Preterm Birth and Necrotizing Enterocolitis Alter Gut Colonization in Pigs

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ABSTRACT: Necrotizing enterocolitis (NEC) in preterm neonates is dependent on bacterial colonization, but it remains unclear whether a particular microbiota or specific pathogens are involved. We hypothesized that gut colonization differs between preterm and term neonates and that overgrowth of *Clostridium perfringens* predisposes to NEC. By using terminal-RFLP and FISH, we characterized the gut microbiota of preterm, caesarean-delivered, formula-fed pigs ($n = 44$) with or without NEC and of formula- or colostrum-fed term, and vaginally born pigs ($n = 13$). A different microbiota with high *C. perfringens* abundance was observed in preterm pigs with NEC compared with healthy individuals. However, immunization against *C. perfringens* toxins did not prevent NEC, and *C. perfringens* inoculation (3.6×10^8 cfu/d) failed to induce NEC ($n = 16$), whereas prophylactic broad-spectrum antibiotics treatment prevented NEC ($n = 24$). Colonization in both groups of term pigs differed from preterm pigs and was dominated by *Lactobacilli* spp. In conclusion, gestational age (GA) and NEC influence neonatal gut colonization, whereas diet has minor effects. *C. perfringens* is more abundant in pigs with NEC but rather as a consequence than a cause of disease. The general bacterial load and underdeveloped gut immune responses in preterm neonates seem more important for NEC development than specific pathogens. (*Pediatr Res* **69:** 10–16, 2011)

Bacterial colonization of the immature intestine is a contributing factor to the development of necrotizing enterocolitis (NEC), a severe intestinal disease in preterm infants. Preterm neonates are generally less exposed to maternal contact and breast milk, which is considered to initiate a balanced gut colonization that provides immunological tolerance and reduced intestinal inflammation (1). Observational studies show that compared with full-term birth and mother's milk feeding, preterm birth and formula feeding predispose to a different fecal microbiota and to NEC (2,3). Hospital environments and delivery method affect the microbiota but have not consistently been linked with NEC in infants (4,5) or pigs (6). Reduced digestive functions and motility of the immature intestine result in accumulated undigested food, which predisposes to bacterial overgrowth and excessive fermentation. Infant NEC is often associated with abundant *Escherichia coli*, *Klebsiella*, *Streptococcus*, and *Clostridium* spp. (7,8). Although no single pathogen has been identified (9–11),

Clostridium perfringens has been associated with NEC in infants and pigs (12–14).

A general risk of infection and the association between bacterial overgrowth and NEC have led to widespread use of antibiotics for preterm infants. However, prolonged antibiotic administration results in a microbiota dominated by fewer species that potentially predispose to NEC (11,12). It remains unknown whether short-term broad-spectrum antibiotic treatment, i.v. and/or orally, will prevent NEC (15).

To elucidate how gut colonization is affected by gestational age (GA), diet, and delivery mode and the relationship with NEC risk, we characterized the luminal- and tissue-associated gut microbiota in preterm and term pigs using culture-dependent methods and molecular methods targeting the bacterial 16S rRNA gene: terminal RFLP (T-RFLP) and FISH. We hypothesized that gut colonization differs between caesarean-delivered preterm pigs and pigs born vaginally at term. We predicted that *C. perfringens* is a dominating species in preterm pigs with NEC and that immunization against *C. perfringens* toxins or inoculation with *C. perfringens* alters NEC incidence. Finally, we hypothesized that prophylactic broad-spectrum antibiotics inhibit gut colonization and prevent NEC in preterm pigs.

MATERIALS AND METHODS

Study 1: gut colonization in preterm and term pigs. Thirteen term pigs from one sow (Large White × Danish Landrace × Duroc) were vaginally born at term (114 -d gestation). The pigs either remained with the sow to suckle colostrum (MAT-C, $n = 7$) or were transferred to temperature- and oxygen-controlled incubators (Air-Shields, Hatboro, PN), fitted with orogastric catheters as previously described (14) and fed formula (MAT-F, $n = 6$). All term pigs were killed for sample collection 38–43 h after birth. According to our standard protocol (14), 44 preterm pigs from three sows were delivered by caesarean section at 91–93% gestation, transferred to incubators, and fitted with vascular catheters (4 Fr) into the umbilical aorta and with 6-Fr orogastric catheters and fed with infant formula (15 mL/kg/3 h). Preterm pigs were stratified after weight into three treatment groups as follows: 1) untreated controls ($n = 22$); 2) TOXICOL pigs ($n = 4$) that within 12 h of age were given serum [15 mL/kg intraarterial (i.a.)] from a pregnant sow immunized with toxins from *C. perfringens* type C and *E. coli* antigens (Schering-Plough, Farum, Denmark); and 3) COLERADO pigs ($n = 18$) given antiserum with anti-toxins for *C. perfringens* type C and D (2.5 mL/kg i.a.) and 2.5 mL p.o., CO Serum Company, Denver, CO). The pigs were continuously evaluated for clinical NEC symptoms such as lethargy, abdominal distention, and bloody diarrhea. Two days after birth, pigs were killed (pentobarbiturate, i.a.) for sample collection unless significant NEC symptoms developed beforehand.

Abbreviations: i.a., intraarterial; **NEC,** necrotizing enterocolitis; **TPN,** total parenteral nutrition; **T-RFLP,** terminal RFLP; **T-RF,** terminal restriction fragment

All studies were approved by the National Committee on Animal Experimentation, Denmark.

Study 2: C. perfringens type A inoculation. Sixteen caesarean-delivered preterm pigs from two sows were fitted with umbilical and orogastric catheters as described above. Total parenteral nutrition (TPN) was administered for 2 d as previously described (16) before full formula feeding (15 mL/kg$^-$/3 h) for 36–48 h until killing and sample collection. The pigs were randomized after weight into controls ($n = 10$) and pigs inoculated with 3.6×10^8 *C. perfringens* type A (($n = 6$). After study 1, multiplex PCR targeting the genes for α, β, $\beta 2$, ϵ, and ι toxins (17) identified *C. perfringens* type A as the only *C. perfringens* type isolated from preterm pigs across several previous studies (data not shown). *C. perfringens* type A isolated from a previous NEC pig was grown anaerobically overnight in brain heart infusion (BHI) broth (National Veterinary Institute, Copenhagen, Denmark). At 4, 10, 22, and 26 h postpartum, 1 mL *C. perfringens* type A culture was given with 1 mL formula to preterm pigs *via* the oral catheter to a total daily dose of 3.6×10^8 cfu. Bacterial density (cfu/mL) was determined by anaerobic plating on *C. perfringens* selective tryptose sulfite cycloserine (TSC) plates (National Veterinary Institute, Copenhagen, Denmark).

Study 3: prophylactic antibiotics. Caesarean-delivered preterm pigs from two sows given first TPN then formula, as described above, were allocated into controls ($n = 13$) and a treatment group receiving oral and systemic broad-spectrum antibiotics, frequently used in NICU (ampicillin, gentamicin, metronidazole, ANTI, $n = 11$). Ampicillin (totally 200 mg/kg/d) was given twice daily by oral (0.5 mL/kg: 500 mg tablets dissolved in 5 mL saline; NordMedica, Copenhagen, Denmark) and i.m. administration (0.175 mL/kg: 1 g dissolved in 3.5 mL saline; Pentrexyl, Bristol-Myers Sqibb, Bromma, Sweden). Gentamicin (totally 2.5 mg/kg/d) was given once daily orally and i.m., with 2.5 mg/kg/d per administration route (0.1 mL/kg: 50 mg/mL diluted in saline to 25 mg/mL; KU-LIFE Pharmacy, Copenhagen, Denmark). Metronidazole (totally 40 mg/kg/d) was administered by two oral doses (0.25 mL/kg: 40 mg/mL; Flagyl; Sanofi Aventis, Hørsholm, Denmark) and two i.m. injections (10 mg/kg/d; 5 mg/mL; Actavis, Hafnarfjordur, Island). Systemic and oral antibiotics were given simultaneously together with formula (3 mL/kg). Control pigs were injected with saline.

Tissue collection and pathological evaluations. For all three studies, five regions of the gastrointestinal organs (stomach, proximal, middle, and distal small intestine, and colon) were evaluated for NEC lesions according to our macropathological scoring system: $1 =$ absence of lesions, $2 =$ local hyperemia, inflammation, and edema, $3 =$ hyperemia, extensive edema, and local hemorrhage, $4 =$ extensive hemorrhage, $5 =$ local necrosis and pneumatosis intestinalis, and $6 =$ extensive necrosis and pneumatosis intestinalis. NEC was defined as a score of minimum 3 in minimum one region (16).

For study 1, sections of distal small intestine were formalin fixated and paraffin embedded for mucosal morphometry analyses, FISH, and HE staining (16). Histomorphological measurements of 10 full length villi and 10 crypts for each pig were performed by the software of SoftWoRx Explorer Version 1.1 (Applied Precision, Issaquah, WA) on scanning images of 3-μm cross sections (ArrayWoRx^e microarray scanner, Applied Precision).

Microbiology. Luminal contents from distal small intestine (study 1) or cecum (studies 2 and 3) were sampled and stored at 4°C until microbiological characterization. Small intestinal contents were cultured anaerobically on calf blood agar plates (SSI Diagnostika, Hillerød, Denmark) and TSC medium for enumeration of total anaerobes and *C. perfringens*, respectively. Cecum contents were enumerated after anaerobic and aerobic cultivation on calf blood agar. Colonies with different morphology (studies 1 and 3) were selected for 16S rDNA sequencing (Agowa, Berlin, Germany) using universal PCR primers and sequence primers S-D-Bact-0008-a-S-20 and S-*-Univ-0519-a-A-18 (18) (DNA Technology, Aarhus, Denmark) as previously de-

scribed (18). The chromatograms were assembled in BioNumerics Version 4.5 (Applied Maths, Kortrijk, Belgium) and blasted for identification (http://www.ncbi.nlm.nih.gov).

The microbiota of small intestinal contents (study 1) and of distal small intestinal tissue (study 3) was characterized by T-RFLP as previously described (18). Extracted DNA (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany) were adjusted to 5 μg/mL (NanoDrop Technologies, Wilmington, DE), PCR amplified, and digested with restriction enzyme *Cfo*I (New England Biolabs, Ipswich, MA) before denaturation and loading on denaturing polyacrylamide gels for sequencing (ABI PRISM 377 DNA; PE Biosystems) as previously described (18). The resulting terminal restriction fragments (T-RFs) were aligned against internal standards (PE GeneScan 500 ROX Size Standard; Applied Biosystems, Warrington, United Kingdom, and PE GENEFLO 625 DNA Ladder ROX Size Standard; CHIMERx, Milwaukee, WI) in BioNumerics Version 4.5 (Applied Maths). For standardization, T-RF intensity relative to total sample intensity was calculated, and identity of dominating T-RFs was suggested by *in silico* digest in the MiCA software (http://mica.ibest.uidaho.edu/digest.php) using the RDPII database. Only T-RFs with a mean intensity >0.01 were presented graphically. Another T-RFLP using restriction enzymes *Msp*I and *Rsa*I (20 U; New England Biolabs) was performed on selected samples. Dominating T-RFs from the two T-RFLP analyses were compared with validate and increase resolution of the identified *Cfo*I-T-RFs.

FISH with a general 16S rRNA bacterial DNA-probe labeled with red isothiocyanate derivative (Cy3, Table 1) was performed on cross sections of distal small intestine from pigs in study 1 as described previously (14). Tissue slides were scanned (ArrayWoRx^e microarray scanner; Applied Precision) and given a FISH score based on abundance and position of bacterial microcolonies: $1 =$ no or very few microcolonies, $2 =$ few microcolonies, $3 =$ abundant colonies in the intestinal lumen, and $4 =$ abundant colonies closely adhering to the mucosa. Tissue sections with visible microcolonies ($n = 30$) were further hybridized with a *C. perfringens*-specific Cy3-labeled probe (Table 1) and given *C. perfringens*-FISH scores as above. Selected species or genera identified by cultivation or T-RFLP were further investigated by FISH with specific probes either previously published (14,18,19) (Table 1) or designed using the software ARB (http://www.arb-home.de) and the database Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (Table 1). Affinity and specificity were tested by hybridization of porcine lung tissue infected with pure culture and by BLAST on the NCBI homepage, respectively.

Calculations and statistical analyses. All results are presented as means and SEM. Group differences in NEC incidences were tested using Fisher exact test (SAS/STAT Version 9.1; SAS Institute, Cary, NC). Group differences in bacterial enumeration were tested using t test, and the mean relative T-RF intensities were tested using Wilcoxon Monte Carlo Estimates Exact Test in SAS. Based on overall similarity of all T-RFs, similarity (Dice) coefficient and principal component analysis (PCA) was generated in BioNumerics Version 4.5 (Applied Maths). All remaining parameters were tested with a two-way ANOVA using the SAS PROC MIXED procedure with treatment, diet, and sex as fixed variables and pigs and sows as random variables. For all analyses, $p < 0.05$ was the critical level of significance.

RESULTS

Clinical Outcomes

Study 1. Some of the preterm pigs developed NEC symptoms 23 h after introduction of enteral feeding (12/44, 27%),

Table 1. *Oligonucleotide probes* used for visualization of bacteria by FISH*

Probe (dye)	Target bacteria	Sequence	Reference or strain used for affinity test
S-D-bact-0338 (CY3 and FITC)	General bacteria	5′-GCTGCCTCCCGTAGGAGT-3′	Ref. 18
S-S-Cperfring-185 (FITC)	*Clostridium perfringens*	5′-TGGTTGAATGATGATGCC-3′	Ref. 14
L-S-Kpneu-1705 (Cy3)	*Klebsiella pneumoniae*	5′-TACACACCAGCGTGCCTT-3′	Isolate verified by sequencing
L-S-Efaecium-347 (Cy3)	*Enterococcus faecium*	5′-GTGTCTTCCACATTTCGT-3′	Type strain ATCC 19434
S-S-Acal-446 (Cy3)	*Acinetobacter calcoaceticus*	5′-AATACTAGTAGCCTCCTC-3′	Isolate verified by sequencing
S-Salac-0078 (Cy3)	*Streptococcus alactolyticus*	5′-CAAAAGAAGCAAGCTCC-3′	Type strain ATCC 43077
LGC354A-lactob (FITC)	*Lactobacillus* spp.	5′-TGGAAGATTCCCTACTGC-3′	Ref. 19

Name, dye, target, sequence, and strain used for affinity test are shown.
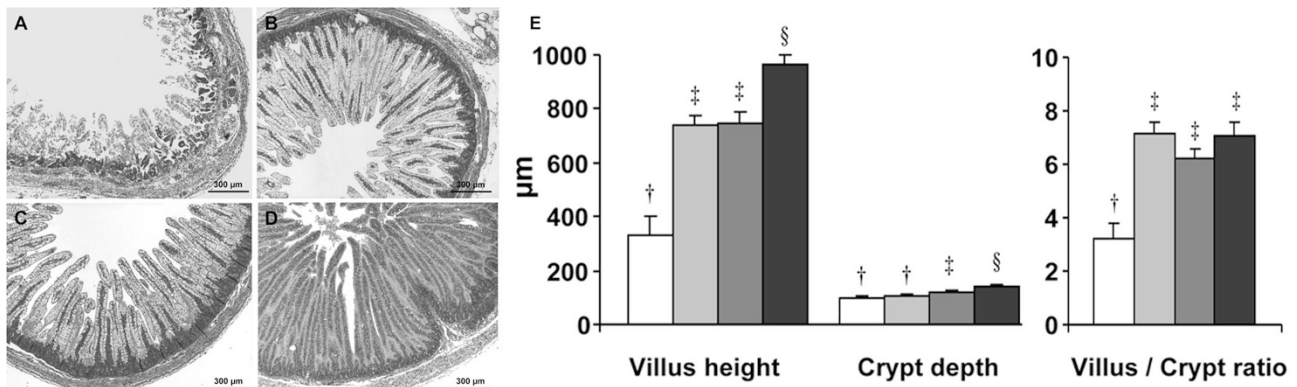
* All probes obtained from MWG-BIOTECH.

Figure 1. Cross sections of distal small intestine (HE stain, 25× magnification) from a preterm NEC pig (*A*), a healthy preterm pig (*B*), formula-fed term pig (*C*), and a colostrum-fed term pig (*D*). Histomorphometry of distal small intestine of preterm pigs with NEC ☐, preterm healthy pigs ☐, term formula pigs ☐, and term colostrum pigs ■ (*E*). Means ± SEM. Different superscript symbols indicate significant difference.

and NEC pathology was confirmed at necropsy. In pigs with NEC, histopathology of the distal small intestine revealed atrophic mucosa with stunted to disintegrated villi, altered mucosal proportions, hemorrhage, and separation of submucosa, muscularis, and serosa (Fig. 1). Pigs with NEC had a significantly higher mean NEC score across all gastrointestinal regions than healthy preterm pigs (3.8 ± 0.4 *versus* 1.1 ± 0.0, $p < 0.05$). Immunization against *C. perfringens* toxins did not prevent NEC because incidences for TOXICOL, COLERADO, and control pigs (27%, 6/22 for immunized and control pigs), and NEC scores were not different ($p > 0.5$).

Study 2. *C. perfringens* type A inoculation did not induce a higher incidence of NEC among *C. perfringens* type A pigs (1/6) *versus* control pigs (1/10). Subclinical intestinal lesions not specific for NEC were observed in two *C. perfringens* type A pigs and in one control pig (scores = 2).

Study 3. No ANTI pigs (0/11) and 11 control pigs (85%) were diagnosed with NEC. However, minor intestinal lesions (score = 2) were observed in several ANTI pigs (9/11) in 1–4 gut regions.

Microbiology

Study 1. There was no microbiological response to immunization (data not shown). Because clinical effects were also absent, TOXICOL and COLERADO pigs were pooled with the control pigs for further analyses. Instead, comparisons were made between preterm pigs that were healthy ($n = 32$) or had NEC ($n = 12$). These groups were further compared with the two groups of term pigs, which irrespective of diet did not develop NEC.

Numbers of total anaerobic bacteria did not differ between NEC and healthy preterm pigs ($4.1 \times 10^9 \pm 2.0 \times 10^9$ *versus* $2.4 \times 10^9 \pm 1.4 \times 10^9$ cfu/g, $p = 0.5$), but NEC pigs tended to have more *C. perfringens* than healthy preterm pigs ($2.3 \times 10^9 \pm 1.8 \times 10^9$ *versus* $1.3 \times 10^8 \pm 6.5 \times 10^7$ cfu/g, $p = 0.1$). FISH and subsequent scanning of small intestinal tissue (Fig. 2*A* and *B*) visualized bacterial abundance *in situ* and revealed that pigs with NEC had higher FISH scores of both general bacteria and *C. perfringens* compared with healthy preterm pigs (Table 2). Based on 97–100% similarity in 16S rDNA sequence, 13 species were identified from small intestinal content (GenBank accession numbers in brackets): *Acinetobacter calcoaceticus* (GQ483513), *Bacillus cereus* (GQ483514), *C. perfringens* (GQ483515), *Enterobacter* sp. [*E. coli* or *Shigella* sp. (GQ483516)], *Enterobacter homaechei* (GQ483517), *Enterococcus faecium* (GQ483518), *Klebsiella pneumoniae* (GQ483519)*, Lactobacillus sakei* (GQ483520), *Leuconostoc mesenteroides* (GQ483521)*, Staphylococcus aureus* (GQ483522), *Staphylococcus chromogenes* (GQ483523), *Streptococcus alactolyticus* (GQ483524), and *Streptococcus*
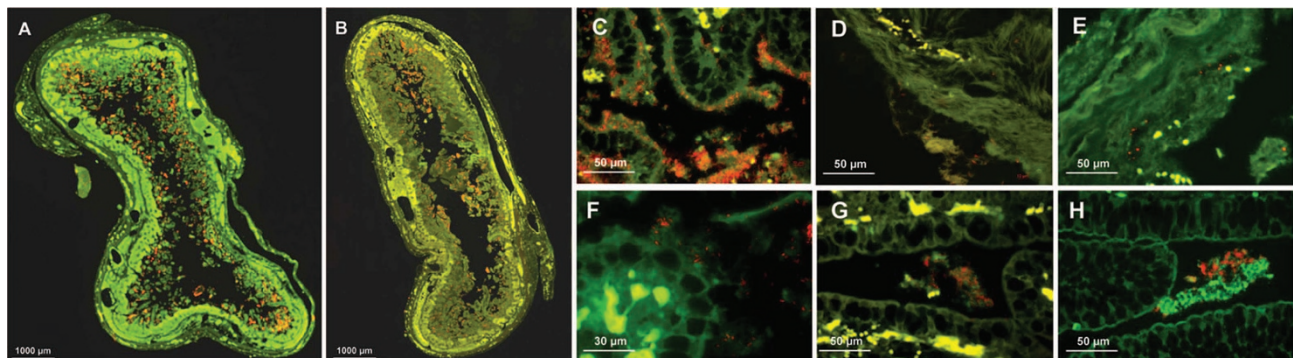

Figure 2. FISH of distal small intestinal cross sections. Scanning images visualize abundances of general bacteria (*A*) and *C. perfringens* (*B*) in preterm NEC pigs. Microscopy images (all 400× magnification) demonstrate close mucosal association (*C*) and epithelial translocation (*D*) of *C. perfringens* and presence of *E. faecium* (*E*), *K. pneumoniae* (*F*), and *S. alactolyticus* (*G*) in preterm pigs and *Lactobacillus* spp. in term pigs (*H*). Probes were labeled with red Cy3 except for *Lactobacillus* spp. labeled with green FITC.

Table 2. *Characteristics of preterm and term pigs from study 1*

	Preterm pigs		Term pigs	
	NEC	Healthy	Formula	Colostrum
Birth weight (g)	1175 ± 56†	1022 ± 40‡	1331 ± 131†	1382 ± 133†
Weight gain (g)	−217 ± 97†	−70 ± 7†	52 ± 16‡	228 ± 35§
FISH score: general bacteria	3.2 ± 0.3†	1.8 ± 0.1‡	2.5 ± 0.0†‡	2.0 ± 0.2‡
FISH score: *C. perfringens*	2.6 ± 0.4†	1.5 ± 0.2‡	1.3 ± 0.3†‡	1.2 ± 0.2‡
Villous height (μm)	330 ± 70†	738 ± 37‡	745 ± 47‡	964 ± 35§
Crypt depth (μm)	101 ± 8†	107 ± 2†	122 ± 3‡	141 ± 7§
Villous/crypt ratio	3.2 ± 0.6†	7.1 ± 0.4‡	6.2 ± 0.4‡	7.1 ± 0.5‡

See text for explanation of FISH scores. Values are represented as mean ± SEM.
Means not sharing same superscript symbol are significantly different ($p < 0.05$).

Table 3. *Average number of T-RFs (an index of diversity) and the similarity index (Dice coefficient) after CfoI digest in study 1*

	Preterm pigs			Term pigs		
	All preterm	NEC (*n* = 12)	Healthy (*n* = 32)	All term	Colostrum (*n* = 7)	Formula (*n* = 6)
T-RFs	16.5*	12.7†	17.9*	23.6‡	25.9‡	20.9*‡
Dice coefficient	47.9 ± 0.5†	56.3 ± 1.8*‡	47.9 ± 0.7†	54.0 ± 1.3*	62.3 ± 2.2‡	59.6 ± 2.8*‡

Values are represented as mean ± SEM. Averages not sharing the same superscript symbols are significantly different. The Dice coefficient indicates the overall microbiota similarity using the average number of T-RF's common for two pigs within the same group. A high coefficient indicates a high degree of similarity.

dysgalactiae (GQ483525). FISH on small intestinal tissue with specific probes confirmed the presence of several of *C. perfringens*, *E. faecium*, *K. pneumonia*, *S. alactolyticus*, and *Lactobacillus* spp. (Fig. 2C–H).

T-RFLP with restriction enzyme *CfoI* revealed totally 105 different T-RFs ranging from 62–632 bp. Preterm pigs had in average 16.5 ± 0.9 different T-RFs, and totally, 80 different T-RFs represented the group. In average, less numbers of T-RFs were observed in the preterm NEC *versus* preterm healthy pigs and term pigs (Table 3), indicating low bacterial diversity in this group. A mean of 23.6 ± 2.0 per pig and totally 72 different T-RFs were observed in the term group. Diversity was generally higher in vaginally born term pigs compared with cesarean-delivered preterm pigs, although formula-fed term pigs and healthy preterm pigs were not significantly different (Table 3). Different colonization patterns between NEC and healthy preterm pigs, between preterm and term pigs, and to a lesser extent between formula- and colostrum-fed term pigs were revealed as spatial separation in the PCA plot (Fig. 3). High interindividual variation was noted among the healthy preterm pigs and coincided with a significantly lower mean similarity coefficient for healthy preterm pigs compared with the other groups (Table 3). Dice coefficients between different groups of pigs were relatively low (data not shown) indicating low microbiota similarity between different groups of pigs. Graphical presentations of dominating T-RFs reveal the most important differences between groups of pigs (Fig. 4), with *C. perfringens* dominant in preterm pigs with NEC and *Lactobacillus* spp. dominant in both groups of term pigs.

T-RFLP with *Msp*I and *Rsa*I resulted in 145 different T-RFs ranging from 60 to 604 bp (data not shown). Comparing T-RFs intensities and distributions from both T-RFLP analyses proposed identity of the dominating *CfoI*-T-RFs (Table 4). In short, T-RFs probably representing *C. perfringens*, *E. faecium*, *K.*

pneumoniae, and *A. calcoaceticus* were dominant in preterm pigs. Increased *C. perfringens* density in NEC pigs represented the main difference between healthy pigs and NEC pigs. T-RFs probably representing *Lactobacillus* spp. dominated in all term pigs and to some extent in healthy preterm pigs (Fig. 4).

Study 2. *C. perfringens* type A-inoculated pigs had higher numbers of *C. perfringens* than control pigs (1.1×10^9 ±
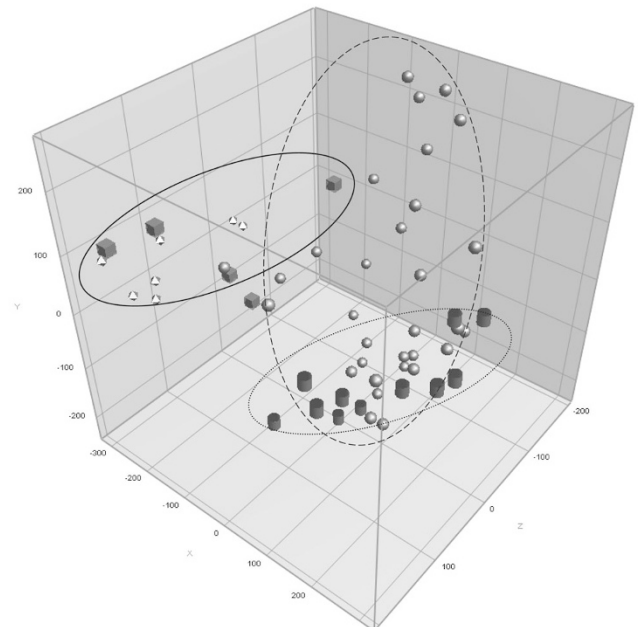

Figure 3. PCA analysis of T-RFLP results shows a three-dimensional separation of pigs according to overall similarity of the small intestinal microbiota. Clustering (indicated by different ellipses) indicates high similarity whereas separation indicates low similarity. Marbles (indicated by broken line), preterm healthy pigs; barrels (indicated by dotted line), preterm NEC pigs; boxes, term formula-fed pigs; and diamonds, term colostrum-fed pigs (both groups of term pigs indicated by solid line).
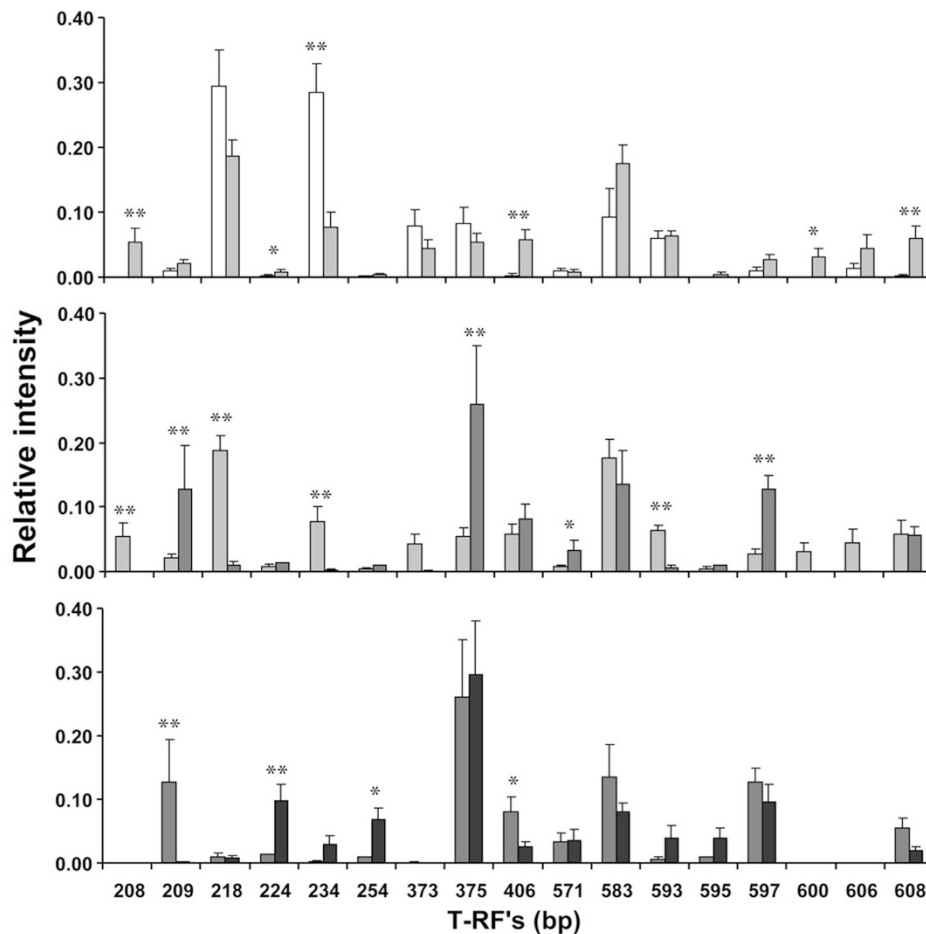
Figure 4. Relative intensity of dominating T-RFs in preterm pigs with NEC ☐, preterm healthy pigs ☐, term formula-fed pigs ☐, and term colostrum-fed pigs ■. Means ± SEM. Stars indicate group differences (*$p < 0.05$ and **$p < 0.01$).

Table 4. *Proposed identification of dominating T-RFs (study 1) was based on comparing between the two analyses (CfoI and MspI + RsaI) T-RFs intensities and overlapping distribution among pigs*

Observed T-RFs				
*Cfo*I	*Msp*I + *Rsa*I	Possible identification	Predominant in	Confirmed with FISH
208	490	*Acinetobacter calcoaceticus*	Preterm pigs, healthy	No
209	154	*Turicibacter* spp.	Term pigs, formula fed	Not performed
218	73	*Enterococcus faecium*	Preterm pigs, all	Yes, but at lower abundance
224	180	*Lactobacillus delbrueckii*	Term pigs, colostrum fed	Yes, at genus level
234	453	*Clostridium perfringens*	Preterm pigs, NEC	Yes
254	180	*Lactobacillus delbrueckii*	Term pigs, colostrum-fed	Yes, at genus level
373	496	*Klebsiella pneumoniae*	Preterm pigs, all	Yes
375	429	*Escherichia coli*	Term pigs, all	Not performed
406	572	*Lactobacillus fermentum/rennanqilfy*	Preterm (healthy) and term pigs	Yes, at genus level
583	496/560	*Streptococcus alactolyticus*	Preterm and term pigs	Yes
597	572	*Lactobacillus acidophilus/brevis/rennanqilfy/plantarum*	Term pigs, all	Yes, at genus level
600	572	*Lactobacillus* spp.	Preterm (healthy)	Yes, at genus level
606	572	*Lactobacillus* spp.	Preterm (healthy)	Yes, at genus level
608	572	*Lactobacillus* spp./*Weissella* spp.	Preterm (healthy)	Yes, at genus level

All except *Lactobacilli* spp. and *Turicibacter* spp. were isolated from intestinal content.

3.2×10^8 *versus* $1.4 \times 10^8 \pm 8.8 \times 10^7$ cfu/mL, $p < 0.01$). Numbers of total anaerobic bacteria did not differ.

Study 3. Compared with controls, ANTI pigs had less total bacteria (aerobes: $2.6 \times 10^5 \pm 1.1 \times 10^5$ *versus* $7.5 \times 10^9 \pm 1.4 \times 10^9$, cfu/mL and anaerobes: $2.6 \times 10^5 \pm 1.1 \times 10^5$ *versus* $7.6 \times 10^9 \pm 1.4 \times 10^9$ cfu/mL, both $p < 0.0001$). Six Gram-positive species were isolated from ANTI pigs: *B.*

cereus (GQ483526), *Clostridium paraputrificum* (GQ483527), *C. perfringens* (GQ483528), *Cronobacter sakazakii* (GQ483529), *E. faecium/hirae* (GQ483530/GQ483531), and *Staphylococcus pasteuri* (GQ483532). PCR product was obtained from only four of 11 ANTI pigs, and to avoid misleading conclusions, no detailed analysis of the T-RFLP results was done from this study.

DISCUSSION

The gut microbiota is important for NEC development in preterm neonates, but it remains unclear whether specific pathogens play a direct role and whether manipulation of the microbiota can alter NEC resistance. By using NEC-sensitive formula-fed preterm pigs, we demonstrate a different intestinal microbiota between formula-fed preterm and term pigs reared in the same environment. A more variable microbiota in healthy preterm pigs than in term pigs suggest that the immature gut facilitates distinct colonization patterns. Consistent with observations in infants (4,11), NEC development was associated with decreasing microbial diversity and overgrowth of *C. perfringens*. The *C. perfringens* overgrowth occurred as a response to disease, rather than as a cause of disease, because *C. perfringens* inoculation failed to induce NEC, and *C. perfringens* toxin immunization failed to protect against NEC. Bacterial load, more than the specific species composition, may be more important for NEC development, as indicated by the absence of NEC after broad-spectrum antibiotics use. Immature intestinal defense in preterm individuals leads to adverse host responses to a bacterial load that does not adversely affect term individuals.

Observations in infants (9,13), preterm pigs (14,16), and quails (20) propose a role for *C. perfringens* in NEC pathogenesis. Furthermore, a NEC-like disease in newborn production pigs (necrotizing enteritis) is prevented by providing colostrum or serum from sows immunized with *C. perfringens* type C and D toxins (21), and this stimulated our interest in the possible influence of *C. perfringens* types C and D in NEC pathogenesis. T-RFLP and FISH revealed that overgrowth of *C. perfringens* in preterm NEC pigs accounted for the main colonization difference between healthy and diseased pigs. However, in contrast to previous results (14), toxicol-treatment of preterm pigs did not prevent NEC and effects were similarly absent after Colorado-serum treatment. Because later analysis revealed that only *C. perfringens* type A is present in our preterm pigs, this was chosen for inoculation in study 2, but NEC incidence did not increase. These observations collectively reveal that *C. perfringens* is not a causative pathogen.

T-RFLP revealed a similar species pattern in the distal small intestine of healthy and NEC preterm pigs. The main colonization difference was related to altered abundances of general bacteria and specific species. FISH revealed overgrowth of general bacteria and *C. perfringens* in preterm pigs with NEC. Hence, an inappropriate microbiota does not alone induce NEC, but possibly high abundance of specific species and high bacterial load are required for NEC, together with intestinal insults such as ischemia and hypoxia. Several of the six bacterial species that escaped our prophylactic antibiotics treatment are commonly associated with human infections such as gaseous gangrene (*C. paraputrificum*), neonatal meningitis (*E. faecium*), food poison (*B. cereus*), and infections and NEC in preterm infants (*C. sakazakii*) (22,23). Overgrowth of these species could potentially result in intestinal inflammation and necrosis as suggested from the observed low-grade lesions. This is corroborated by the association in human infants among NEC antibiotic treatment and low fecal bacterial diversity (11,12). Therefore, broad-spectrum antibiotics treatment may prevent NEC short-term but have negative colonization effects with increased risk of NEC in the long-term.

We compared preterm caesarean delivered, formula-fed pigs with term formula- or colostrum-fed pigs after spontaneous delivery. This is clinically relevant because of an increasing proportion of caesarean-delivered preterm infants (4,24) but hampers firm conclusions regarding the effects of GA on colonization. However, a different colonization has been observed between vaginally- and caesarean-delivered neonates but has not been correlated with NEC or neonatal survival in preterm infants or pigs (4,6,24). Thus, factors regarding host responses and feeding procedures in preterm neonates are probably more important for initial disease progression than the exact nature of the gut microbiota. Vaginally delivered term pigs that are reared with the sow are spontaneously colonized with maternal and environmental bacteria, presumably resulting in a natural and optimal microbiota (1,4,25). In this study, this included higher abundance of *Lactobacillus* spp. and *E. coli* (T-RFs 224 and 254, 597 and 375), where the main differences between the term groups were within the *Lactobacillus* genera. Hence, this study shows that different diets and environments do not markedly affect intestinal colonization in term neonates and highlights the importance of the age-related host-responses rather than of diet or environment.

Compared with previous culture-independent studies on preterm infants and pigs that reported 10–36 different species (4,11,13,26,27), T-RFLP revealed higher total bacterial diversity in the contents of distal small intestine of preterm pigs in this study. Previously, T-RFLP on distal intestinal tissue was performed to characterize the gut microbiota of preterm pigs and reflected only the mucosa-associated bacteria (14,16). This study focused on luminal bacteria, which affect the host by modifying the luminal environment by metabolite production (*e.g.* short-chain fatty acids, organic acids, antimicrobial compounds, toxins, and immunomodulatory factors) (5,6). Low fecal microbial diversity in preterm infants may result from highly hygienic procedures and common use of antibiotics in the NICUs.

Pigs and humans share similar developmental characteristics of the gastrointestinal tract, but at birth, gut structure, function, and immunity are less mature in pigs, than in infants (28), and 10% preterm delivery is associated with significant developmental immaturity. Shortly after formula feeding, $\sim$50% of preterm pigs spontaneously develops NEC and represents a valuable tool to study infant NEC in a highly controlled environment. Preterm pigs fed with colostrum show a NEC incidence of 5–10% (14,16), similar to that in preterm infants. However, the NEC-protective effects of colostrum are possibly not caused by microbiological effects because this study suggest that diet and environment had only marginal effect on the composition of the term gut microbiota. Also, in preterm pigs, the gut microbiota is not clearly related with diet although it is required for NEC (14,16). Therefore, on the basis of this study, we suggest that preterm delivery facilitates a distinct pattern of gut colonization that in combination with immature host immune responses lead to NEC in both pigs and infants.

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