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Early Administration of Probiotics Alters Bacterial Colonization and Limits Diet-Induced Gut Dysfunction and Severity of Necrotizing Enterocolitis in Preterm Pigs[1–3]

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Abstract

Following preterm birth, bacterial colonization and enteral formula feeding predispose neonates to gut dysfunction and necrotizing enterocolitis (NEC), a serious gastrointestinal inflammatory disease. We hypothesized that administration of probiotics would beneficially influence early bacterial colonization, thereby reducing the susceptibility to formula-induced gut atrophy, dysfunction, and NEC. Caesarean-delivered preterm pigs were provided total parenteral nutrition (1.5 d) followed by enteral feeding (2 d) with porcine colostrum (COLOS; $n = 5$), formula (FORM; $n = 9$), or formula with probiotics (FORM-P; *Bifidobacterium animalis* and *Lactobacillus*: *L. acidophilus*, *L. casei*, *L. pentosus*, *L. plantarum*; $n = 13$). Clinical NEC scores were reduced ($P < 0.05$) in FORM-P (2.0 ± 0.2) and COLOS groups (1.7 ± 0.5) compared with FORM pigs (3.4 ± 0.6). Lower NEC scores were associated with elevated intestinal weight, mucosa proportion, villus height, RNA integrity, and brush border aminopeptidase A and N activities, and lower gastric organic acid concentration in the FORM-P and COLOS groups ($P < 0.05$). Diversity of the mucosa-associated bacteria in the distal small intestine was similar among formula-fed pigs, yet the abundance of specific bacterial groups differed between FORM-P and FORM pigs. FORM-P pigs had lower colonization density of a potential pathogen, *Clostridium perfringens*, and had commensal *Lactobacillus* bacteria more closely associated with enterocytes along the villus-crypt axis relative to FORM pigs. These results suggest that probiotic administration immediately after birth promotes the colonization of a beneficial commensal microbiota capable of limiting the formula-induced mucosal atrophy, dysfunction, and pathogen load in preterm neonates, thereby reducing the incidence and severity of NEC. J. Nutr. 138: 1437–1444, 2008.

Introduction

Necrotizing enterocolitis (NEC)[8] is the most serious gastrointestinal disease afflicting preterm neonates. Bacterial colonization is considered a prerequisite for NEC and, combined with prematurity and enteral formula feeding, predisposes the neonate to this severe inflammatory condition. Many theories exist regarding the role of bacteria in NEC development, including the involvement of a specific causative pathogen (1,2), an inappropriate immune response to general bacterial colonization (3), and mucosal injury caused by excessive nutrient fermentation (4). We have recently shown, using a germ-free pig model, that the initial bacterial colonization immediately after birth is a prerequisite for formula-induced mucosal atrophy and dysfunction in preterm pigs and that only pigs experiencing this atrophic response are susceptible to NEC (5).

Probiotic bacteria have been described as possessing preventive or therapeutic potential for bacterial-mediated gastrointestinal tract (GIT) diseases such as irritable bowel syndrome (6), pouchitis (7), and diarrhea (8). Recently, the effectiveness of probiotic administration to human infants at risk of developing NEC has been reported (4,9). Although these studies convincingly show a reduction in the clinical outbreak of NEC, little is known about how probiotics affect gut structure, function, or

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[3] Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
[8] Abbreviations used: Ap, aminopeptidase; bp, base pair; BW, birth weight; CFU, colony-forming unit; COLOS, colostrum fed; S_D, Dice similarity coefficient; FISH, fluorescent in situ hybridization; FORM, formula fed; FORM-P, formula and probiotic fed; GIT, gastrointestinal tract; MCT, medium chain triglyceride; NEC, necrotizing enterocolitis; RIN, RNA integrity number; SI, small intestine; TPN, total parenteral nutrition; T-RFLP, terminal-restriction fragment length polymorphism.
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other colonizing bacteria and how this may lead to the prevention of the inflammatory responses in NEC. In the neonatal period, the GIT has relatively low bacterial density, stability, and species diversity (10), particularly following caesarean section and total parenteral nutrition (TPN) (11). This provides an opportunity for probiotics to establish a synergistic relationship within the highly sensitive GIT of premature neonates at risk of developing NEC. We hypothesized that oral administration of probiotics (4 *Lactobacillus* and 1 *Bifidobacterium* species) starting immediately after caesarean section delivery would alter the initial mucosa-associated colonization pattern of preterm formula-fed neonates and thereby reduce the bacterial-dependant mucosal atrophy and GIT dysfunction preceding NEC. Because many studies have shown these probiotic species to be capable of conferring a positive effect on gut physiology, coupled with the understanding that different species fulfill different niches in the gut, we combined the probiotics in a mixture to maximize the potential affect. Our aims were to test whether probiotics addition would: *1*) reduce NEC incidence and severity in a well-established preterm pig model; *2*) improve intestinal mucosal structure and digestive function; and *3*) alter the mucosa-associated bacteria communities in the distal small intestine (SI) and colon. Results from formula-fed pigs supplemented with probiotics were compared with those from control formula-fed pigs and colostrum-fed pigs, because the latter have previously been shown to exhibit a markedly reduced NEC incidence and severity (5).

Materials and Methods

Surgical preparation of sows and newborn piglets. Twenty-eight preterm piglets were obtained from 2 litters delivered by caesarean section at 107 d of gestation (Duroc × Yorkshire × Danish Landrace, Research Station Sjælland II, Denmark; term, 116 ± 2 d). Derivation, rearing, and catheterization of piglets was performed as previously described (5). The National Committee on Animal Experimentation in Denmark approved all procedures.

Nutrient solutions and treatment protocol. The TPN solution used was prepared as previously described and was formulated to meet the nutrient requirements of preterm pigs (12). The TPN solution was infused continuously for 36 h and passive immunization was provided by administration of adult porcine serum, as previously described (11).

The enteral formula diet was made from 3 commercially available products used for feeding infants 0–2 y of age [per liter of water: 80 g Pepdite 2–0, 70 g Maxipro, and 75 mL Liquigen- medium-chain triglyceride (MCT), all products kindly donated by SHS International] and described previously (13,14). The energy and protein concentrations of the formula were designed to match the composition of porcine milk during lactation. Porcine colostrum was collected manually from different sows (Large White × Landrace, Research Station Sjælland II, Denmark) within 6 h of completed farrowing and stored at −20°C until used.

Immediately after birth, pigs were randomly assigned to 3 treatment groups defined by their enteral feeding regimen [15 mL/(kg body weight (BW)$^{-1}$· 3 h)]: porcine colostrum (COLOS, $n = 5$), control formula (FORM, $n = 9$), or formula plus a probiotic mixture (kindly donated by Chr. Hansen, A/S) of *Bifidobacterium animalis* (DSM15954) and 4 *Lactobacillus* species: *acidophilus* (DSM13241), *casei* (ATCC55544), *pentosus* (DSM14025), and *plantarum* (DSM13367) (FORM-P; $n = 13$). 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 5×10^9 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. Individual housing and separate feeding supplies were used to prevent cross contamination of bacteria between treatment groups.

Throughout the experiment, potential clinical signs of NEC (feeding intolerance, abdominal distension, hemorrhagic diarrhea, and respiratory distress) were recorded every 3 h. To minimize variation resulting from age differences, piglets were scheduled to be killed 40–48 h after the start of oral feeding unless discomfort and clinical signs of NEC required euthanasia at an earlier time point.

Tissue collection. At tissue collection, pigs were killed (sodium pentobarbital, 200 mg/kg BW, administered intra-arterially through the umbilical catheter), the GIT was evaluated macroscopically for signs of NEC, and tissue samples were collected and stored as previously described (11). For in situ hybridization analysis, tissue samples were fixed in 4% paraformaldehyde for 48 h or Carnoy's fluid [6:3:1 mixture of ethanol: chloroform:acetic acid on a volume basis (15)] for 6–8 h and then all samples were transferred to 70% ethanol and stored at 4°C until later histological preparation.

Gut morphology and enzyme assays and proinflammatory cytokine concentrations. Distal intestinal villus and crypt depth (16) and activities of brush border peptidases [aminopeptidase A (ApA), aminopeptidase N (ApN)] and disaccharidases (lactase, maltase) were all measured as previously described (17). Porcine interleukin (IL)-6 and interferon (IFN)γ of distal SI whole tissue homogenate was determined by an R & D DuoSet ELISA (R & D Systems, catalogue no. DY686) as previously described (11).

Microbiology, short chain fatty acid concentrations, and stomach pH. Culture-based microbiology was performed to enumerate densities of selected bacterial groups associated with stomach contents, distal SI mucosal scrapings obtained by scraping a segment of the distal SI, and colon contents as previously described (11). Furthermore, the bacterial assemblage of the distal SI and colon was analyzed by terminal-restriction fragment length polymorphism (T-RFLP) using whole-tissue homogenates as previously described in detail (11). Estimates of variation in bacterial colonization patterns within and between treatment groups were made based on pairwise comparisons of T-RF using the Dice similarity coefficient (S_D) calculation in the Bionumerics software package as described previously (18). Generally, there is a direct relationship between the S_D and the similarity of 2 compared pigs, with 2 identical T-RFLP profiles having a similarity coefficient of 1.00 (100% similarity). Identification of specific bacteria characterized by T-RF was done in silico by inserting primer sequences and restriction enzymes into the MiCA software (19) using the RDPII database (20).

Finally, the location of bacteria associated with the distal SI mucosa was investigated using fluorescent in situ hybridization targeting 16S rRNA. Fixed tissue samples were analyzed from all pigs in all 3 treatment groups. The samples were sectioned (5 μm) and mounted on SuperFrost/plus slides (Menzel-Gläser). The slides were deparaffinized in xylene and dehydrated in 100% alcohol for 30 min before hybridization. Tissue sections were then treated with 3 g/L Lyzozyme (Roche Diagnostics) in PBS for 10 min at room temperature and washed in PBS. The hybridization was carried out at 45°C with 40 μL of hybridization buffer [100 mmol/L Tris (pH 7.2), 0.9 mol/L NaCl, 0.1% sodium dodecyl sulfate] and 200 ng of probe for 16 h in a Sequenza Slide Rack (Thermo Shandon). The samples were then washed in 100 mL of warmed (45°C) hybridization buffer for 15 min and subsequently in 100 mL of warmed (45°C) washing solution [100 mmol/L Tris (pH 7.2), 0.9 mol/L NaCl] for an additional 15 min and finally rinsed in water and air dried. The oligonucleotide probes used in this study are listed in **Supplemental Table 1**. The oligonucleotide probes designed in this study were selected using the software ARB (21). It was not possible within 16S rRNA to design a probe that could distinguish between *L. pentosus* and *L. plantarum*; therefore, a single probe targeting both bacterial species was designed. The oligonucleotide probes (MWG-BIOTECH AG) were 5′ labeled with either fluorescein isothiocyanate or the isothiocyanate derivative Cy3. Probe validation was performed by injection of pure cultures of *B. animalis*, *L. casei*, *L. acidophilus*, *L. plantarum*, and *L. pentosus*, respectively, from MRS broth medium (Oxoid) intrabronchially into sterile pig lung, fixed in either 10% neutral buffered formalin or Carnoy's fixation. There was no cross-hybridization between the probes in either Carnoy's or formalin-

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fixed tissue and all species-specific probes produced strong signal in the control tissue with pure cultures. The samples were rinsed in water, air dried, and mounted in Vectashield (Vector Laboratories) for epifluorescence microscopy. An Axioimager M1 epifluorescence microscope equipped for epifluorescence with a 100-W HBO lamp and filter sets 43 and 38 were used to visualize CY3 and fluorescein, respectively. Images were obtained using an AxioCam MRm version 3 FireWiremonocrome camera and the software AxioVision version 4.5 (Carl Zeiss). Images were processed for display by using Photoshop software (Adobe).

The concentrations of short chain fatty acids (formic, acetic, propionic, buturic, lactic, benzoic, iso-valeric, valeric, iso-capronic, capronic, heptane, and sorbine acids) in stomach contents were measured as described previously (22) using GC. The pH of stomach contents was measured using a Corning M240 pH meter (Philip Harris Scientific).

Intestinal RNA integrity and cytokine expression. Total RNA was isolated from distal SI tissue using the RNeasy Midi kit (Qiagen), as described in the manufacturer's protocol. RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity was determined by the A_{260}:A_{280} ratio (GeneQuant Pro; Biochrom). RNA integrity was measured on the Agilent 2100 bioanalyzer, using the Nanochip 6000 (Agilent Technologies) instead of conventional agrose gel electrophoresis because of the increased sensitivity to detect degraded RNA (23). Data are presented as RNA integrity numbers (RIN). As previously described (23), RIN vary from 1–10 with a RIN of 1 representative of completely degraded RNA, whereas a RIN of 10 is achieved for intact RNA. Extracted RNA was converted into first-strand cDNA by reverse transcription of 1 μg of total RNA using QuantiTect Reverse Transcription (Qiagen) according to the manufacturer's instructions (total volume, 20 μL) and stored at −20°C.

The relative gene expression of 4 different cytokines IL-1β, IL-10, tumor necrosis factor α, and IFNγ was determined for all treatment groups to indicate an inflammatory response. Quantitative PCR of mRNA was performed as previously described, with minor modifications (24). Briefly, Ampliqon RealQ-PCR Master Mix kit (Bie & Berntsen) was mixed with 70–200 ng of template cDNA and gene of interest-specific primers (**Supplemental Table 2**) were designed with Primer3 (25) and synthesized (TAG Copenhagen). Using the RotorGene 3000 Detection System (Corbett Research), cycle conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles with denaturation for 15 s at 95°C and annealing/elongation for 1 min at 59–62°C, and all reactions were performed in triplicate. In each run, a 3-fold serial dilution standard curve of nonexperimental lung tissue was used to assign relative concentrations to the samples. The stability of the 5 housekeeping genes (24) was determined using geNorm (26). A gene expression normalization factor was determined by geNorm based on the mean of the 3 most stable housekeeping genes (Supplemental Table 2; β-actin and GAPDH excluded) and used to calculate gene expression for all samples with a NEC score < 5. To compare the fold change in gene expression between the treatment groups, normalized gene expression values for FORM-P and COLOS were compared with FORM (set to 1).

Statistical analysis. The data were analyzed by a 2-way ANOVA using the MIXED procedure of SAS (SAS/STAT version 8.1, SAS Institute). Treatment (FORM, FORM-P, and COLOS) and intestinal region (proximal, middle, and distal) were considered as fixed effects and pig and litter were included as random effects. The results in tables and figures are given as the least square means (LSmeans) ± SEM and differences between 2 means were tested by the least significant difference test. NEC incidence was determined by χ^2 analysis. $P = 0.05$ was used as the critical level of significance for all evaluations.

Results

Clinical observations. In the FORM group, 89% (8/9) of pigs developed NEC (NEC score ≥1.50) and had a NEC severity score (**Table 1**) of 3.4 ± 0.6. In comparison, 69% (9/13) of FORM-P pigs developed NEC and had a lower NEC severity score (2.0 ± 0.2; $P < 0.05$). Five of the FORM and one of the FORM-P pigs had to be killed within 15–30 h from the start of enteral nutrition due to increasing severity of clinical NEC symptoms. Relative to the formula-fed pigs, both NEC severity (1.8 ± 0.5) and NEC incidence (40%, 2/5) decreased in COLOS pigs ($P < 0.05$).

Gut weights and morphology. Compared with the FORM pigs, the relative weight of the SI (Table 1) was higher (+28%; $P < 0.05$) in FORM-P pigs, with the greatest difference for the proximal SI (+41%). The probiotics also specifically reduced atrophy of the SI mucosa (Table 1) as indicated by a higher proportion of mucosa in all SI regions of the FORM-P pigs compared with FORM pigs (+6–17%; $P < 0.05$). These effects on SI morphology were further documented by the longer villi (+27%; $P < 0.05$) in FORM-P pigs and crypt depths were similar (Table 1). The trophic response of the SI mucosa to colostrum feeding was evident, as COLOS pigs showed increases in SI mucosal proportions (+15%; $P < 0.05$) and distal villus height (+72%; $P < 0.05$) and a moderate increase in SI weight (+24%; $P = 0.11$) compared with FORM pigs. COLOS pigs differed only from FORM-P pigs in increased villus height (+34%; $P < 0.05$) and decreased crypt depth (−14%; $P < 0.05$). Colon weights of all formula-fed pigs (8.5 ± 0.4 g/kg) were higher than in COLOS pigs (5.0 ± 0.9 g/kg; $P < 0.05$) and stomach weights of FORM pigs (9.8 ± 1.4 g/kg) tended to be higher than in FORM-P (7.2 ± 0.9 g/kg; $P = 0.13$) and COLOS (5.7 ± 1.5 g/kg; $P = 0.06$) pigs. SI length (311 ± 15 cm/kg) and weights (g/kg BW) of heart (8.3 ± 0.6), liver (27.8 ± 0.5), spleen (2.1 ± 0.1), kidney (9.1 ± 0.2), and lung (17.7 ± 0.3) did not differ among the groups.

Brush border enzymes. Both ApA and ApN activities were ~50% greater in FORM-P pigs compared with FORM pigs (Table 1) and COLOS pigs showed even higher activities (+100–200%; $P < 0.05$). In all diet groups, ApA and ApN activities were lowest in the proximal region and increased ~50–100% in both middle and distal SI regions. In contrast, brush border disaccharidase activities were entirely dependent on diet type; FORM and FORM-P pigs did not differ in lactase or maltase activities (Table 1), whereas enzyme activities were markedly higher in COLOS pigs (+100–200%; $P < 0.05$). In all diet groups, lactase and maltase levels were ~25–100% greater in the middle and distal SI regions compared with the proximal SI.

TABLE 1 NEC severity and small intestinal structural and functional indices of preterm piglets fed FORM, FORM-P, or COLOS[1]

Treatment group	n	NEC severity	SI weight	Mucosa	Villus height	Crypt depth	Lactase	Maltase	ApA	ApN
			g/kg BW	%	$\mu m \times 100$		U/g tissue			
FORM	9	3.4 ± 0.6[a]	27.4 ± 2.5[b]	55.8 ± 1.6[b]	4.9 ± 0.5[c]	1.4 ± 0.8[ab]	6.0 ± 1.3[b]	1.1 ± 0.1[b]	2.0 ± 0.3[b]	3.2 ± 0.6[c]
FORM-P	13	2.0 ± 0.2[b]	35.2 ± 1.9[a]	67.3 ± 1.3[a]	6.2 ± 0.3[b]	1.5 ± 0.5[a]	7.1 ± 1.1[b]	1.4 ± 0.1[b]	3.0 ± 0.2[a]	4.8 ± 0.4[b]
COLOS	5	1.7 ± 0.5[b]	33.9 ± 3.0[ab]	70.4 ± 2.1[a]	8.3 ± 0.5[a]	1.3 ± 0.9[b]	21.6 ± 1.8[a]	2.6 ± 0.2[a]	3.7 ± 0.4[a]	9.3 ± 0.7[a]

[1] Values are LSmeans ± SEM. Means in a column with superscripts without a common letter differ, $P < 0.05$.

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Short chain fatty acid concentrations and stomach pH. Total organic acid concentrations in stomach contents were 80–130% greater in FORM pigs than in FORM-P and COLOS pigs (**Table 2**; $P < 0.05$). Octanoic acid accounted for 33% of the total organic acid concentration in FORM pigs, but concentrations were significantly lower in FORM-P pigs, accounting for only 26% of total organic acids (Table 2). Only low concentrations of octanoic acid were measured in the complete formula diet (1.1 mmol/kg) or the Liquigen-MCT component of the formula (2.3 mmol/kg). Seven pigs had clinical NEC in the stomach (5 FORM, 1 FORM-P, 1 COLOS pig) and total organic acid levels for these pigs were elevated compared with pigs without stomach NEC (~125% for FORM and FORM-P, 9-fold for COLOS). Lactic acid concentration was ~40% lower in FORM and FORM-P pigs than in COLOS pigs ($P < 0.05$). Acetic and butyric acid concentrations were ~1.4-fold greater in FORM and COLOS pigs than in FORM-P pigs ($P < 0.05$). Propionic acid was not detected in the stomach contents of pigs in any group. The mean pH of stomach contents (Table 2) was significantly higher in FORM than in COLOS pigs, with intermediate values in FORM-P pigs.

Microbiology. Semiselective culturing showed that differences in both bacterial density and the relative proportions of the culturable bacteria existed between treatment groups. Total anaerobic bacteria (range, 10^8–10^{10} CFU/g contents) and coliform bacteria (range, 10^4–10^{10} CFU/g contents) were similar for all treatment groups (**Fig. 1**), with the exception of a higher density of total anaerobes in the colon contents of COLOS pigs compared with FORM-P ($P = 0.15$) and FORM pigs ($P < 0.05$). For the distal SI and colon contents, clostridial density was lower in FORM-P pigs than in FORM pigs ($P < 0.05$; Fig. 1). Interestingly, both lactic acid and *Lactobacillus* bacteria were generally more abundant in FORM-P pigs compared with FORM pigs, yet intermediate for COLOS pigs. The relative proportions of *Lactobacillus*/clostridial bacteria in FORM-P pigs were markedly higher in the stomach (45%; $P < 0.05$) and distal SI (27%; $P < 0.05$) and tended to be greater in the colon ($P = 0.1$) compared with FORM pigs. Similarly, at each location, the proportion of lactic acid bacteria:clostridial bacteria was greater in FORM-P pigs compared with FORM pigs (+15–40%; $P < 0.05$). FORM-P pigs also had lower ($P < 0.05$) densities of enterococci in the stomach and distal SI compared with FORM pigs, whereas COLOS pigs showed intermediate levels. However, in the colon of COLOS pigs enterococci levels tended to be greater than in both formula-fed groups ($P < 0.1$).

T-RFLP analysis of whole tissue homogenates from the distal SI and colon demonstrated marked differences in bacterial communities between treatment groups. In the distal SI, a total of 36 identifiable T-RF were identified between 60 and 600 bp. The number of identifiable T-RF did not differ between FORM and FORM-P pigs but was significantly lower in COLOS pigs (**Table 3**). The S_D revealed that individuals within treatment groups (i.e. FORM vs. FORM) had more similar bacterial diversity than individuals from different treatment groups (i.e. FORM vs. FORM-P; Table 3). Diet influenced bacterial diversity as indicated by similar S_D for all formula-fed pigs but S_D that were significantly different from COLOS pigs ($P < 0.05$). Despite the similar degree of bacterial diversity among the formula-fed pigs, probiotic administration selectively increased ($P < 0.05$) the relative density of bacteria at T-RF 209, 549, and 551 bp, while decreasing the relative density of bacteria at T-RF 233 bp compared with FORM pigs (**Fig. 2***A*). Bacteria at T-RF 599 bp were identified in only FORM-P pigs, and based on in silico digestion of the probiotic 16S rDNA sequences, the 4 *Lactobacillus* probiotic species would be represented by a T-RF of 599 bp. In the colon, a total of 34 T-RF were identified

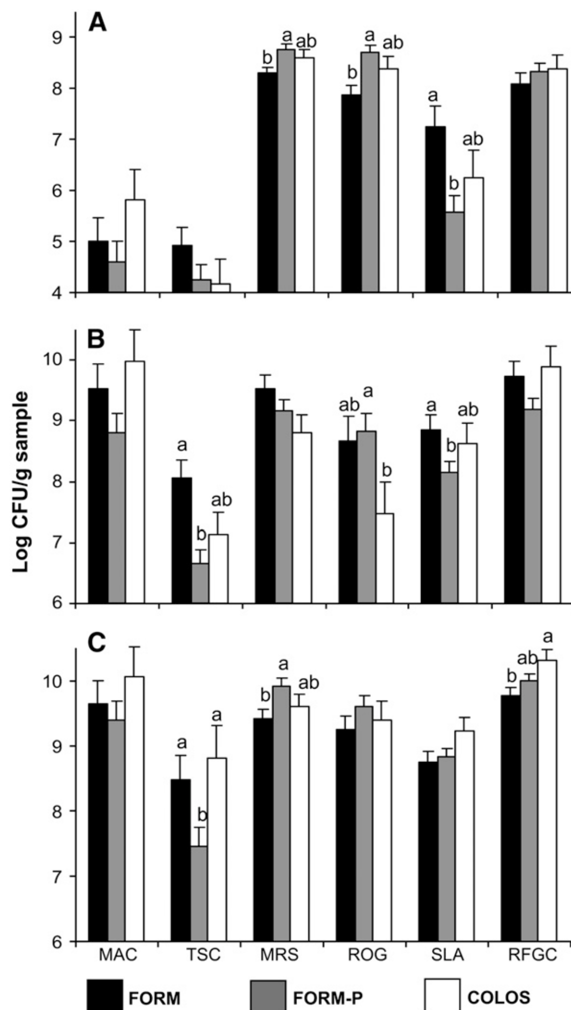

FIGURE 1 Semiselective culturing of bacteria from stomach contents (*A*), distal SI mucosal homogenates (*B*), and colon contents (*C*) of preterm piglets fed FORM, FORM-P, or COLOS. Plating media selective for enterobacteria and coliforms (MAC), clostridium (TSC), lactic acid bacteria (MRS), lactobacilli (ROGOSA), enterococci (SLA), and total anaerobes (RFGC). Values are LSmeans ± SEM, n = 9 (FORM), 13 (FORM-P), and 5 (COLOS). Means without a common letter differ, $P < 0.05$.

TABLE 2 Organic acid concentrations and pH of stomach contents from preterm piglets fed FORM, FORM-P, or COLOS[1]

Treatment group	n	Total organic acids	Octanoic acid	Lactic acid	Acetic + butyric acid	pH
		mmol/kg contents				
FORM	9	296 ± 42[a]	96.8 ± 17.2[a]	29.1 ± 7.6[b]	5.8 ± 1.9	3.4 ± 0.2[a]
FORM-P	13	161 ± 29[b]	51.3 ± 14.3[b]	35.8 ± 5.1[b]	2.4 ± 0.7	3.2 ± 0.2[ab]
COLOS	5	129 ± 24[b]	2.0 ± 1.2[c]	56.7 ± 14.3[a]	5.7 ± 1.3	2.6 ± 0.1[b]

[1] Values are LSmeans ± SEM. Means in a column with superscripts without a common letter differ, $P < 0.05$.

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between 60 and 600 bp. The number of identifiable T-RF was lower in FORM pigs than in COLOS pigs ($P < 0.05$) and was intermediate in FORM-P pigs, and bacterial diversity was the least homogenous among FORM pigs (i.e. FORM vs. FORM), intermediate for COLOS pigs, and most homogenous for FORM-P pigs (Table 3). Similar to the distal SI, diet influenced the bacterial diversity in the colon, as shown by more similar S_D between FORM and FORM-P pigs compared with formula-fed and COLOS pigs. Mean relative intensities of T-RF differed among treatment groups (Fig. 2*B*), although significant differences were detected in relatively few T-RF. Specifically, FORM-P pigs tended to show higher intensity for T-RF 207, 209, 231, and 599 bp and lower intensity for T-RF 583 bp ($P < 0.1$) compared with FORM pigs, whereas COLOS pigs showed marked increases in T-RF 231, 375, and 571 bp compared with all formula pigs. Of the all the T-RF detected in the distal SI and colon, T-RF 583, 375, 233, and 209 bp were among the most abundant in both locations. According to the available T-RFLP libraries (RDP II database), many bacteria of different taxonomical groups (genus and species) can share these T-RF. Thus, identification of bacteria responsible for these T-RF would require further isolation, cloning, and genetic sequencing. The exception is T-RF 233, which is almost exclusively identified as Clostridium, or more specifically, *Clostridium perfringens*. Data from this laboratory (our unpublished data) have repeatedly identified *C. perfringens* in cecal contents collected from formula-fed pigs with an increase in T-RF 233 bp.

Hybridization with a general eubacterial probe showed that in healthy FORM-P pigs, bacteria were located along the entire villus-crypt axis and in close association with the enterocytes (**Fig. 3***B*), whereas bacteria in FORM (Fig. 3*A*) and COLOS pigs (Fig. 3*C*) were distributed more among the villus tips and in the intestinal lumen. Subsequent hybridization with a general *Lactobacillus* probe identified that lactobacilli were among these colonizing bacteria (Fig. 3*D–F*). Probes targeted specifically toward the probiotic strains did not identify probiotics in either FORM (Fig. 3*G*) or COLOS pigs (Fig. 3*I*) but did identify probiotic species colonizing the intestine of FORM-P pigs (Fig. 3*H*). Probiotic *L. acidophilus* was detected along the villus-crypt axis in FORM-P pigs (Fig. 3*J*), whereas *B. animalis* and *L. casei* were only minimally detected (data not shown) and *L. pentosus* and *L. plantarum* were below detection levels.

Intestinal RNA integrity and cytokine expression and concentration. RNA purity absorbance ratios (A_{260}:A_{280} and A_{260}:A_{230}) showed that all treatment groups had similar levels of

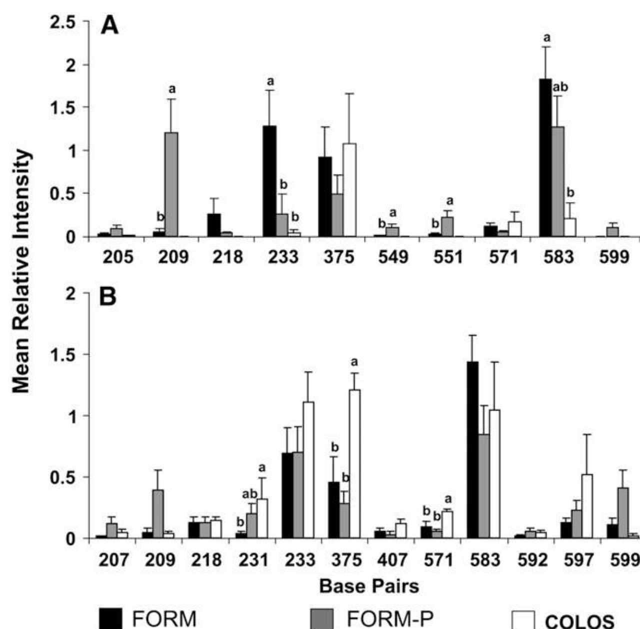

FIGURE 2 Relative fluorescent intensity of dominant T-RF determined by T-RFLP analysis of distal small intestinal (*A*) and colon (*B*) whole tissue homogenates of preterm pigs fed FORM, FORM-P, or COLOS. Values are LSmeans ± SEM, n = 9 (FORM), 13 (FORM-P), and 5 (COLOS). Means without a common letter differ, $P < 0.05$.

highly purified RNA (1.83 ± 0.01 and 1.88 ± 0.06). However, high RNA purity did not always correspond to high RNA integrity and even degraded RNA had high purity values. FORM pigs showed lower RIN values than pigs from the other 2 groups (5.0 ± 0.5 vs. 7.2 ± 0.4; $P < 0.01$). IL-6, IFNγ, IL-1β, and IL-10 gene expressions, regardless of RIN, did not differ among the groups (data not shown), likely due to the few samples ($n = 11$) with sufficient quality mRNA (RIN > 7.0). Proinflammatory cytokine IL-6 was detected only in formula-fed pigs, with concentrations ranging from 6 to 52 × 10^3 μg/L tissue homogenate. Similarly, IFNγ did not differ between formula-fed groups (160 ± 60 μg/L tissue homogenate) and was detectable in only a single COLOS pig.

Comparisons of NEC and healthy pigs across all treatments. Physiological differences existed between pigs diagnosed as NEC pigs ($n = 19$) and healthy pigs ($n = 8$). Compared

TABLE 3 Mean number of T-RF and S_D for distal SI and colon tissue homogenates for preterm piglets fed FORM, FORM-P, or COLOS[1,2]

				S_D within or between treatments[4]		
GIT region	Treatment	n	T-RF[3]	FORM	FORM-P	COLOS
Distal SI	FORM	9	11.4 ± 1.6[a]	47.1 ± 3.1[a]	41.7 ± 1.7[a]	34.4 ± 2.2[b]
	FORM-P	13	12.6 ± 1.0[a]	—	43.0 ± 2.1[a]	29.2 ± 1.4[c]
	COLOS	5	6.2 ± 1.3[b]	—	—	44.4 ± 3.8[a]
Colon	FORM	9	16.4 ± 0.8[b]	67.8 ± 1.4[b]	68.1 ± 1.0[b]	62.7 ± 1.4[c]
	FORM-P	13	16.7 ± 0.9[ab]	—	74.4 ± 1.1[a]	59.1 ± 1.5[c]
	COLOS	5	19.6 ± 1.3[a]	—	—	73.9 ± 3.7[ab]

[1] Values are LSmeans ± SEM.
[2] S_D represent pairwise comparisons of T-RF between 2 random individuals, originating either from the same treatment group or from different treatment groups.
[3] For each region (distal SI and colon), means in a column with superscripts without a common letter differ, $P < 0.05$.
[4] For each region (distal SI and colon), means within columns and rows with superscripts without a common letter differ, $P < 0.05$.

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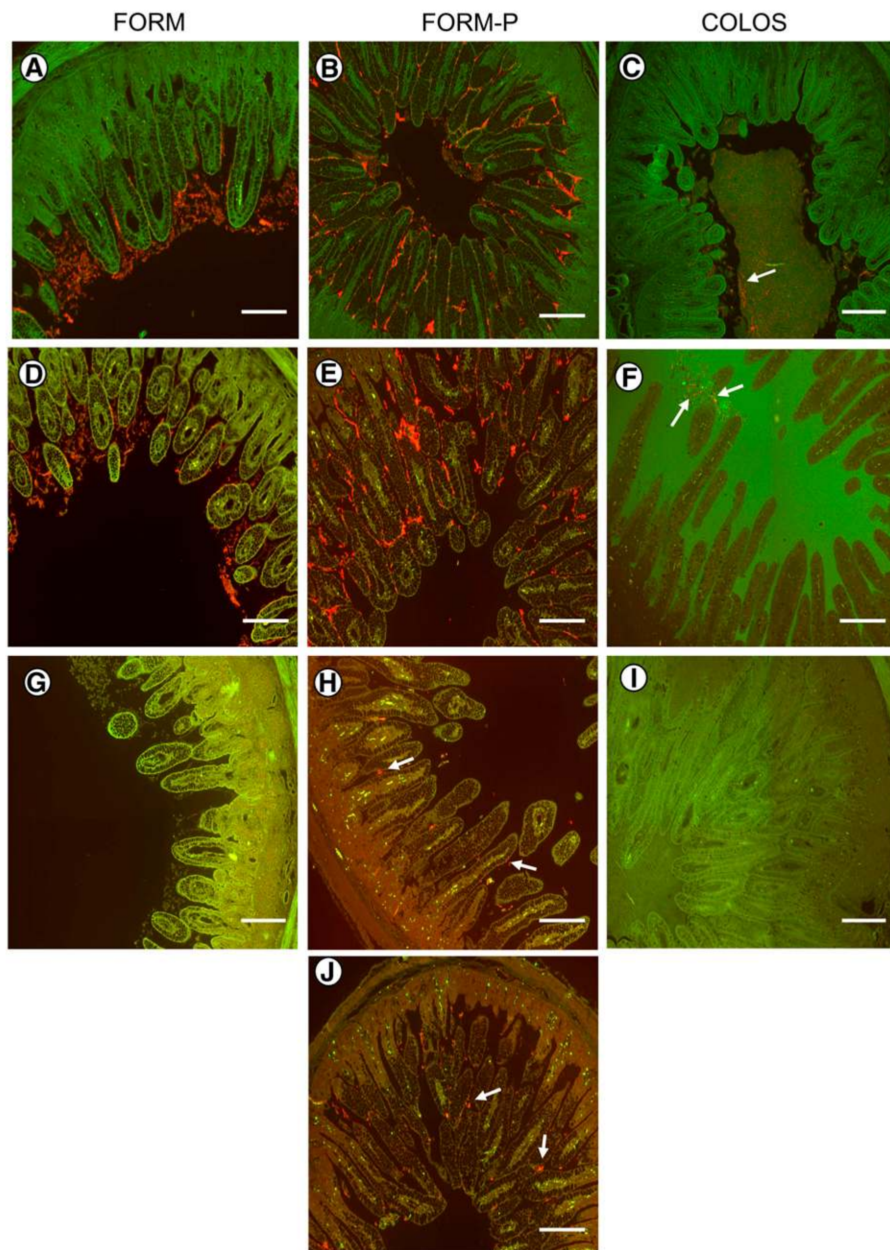

FIGURE 3 Representative in situ hybridization pictures of Carnoy's fixed distal small intestinal sections of healthy preterm pigs fed FORM, FORM-P, or COLOS. In COLOS pigs (*C*), a general bacterial probe (red fluorescence) showed hybridization of bacteria mostly contained within a mucosa-associated biofilm, whereas bacteria were present along the tips of the villi in FORM pigs (*A*) and along the entire villus-crypt axis of FORM-P pigs (*B*). A genus-specific *Lactobacillus* probe (red fluorescence) identified lactobacilli as being a dominant genus of bacteria in FORM (*D*) and FORM-P pigs (*E*) but less dominant in COLOS pigs (*F*). Individual probes targeting the 5 probiotic bacterial strains produced no signal in FORM (*G*) and COLOS pigs (*I*) but identified probiotic bacteria in relatively low numbers along the length of the villus in FORM-P pigs (*H*). Among these, *L. acidophilus* (red fluorescence) was identified as the most dominant probiotic bacteria in the FORM-P pigs (*J*). Scale bar = 200μm.

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with healthy pigs, the intestines of NEC pigs had reduced mucosal mass (−5%; $P < 0.05$), decreased ApN (−29%; $P < 0.05$) and disaccharidase activities (−23–30%; $P < 0.1$), and increased IFNγ concentrations (+82%; $P < 0.05$). Similarly, analysis of high quality mRNA (RIN ≥7) showed that pigs suffering from even a moderate degree of NEC ($n = 5$) had increased expression of IFNγ (+192%; $P < 0.05$) compared with healthy pigs ($n = 6$). Microbiological differences also existed between healthy and NEC pigs. T-RFLP analysis showed that T-RF 233 bp (corresponding to *C. perfringens*) was increased 32-fold in the distal SI of NEC pigs compared with healthy pigs ($P < 0.0001$).

Discussion

After birth, the immature GIT must adapt to the intake of complex enteral nutrients, bacterial colonization, and the resultant mucosal immune stimulation from both dietary and bacterial antigens. At times, aberrant bacterial growth results from suboptimal diets, and such diet-bacteria interactions have been shown to be particularly detrimental following formula feeding, leading to an increased incidence of inflammatory lesions and NEC (3,27). In this study, we demonstrate that administration of probiotics to preterm pigs exposed to TPN and subsequent enteral formula feeding had reduced diet-induced mucosal atrophy, dysfunction, and NEC severity. We also show that probiotics promote the growth of nonprobiotic *Lactobacillus* and decreased the levels of the potential pathogen, *C. perfringens*. Thus, this study demonstrates that probiotics administered immediately after delivery can improve intestinal function and health of neonates susceptible to NEC by promoting the early colonization of a beneficial microbiota. Such effects may be particularly important following caesarean delivery and parenteral nutrition, 2 components that delay the neonatal acquisition of bacteria.

The early onset and increased severity of clinical symptoms in FORM pigs, relative to FORM-P and COLOS groups, resulted in several of these pigs being killed prior to the predetermined trial completion time. Also compared with the other groups, FORM pigs had a marked increase in the incidence of NEC in

the stomach. This corresponded with a high total organic acid concentration, with octanoic acid being the most abundant organic acid. Octanoic acid is naturally found in coconut oil and the formula diet used in the current study contained coconut oil as part of the Liquigen-MCT component. Surprisingly, despite the elevated levels of octanoic acid in both formula groups, only small amounts of this organic acid were detected in the formula diet. This suggests that the octanoic acid detected in the stomach contents is a metabolite resulting from gastric digestion and fermentation of the MCT fraction of the formula diet, and the high concentration of octanoic acid may be responsible for the higher degree of stomach NEC. Interestingly, gastric octanoic and lactic acid were significantly lower in FORM-P pigs than in FORM pigs. The ability of the probiotics to decrease the organic acid concentration in the gastric lumen could be an important protective effect, yet it will be important to investigate whether probiotics are capable of similar effects in the SI and colon of preterm neonates, particularly because these are the sites most vulnerable to mucosal damage caused by excessive organic acid production (28,29).

Mucosal proportions, villus morphology, and relative intestinal weights are indicators of the mucosal architecture and were all improved in formula-fed pigs administered probiotics. Brush border peptidases, but not disaccharidases, were also improved in pigs receiving probiotics and this supports our previous results that neonatal aminopeptidase activities are more sensitive to bacterial colonization and inflammation, whereas disaccharidases are mainly diet dependant (5). Thus, the beneficial effects of probiotics are at least partly mediated via effects on intestinal integrity and digestive function.

The enumeration of enterococci, coliform, and clostridia bacteria in the preterm neonate is particularly relevant, because growth of these genera of bacteria is typically stimulated by formula feeding (10,30) and have been identified as potential pathogens in preterm neonates (1,3,31). The lower abundance of these bacteria in the stomach, distal SI, and colon of FORM-P pigs, compared with FORM pigs, supports the notion that probiotics inhibit the growth of potential NEC-related pathogens. In general, the distal SI mucosa was densely colonized by both aerobic and anaerobic bacteria (10^7–10^{10} CFU/g mucosa), although few bacterial species predominated as indicated by T-RFLP analysis. *Lactobacillus* bacteria were relatively abundant in the distal SI mucosa, but the relative intensity of T-RF 599 bp was low, indicating that administered probiotic strains of *Lactobacillus* were a minor subpopulation of the mucosa-associated bacteria. This suggests that probiotic bacteria were unable to colonize and proliferate as efficiently as other commensal bacteria. Nevertheless, probiotic administration to formula-fed pigs significantly altered the abundance of several bacterial populations as indicated by relative shifts in lactic acid and lactobacillus proportions and as shown by changes in the relative intensity of dominating species at T-RF 209 and 233 bp. Available T-RFLP libraries (RDP II database) indicate that T-RF 233 bp represents *Clostridium* species, in particular *C. perfringens*, and T-RF 233 bp was dramatically suppressed in FORM-P pigs compared with FORM pigs. This marked decrease in T-RF 233 bp corresponded with the decrease in cultured clostridial species colonizing the distal SI mucosa of FORM-P pigs. Interestingly, *Clostridium* species have repeatedly been suspected of being involved in the pathophysiology of NEC (1–3,32) and we showed that *C. perfringens* (T-RF 233 bp) increased 32-fold in pigs with NEC compared with healthy pigs. Although our findings do not prove a causal relationship between increased density of *Clostridium* species and NEC, they do suggest that probiotic administration is capable of reducing both NEC and potential pathogens by altering the intestinal bacterial community.

The ability of probiotic administration to alter the distal SI microbiota was further demonstrated after hybridization of all bacteria with the general 16S rRNA probe. Bacteria were present in higher density and were more completely distributed along the entire villus-crypt axis in FORM-P pigs than in FORM pigs, suggesting a higher degree of direct bacterial-host interaction in FORM-P pigs. Hybridization with a genus-specific *Lactobacillus* probe and probiotic specific probes demonstrated that this increased density of bacteria was predominantly due to an increase in colonizing *Lactobacillus* bacteria and that only a minor proportion of these bacteria were identified as probiotic bacteria, of which *L. acidophilus* was the most abundant.

To determine whether the improved intestinal function observed in FORM-P pigs compared with FORM pigs was related to differences in the intestinal inflammatory response, protein levels and gene expression of several pro- and antiinflammatory cytokines were measured. Proinflammatory cytokine abundance measured in intestinal tissues indicated similar levels of IL-6 and IFNγ for both formula groups, whereas a comparison of healthy and diseased pigs showed higher IFNγ in pigs with NEC. Generally, detection of these proinflammatory markers was present in only formula-fed pigs, suggesting that colostrum is capable of suppressing the inflammatory response, whereas probiotic administration improved intestinal function by other mechanisms. Surprisingly, these data showed poor correlation with the gene expression profiles of pro- and antiinflammatory cytokines. This poor correlation likely reflects that gene expression analysis was limited to pigs with high quality RNA. RNA integrity was significantly reduced in the FORM pigs, leading to the exclusion of many pigs from the gene expression analysis. Further, the lowered RIN values in the FORM group suggest that these pigs were experiencing increased necrosis-induced RNA degradation prior to sample collection. This observation supports the increased clinical NEC severity in FORM pigs compared with FORM-P and COLOS pigs. Previous NEC animal model studies have recognized the difficulty in measuring gene expression in diseased tissues and have also excluded samples prior to analysis depending on the degree of necrosis (33). Our study demonstrates that RNA purity is insufficient to validate RNA for subsequent gene expression analysis and that determination of RIN values is a necessary and sensitive marker of RNA quality (34) in diseased tissues.

Reduced NEC incidence with probiotics has also been reported in other animal models of NEC (1,27). While these studies are informative, the strength of the preterm pig model used in this study is that the clinical and histological characteristics of NEC closely resemble those in humans and that these symptoms develop spontaneously after preterm birth and formula feeding. Further, the piglet GIT clearly has a high degree of anatomical and physiological similarity with the infant GIT that may make studies involving microbial-associated disease more comparable. Also, the implementation of a TPN period just after birth is an important advancement in this preterm NEC model. Not only is TPN administration clinically relevant, as a period of TPN is commonly employed in neonatal wards, but work from this laboratory (our unpublished data) has shown that TPN tends to increase the sensitivity of the preterm gut to intestinal dysfunction and NEC. Finally, the TPN period allows for frequent probiotic administration prior to the start of enteral feeding in a highly sensitive biological model of immaturity. In conclusion, our results have shown that probiotic administration to preterm pigs subjected to TPN and formula feeding can beneficially influence the establishment of a com-

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mensal microbiota capable of reducing gut mucosal atrophy, dysfunction, and pathogen colonization that all contribute to the intestinal conditions that lead to NEC.

References (35–38) are cited in the online supporting data.

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