directly induce NEC.

We demonstrate the presence of a NEC-associated gut microbiota within the first days of life if not in the first meconium. Previously it was thought that the in utero environment was largely sterile and that a fetus was not colonized with bacteria until the time of birth.<sup>5</sup> Recently it has been suggested that the colonization process can

sometimes well begin before delivery, and has for example been associated with preterm delivery.<sup>5,39</sup> With the results of the present study we share this hypothesis that (yet unrevealed) factors during the first days of life or even in utero could play an important role in the formation of a more healthy or a more NEC-associated microbiota. While maternal factors were not found to be associated with the development of a NEC-associated gut-microbiota we did find an association between early enteral nutrition and increased abundances of (protective) lactate-producing bacteria in post-meconium samples. This also holds true for breast milk. We could only analyze data concerning the feeding regime in about two thirds of the patients due to the inclusion of patients from a double blind prospective early nutrition study. That we nonetheless observed these strong differences in abundances of lactate-producing bacteria in a small group of patients only strengthens our observation. Our findings therefore confirm the hypothesis that both early enteral nutrition after birth and breast milk play a pivotal role in protecting the intestine against development of NEC.<sup>40</sup> Early commencement of breast milk and subsequently predominantly giving breast milk likely stimulates lactate producing bacteria in neonates with a low gestational age in a similar way as breast milk normally stimulates bifidobacteria in neonates with a more normal GA.<sup>40</sup> Protection against colonization with pathogenic bacteria is thought to be achieved by providing lactate producers, such as bifidobacteria, with human oligosaccharides.<sup>26-28</sup>

This study points at the clinical relevance of analysis of meconium in preterm neonates at risk for NEC to detect NEC-associated bacteria. If *C. perfringens* and other NEC-associated bacteria, possibly also *Clostridium butyricum*, as shown by Cassir et al.,<sup>26</sup> are found within gut microbiota of a neonate, this neonate could then subsequently be treated with more targeted antibiotic therapies. Apart from breast milk a proper microbial development of the gut microbiota could be stimulated in the future with the use of pre- or probiotics. Larger trials are needed to further investigate the microbiota in both fecal samples and resection specimens in children at high risk for NEC and who develop NEC. Based on that data we could then outline an optimal and individual pre- or probiotic strategy for neonates at risk.

## Conclusion

This study demonstrates the presence of a NEC-associated gut microbiota already within the first days of life in premature children at risk for NEC. We observed an association between *C. perfringens* and *B. dorei* in the meconium and NEC development. Our data suggests that (yet unrevealed) factors during the first days of life, or even in utero, could be associated with the formation of a NEC-associated microbiota. Also, this was the first study showing that *B. dorei* in the meconium was associated with an increased mortality, suggesting that microbiota analysis

could also be used as a predictive tool. In post-meconium fecal samples, increased numbers of staphylococci, and not bifidobacteria, were negatively associated with NEC. Abundance of lactate-producing bacteria was associated with an early commencement of enteral nutrition and with breast milk. Our study suggests causality, however this is not proven. These findings should be verified by testing for NEC induced infection with *C. perfringens* in animal models, possibly in combination with targeted antibiotics treatment. Alternatively, it should be investigated whether protection against NEC development can be established by increasing the prevalence of lactate producers via the use of pre-, pro- or postbiotics.

### **Acknowledgments**

We would like to thank all the parents of subsequent neonates for participation in the study. Also, we would like to thank all the staff and students who were involved in the inclusion and sample collection of the study. We greatly acknowledge the help of C. Bus-Spoor and R.H.J. Tonk for technical assistance of the study, and S.K.A. David for editing the English language in the manuscript.



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## Supplements

**SUPPLEMENTAL TABLE S1:****Nucleotide sequences of primers used in the construction of libraries for Illumina sequencing**

V3_F_modified	aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNCC TACGGGAGGCAGCAG
V4_1R	caagcagaagacggcatacagagatCGTGATgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_2R	caagcagaagacggcatacagagatACATCGgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_3R	caagcagaagacggcatacagagatGCCTAAgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_4R	caagcagaagacggcatacagagatTGGTCAgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_5R	caagcagaagacggcatacagagatCACTGTgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_6R	caagcagaagacggcatacagagatATTGGCgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_7R	caagcagaagacggcatacagagatGATCTGgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_8R	caagcagaagacggcatacagagatTCAAGTgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_9R	caagcagaagacggcatacagagatCTGATCgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_10R	caagcagaagacggcatacagagatAAGCTAgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_11R	caagcagaagacggcatacagagatGTAGCCgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_12R	caagcagaagacggcatacagagatTACAAGgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_13R	caagcagaagacggcatacagagatCGTACTgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_14R	caagcagaagacggcatacagagatGACTGAgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_15R	caagcagaagacggcatacagagatGCTCAAgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT
V4_16R	caagcagaagacggcatacagagatTCGCTTgtgactggagttcagacgtgtgctcttccgatct GGACTACHVGGGTWCTAAT

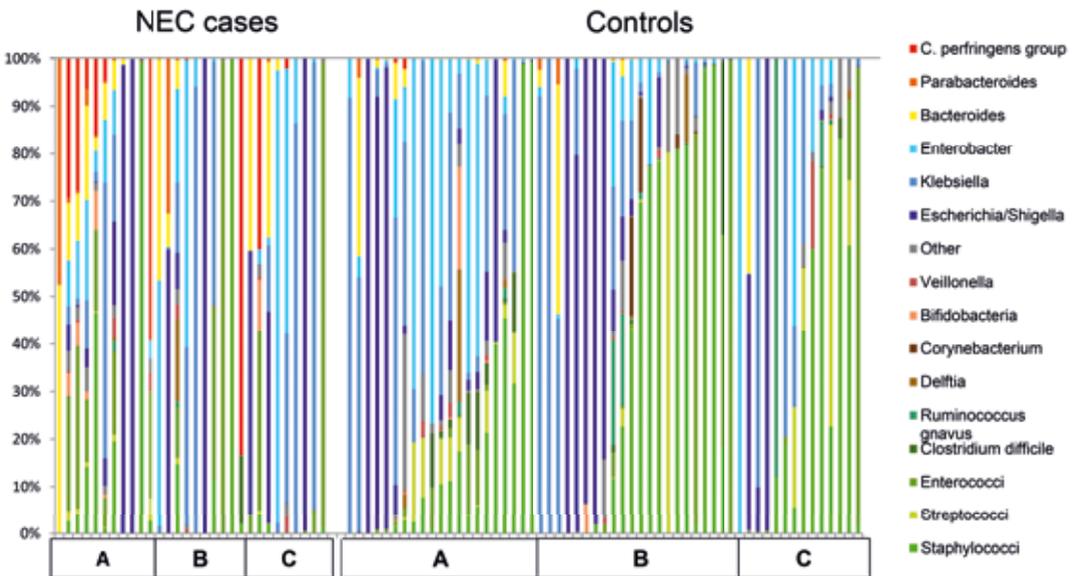
Lowercase letters denote adapter sequences necessary for binding to the flowcell, underlined lowercase are binding sites for the Illumina sequencing primers, bold uppercase highlight the index sequences (additional indexes can be found in the paper by Bartram et al.). Regular uppercase are the V3 and V4 region primers (341F on for the forward primer and 806R for the reverse primers). The inclusion of four maximally degenerated bases (“NNNN”) maximizes diversity during the first four bases of the run. Diversity is important for identifying unique clusters and base-calling accuracy.

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**SUPPLEMENTAL FIGURE 1:****Gut microbiota composition in necrotizing enterocolitis- and control samples per time point.**

The relative abundances of the microbial groups are shown per sample per time point (Samples (A) represent meconium samples and samples (B) and (C) represent the last two samples before NEC development).

**SUPPLEMENTAL DATA FILE 1:****PCR & MiSeq preparation**

Reaction conditions consisted of an initial 94 °C for 3 min followed by 32 cycles of 94 °C for 45 sec, 50 °C for 60 sec, and 72 °C for 90 sec, and a final extension of 72 °C for 10 min. An agarose gel confirmed the presence of product (band at ~465 base pairs) in successfully amplified samples. The remainder of the PCR product

(~45  $\mu$ l) of each sample was mixed thoroughly with 25  $\mu$ l Agencourt AMPure XP magnetic beads and were incubated at room temperature for 5 minutes. Beads were subsequently separated from the solution by placing the tubes in a magnetic bead separator for 2 minutes. After discarding the cleared solution the beads were washed twice by resuspending the beads in 200  $\mu$ l freshly prepared 80% ethanol, incubating the tubes for 30s in the magnetic bead separator and subsequently discarding the cleared solution. The pellet was subsequently air dried for 15 minutes and resuspended in 52.5  $\mu$ l 10 mM Tris HCl pH 8.5 buffer. 50  $\mu$ l of the cleared up solution is subsequently transferred to a new tube. The DNA concentration of each sample was done using a Qubit® 2.0 fluorometer ([www.invitrogen.com/qubit](http://www.invitrogen.com/qubit)) and the remainder of the sample was stored at -20°C until library normalization °C. Library normalization was done the day before running samples on the MiSeq by making 2 nM dilutions of each sample. Samples were pooled together by combining 5  $\mu$ l of each diluted sample. 10  $\mu$ l of the sample pool and 10  $\mu$ l 0.2 M NaOH were subsequently combined and incubated for 5 minutes to denature the sample DNA. To this, 980  $\mu$ l of the HT1 buffer from the MiSeq 2x300 kit is subsequently added. A denatured diluted PhiX solution was made by combining 2  $\mu$ l of a 10 nM PhiX library with 3  $\mu$ l 10 mM Tris HCl pH 8.5 buffer with 0.1% Tween 20. These 5  $\mu$ l were mixed with 5  $\mu$ l 0.2 M NaOH and incubated for 5 minutes at room temperature. These 10  $\mu$ l were subsequently mixed with 990  $\mu$ l HT1 buffer. 150  $\mu$ l of the diluted sample pool was combined with 50  $\mu$ l of the diluted PhiX solution and is further diluted by adding 800  $\mu$ l HT1 buffer. 600  $\mu$ l of the prepared library was loaded into the sample loading reservoir of the MiSeq 2x300 cartridge.