Enteral but not parenteral antibiotics enhance gut function and prevent necrotizing enterocolitis in formula-fed newborn preterm pigs

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EVERY YEAR, APPROXIMATELY 15 million infants are born preterm worldwide (i.e., before the completion of 37 wk of gestation), comprising 11% of all pregnancies (42). These infants have an immature gastrointestinal (GI) tract and immune system and are therefore susceptible to infection and intestinal diseases, such as necrotizing enterocolitis (NEC). NEC is the most common GI complication affecting 7% of very low birth weight infants (500-1,500 g) with a mortality rate of 20–30% (31). The etiology and pathogenesis of NEC are still incompletely understood, but prematurity, aggressive enteral nutrition, and abnormal gut bacterial colonization are important predisposing factors (37).

Breast milk is the most NEC-protective diet for preterm infants (43), but lactation is often delayed and mother’s milk is frequently inadequate for enteral nutrition after preterm delivery within the first week. Conversely, alternatives to breast milk, such as infant formula, are associated with increased NEC incidence (6–10 times, 28). Therefore, it is critical to identify medical and nutritional strategies to protect formula-fed preterm infants against NEC development. Modulations of the gut microbiota with pre- and probiotics may decrease NEC risk, but important questions related to type and dose of different products and their optimal time of administration remain unclear (10, 18, 23, 46).

Intravenous antibiotics are frequently used in neonatal intensive care units (NICUs) to prevent and treat bacterial infections caused by invasion via indwelling catheters or via bacterial translocation from the immature gut or other body surfaces (27). Likewise, intravenous antibiotics are commonly applied as a treatment following the onset of NEC (31). A systematic review of five studies including 456 preterm infants revealed that enteral antibiotics reduced NEC incidence and NEC-related deaths in low birth weight infants (5). However, these studies have not led to a widespread use of prophylactic enteral antibiotics for preterm infants, in part attributable to uncertainties regarding product, time, doses, and efficacy to promote intestinal health. Other important reservations are the increased risk of NEC after long-term use of antibiotics and the possible development of antibiotic resistance (1, 11, 22). Regardless, it remains unknown how a few days of neonatal antibiotics affect host response, antibiotic resistance, and total need for antibiotics during hospitalization.

The preterm pig is a preclinical model of preterm infants in which the immature gut is highly sensitive to both microbial colonization and formula feeding. Thus preterm pigs fed rapidly advancing volumes of enteral formula within the first week after birth by caesarean section at 90–92% gestation have a high incidence of spontaneous NEC-like lesions (38). Even a gradual introduction of formula feeds causes mild NEC le-
sions, whereas preterm pigs fed formula under germ-free conditions or in utero show no signs of NEC (3, 37). This underlines the critical role of the early gut colonization to induce NEC. Immaturity of the gut immune system may explain that enhanced bacterial colonization after vaginal birth or probiotics administration may increase NEC sensitivity (10, 40), whereas an immune-modulatory milk diet, like bovine colostrum, is protective (39). Similarly, combined administration of prophylactic enteral and parenteral broad-spectrum antibiotics during the first days after preterm birth effectively prevents NEC in formula-fed pigs (20). It is not known, however, whether both routes of administration are required to achieve this protection. Enteral antibiotics have profound effects on gut luminal bacteria but may also be absorbed into the blood stream, depending on the pharmacokinetics of each product and transport from the intestinal lumen. Antibiotic effects in the gut lumen will also reduce the formation of bacterial fermentation products (20). Parenteral antibiotics are distributed to vascular beds and tissues according to their pharmacokinetics, followed by metabolism and excretion via urine and bile (13). In this manner, both enteral and parenteral antibiotics have local as well as systemic effects, depending on the specific product. Regardless, little is known about the possible benefits or adverse effects of enteral vs. parenteral use of antibiotics for preterm infants.

Based on the apparent detrimental effects of rapid bacterial colonization for NEC development in newborn preterm pigs, we hypothesized that broad-spectrum antibiotics given enterally during the immediate postnatal period would be superior to parenteral administration in preventing early, formula-induced NEC symptoms. To test this hypothesis, we investigated intestinal structure, function, microbiology, and mucosal immunity in preterm pigs administered a combination of three commonly used antibiotics enterally or parenterally for 5 days after birth. The data on blood hematology and effects on systemic immunity have been published as a separate report, in which we explain that enhanced bacterial colonization after vaginal birth or probiotics during the first days after preterm birth effectively prevents NEC in formula-fed pigs (20). The enteral formula diet was made from three commercial products for feeding infants (per liter of water: 75 g Liquiden MCT, 80 g Peptide, and 70 g Arla DI-9224; from Nutricia, Allerød, Denmark, and Arla Food Ingredients, Viby J, Denmark, respectively). From previous studies, we know that high levels of maltodextrin in the Pepdite product often increase NEC sensitivity, but NEC incidence in preterm pigs varies among experiments and periods even using the same diet (similar to conditions in the human neonatal clinic). MEN was initiated within 5 h of delivery as boluses of 3 ml/kg every 3 h on days 1 and 2. In the morning of day 3, PN was stopped, and total enteral nutrition was given as boluses of 15 ml/kg every 3 h until euthanasia and tissue collection on day 5.

**MATERIALS AND METHODS**

**Animals and study design.** Forty-nine preterm piglets were delivered from three sows (Large White × Danish Landrace × Duroc) by cesarean section at day 106 of gestation (i.e., 90–92% of expected gestational length). Delivery and housing of the preterm piglets were performed as previously described (20, 38). In brief, the newborn preterm piglets were immediately transferred to the pig NICU and reared in temperature- and oxygen-regulated incubators. Pigs were weighed and fitted with umbilical arterial and orogastric catheters and then provided passive immunization with maternal plasma (16 ml/kg body wt (BW) during the first 24 h) as previously described (4). Pigs were block randomized according to birth weight and sex into three groups: pigs receiving broad-spectrum antibiotics through the umbilical arterial catheter (parenteral route, PAR, n = 17) or orogastric catheter (enteral route, ENT, n = 16) or pigs receiving saline control via the orogastric catheter (CON, n = 16). A combination of ampicillin (30 mg/kg BW 3 times daily), gentamicin (2.5 mg/kg BW twice daily), and metronidazole (10 mg/kg BW 3 times daily), formulated for enteral and parenteral use, was given in identical doses to target a broad range of microorganisms. Selection of antibiotics and doses were adapted from previous studies (7) and from their common use for preterm infants in Denmark. Parenteral antibiotics comprised ampicillin (Pentrexyl; Bristol-Myers Squibb, Solna, Sweden), gentamicin (B. Braun Medical, Melsungen, Germany), and metronidazole (B. Braun Medical). Enteral antibiotics comprised ampicillin (Pembritin; Chemidex Pharma, Surrey, United Kingdom), gentamicin (Genotin Vet; ScanVet, Fredensborg, Denmark), and metronidazole (Flagyl; Sanofi Aventis, Horsholm, Denmark). Animals were treated in accordance with the Animal Experimentation Act of Denmark, which is in accordance with the Council of Europe Convention ETS 123. The study was approved by the National Animal Experimentation Board.

**Nutrient solutions and feeding protocol.** All pigs were initially provided parenteral nutrition (PN) via the umbilical catheter and minimal enteral nutrition (MEN) via the orogastric tube. The PN solution was based on a commercial product (Kabiven, Fresenius Kabi, Runcorn, Cheshire, United Kingdom) and adjusted in composition to meet the nutritional requirements for piglets (20). The enteral formula diet was made from three commercial products for feeding infants (per liter of water: 75 g Liquiden MCT, 80 g Peptide, and 70 g Arla DI-9224; from Nutricia, Allerød, Denmark, and Arla Food Ingredients, Viby J, Denmark, respectively). From previous studies, we know that high levels of maltodextrin in the Pepdite product often increase NEC sensitivity, but NEC incidence in preterm pigs varies among experiments and periods even using the same diet (similar to conditions in the human neonatal clinic). MEN was initiated within 5 h of delivery as boluses of 3 ml/kg every 3 h on days 1 and 2. In the morning of day 3, PN was stopped, and total enteral nutrition was given as boluses of 15 ml/kg every 3 h until euthanasia and tissue collection on day 5.

**Clinical observation and in vivo tests.** Pigs were weighed daily and closely monitored throughout the study. Once clear NEC symptoms appeared (distended abdomen, bloody diarrhea, lethargy, respiratory distress), the pigs were immediately anesthetized and subsequently euthanized with an intracardiac overdose of pentobarbital (9). Anesthesia was performed by intramuscular injection of a combination of anesthetic and analgesic compounds including zolazepam + tiletamine (Zoletil 50; Virbac, Kolding, Denmark), xylazine (20 mg/ml, Norcaryl; MSD Animal Health, Ballerup, Denmark), ketamine (100 mg/ml, Ketaminol; MSD Animal Health), and butorphanol (10 mg/ml, Torbugesic; ScanVet). A fecal score was given twice daily by assessing fecal consistency and volume as previously described (35). On day 4, an in vivo intestinal galactose absorption test was performed by giving an oral bolus (15 ml/kg) of 5% galactose in 0.9% NaCl, followed by blood sampling 20 min after administration (20). Obtained plasma after centrifugation of blood samples was stored at –20°C until analysis. To assess intestinal permeability, pigs received an oral bolus of 5% lactulose and 5% mannitol (15 ml/kg) exactly 3 h before euthanasia (20). Urine was collected by cystocentesis at euthanasia and stored at –20°C until analysis. Concentrations of lactulose and mannitol in urine and galactose in plasma were analyzed as previously described (35).

**Blood sampling and analysis, tissue collection, and NEC evaluation.** On day 5, blood sampling was performed by cardiac puncture in anesthetized pigs just before euthanasia for plasma biochemistry using the Advia 1800 Chemistry systems (Siemens, Erlangen, Germany). Tissues were then collected as previously described (9, 35). Five regions of the GI tract (stomach, proximal, middle and distal small intestine, and colon) were evaluated for macroscopic pathological NEC lesions (20). Each of the five regions was given an NEC severity score ranging from 1 (no lesions) to 6 (severe and extensive pathological changes including pneumatosis intestinalis, hemorrhage, and necrosis). Pigs were designated as NEC positive when a minimum disease score of 3 in at least one region was noted. Organs were weighed, and the content of each intestinal region was carefully emptied and stored at –80°C for microbiology and organic acid analysis. Intestinal tissues of each region were snap frozen and stored at –80°C to evaluate enzymatic activities and levels of inflam-
matory mediators. Two pieces of each intestinal region were fixed in 4% formalin for later histological analysis.

**Intestinal morphology, enzymatic activity, inflammatory cytokines, and organic acids.** Formalin-fixed tissue samples from the proximal, middle and distal small intestine, and colon were routinely processed and embedded in paraffin. Samples were cut into 2–4-μm cross sections and stained with hematoxylin and eosin. Villus height and crypt depth were measured in representative cross sections of each small intestinal region, as previously described (20). Goblet cell density in the colon was quantified following staining with Alcian blue (at pH 2.5) and periodic acid-Shiff. The goblet cell density was calculated using a stereological approach as the area of goblet cells relative to the total area of the tunica mucosa.

Tissues of the proximal, middle, and distal intestine were homogenized for measurement of brush-border enzyme activities (lactase, maltase, sucrase, aminopeptidase N, aminopeptidase A, and dipetidyl peptidase IV), as previously described (26). Samples of luminal content from the colon were used to measure organic acid concentration by gas chromatography according to a previous protocol (6). IL-8 levels in the distal small intestine were analyzed by ELISA (R&D Systems, Abingdon, Oxfordshire, United Kingdom) following the manufacturer’s instructions (26) and expressed as picograms per milligram of wet tissue.

**Microbial analysis of gut contents.** Total DNA of the colon and distal small intestinal content was extracted, and the total colonic bacterial load was quantified by qPCR by a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using *Escherichia coli* K12 as positive control and standard as previously described (16). Distal intestinal and colonic microbiota composition was determined by tag-encoded 16S rRNA gene MiSeq-based (Illumina, San Diego, CA) high-throughput amplicon sequencing (7, 36). Sequencing data were analyzed using the QIIME pipeline (Quantitative Insights Into Microbial Ecology), and analysis of similarity (ANOSIM) was applied to evaluate differences in bacterial distribution among the groups as previously reported (7).

**Bacterial analysis in the intestinal mucosa by fluorescence in situ hybridization.** Bacterial abundance adhering to the intestinal mucosa was analyzed by fluorescence in situ hybridization (FISH) using fixed and paraffin-embedded cross sections (3 μm). For hybridization, a general probe targeting bacterial 16S rRNA (Alexa Fluor 555-labeled oligonucleotide probes; Eurofins MW Operon, Ebensburg, Germany; sequence: 5′-GCTGCCCTCCGTAGGAT-3′) was used and visualized by a fluorescence microscope (30). The fluorescence signal in the tissues was scored from 0–6, by a blinded observer using grades of bacterial density (0, no bacteria; 1, very few bacteria; 2, few spread bacteria; 3, bacteria spread in the whole section; 4, small bacterial colonies spread in the whole section; 5, large colonies; 6, widespread overgrowth with large colonies).

**Antibiotic-associated microbial resistance in gut content.** MacConkey and Slanetz-Bartley agar media are selective for Enterobacteriaceae and *Enterococcus* spp., respectively, and were used in this study to quantify total number and resistant fractions of indicator bacteria in the intestine. Approximately 1 g of frozen distal intestinal and caecum content was thawed and used to prepare 10-fold dilutions in physiological saline. Twenty microliters of each dilution was transferred in duplicate to six different agar plates: three MacConkey agar plates (Oxoid, Basingstoke, Hampshire, United Kingdom) including one without antibiotics and two supplemented with either 32 mg/l ampicillin or 4 mg/l gentamicin; three Slanetz-Bartley agar plates (Oxoid), including one without antibiotics and two supplemented with either 16 mg/l ampicillin or 256 mg/l gentamicin. Antimicrobial concentrations were selected based on MIC wild-type distribution (www.EUCAST.org) to select resistant bacteria. Purple (MacConkey) and pink-purple (Slanetz-Bartley) colonies were counted following the recommendations by the Nordic Committee on Food Analysis (33) after 24 h of incubation at 37°C (MacConkey) or 42°C (Slanetz-Bartley) to determine mean bacterial densities (CFU/g). From each sample, one or more colonies representing both MacConkey and Slanetz-Bartley agar were identified to the genus level by MALDI-TOF mass spectrometry (VITEK MS; BioMerieux, Craponne, France).

**Statistical analysis.** NEC incidence among the three groups was analyzed by Fisher’s exact test (JMP, SAS Institute, Cary, NC). The NEC severity score and FISH score were compared using the Kruskal Wallis test (JMP). The remaining continuous data were analyzed by linear mixed model (JMP) with treatment, litter, and sex as the fixed variables. Tukey’s post hoc test was performed to compare each pair of treatments. Values are presented as means ± SE. Means with *P* values <0.05 were regarded as statistically significant.

**RESULTS**

**Clinical observations, NEC lesions, growth, and plasma biochemistry.** A total of 49 pigs of the initial 64 pigs were tissue sampled, whereas 15 pigs were euthanized and excluded from the study during the first 2 days because of severe respiratory distress or catheter-related problems. Diarrhea occurred in all three groups within 24 h after the transition to full enteral feeding on day 3. Although there were no differences in fecal score among the groups, bloody diarrhea was only observed in CON pigs. Five CON pigs and one PAR pig were euthanized and sampled before the end of the study due to clinical signs of NEC.

At tissue collection, 63% of CON pigs were diagnosed with NEC (10/16), which was similar to the group with parental administration of antibiotics (59%, 10/17). In contrast, enteral administration of antibiotics (ENT) completely prevented NEC lesions (0%, 0/16) (ENT vs. CON and PAR, both *P* < 0.001, Fig. 1A). Accordingly, average NEC severity score of the whole gastrointestinal tract was lower in ENT pigs vs. CON and PAR pigs (*P* < 0.01, Fig. 1B). NEC lesions in this study were mainly observed in the colon (Fig. 1C), whereas lesions in the small intestine were limited (Fig. 1D).

There were no differences among groups in birth weight and weight at necropsy, but PAR showed higher BW gain than CON (*P* < 0.05, Table 1). ENT pigs had lower relative stomach weight and higher weights of the middle and distal small intestine, relative to CON pigs (all *P* < 0.05). Finally, CON pigs had a tendency to have lower kidney weight (*P* = 0.06) and higher liver weight (*P* = 0.08) than the other two groups. Blood biochemistry values were similar among groups (Table 2), except for elevated total plasma protein in CON vs. PAR pigs (*P* < 0.05) and a tendency to have lowered albumin and creatinine levels in ENT pigs, compared with the other groups (*P* = 0.07–0.08).

**Intestinal nutrient absorption, permeability, morphology, and digestive enzymes.** Galactose absorption and intestinal permeability (shown as the urinary ratio of lactulose/manitol) were not different among the three groups (Fig. 2, A and B). However, the ENT pigs had a lower permeability value than the pooled mean value from the CON and PAR groups (0.027 ± 0.022 vs. 0.094 ± 0.016, *P* < 0.05, Fig. 2B).

In the distal small intestine, PAR pigs had higher villus heights than CON pigs, and both ENT and PAR pigs had a greater villus height/crypt depth ratio than CON pigs (*P* < 0.05, Fig. 2, C–E). In the middle small intestine, ENT pigs also showed a strong trend toward greater villus height/crypt depth ratio than CON pigs (*P* = 0.06, Fig. 2D). The NEC lesions in CON and PAR pigs were mainly observed in the colon (Fig. 1C), but severe lesions were also observed especially in the
distal part of the small intestine, as shown by villi undergoing necrosis, strong hyperemia, and vascular stasis, in contrast to the healthy mucosa in ENT pigs (Fig. 2E).

There were few differences in brush-border enzyme activities among the groups across all three intestinal regions, although maltase activity was generally higher in ENT vs. CON pigs ($P < 0.05$ as analyzed across the 3 regions, Fig. 3). Specifically in the middle small intestine, maltase activity also tended to be higher in ENT vs. PAR pigs ($P = 0.07$).

### Table 1. Body and relative organ weights for piglets

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of pigs</th>
<th>CON</th>
<th>PAR</th>
<th>ENT</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight, g</td>
<td>$885 \pm 52$</td>
<td>$938 \pm 52$</td>
<td>$906 \pm 52$</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Weight at necropsy, g</td>
<td>$919 \pm 60$</td>
<td>$1075 \pm 60$</td>
<td>$997 \pm 59$</td>
<td>0.21</td>
<td></td>
</tr>
</tbody>
</table>

Values (means $\pm$ SE) not sharing the same letters are significantly different.

**Inflammatory cytokines, colonic mucin-containing goblet cells, and organic acid.** IL-8 levels in the distal small intestine were markedly reduced in ENT vs. CON pigs ($P < 0.05$, Fig. 4A) with intermediate values in PAR pigs. In the colon, the density of mucin-containing goblet cells in the epithelium was higher in ENT than in CON pigs ($P < 0.01$, Fig. 4, B and C) with intermediate values in PAR pigs. Figure 4C illustrates representative colonic sections stained with Alcian blue and periodic acid-Shiff showing pink mucin-containing goblet cells.

### Table 2. Plasma biochemistry at euthanasia

<table>
<thead>
<tr>
<th>Group</th>
<th>Albumin, g/l</th>
<th>Total protein, g/l</th>
<th>Alkaline phosphatase, U/l</th>
<th>Alanine transaminase, U/l</th>
<th>Total protein, g/l</th>
<th>Bilirubin, mmol/l</th>
<th>Creatinine, mmol/l</th>
<th>Iron, mmol/l</th>
<th>Ionized phosphate, mmol/l</th>
<th>Aspartate aminotransferase, U/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>$12.5 \pm 0.4$</td>
<td>$29.5 \pm 0.6^{a}$</td>
<td>$19.8 \pm 1.4^{a}$</td>
<td>$0.5 \pm 0.4$</td>
<td>$55.3 \pm 2.8$</td>
<td>$2.4 \pm 0.1$</td>
<td>$53.5 \pm 2.8$</td>
<td>$6.0 \pm 0.8$</td>
<td>$1.2 \pm 0.2$</td>
<td>$43.7 \pm 29.2$</td>
</tr>
<tr>
<td>PAR</td>
<td>$11.6 \pm 0.3$</td>
<td>$27.5 \pm 0.6^{a}$</td>
<td>$20.4 \pm 1.4$</td>
<td>$0.5 \pm 0.6$</td>
<td>$56.8 \pm 2.7$</td>
<td>$2.4 \pm 0.1$</td>
<td>$56.8 \pm 2.7$</td>
<td>$5.8 \pm 0.8$</td>
<td>$1.4 \pm 0.1$</td>
<td>$108.7 \pm 1.0$</td>
</tr>
<tr>
<td>ENT</td>
<td>$11.6 \pm 0.2$</td>
<td>$27.6 \pm 0.6^{a}$</td>
<td>$17.9 \pm 1.4$</td>
<td>$0.5 \pm 0.5$</td>
<td>$48.2 \pm 2.6$</td>
<td>$2.6 \pm 0.1$</td>
<td>$48.2 \pm 2.6$</td>
<td>$7.4 \pm 0.8$</td>
<td>$1.2 \pm 0.5$</td>
<td>$108.0 \pm 2.8$</td>
</tr>
</tbody>
</table>

Values (means $\pm$ SE) not sharing the same letters are significantly different.
Both ENT and PAR pigs showed much lower concentrations of colonic organic acids than CON pigs for total organic acids \( (P < 0.0001) \), lactate \( (P < 0.0001) \), and butyrate \( (P < 0.01, \text{Fig. 4B}) \). ENT pigs also had lower levels of acetate than CON pigs \( (P < 0.05) \).

**Gut microbiota.** Total bacterial load in the colon, shown by \( \log_{10} \) of 16S rRNA copies, was similar between CON and PAR pigs but was reduced 100-fold in ENT pigs \( (P < 0.01, \text{Fig. 5A}) \), as also reported previously \( (32) \). Sequencing data revealed that the relative abundance of predominant genera in each treatment group was similar between distal intestinal and colon content \( (\text{Fig. 5B}) \), as determined by weighted UniFrac distance analysis \( (\text{ANOSIM}, r = -0.03, P = 0.86) \). In CON pigs, the microbiota was dominated by 80–90% of Gram-positive bacteria, mainly *Enterococcus* spp. (purple color) and *Clostridium* spp. (green color). In contrast, Enterobacteriaceae (Gram-negative, red color) were dominating (relative abundance 80–90%) in ENT and PAR pigs. ANOSIM analysis showed a significant difference in gut bacterial distribution between CON vs. ENT pigs \( (r = 0.64, P = 0.003) \), as well as between CON vs. PAR pigs \( (r = 0.71, P = 0.001) \). FISH scores across the three regions of the small intestine were consistently lower in ENT vs. CON pigs and also lower.
for PAR pigs, relative to CON, in the distal region ($P < 0.05$, Fig. 5C). A representative photomicrograph of a FISH analysis with a general bacterial probe in red fluorescence in the distal small intestine is shown in Fig. 5D.

**Antimicrobial resistance.** All the tested colonies from Slanetz-Bartley media were identified as *Enterococcus faecium*, whereas colonies from MacConkey media were either *Klebsiella oxytoca* or *Enterobacter cloacae* (coliform species within the Enterobacteriaceae family). Within each of the three groups, total and resistant bacterial counts in the content samples were similar between the distal intestine and cecum (Fig. 6). Based on Slanetz-Bartley medium without added antibiotics, total *Enterococcus* load differed among all three pig groups with the highest counts shown in CON pigs, followed by PAR pigs, and negligible counts in ENT pigs (detection limit of 500 CFU/g, dashed line, Fig. 6, A and B, $P < 0.05$). This indicates that prophylactic antibiotics efficiently eliminated Gram-positive bacteria like *Enterococcus*, consistent with the sequencing data. Ampicillin- and gentamicin-resistant *Enterococcus* were either absent or present in low numbers close to the detection limit of 500 CFU/g, indicating that there was no antibiotics-resistant *Enterococcus* in the gut in any of the three pig groups. Total and resistant coliform load was lower in ENT vs. CON and PAR pigs ($P < 0.05$, Fig. 6, C and D), verifying the effect of enteral antibiotics on coliform gut bacteria. Counts of ampicillin- and gentamicin-resistant coliforms were similar to total coliform counts irrespective of treatment group. This indicates that there was no increase in antibiotics-resistant bacteria in the gut following administration of enteral or parenteral antibiotics to preterm pigs within the time span of the experiment.

**DISCUSSION**

Excessive gut bacterial colonization is an important predisposing factor for NEC in preterm infants, and high loads are associated with NEC development in animal models (37). A few older studies suggest that enteral antibiotics protect infants against NEC (5), but standard prophylactic use of either enteral or parenteral antibiotics is not recommended because of concerns for promoting antibiotic resistance and an unnecessary treatment of infants with a low risk of NEC. Furthermore, the NEC risk and intestinal dysbiosis may actually increase after prolonged use of antibiotics (1, 11). We speculate that enteral antibiotics used only during the first few days after preterm birth may be beneficial to delay gut bacterial colonization and allow the immature intestine to adapt structurally, functionally, and immunologically to the transition into postnatal life. As suggested by our parallel studies into systemic immunity (32), a short delay of gut bacterial colonization may also improve maturation of systemic immunity and the resistance toward late-onset sepsis. In this manner, early short-term, prophylactic antibiotics may avoid extended use of antibiotics later and thus reduce overall use of antibiotics. However, it is important to note that the intestine of preterm, formula-fed pigs may be even more immature and sensitive to bacterial colonization shortly after birth than the intestine of preterm infants receiving mother’s own milk or mixed diets (38). The translational
relevance of the results may therefore be highest for extremely preterm infants fed formula.

Using an identical rearing protocol, we previously reported that a combined administration of both enteral and parenteral antibiotics completely prevented NEC development within the first week after preterm birth, and the pigs did not have any symptoms of infections or NEC until day 10, after cessation of antibiotics treatment on day 5 (20). The present results suggest that enteral, but not parenteral, antibiotics reduced gut bacterial colonization and protected preterm pigs against NEC. We also demonstrate that the reduced gut bacterial colonization in ENT pigs reduced the levels of colonic organic acids (especially lactate), which have repeatedly been shown to be elevated in association with NEC, reflecting excessive nutrient fermentation (20, 26, 38). ENT pigs also had higher intestinal weight, digestive capacity, and villus height/crypt depth ratio and lower bacterial adherence, all indicative of enhanced intestinal maturation, relative to CON pigs (26). With this information combined, we suggest that reducing bacterial colonization by using enteral antibiotics within the first few days after preterm birth may protect and support the intestine to mature because there is less interference from colonizing microbes and their fermentation products. Future studies are required to determine whether prophylactic use of enteral antibiotics immediately

Fig. 4. Inflammatory cytokine, mucin-containing goblet cells, and organic acids. A: IL-8 in the distal small intestine. B: goblet cell density in colon. C: representative Alcian blue- and periodic acid-Shiff-stained colonic sections of CON, PAR, and ENT pigs with pink mucin-containing goblet cells. D: organic acids in the colon content; \( n = 16–17 \)/group. Values (means ± SE) not sharing the same letters are significantly different (\( P < 0.05 \)).
after preterm birth can lower the overall infection risk (including late-onset sepsis) and total use of antibiotics during the entire hospitalization period.

Enterococcus and Clostridium spp. have been shown to be associated with NEC development in preterm pigs (4, 37), and the reduced abundance of these species and their fermentation products in ENT pigs is consistent with our previous study on simultaneous enteral and parenteral antibiotic administration (20). The apparently higher susceptibility of Enterococcus to antibiotics, relative to Enterobacteriaceae, was confirmed by 16S rRNA gene high-throughput amplicon sequencing. This explains that the intestine of ENT pigs had few Enterococcus spp. and was colonized mainly by Enterobacteriaceae. The data also suggest that Gram-positive bacteria, in particular Enterococcus spp., may be the main bacteria group that affects the immature gut at this time and potentially leads to bacteremia via bacterial translocation across the gut barrier to the blood stream, as indicated in our study on systemic immunity (32).

The cultures of frozen intestinal samples gave a preliminary indication of the risk for development of bacterial resistance genes following antibiotic treatment. For both coliforms and enterococci, there was no apparent selection of resistance to ampicillin and gentamicin, irrespective of the antibiotic administration route. More studies are required to confirm this, but the results indicate that short-term prophylactic enteral antibiotics for newborns may not necessarily increase antibiotic resistance, at least not within the first week. It is difficult to predict the effect on later resistance, as this may be influenced by many factors, including the relative fitness of resistant vs. susceptible strains, and by the efficiency of horizontal transfer of resistance genes (21).

It is interesting that PAR pigs did not have reduced total bacterial load in the gut despite that the microbiota composition was clearly different from that in CON pigs. In preterm neonates, the transfer of specific antibiotic compounds to and from the gut and their luminal and systemic metabolism are not necessarily the same as in adults. In our study, CON pigs were dominated by Gram-positive bacteria (80% Enterococcus and Clostridium), whereas PAR pigs were mainly colonized with Gram-negative bacteria (80% Enterobacteriaceae). Microbiology data in ENT pigs confirmed that the used broad-spectrum antibiotics efficiently eliminated Gram-positive bacteria. It is possible that a part of the parenteral antibiotics (especially metronidazole) was metabolized by liver and then excreted into the bile, which may have depressed the Gram-positive bacteria in the gut lumen of PAR pigs (13). Likewise, the increased proportion of Gram-negative bacteria in the gut of PAR pigs, relative to CON pigs, may thus be a result of an altered interaction between Gram-positive and -negative bacteria after a major proportion of Gram-positive bacteria was depressed by the luminal effects of the parenteral antibiotics. Despite the wide difference in Gram-positive vs. Gram-negative bacteria in PAR and CON pigs, these groups had similar NEC incidence, suggesting that bacterial density is more important for early...
NEC development than Gram-negative vs. Gram-positive bacterial composition. The results contrast with those in young rodents in which induced NEC lesions are related to an excessive inflammation by pathways activated by Gram-negative bacteria (14, 15).

In addition to the suppression of bacterial colonization and enhancement of intestinal function and maturation, ENT pigs also showed lower inflammatory cytokine IL-8 concentration in the distal small intestine, lower stomach and liver weight, and lower total plasma protein, relative to the other two groups. Collectively, these parameters indicate reduced inflammation in ENT pigs. NEC pathogenesis is associated with damaged intestinal barrier (2), and we found that ENT pigs had lower urinary lactulose/mannitol ratio, an indicator of intestinal permeability, than the other two groups. This was consistent with findings in the previous antibiotic study with simultaneous enteral and parenteral antibiotic administration (20). Increased intestinal permeability may be derived from either the gut immaturity or damaged intestinal barrier. In other words, low intestinal permeability in ENT pigs may be due to greater intestinal maturation to facilitate gut closure and less damaged gut tissues. Increased stomach and liver weights in affected preterm pigs are commonly associated with NEC lesions, edema, and elevated inflammatory responses (26). Consistent with this, CON pigs also had poor weight gain and high total plasma protein content, possibly reflecting a higher degree of dehydration, compared with ENT pigs. Overall, the intestines in CON pigs were affected in a similar manner as the control pigs in the earlier study (20), as evidenced by similar villus heights and levels of brush-border enzymes, organic acids, and IL-8. However, in contrast to this earlier study, enteral antibiotics did not exert marked effects on intestinal villus heights, permeability, or brush-border enzymes. It may be important to compare enteral antibiotics with the combined parenteral and enteral administration of antibiotics in a future study to conclude whether the combined treatment is more effective than enteral antibiotics alone.

The ENT pigs showed a marginal increase in intestinal maltase activity, indicating slightly enhanced carbohydrate digestive capacity. Maldigestion of maltodextrin has been shown to cause NEC in preterm pigs (44), probably attributable to the stimulation of bacterial overgrowth and fermentation activity triggering inflammation and tissue damage in the lower parts of the gut where microbial density is high. Consistent with this, enteral administration of antibiotics also dampened IL-8 levels in the distal small intestine, similar to the effects also seen when preterm pigs are fed more protective diets, such as bovine colostrum or donor human milk (19, 26). It is possible that the low gut bacterial load induced by enteral antibiotics acts in a manner similar to that of protective milk diets to reduce the exposure of gut epithelium and immune cells to microbial antigens, thereby limiting the inflammatory response, cytokine production, and tissue damage.
The density of mucin-containing goblet cells may reflect the ability of the intestine to form a mucus layer to protect the intestine from bacterial invasion and translocation, and both commensal and pathogenic bacteria can accelerate colonic mucin secretion (12). Germ-free conditions reduce the number of colonic goblet cells and thickness of the mucus layer in rodents (12). In contrast, we showed a much higher colonic goblet cell density in ENT pigs despite that they were colonized with 100-fold fewer bacteria than CON pigs. This may suggest low mucin synthesis or excessive mucin release into the gut lumen stimulated by a high bacterial load in CON pigs. If the protective mucus layer is not maintained, the epithelial layer is exposed to luminal microbes, leading to higher intestinal permeability and increased risk of bacterial translocation.

The concentrations of organic acids in the colon reflect the degree of bacterial fermentation utilizing carbohydrates and proteins, which are not absorbed in the small intestine (17, 45). In this study, levels of organic acids followed the trend of bacterial load in the colon. ENT pigs had low values but appeared to have similar hexaspis-absorbptive and protein-digestive capacities, relative to the other two groups. In all groups, the relatively low intestinal maltase and sucrase activities, compared with lactase activity, which are common in preterm pigs (10, 19, 25, 34, 41, 44), may partly explain that preterm pigs easily have diarrhea when fed with formulas containing high amounts of maltodextrin. Appropriate selection of formula ingredients to help modulate the gut microbiota, potentially in combination with probiotics, may be important to control indigestible nutrients and avoid excessive nutrient fermentation after cessation of antibiotics. High amounts of organic acids in the colon are, not only an indicator of intense fermentation of unabsorbed nutrients and promotion of bacterial overgrowth, but are also able to induce colitis and mucosal injury, as shown in rats (24, 29).

In conclusion, our study provides new knowledge in that enteral antibiotics applied in the immediate postnatal period are superior to parenteral antibiotics in preventing NEC development in formula-fed newborn preterm pigs. In addition, evidence from gut tissue and microbiological analyses suggests that early usage of enteral antibiotics may be beneficial in terms of stimulation of intestinal maturation and digestive capacity and delayed gut colonization, thereby lowering the risk of bacterial translocation and tissue damage, at least in the short term and in the absence of the gut-protective effects of mother’s milk.

Just after preterm birth, the immature intestine may be relatively intolerant to fast bacterial colonization and a high bacterial load (8). Preterm newborns may, therefore, benefit from a delayed bacterial colonization in the first few days after birth, allowing better maturation of the intestine and gut immune system. It is possible that such effects are most pronounced with formula feeding in the absence of mother’s milk. Future studies are required to determine whether neonatal enteral antibiotics provide preterm neonates with a better resistance to infections, such as late-onset sepsis, and whether that in turn reduces antibiotics use later. It is also important to study whether and how the effects of systemic and enteral antibiotics may interact with the simultaneous or later administration of probiotics, or with the exposure to fetal inflammation and bacterial invasion of the amniotic cavity after maternal chorioamnionitis. Collectively, our results may justify a preliminary clinical trial in preterm infants to investigate the effects of early enteral antibiotics on intestinal maturation, immunity, and NEC, combined with careful consideration of antibiotics-resistant bacteria, later gut bacterial dysbiosis, and total use of antibiotics.

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Disclosures

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Author Contributions


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