Necrotizing Enterocolitis in Preterm Pigs Is Associated with Increased Density of Intestinal Mucosa-Associated Bacteria Including Clostridium perfringens

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Necrotizing enterocolitis in preterm pigs is associated with increased density of intestinal mucosa-associated bacteria including *C. perfringens*

Short title: Tissue-associated bacteria and NEC

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Key words: Animal model, Cell culture, Gene expression, Necrotizing enterocolitis, Microbiota, Quantitative PCR, *Clostridium perfringens*

Abbreviations: NEC, necrotizing enterocolitis; FISH, fluorescence *in situ* hybridization; PBS, phosphate buffered saline; MOI, multiplicity of infection; SEM, standard error of mean; RIN, RNA integrity number

Abstract

Background: Necrotizing enterocolitis (NEC) is associated with changes in the luminal gut microbiota. It is not known whether the mucosa-associated microbiota is affected by NEC and stimulates inflammatory lesions. *Objective:* We hypothesized that the density of the mucosa-associated microbiota correlates with NEC severity in preterm pigs and that *C. perfringens*, which has been associated with NEC in preterm infants, is stimulating the expression of immune genes in intestinal epithelial cells. *Methods:* First, we determined the density of total bacteria and *C. perfringens* in the distal small intestinal mucosa of 58 NEC- and healthy preterm pigs using quantitative PCR. Next, we analyzed in IPEC-J2 cells the effect of different infection densities of *C. perfringens* type A on the expression of genes related to intestinal function and immune response. *Results:* Total bacterial and *C. perfringens* densities were higher in NEC- versus healthy pigs, and correlated positively with NEC severity. In IPEC-J2 cells expression levels of immune-related genes (*CCL5*, *NFKBIA*, *IL8*, *IL1RN*, and *TNFAIP3*) increased, while the expression of the sodium/glucose co-transporter (*SLC5A1)* decreased, with increasing density of *C. perfringens*. *Conclusions:* The density of mucosa-associated bacteria, and specifically *C. perfringens*, may stimulate the progression of NEC in preterm pigs. *C. perfringens* affects newborn porcine intestinal epithelial cells by changing their immune gene expression patterns, which may enhance the inflammation and development of lesions in the immature intestine.

Introduction

An unbalanced intestinal microbiota is a risk factor for necrotizing enterocolitis (NEC), but its exact contribution remains unclear. In general, studies show that infants developing NEC have a different gut microbiota than infants staying healthy [1]. Differences include lower bacterial diversity, higher density of total bacteria and increased numbers of Proteobacteria like *E. coli* and *Klebsiella* [2–5],

but also of *Clostridium spp.* including *C. perfringens* [6,7; ?ny kilde/Sim et al 2015?], which may lead to a more severe and often lethal disease progression than other NEC-related pathogens [7;schlapbach?]. Thus, pathogens may at a certain threshold alone or together with other members of the microbiota stimulate NEC development. Still, in other studies only minimal changes are observed [8,9]. Therefore, cause and effect are difficult to separate.

The majority of intestinal microbiota analyses have relied on fecal samples to reflect the intestinal microbiota, and only few studies have included intestinal tissue or contents [3,7,8]. This may add further to the ambiguity regarding the composition of the microbiota due to differences in sample material [10]. The bacteria that are most relevant to NEC and directly affects intestinal epithelial cells may be those in close contact with the intestinal epithelial cells, the mucosa-associated microbiota. Access to intestinal samples from preterm infants is obviously difficult. Hence, the mucosa-associated microbiota is best investigated in appropriate animal models of NEC, coupled with cell studies. To provide novel insight into the association between NEC and the density of the mucosa-associated microbiota, and the transcriptional immune response of epithelial cells to *C. perfringens*, we used a preterm pig model of NEC [11] and a porcine intestinal IPEC-J2 cell line [12]. We hypothesized that NEC severity would correlate with the density of the distal small intestinal mucosa-associated bacteria. Although the etiology of NEC is multifactorial and several bacterial pathogens have been associated with the disease [2–7], we choose *C. perfringens* for more in-depth analysis. *C. perfringens* has repeatedly been associated with NEC in preterm pigs [reviewed in 13] and in preterm infants [6,7; ?ny kilde/Sim et al 2015?], where it often lead to a fulminant disease course [7; schlapbach?]. Furthermore, it is know to produce a large number of toxins and is a common cause of severe diseases including enteric disease in humans and animals [reviewed in Hatheway 1990]. (Thomas et al 1984?). The tissue samples originated from previous studies of diet-dependent differences in NEC-sensitivity and the mucosa-associated microbiota [14–

16]. Therefore, the present study included 58 preterm pigs to investigate the association between the microbiota and NEC across diets. Furthermore, we investigated the effect of increasing numbers of *C. perfringens* on immune gene expression pattern in IPEC-J2 cells. This cell line is derived from the jejunum of a neonatal, unsuckled pig [12], and is a suitable *in vitro* model for newborn epithelial cells [17]. Our choice of genes was based on our previous diet studies in preterm pigs [17,18] and studies on the effects of pathogens on IPEC-J2 cells [12].

Materials and methods

Intestinal microbiota analyses

Fifty-eight preterm pigs were delivered by caesarean section, and surgical procedures, rearing, diet intervention, euthanasia and tissue evaluation and collection have been described previously [14–16]. The pigs were fed the following enteral diets: porcine colostrum (n = 5), formula (n = 9), formula + probiotics (n = 13) [14], formula containing lactose (n = 11) or maltodextrin (n = 11) as the principal carbohydrate source [15], or formula with a casein:whey-ratio of 60:40 (n = 9) [16]. Information on the composition of the diets can be found in table 1. After euthanasia, NEC was blinded evaluated in the stomach, proximal-, middle-, and distal small intestine, and colon using a score ranging from 1 (no or minimal focal hyperaemic gastroenterocolitis) to 6 (severe extensive hemorrhagic and necrotic gastroenterocolitis). A score of minimum three in one intestinal region was defined as a case of NEC. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

To get an approximation of the mucosa-associated microbiota for both FISH and quantitative PCR, the luminal content was gently removed by squeezing along the length of the intestine and the remaining tissue used for analysis. To visualize the association between the microbiota and the intestinal tissue, fluorescence *in situ* hybridization (FISH) was performed on distal small intestinal

samples without luminal content from two of the studies [15,16] as previously described [14]. Quantitative PCR was performed on distal small intestinal samples without luminal content that was collected as full thickness tissue samples of 3 cm, snap frozen in liquid nitrogen and stored at -80°C until analysis. DNA from the tissue samples was extracted using the QIAamp DNA mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. Quantification of total bacteria [19] and *C. perfringens* [20] was performed according to previous studies, but with minor modifications. Briefly, the reaction mixture (25 µl) for total bacteria included 25 ng extracted DNA, 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Nærum, Denmark), 0.9 µM of each primer (0.09 µM of primer 8FB) and 0.2 µM TaqMan probe. For *C. perfringens*, 20 µl reaction mixture included 20 ng extracted DNA, 2× Taqman Universal PCR Master Mix (Applied Biosystems), 0.25 mg/ml bovine serum albumin (Applied Biosystems), 1 µM of each primer and 0.1 µM TaqMan probe. Primers and probes (table 2) were synthesized at DNA Technology, Aarhus, Denmark. Quantitative PCR was performed on a RotorGene 3000 Detection System (Corbett Life Science, Sydney, Australia) under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, 60°C for 45 s, 65°C for 15 s, and 72°C for 15 s for total bacteria, and 95°C for 10 min, 45 cycles at 94°C for 10 s, 55°C for 20 s, and 70°C for 10 s for *C. perfringens*. Standard curves were generated using *C. perfringens* DNA (NCTC 10240, National Veterinary Institute, Frederiksberg C, Denmark) ranging over five (total bacteria) and six (*C. perfringens*) tenfold dilutions from the limit of detection (total bacteria, 0.1 pg DNA/µl and *C. perfringens*, 0.0125 pg DNA/µl). Cycle threshold was determined using the Rotor-Gene 3000 data analysis software (Corbett Life Science) using the Auto-Find Threshold function. Standards and samples were run in triplicates, and every reaction plate included one non-template control in triplicate. Due to lack of sample material, samples from 49 pigs were included in the total bacteria assay, while samples from all 58 pigs were included in the *C. perfringens* assay. The results were

calculated as relative quantities measured as pg DNA/25 ng of extracted total DNA (total bacteria) and pg DNA/20 ng of extracted total DNA (*C. perfringens*).

Gene expression in IPEC-J2 cells during *C. perfringens* infection

IPEC-J2 cells [12] were maintained as previously described [17]. *C. perfringens* type A (NCTC 10240, National Veterinary Institute) was cultured (16 h; 37°C; anaerobic; brain heart infusion broth, SSI diagnostics, Hillerød, Denmark), washed twice in Dulbecco's phosphate buffered saline (PBS, Sigma-Aldrich, Brøndby, Denmark) and resuspended in growth medium that was prepared according to [17], but without antibiotics. For the experiment, IPEC-J2 cells were grown in 6-well plates (Corning Costar cell culture plates, Sigma-Aldrich) until near-confluence was achieved and incubated in growth medium without antibiotics for 24 h. They were infected for 2 h with *C. perfringens* at multiplicity of infection (MOI; n = 5) = 0, 10, 20 and 50 determined by OD600 measurement. Hereafter, the supernatant was collected, and the cells washed (PBS) and harvested (1× trypsin:EDTA, Sigma-Aldrich). The cells and supernatant were centrifuged (10 min; 1000 rpm; 4°C), and the cell pellet stored at -80°C. The gene expression analysis was done as previously described using reverse transcription quantitative real-time PCR [17,21]. Genes are denoted by their gene symbol and information on primers for reference genes and genes showing significant differences can be found in table 3, while information on the 48 primer pairs analyzed have been published previously [17]. *RPL13A* and *ACTB* were the most stably expressed reference genes of 5 candidate genes, and used to normalize all samples in GenEx5 (MultiD Analyses AB, Göteborg, Sweden). After normalization, quantification cycle was converted to relative quantities. Relative expression of the sample with the lowest level of expression was scaled to 1 for each primer assay.

Statistical analyses

The density of total bacteria and *C. perfringens* was analyzed using a Mann-Whitney test with a Dunn's multiple comparison post-hoc test in GraphPad Prism (Version 5.02, La Jolla, CA, USA) and the results considered significant when $p < 0.05$. Correlation analysis was performed using Spearman correlation analysis in GraphPad Prism, and correlations were considered significant if $\rho < -0.5/> 0.5$, equal to $p < 0.001$. Analysis of the gene expression in IPEC-J2 cells was performed as previously described [17]. Data was $\log_2$ transformed and tested with a one-way ANOVA with a Tukey-Kramer's post-hoc test (GenEx5, MultiD Analyses AB). Gene expression was considered significant if $p < 0.05$ and relative gene expression differences were > 2.0-fold between the groups.

Results

Intestinal microbiota analyses

Visual inspection of FISH images showed a higher number of bacterial micro-colonies associated with the mucosa in NEC- than healthy pigs. *C. perfringens* was part of the micro-colonies, which were found along the length of the villi and down to the crypts in NEC- and healthy pigs. Quantitatively, total bacterial density was higher in NEC- (n = 34) compared with healthy pigs (n = 15), and the same was observed for the density of *C. perfringens* when comparing NEC- (n = 38) with healthy pigs (n = 20, figure 1A,B). The correlation between the density of total bacteria and the distal small intestinal NEC score was $\rho = 0.440$, $p < 0.01$ (figure 1C) mainly driven by the low NEC score pigs. An even more positive correlation was found between the distal small intestinal NEC score and the density of *C. perfringens* ($\rho = 0.687$, $p < 0.001$, figure 1D). Finally, a positive correlation between the densities of total bacteria and *C. perfringens* was observed ($\rho = 0.585$, $p < 0.001$).

Gene expression in IPEC-J2 cells during *C. perfringens* infection

The *in vitro* experiment showed that the cellular response of IPEC-J2 cells changed with increasing number of *C. perfringens* type A. A small effect was observed on the RNA integrity, measured by RNA integrity number (RIN), as this decreased from MOI = 0 (mean RIN = 9.98 ± 0.02) to MOI = 20 (mean RIN = 9.38 ± 0.25). The largest effect was seen at MOI = 50 since a RIN for only one replicate was obtainable, which indicated a high degree of RNA degradation resulting from cell lysis (figure 2A). To assure the most accurate results, the MOI = 50 group and one MOI = 10 replicate were excluded in the statistical analysis as large differences in RIN may affect the results. Of the 22 genes, passing the data evaluation (table 3), six were differentially expressed between the MOI groups (figure 2B). The expression of *CCL5, NFKBIA, IL8* and *TNFAIP3*, encoding proteins involved in inflammation, was up-regulated in MOI = 10 compared with MOI = 0. Furthermore, *NFKBIA* and *TNFAIP3* expression was higher at MOI = 20 compared with MOI = 0. The expression of *CCL5* and *IL8* decreased at MOI = 20 to levels between MOI = 0 and MOI = 10, while the decrease in *IL1RN* resulted in a difference between MOI = 10 and MOI = 20. The expression of *SLC5A1* (sodium/glucose co-transporter) decreased with increasing MOI.

Discussion

In this study, we observed an association between NEC severity and the density of total bacteria, including *C. perfringens* Type A, in the distal small intestinal mucosa of preterm pigs. The results indicate that the mucosal bacterial density is a factor associated with the progression of NEC. Even though this study does not clarify the exact contribution of the microbiota to the pathogenesis, we speculate that reaching a certain threshold of bacterial contact stresses the immature intestinal epithelium and set off the inflammatory process towards NEC. Although 14 of the 38 pigs with NEC did not have lesions in the distal small intestine, but in another intestinal region, the density of total bacteria in the distal small intestine was similar to pigs with NEC in this region (data not

shown). We speculate that the increased density of bacteria in the distal small intestine might reflect the bacterial density in other regions of the intestine, and in the initial phase of NEC, another region was stressed before the distal small intestinal region and the inflammatory process towards NEC initiated here. The NEC-inducing effect of mucosal bacteria may interact with diet factors, because a high intake of poorly digestible milk diets would likely increase bacterial proliferation and metabolism. The results are in accordance with studies in preterm infants and pigs showing an association between NEC and the density of both total bacteria [3,4] and *C. perfringens* [6,7,13]. The association between specific bacteria and NEC lesions could be due to increased density of total bacteria, which was observed by the positive correlation between the density of total bacteria and *C. perfringens*. However, the induction of intestinal injury by specific pathogens may still be significant. Bjørnvad *et al*. [22] found that *C. perfringens* invaded the tissue more deeply in NEC- compared with healthy pigs. In the present study, FISH observations showed bacteria closely associated with the surface of the intestinal epithelium, and invasive pathogens could therefore potentially affect the epithelial cells directly.

Gene expression changes in IPEC-J2 cells exposed to *C. perfringens* were observed by an increased expression of inflammatory factors concomitant with a decrease in *SLC5A1*. In contrast, other genes like *CD14*, *IL6*, *IL18* and *TLR4*, involved in the innate immune response, were not affected. Genes encoding pro-inflammatory factors, *IL8* and *CCL5*, and anti-inflammatory factors, *IL1RN*, *NFKBIA* and *TNPAIP3*, were affected by the increased numbers of *C. perfringens*, and indicate that increased bacterial level initiates an inflammatory response. A concomitant up-regulation of IL-8 and IL-10 in plasma was also observed in preterm infants in response to severe NEC [23]. Likewise, *CCL5*, *IL8* and *NFKBIA* were up-regulated with increasing NEC in preterm pigs [24, unpublished data] generating further evidence for the applicability of IPEC-J2 cells as a low cost model for the neonatal intestine. In preterm pigs given oral antibiotics NEC is prevented and *IL8* and other

immune-related genes are down-regulated [25]. The decrease in *SLC5A1* expression indicates that absorptive functions are compromised during *C. perfringens* infection. This could cause accumulation of nutrients available for bacterial fermentation and lead to bacterial overgrowth and cytotoxic levels of metabolites. Conversely, providing milk diets that easily exceed the immature digestive capacity of preterm newborns may provide substrates for bacterial fermentation. In quails, excessive luminal fermentation by *Clostridium* species leads to NEC-like lesions [26]. Bacterial toxins may also have a cytotoxic effect, and a rapid up-regulation of toxins by *C. perfringens* type C when in close contact with Caco-2 cells was observed [27]. However, in preterm infants no positive correlation was found between *C. perfringens* α-toxins and NEC severity [7]. Further studies should investigate if the observed changes in gene expression of the epithelial cells are unique to *C. perfringens* and if differences, for example production of metabolites, between pathogens involved in NEC are associated with different disease courses.

In conclusion, mucosa-associated bacterial density is associated with NEC severity in preterm pigs. Furthermore, specific pathogens may play a role in NEC development that was shown in this study by the association between NEC severity and the density of *C. perfringens*, and by the ability of this pathogen to induce changes in immune response genes of intestinal epithelial cells *in vitro*. It is highly likely, that increased bacterial density in close association with the intestinal epithelium also plays an important role for NEC development in preterm infants, both in its initiation and progression to severe mucosal lesions. Our results suggest that preventive measures against NEC might be directed towards stimulating the mucosal barrier against bacterial attachment, coupled with provision of diets that minimize maldigestion and substrates available for fermentation. This could include stimulation of intestinal mucus production by highly digestible and immune-modulating milk diets [28] or by decreasing bacterial density using oral antibiotics [25], as shown in preterm pig studies.

References

(1) Neu J: Necrotizing enterocolitis: The mystery goes on. Neonatology 2014;106:289–295.

(2) Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, Antonopoulos DA, Chang EB, Claud EC: 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3:944–954.

(3) Bucher BT, McDuffie LA, Shaikh N, Tarr PI, Warner BB, Hamvas A, White FV, Erwin CR, Warner BW: Bacterial DNA content in the intestinal wall from infants with necrotizing enterocolitis. J Pediatr Surg 2011;461029–1033.

(4) Jenke AC, Postberg J, Mariel B, Hensel K, Foell D, Dabritz J, Wirth S: S100A12 and hBD2 correlate with the composition of the fecal microflora in ELBW infants and expansion of E. coli is associated with NEC. Biomed Res Int 2013;2013:150372.

(5) Torrazza RM, Ukhanova M, Wang X, Sharma R, Hudak ML, Neu J, Mai V: Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. PLoS One 2013;8:e83304.

(6) De La Cochetiere MF, Piloquet H, des Robert C, Darmaun D, Galmiche JP, Roze JC: Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. Pediatr Res 2004;56:366–370.

(7) Dittmar E, Beyer P, Fischer D, Schafer V, Schoepe H, Bauer K, Schlosser R: Necrotizing enterocolitis of the neonate with Clostridium perfringens: diagnosis, clinical course, and role of alpha toxin. Eur J Pediatr 2008;167:891–895.

(8) Smith B, Bodé S, Petersen BL, Jensen TK, Pipper C, Kloppenborg J, Boye M, Krogfelt KA, Mølbak L: Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol 2011;11:73.

(9) Normann E, Fahlen A, Engstrand L, Lilja HE: Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. Acta Paediatr 2013;102:129–136.

(10) Romano-Keeler J, Moore DJ, Wang C, Brucker RM, Fonnesbeck C, Slaughter JC, Li H, Curran DP, Meng S, Correa H, Lovvorn HN III, Tang YW, Bordenstein S, George AL Jr, Weitkamp JH: Early life establishment of site-specific microbial communities in the gut. Gut Microbes 2014;5:192–201.

(11) Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjørnvad CR, Thymann T, Grondahl ML, Hansen AK, Jensen SK, Boye M, Mølbak L, Buddington RK, Weström BR, Holst JJ, Burrin DG: Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology 2006;130:1776–1792.

(12) Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, Tedin K, Wieler LH: Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 2006;125:293–305.

(13) Cilieborg MS, Boye M, Sangild PT: Bacterial colonization and gut development in preterm neonates. Early Hum Dev 2012;88:S41–S49.

(14) Siggers RH, Siggers JL, Boye M, Thymann T, Mølbak L, Leser TD, Jensen BB, Sangild PT: Early administration of probiotics alters bacterial colonization and limits diet-induced gut dysfunction and severity of necrotizing enterocolitis in preterm pigs. J Nutr 2008;138:1437–1444.

(15) Thymann T, Møller HK, Stoll B, Støy ACF, Buddington RK, Bering SB, Jensen BB, Olutoye OO, Siggers RH, Mølbak L, Sangild PT, Burrin DG: Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs. Am J Physiol Gastrointest Liver Physiol 2009;297:G1115–G1125.

(16) Thymann T, Støy ACF, Bering SB, Mølbak L, Sangild PT: Casein addition to a whey-based formula has limited effects on gut function in preterm pigs. J Anim Sci 2012;90:378–380.

(17) Støy ACF, Heegaard PMH, Sangild PT, Østergaard MV, Skovgaard K: Gene Expression analysis of the IPEC-J2 cell line: A simple model for the inflammation-sensitive preterm intestine. ISRN Genomics 2013;2013:980651.

(18) Cilieborg MS, Skovgaard K, Nørgaard LM, Sangild PT, Boye M: Intestinal colonization, gut function and inflammatory responses are moderately influenced by gestational age at birth. Abstract from 14th International Symposium on Microbial Ecology 2012.

(19) Palmer C, Bik EM, Digiulio DB, Relman DA, Brown PO: Development of the human infant intestinal microbiota. PLoS Biol 2007;5:1556–1573.

(20) Wise MG, Siragusa GR: Quantitative detection of Clostridium perfringens in the broiler fowl gastrointestinal tract by real-time PCR. Appl Environ Microbiol 2005;71:3911–3916.

(21) Skovgaard K, Cirera S, Vasby D, Podolska A, Breum SØ, Dürrwald R, Schlegel M, Heegaard PMH: Expression of innate immune genes, proteins and microRNAs in lung tissue of pigs infected experimentally with influenza virus (H1N2). Innate Immun 2013;19:531–544.

(22) Bjørnvad CR, Thymann T, Deutz NE, Burrin DG, Jensen SK, Jensen BB, Mølbak L, Boye M, Larsson LI, Schmidt M, Michaelsen KF, Sangild PT: Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092–G1103.

(23) Edelson MB, Bagwell CE, Rozycki HJ: Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. Pediatrics 1999;103:766–771.

(24) Støy ACF, Heegaard PMH, Thymann T, Bjerre M, Skovgaard K, Boye M, Stoll B, Schmidt M, Jensen BB, Sangild PT: Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs. Clin Nutr 2014;33:322–329.

(25) Jensen ML, Thymann T, Cilieborg MS, Lykke M, Mølbak L, Jensen BB, Schmidt M, Kelly D, Mulder I, Burrin DG, Sangild PT: Antibiotics modulate intestinal immunity and prevent necrotizing enterocolitis in preterm neonatal piglets. Am J Physiol Gastrointest Liver Physiol 2014;306:G59–G71.

(26) Waligora-Dupriet AJ, Dugay A, Auzeil N, Huerre M, Butel MJ: Evidence for clostridial implication in necrotizing enterocolitis through bacterial fermentation in a gnotobiotic quail model. Pediatr Res 2005;58:629–635.

(27) Vidal JE, Ohtani K, Shimizu T, McClane BA: Contact with enterocyte-like Caco-2 cells induces rapid upregulation of toxin production by Clostridium perfringens type C isolates. Cell Microbiol 2009;11:1306–1328.

(28) Puiman PJ, Jensen M, Stoll B, Renes IB, de Bruijn AC, Dorst K, Schierbeek H, Schmidt M, Boehm G, Burrin DG, Sangild PT, van Goudoever JB: Intestinal threonine utilization for protein and mucin synthesis is decreased in formula-fed preterm pigs. J Nutr 2011;141:1306–1311.

(29) Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA: Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 1990;56:1919–1925.

Figure legends

Figure 1: (A) Association between NEC and the density of total bacteria and (B) the density of *C. perfringens* in NEC- (NEC-pigs) and healthy pigs (HEAL-pigs) determined by quantitative PCR. Significant differences are indicated as * for $p < 0.05$ and *** for $p < 0.001$. Since two different quantitative PCR assays have been used for quantification of total bacteria and *C. perfringens* figure (A) and (B) cannot be directly compared. Spearman correlations between distal small intestinal NEC score and the density of (C) total bacteria and (D) *C. perfringens*.

Figure 2: (A) RNA degradation in IPEC-J2 cells measured by the RNA integrity number (RIN) and (B) relative fold changes (mean + standard error of mean) of genes significantly different expressed in IPEC-J2 cells stimulated with *C. perfringens* at multiplicity of infection (MOI) = 0 (white bars), 10 (light grey bars) and 20 (black bars). Different superscript letters (a,b) indicate significant differences, $p < 0.05$ and fold change > 2.

Tables

Table 1: Diet composition in macronutrient content pr litre. All diets were fed as a bolus (15ml/kg body weight) every 3 h.

Diet	Energy, kJ	Protein, g		Carbohydrates, g			Fat, g
		Whey	Casein	Maltodextrin	Lactose	Other	
Sow colostrum[#]							
Formula[##]	4151	67	0	45		9	61
Formula with probiotics[##]	4151	67	0	45		9	61
Formula with lactose[###]	4648	63	0	8	48	2	70
Formula with maltodextrin[###]	4634	62	0	55	0	6	70
Formula Casein:whey ratio of 60:40[###]	4620	25	37	55	0	6	70

Porcine colostrum was collected manually from sows (Large White x Landrace, Research Station Sjælland II, Denmark) within 6 h of completed farrowing and stored at -20°C until used. Values for the composition of colostrum, see Sangild & Xu, 2004. Sangild PT & Xu RJ (2004) Colostrum. In Encyclopedia of Animal Science, pp. 1–3 [WG Pond and AW Bell, editors]. New York: Marcel Dekker.

Ingredients used: Pepdite, Maxipro and Liquigen-MCT, all products kindly donated by SHS International, Liverpool, UK. The probiotic mixture (kindly donated by Chr. Hansen, A/S) consisted of Bifidobacterium animalis (DSM15954) and 4 Lactobacillus species: acidophilus (DSM13241), casei (ATCC55544), pentosus (DSM14025), and plantarum (DSM13367). Probiotics were reconstituted in 1 % peptone-water and each strain was included at 10^9 colony-forming units (CFU)/g of viable lyophilized bacteria for a total concentration of 5 x 10^9 CFU/3 mL peptone-water. Boluses of probiotics or peptone-water placebo were administered (2 mL/ kg BW) every 6 h during the TPN period and every 3 h during the enteral phase. Boluses of probiotics were reconstituted fresh prior to every bolus administration.

Ingredients used: Seravit, Liquigen medium-chain triglyceride, and Calogen long-chain triglyceride (Nutricia, Allerød, Denmark); Variolac and Lacprodan alpha-15 and Miprodan (ARLA Foods Ingredient, Viby, Denmark); and Polycose (Abbott Nutrition, Columbus, OH).

Table 2. Oligonucleotide sequences of primers and probes used for quantification PCR and visualization (fluorescence *in situ* hybridization) of total bacteria and *C. perfringens* in distal small intestinal tissue of preterm pigs.

Primer/probe name	**Oligonucleotide sequence (5'-3')**	**Reference**
Quantitative PCR		
Total bacteria		
Forward primers	8FM: AGAGTTTGATCMTGGCTCAG	[19]
	8FB: AGGGTTCGATTCTGGCTCAG	
Reverse primer	Bact515R: TTACCGCGGCKGCTGGCAC	
TaqMan probe	Bact338K: [FAM]CCAKACTCCTACGGGAGGCAGCAG[TAMRA]	
C. perfringens		[20]
Forward primer	CPerf165F: CGCATAACGTTGAAAGATGG	
Reverse primer	CPerf269R: CCTTGGTAGGCCGTTACCC	
TaqMan probe	CPerf187F: [FAM]TCATCATTCAACCAAAGGAGCAATCC[TAMRA]	
Fluorescence *in situ* hybridization		
Total bacteria	S-D-bact-0338-a-A-18: [FITC]GCTGCCTCCCGTAGGAGT	[29]
C. perfringens	S-S-Cl.perf.-185-a-A-18: [Cy3]TGGTTGAATGATGATGCC	[11]

Table 3. Gene symbol, protein name, forward (F) and reverse (R) primer sequences, amplicon length and primer efficiency for reference genes and genes of interest in IPEC-J2 cells during *C. perfringens* infection.

Gene symbol	Protein	Sequence (5´- 3´)	Amplicon length	Efficiency
Reference genes				
ACTB	β-actin	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76	0.93
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76	0.85
Genes of interest showing significant difference between groups				
CCL5	Chemokine (C-C motif) ligand 5	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCCTCCATCCTAGC	121	0.92
IL1RN	Interleukin 1 receptor antagonist	F: TGCCTGTCCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC	90	0.98
IL8	Interleukin 8	F: TTGCCAGAGAAATCACAGGA R: TGCATGGGACACTGGAAATA	78	0.80
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	F: GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTTAGACACTTTCC	85	0.88
SLC5A1	Sodium/glucose co-transporter	F: CTGCAAGAGAGTCAATGAGGAG R: CCGGTTCCATAGGCAAACT	99	0.95
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	F: CCCAGCTTTCTCTCATGGAC R: TTGGTTCTTCTGCCGTCTCT	113	0.90
Genes of interest not showing significant difference between groups				
APOA1	Apolipoprotein A-I	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC	86	0.81
C3	Complement component 3	F: ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG	76	0.87
CD14	CD14 molecule	F:GGGTTCCTGCTCAGATTCTG R: CCCACGACACATTACGGAGT	164	0.83
CLDN3	Claudin 3	F: ATCGGCAGCAGCATTATCAC R: ACACTTTGCACTGCATCTGG	94	0.87
CXCL10	Chemokine (C-X-C motif) ligand 10	F: CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTGTTCGAGGA	141	0.82
DEFB1	Defensin, beta 1	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109	0.90
DEFB4A (*DEFB2*)	Defensin, beta 4A	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99	0.83
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	F: ACACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71	0.88
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	F: CTCGCACAAGGAGACATGAA R: GGGTAGCCCAGTTTTTGTCA	97	0.88
IL18	Interleukin 18	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100	0.86
IL6	Interleukin 6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	116	085
MUC1	Mucin 1	F: GGATTTCTGAATTGTTTTTGCAG R: ACTGTCTTGGAAGGCCAGAA	116	0.81
OCLN	Occludin	F: CGGTGAGAAGATTGGCTGAT R: TTTCAAAAGGCCTGGATGAC	100	0.85
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b, regulatory subunit 1	F: GCAAACTGGCTACTGTGTGAAG R: GCACAGTCTGGTCATTGGAA	113	0.83
TGFB1	Transforming growth factor, beta 1	F: GCAAGGTCCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG	97	0.81
TLR4	Toll-like receptor 4	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145	0.81