Elective cesarean delivery affects gut maturation and delays microbial colonization but does not increase necrotizing enterocolitis in preterm pigs

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Siggers RH, Thymann T, Jensen BB, Mølbak L, Heegaard PM, Schmidt M, Buddington RK, Sangild PT. Elective cesarean delivery affects gut maturation and delays microbial colonization but does not increase necrotizing enterocolitis in preterm pigs. Am J Physiol Regul Integr Comp Physiol 294: R929–R938, 2008. First published December 26, 2007; doi:10.1152/ajpregu.00705.2007.—Although preterm birth and formula feeding increase the risk of necrotizing enterocolitis (NEC), the incidence of cesarean section (CS) and vaginal delivery (VD) are unknown. Therefore, gut characteristics and NEC incidence and severity were evaluated in preterm pigs (92% gestation) delivered by CS or VD. An initial study showed that newborn CS pigs (n = 6) had decreased gastric acid secretion, absorption of intact proteins, activity of brush-border enzymes and pancreatic hydrolases, plasma cortisol, rectal temperature, and changes in blood chemistry, indicating impaired respiratory function, compared with VD littersmates (n = 6). In a second experiment, preterm CS (n = 16) and VD (n = 16) pigs were given total parenteral nutrition (36 h) then fed porcine colostrum (VD-COL, n = 6; CS-COL, n = 6) or infant milk formula (VD-FORM, n = 10; CS-FORM, n = 10) for 2 days. Across delivery, FORM pigs showed significantly higher NEC incidence, tissue proinflammatory cytokines (IL-6, IL-10), Clostridium colonization, and impaired intestinal function, compared with COL pigs. NEC incidence was equal for CS (6/16) and VD (6/16) pigs, CS pigs had decreased bacterial diversity and density, higher villus heights, and increased brush-border enzyme activities (lactase, aminopeptidases) compared with VD pigs. In particular, VD-FORM pigs showed reduced mucosal proportions, reduced lactase and aminopeptidases, and increased proinflammatory cytokine IL-6 compared with CS-FORM (P < 0.06). Despite the initial improvement of intestinal and metabolic functions following VD, gut function, and inflammation were similar, or more negatively affected in VD neonates than CS neonates. Both delivery modes exhibited positive and negative influences on the preterm gut, which may explain the similar NEC incidence.

vaginal; cesarean section; bacteria; birth; intestine

PREGNANCY DELIVERY AND THE ASSOCIATED immaturity of organ systems complicate postnatal care of neonates and increase the risk of morbidity and mortality. Infants delivered preterm commonly suffer from postnatal hypoxia, hyperthermia, hemodynamic instability, and gut dysfunction (2, 42). These complications, coupled with poor enteral feeding responses, are associated with an increased risk of developing the severe inflammatory gut disease, necrotizing enterocolitis (NEC). In both infants and preterm pigs, mucosal inflammatory reactions may be present along the entire gut, although they are most commonly located in the distal small intestine (SI) and colon (22, 42). Despite precautions and the initial administration of total parenteral nutrition (TPN), NEC remains the most common gut disease affecting preterm neonates (9).

The ability to induce NEC in several animal models by exposing term neonates to hypoxia and cold stress (1, 13) has fostered speculation that deficiencies in thermoregulation and respiratory capabilities, which are common for preterm infants (3), increase the risk of NEC. If so, the thermoregulatory and respiratory problems that are more common among infants delivered by cesarean section (CS), compared with those born vaginally (VD) (3, 39), may be a determinant of NEC risk. Another consideration is that preterm delivery, and particularly by CS, interrupts the normal glucocorticoid-dependent prenatal development of the gut and other organs (39, 43), thereby compromising the adaptation of neonates to ex utero conditions. Correspondingly, antenatal administration of glucocorticoids is used clinically to improve the functions of the lungs, gut, and other organs of compromised preterm neonates (35, 43). Additionally, the fetal and preterm intestine exhibits a hyper-responsiveness to LPS from gram-negative bacteria (7, 34), and this may lead to an increased risk of gut inflammation. Interestingly, VD reduced the responsiveness of newborn mice to LPS, an effect that was not seen in CS mice (30).

Although the causes of NEC remain elusive, there is agreement that bacterial colonization is essential. The different assemblages of gut bacteria resulting from CS and VD (17) have been related to differences in intestinal functions and health (26) and incidence of NEC (18). However, the influence of birth mode on the mucosa-associated bacteria is not always evident, particularly when fecal samples are used as a surrogate for the entire gut microbiota (31). Interpretation of the microbiological results available from the different studies is confounded further by inconsistencies in gestational age at birth, differences in postnatal diets, environmental conditions, and antibiotic administration. Moreover, the postnatal source of nutrition (breast milk, formula, or TPN) has a pronounced influence on the gut bacteria of infants and has been related to the risk of NEC (31). Consequently, the relationships among birth mode, postnatal diet, and the gut bacteria assemblages remain uncertain, complicating attempts to identify the causes and risk factors for NEC.

The above studies, in conjunction with other findings (18, 28), suggest that caesarean delivery may be a risk factor of

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NEC. Yet, the lack of controlled studies has left this clinically important question unanswered. Therefore, the present study compared gut characteristics of newborn preterm pigs obtained by CS or induced VD from the same sow to minimize variation caused by genetic and gestational differences. A subsequent experiment evaluated the combined influences of delivery mode and diet on gut structure, function, and the resident bacteria, and correlated these findings with the incidence and severity of NEC. This was accomplished by maintaining preterm pigs delivered by CS or VD on TPN for 36 h prior to 48 h of full enteral nutrition using either an optimal diet (porcine colostrum) or a suboptimal diet (infant formula). The use of the preterm pig model for this study is particularly useful, not only because of the anatomical and physiological relevance for the human infant, but also because confounding variables common in human studies (diet, environment, and antibiotics) are avoided.

MATERIALS AND METHODS

Experiment 1: The influence of delivery mode on gut structure and functions at birth. Following a standard protocol (39), a pregnant sow (Large White × Landrace) at 106 days of gestation (term = 115 days) was sedated with azaperone (0.05 ml/kg im; Janssen, Beerse, Belgium). Anesthesia was induced and maintained using thiopental so- (5–10 ml/kg iv; Abbott, North Chicago, IL). A midline incision was made and all pigs in the left uterine horn were removed following ligation and transection of the umbilical cord. These pigs were considered representative of elective CS (n = 6). After closure of the uterine and body walls, parturition was induced by administering 200 µg im of cloprostenol (a PGF₂α analog; Estrumate, Pitman-Moore, Harefield, UK). The remaining pigs in the litter were delivered vaginally 30–36 h postinduction (n = 6).

A blood sample was collected from the umbilical cord artery of each CS and VD pig within 2 min of delivery. Blood pH, P CO₂, P O₂, hemoglobin, and concentrations of Na⁺, Cl⁻, K⁺, Ca²⁺, and glucose were measured using a hemoximeter (Radiometer, Copenhagen, Denmark) and a blood gas and electrolyte analyzer (NOVA Biomedical, Waltham, MA). Plasma cortisol concentrations were determined by ELISA (Biomar Diagnostics, Marburg, Germany). The pigs were then euthanized (pentobarbital sodium; 200 ml/kg iv), and the gut was harvested for analysis within 1–3 h of delivery (see below). All procedures involving the use of animals were approved by the Danish National Committee on Animal Experimentation.

Experiment 2: Effects of delivery mode and diet on gut characteristics and NEC lesions. The second experiment used 32 preterm pigs obtained as described above from four pregnant sows (Large White × Danish Landrace) via CS (n = 16, 106 days ± 2 days) or induced VD (n = 16, 107 ± 2 days). There were no complications associated with delivery of the pigs, but all VD pigs from one of the sows were dead at birth. To compensate for this loss in VD pigs, parturition was induced without prior CS for a separate pregnant sow, and a further eight VD pigs were obtained. Comparison of these VD pigs with VD pigs delivered following prior CS showed no differences for parameters measured in this study, thus all VD pigs were pooled. Immediately after delivery, the CS and VD pigs were placed in individual conventional infant incubators (Air-Shields, Hatboro, PA). Incubator temperature was maintained at 37°C for the first day after birth and was then lowered 1°C every 24 h. Supplemental oxygen was provided for the first 24 h (2–3 l/min). Within 1 h of delivery, each neonatal pig was fitted with an orogastric tube (infant feeding tube 6F, Portex, Kent, UK), and a vascular catheter was inserted through the umbilical artery (6F; Portex). Rectal temperatures were recorded every 30 min for the first 4 h, and every 2 h thereafter until 10 h postpartum. Porcine serum, previously collected from pregnant sows, was administered to the pigs (4 ml/kg at 8 h, 5 ml/kg at 16 h, 10 ml/kg at 22 h postpartum) to provide passive immunization (40).

Feeding protocols. TPN was initiated within 6 h postpartum and was continuously infused via the orogastric catheter for 36 h. The rate of infusion was 4 ml/h/kg for the first 12 h and was increased to 6 ml/kg⁻¹h⁻¹ for the remainder of the TPN period. Following the TPN period, the CS and VD pigs were fed via the orogastric tube (15 ml/kg⁺¹·3 h⁻¹) formula (FORM, n = 20) or porcine colostrum (COL, n = 12). Tissues were harvested for analysis after 48 h of enteral nutrition or when clinical symptoms of NEC were observed. Parenteral nutrition, formula, and porcine colostrum were prepared and collected as previously described (36).

Blood sampling and analysis. Arterial blood samples (2 ml) were collected at 12 and 36 h of the TPN period and at 60 h postpartum (24 h after initiation of enteral feeding) and placed in heparinized syringes. Blood chemistry analyses (pH, P CO₂, P O₂, oxygen saturation, hemoglobin, sodium, calcium, chloride, glucose, lactate) were performed immediately after collection (Radiometer, Copenhagen, Denmark). The remaining blood was transferred to ice-chilled EDTA collection tubes, centrifuged (5,000 g, 10 min, 4°C), and plasma was collected and stored at −20°C. Plasma cortisol concentrations after 12 and 36 h of TPN and at the time of tissue collection were determined by ELISA (Biomar Diagnostics, Marburg, Germany).

Clinical and pathological evaluation. The pigs were evaluated for clinical symptoms of intestinal disease every 3 h (feeding intolerance, stool consistency and color, abdominal distension, and respiratory distress). Euthanasia and tissue collection were performed after presentation of clinical NEC, but before the animal experienced advanced morbidity. All animals remaining after 48 h of enteral feeding were euthanized for tissue collection. Before tissue collection, pigs were sedated [zolazepam and tiletamine HCl (0.02 ml/kg, Zoletil, Virbac, France)] and then euthanized (pentobarbital sodium, 200 mg/kg, both via the vascular catheter). Immediately, the gut was removed and was evaluated macroscopically for signs of NEC. The extent of damage in the stomach, proximal and distal small intestine (SI), and colon was characterized by the magnitude of inflammation, edema, hemorrhage, necrosis, and or pneumatosis intestinalis, using the following scoring system: 1, no or minimal subacute focal hyperemic gastroenterocolitis; 2, mild acute focal gastroenterocolitis; 3, moderate acute locally extensive gastroenterocolitis; 4, severe acute locally extensive hemorrhagic gastroenterocolitis; 5, severe peracute locally extensive hemorrhagic and necrotic gastroenterocolitis; 6, severe peracute extensive hemorrhagic and necrotic gastroenterocolitis. Pigs with an average NEC score ≥1.5 across four regions of the gut (stomach, proximal and distal SI, colon) or a minimum score of three in any location were considered to have clinical NEC.

Tissue collection. Immediately after the evaluation, the entire gut was placed on ice and following a previous protocol (4) was divided into three regions, stomach, SI, and colon. The SI was further divided into three segments of equal length, representing the proximal, middle, and distal SI. Small intestinal tissues to be used for enzyme analyses and 16S rRNA gene microbial identification were immediately frozen in liquid nitrogen and subsequently stored at −80°C. One segment of distal SI was fixed in 4% paraformaldehyde for 48 h and subsequently transferred to 70% ethanol and stored at 4°C until processed for histology. A second segment of distal SI was used to collect mucosa for microbiologic analysis. Another 10-cm segment from each region was used to measure the proportion represented by mucosa, based on a dry matter basis after drying the mucosa and muscularis layers at 60°C for 72 h. Additional segments from each region were placed in cold (2–4°C) mammalian Ringer solution that had been aerated (95% O₂–5% CO₂) or were frozen immediately in liquid nitrogen and stored at −80°C. These were used for immediate and later measurements of glucose and amino acid absorption by intact tissues and brush-border membrane vesicles (BBMV), respectively. After the intestine had been harvested and processed, the contents of the stomach were collected and frozen at −80°C. The
weights of the heart, spleen, liver, kidneys, adrenal glands, pancreas, lungs, and the empty stomach and SI were recorded.

Gut morphology and enzyme assays. Distal intestinal villus and crypt depth (4) and activities of brush-border peptidases (aminopeptidase A, aminopeptidase N) and disaccharidases (lactase, maltase) are all measured as previously described (43). The activities of pancreatic enzymes (amylase, trypsin), stomach chymosin activity, and stomach acidity (pH) were measured following methods described previously (39, 40).

Measurement of nutrient absorption (Experiment 1). Following a previous protocol (5) everted sleeves were prepared from each region for measuring initial rates of carrier-mediated glucose transport and rates of absorption (carrier-mediated and carrier-independent) for leucine, lysine, and proline, which are substrates for the neutral, basic, and imino amino acid transporters.

A standard protocol was used to prepare and evaluate the purity of BBMV prepared from the frozen tissue and to measure rates of nutrient (glucose, lysine, leucine, proline) accumulation (42, 49). The ability of the SI to absorb the protein macromolecules BSA (A-4903, 5.0 g/l; Sigma, St. Louis, MO) and bovine IgG (G-5009, Sigma; 5.0 g/l) was measured in vitro, as previously described (41).

Proinflammatory cytokine concentrations. Porcine IL-6 of distal SI whole tissue homogenate was determined by an R&D DuoSet ELISA (catalog no. DY868; R&D Systems, Abingdon, UK), using goat anti-IL-6 for coating (0.8 mg/ml) and biotinylated goat anti-porcine IL-6 (0.1 mg/ml in 1% BSA (catalog no. A2153; Sigma, St. Louis, MO)). ELISA plates from Nunc (Roskilde, Denmark, type: anti-porcine IL-6 for coating (0.8 mg/ml) were incubated for 20 min at room temperature followed by washing. Plates were blocked with 100 µl/well overnight at room temperature, followed by 3 times washing in PBS containing 0.05% Tween 20. All subsequent washings followed the same protocol. Plates were blocked for 1 h in 1% BSA, 300 µl/well, followed by washing and incubation of samples, diluted twice in 1% BSA. All subsequent incubations were done in 100 µl. A standard preparation of recombinant porcine IL-6 (from the DuoSet kit) was applied in double determination as a twofold dilution row from 20,210 pg/ml in 1% BSA to a minimum concentration of 316 pg/ml. Two wells were used for buffer controls. All samples were incubated for 2 h at room temperature, and then washed and incubated with detection antibody for 2 h. After washing, the plate was incubated with peroxidase-conjugated streptavidin (from the DuoSet kit, diluted 1/200) and incubated for 20 min at room temperature following by washing. Plates were developed with TMB Plus from Kem-En-Tec (Taastrup, Denmark), incubating 10–20 min at room temperature in the dark and then stopped by the addition of 100 µl 0.5 M sulfuric acid to each well. Plates were read in a Thermo Multiskan Ex spectrophotometer (Thermo Scientific, Waltham, MA) at 450 nm subtracting background (540 nm). Sample values for IL-6 were then calculated from the curve fit to the readings of the standard (using Ascent software v. 2.6).

Minimum detection level of IL-6 was 630 pg/ml.

IFN-γ was determined by an in-house sandwich ELISA, similar to previously described (10), using antiporcine IFN-γ from Pierce (catalog no. MP700; Rockford, IL) at 1 µg/ml in 0.1 M carbonate, pH 9.6 for coating and biotinylated anti-porcine IFN-γ from BD Pharmingen (catalog no. MP701B; San Jose, CA) for detection. A standard of pooled porcine plasma was collected from whole blood that had been stimulated with SEB (staphylococcal enterotoxin B), and this standard was calibrated against a recombinant porcine IFN-γ from R&D Systems (Abingdon, UK, catalog no. 985-P1-050). The standard concentration ranged from 4,255 pg/ml to 6 pg/ml. Samples were applied undiluted, 50 µl/well. Readings and calculations were performed as described for IL-6. Minimum detection level of IFN-γ was 8 pg/ml.

Microbiology, short-chain fatty acid (SCFA) concentrations and stomach pH. Culture-based microbiology was performed to enumerate densities of selected bacterial groups associated with stomach contents, fresh mucosa obtained by scraping a segment of the distal SI, and colon contents. The samples were cultured for total anaerobes, lactic acid bacteria, lactobacilli, coliform bacteria, and enterococci (20), as well as for Clostridia (14). Briefly, total anaerobes were cultured on rumen fluid-glucose-cellobiase agar and incubated anaerobically at 38°C for 7 days. Lactic acid bacteria and lactobacilli bacteria were cultured on deMan, Rogosa, Sharp agar and Rogosa agar, respectively, and incubated anaerobically at 38°C for 2 days. Coliform bacteria were cultured aerobically at 38°C for 1 day on McConkey agar, and enterococci bacteria were cultured on Sianetz-Barkley agar aerobically at 37°C for 2 days. Clostridia were enumerated by culturing anaerobically on tryptose sulfite cycloserine agar at 37°C for 1 day. Densities were normalized to grams of contents or mucosa.

We further characterized the bacterial assemblages in the distal SI mucosa using terminal-restriction fragment length polymorphism (T-RFLP). Briefly, 200 mg of distal SI homogenate was added to 2-ml screw-caps tubes containing 350 mg of 100-µm zirconia-silica beads (Biospec Products, Bartlesville, OK) and 600 µl of PBS, and shaken for 4 min at high speed on a minibeat (Biospec Products). Subsequent DNA extraction was performed as previously described (27), with minor modifications. Before DNA purification, the supernatant was treated with proteinase K (Qiagen, Hilden, Germany) at 56°C for 1 h. DNA was then purified using cetyltrimethylammonium bromide (32), and after centrifugation of the supernatant phenol/ chloroform/isooamyloalcohol mixture, the DNA was extracted using a 6% Chexal solution (10%). Bio-Rad, Munich, Germany). The bacterial DNA was stored at −20°C until used for PCR analysis, as previously described (27), with minor modifications. Briefly, the DNA was amplified with the fluorescently labeled 5′ FAM (carboxy-fluorescein-N-hydroxysuccinimide-ester-dimethyl sulfoxide) forward primer S-D-Bact-0008-a-S-20 (5′-AGAGTTTGTATCMTGCTGAG-3′) (27), and the reverse primer S-D-Bact-0266-20 (5′-CGGTT-CAATCTTCATGGTTT-3′) (33) for 30 cycles, digested with 20 U of restriction enzyme CfoI (Boehringer Mannheim, Mannheim, Germany) for 3 h, loaded onto a denaturing polyacrylamide gel for electrophoresis, and analyzed on an automatic sequence analyzer (ABI PRISM 373 DNA sequencer, PE Biosystems, Foster City, CA).

The lengths of terminal-restriction fragments (T-RFs) were analyzed using the BioNumerics software package v. 4.0 (Applied Maths, Austin, TX) with special emphasis on identification of fragments differentially expressed among treatment groups. Sequence data were imported into the program and aligned using the DNA fragment length standard within the samples. For each T-RF within a pig, semiquantitative evaluation of the relative abundance was estimated by calculating the ratio of a specific T-RF intensity relative to the total intensity of all T-RFs.

Estimates of variation in bacterial colonization patterns within and between treatment groups were made based on pairwise comparisons of T-RFs using the Dice similarity coefficients (S2) calculation in the BioNumerics software package as described previously (33). Generally, there is a direct relationship between the S2 and the similarity of two compared pigs, with two identical T-RFLP profiles having a similarity coefficient of 1.00 (100% similarity). Identification of specific bacteria characterized by T-RFs was done in silico by inserting primer sequences and restriction enzymes in the MiCA home page (http://mica.ibest.uidaho.edu/digest.php) using the RDPII database (Release 9, Update 37, Bacterial SSU 16S rRNA).

The concentrations of SCFAs (formic, acetic, propionic, butyric, lactic, benzoic, iso-valeric, valeric, iso-capronic, capronic, heptane, sorbine acids) in stomach contents were measured using gas chromatography, as described previously (24). The pH of the stomach contents was measured using a Corning M240 pH meter (Philip Harris Scientific, Manchester, UK).

Data analysis. Values presented in tables and figures are expressed as means ± SE. The main effects of delivery mode (CS and VD) and diet type (COL and FORM), and possible interactions, were analyzed by PROC GLM program of SAS (SAS Institute, v. 8.2, Cary, NC). When no significant effect of diet or delivery method was detected,
data were pooled and analyzed across delivery modes or diets. In light of variation among fetuses and pigs that originated from different sows, a litter effect was initially included in the model when evaluating the effects of delivery mode and diet. For all tissue parameters, litter effects remained insignificant. When a main treatment effect was detected, the LSD test was used to identify differences between individual means. The univariate procedure (SAS, 1998) was used to detect nutrient accumulation ratios that differed from 1.0. For all comparisons, a probability value of \( P < 0.05 \) was used as the critical level of significance.

RESULTS

Experiment 1: The influence of delivery mode on gut structure and functions at birth. Mean body weight (BW) at birth was identical for CS and VD pigs (1.31 ± 0.09 and 1.30 ± 0.15 kg, respectively), but VD pigs had heavier SI, livers, adrenal glands, and lighter lungs (Table 1). There were no effects of delivery mode on the relative mass of other internal organs (stomach, pancreas, kidneys, spleen, heart). At birth, VD pigs had lower blood pH (7.17 ± 0.02 vs. 7.34 ± 0.03) and higher PCO₂ (66 ± 2 mmHg), ionized potassium (5.27 ± 0.25 vs. 3.95 ± 0.07 mEq/L), and plasma cortisol (193 ± 13 vs. 96 ± 13 nM) (all \( P < 0.05 \)). VD pigs also had lower gastric pH (3.0 ± 0.7 vs. 6.9 ± 0.8; \( P < 0.05 \)), higher activities of trypsin and amylase in pancreatic tissue (Table 1) and higher intestinal sucrase, aminopeptidase N (ApN), and aminopeptidase A (ApA), and aminopeptidase N (ApN) in CS- and VD pigs (5.1 ± 0.09 vs. 11.3 ± 0.06, 0.01). Stomach chymosin levels tended to be higher in VD pigs, relative to CS (\( P = 0.14 \), Table 1), while the intestinal capacity to absorb macromolecules was markedly elevated in VD pigs for both bIgG and BSA (\( P < 0.01 \)).

Rates of glucose transport by intact tissues at 50 mM declined significantly from the proximal to distal SI in both CS and VD pigs (\( P < 0.05 \)). Rates of absorption averaged for the three regions did not differ significantly between the CS and VD pigs for glucose and lysine, but values for CS pigs were lower for leucine and higher for proline (Table 1).

Tracer accumulation ratios for the intact tissues did not differ between CS and VD pigs for glucose (75 ± 8), leucine (17 ± 4), and lysine (14 ± 3), with all exceeding 1.0, indicating a saturable component of absorption was present at 92% of gestation for these three nutrients. The accumulation ratios were directly related to nutrient accumulation by BBMV with the lowest values measured for proline (0.23 ± 0.01 pmol/s⁻¹·mg protein⁻¹), the highest for glucose (23.6 ± 3.5), and intermediate values for leucine (15.3 ± 0.9) and lysine (4.7 ± 0.3). Rates of BBMV uptake did not differ between CS and VD pigs for any of the three nutrients. BBMV proline uptake and the accumulation ratio of CS pigs (0.18 ± 0.01 pmol/s⁻¹·mg protein⁻¹ and 2.9 ± 0.4) was lower compared with VD pigs (0.25 ± 0.02 and 5.8 ± 1.2; \( P < 0.05 \)). This suggests the higher intact tissue uptake for CS pigs was caused by a higher carrier-independent influx of proline.

Experiment 2: effects of delivery mode and diet on gut characteristics and NEC lesions. The incidence of NEC was not affected by mode of delivery and was similar for CS-FORM (50%, 5/10) and VD-FORM (50%, 5/10), and for CS-COL (16%, 1/6) and VD-COL pigs (16%, 1/6). Likewise, the average NEC score did not differ between CS-FORM (2.1 ± 0.3) and VD-FORM pigs (2.5 ± 0.3), or between CS-COL (1.1 ± 0.1) and VD-COL pigs (1.1 ± 0.1; Fig. 1A). Both NEC indices were significantly higher (\( P < 0.05 \)) for FORM relative to COL pigs. The onset of clinical signs of NEC (vomiting, abdominal distension, diarrhea, lethargy, distressed breathing) began within 24–28 h of enteral feeding in 50% of the FORM pigs and resulted in four pigs being euthanized before the full 48-h enteral feeding period. None of the COL pigs had clinical signs of NEC that required euthanasia before the end of the enteral period.

Mean rectal body temperatures at birth for VD pigs (37.3 ± 0.4°C) were higher than for the CS pigs (35.6 ± 0.3°C, \( P < 0.05 \)) and remained significantly higher until 6 h postpartum (Fig. 2). Despite all pigs being placed in heated incubators immediately after birth, body temperatures decreased to minimal values within 30 min postpartum for both CS (34.7 ± 0.3°C) and VD (36.6 ± 0.4°C) pigs. By 1 h postpartum, body temperatures in VD pigs had increased to 37.7 ± 0.4°C and remained at ~37°C for the remainder of the measurement period. In contrast, rectal body temperatures of CS pigs remained below 37°C until at least 8 h postpartum.

Table 1. Organ weights and in vitro activities of intestinal enzymes, pancreatic enzymes, stomach chymosin, and in vitro intestinal uptake rates for nutrients, and two intact proteins in litter-mate preterm pigs born by cesarean section or after induced vaginal birth

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>VD</th>
<th>P Value</th>
<th>Enzymes and Nutrients</th>
<th>CS</th>
<th>VD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>19.6±0.8</td>
<td>22.9±0.2</td>
<td>0.01</td>
<td>Trypsin</td>
<td>0.8±0.06</td>
<td>1.15±0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Lungs</td>
<td>28.7±1.7</td>
<td>20.±1.0</td>
<td>0.01</td>
<td>Amylase</td>
<td>5.1±0.9</td>
<td>11.3±0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>20.8±0.8</td>
<td>24.1±0.7</td>
<td>0.01</td>
<td>Chymosin</td>
<td>2.52±0.29</td>
<td>4.29±1.07</td>
<td>0.14</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.12±0.01</td>
<td>0.14±0.01</td>
<td>0.04</td>
<td>Glucose</td>
<td>3.91±0.33</td>
<td>4.39±0.37</td>
<td>0.35</td>
</tr>
<tr>
<td>Sucrase</td>
<td>0.22±0.02</td>
<td>0.31±0.01</td>
<td>0.01</td>
<td>Leucine</td>
<td>1.51±0.12</td>
<td>1.86±0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Maltaise</td>
<td>1.08±0.05</td>
<td>1.27±0.08</td>
<td>0.09</td>
<td>Lysine</td>
<td>1.55±0.10</td>
<td>1.30±0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Lactase</td>
<td>63.7±6.9</td>
<td>60.5±7.7</td>
<td>0.77</td>
<td>Proline</td>
<td>2.02±0.13</td>
<td>1.51±0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>ApN</td>
<td>5.17±0.42</td>
<td>6.23±0.23</td>
<td>0.04</td>
<td>BSA</td>
<td>40.0±3.4</td>
<td>52.1±3.5</td>
<td>0.01</td>
</tr>
<tr>
<td>ApA</td>
<td>1.31±0.08</td>
<td>1.98±0.22</td>
<td>0.02</td>
<td>bIgG</td>
<td>39.1±4.0</td>
<td>53.4±3.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are given as means ± SE. Organ weights are given in grams per kilogram body weight. Intestinal enzymes [sucrase, maltase, lactate, aminopeptidase A (ApA), and aminopeptidase N (ApN)] are given in units per gram of tissue. Pancreatic enzymes, trypsin and amylase, are given in units per gram. Stomach chymosin is given in micrograms per gram. The nutrients glucose, leucine, lysine, and proline are given in micromoles per gram per minute. The intact proteins BSA and bIgG are given in micrograms per gram. One unit (U) of activity represents one µmol of substrate hydrolyzed per min at 37°C. Values are means or LS means ± SE (n = 6). Intestinal values are analyzed across all three regions (proximal, middle, distal), except intestinal enzyme values which represent averages across the proximal and middle regions. \( P \) values show analysis of variance for effect of treatment across regions.
Blood chemistry values during the TPN period (12 and 36 h postpartum) and after 24 h of enteral feeding (60 h postpartum) are presented in Table 2. Diet type did not alter values during the period of enteral nutrition (P > 0.50 for comparisons of FORM and COL pigs). Therefore, evaluation of delivery mode was made using pooled data for both diet groups. While blood gases and ions were highly responsive to delivery method immediately after birth (experiment 1), few differences remained present during the immediate postnatal period. Despite the low values immediately after birth, CS pigs had consistently higher PCO₂ values (20–30%, P < 0.05) compared with VD pigs, and a trend toward lower O₂sat values (significant at 36 h), indicating reduced respiratory function. At 12 h postpartum, CS pigs also had lower K⁺, Ca²⁺, and Cl⁻ levels compared with VD (P < 0.05). Relative to 12 h, all blood ion levels increased by 36 h in CS pigs, but decreased in VD pigs. Apart from these moderate differences, delivery mode was not associated with any sustained differences in hemodynamics, metabolism (lactate, glucose), or electrolyte balance throughout the experiment. In contrast, plasma cortisol levels were significantly higher in VD relative to CS pigs at 12 h (+38%) and 36 h (+57%) and at time of kill (+43%, all P < 0.05, Table 2). Cortisol levels did not differ for FORM and COL pigs.

**Organ dimensions.** The relative weight of the SI was responsive to delivery mode (Table 1), but the response after TPN and enteral feeding was opposite to those detected at birth (experiment 1). Specifically, both relative SI and colon weights at necropsy for CS pigs (36.5 ± 1.3 and 9.6 ± 0.4 g/kg BW, respectively) exceeded those for VD pigs (28.1 ± 1.8 and 7.6 ± 0.4 g/kg BW, respectively), with limited effects of diet. Relative weights of the spleen and heart were also higher in CS pigs (0.22 ± 0.01 and 0.7 ± 0.2 g/kg BW, respectively) than in VD pigs (0.19 ± 0.01 and 6.8 ± 0.2 g/kg BW, respectively), while the adrenal gland weights were lower (0.19 ± 0.01 vs. 0.22 ± 0.01 g/kg BW, P < 0.05). Across all of the organs, there were no effects of diet on weight, except the lower relative dry mass of mucosa in VD-FORM pigs compared with
Table 2. Blood chemistry values for pigs at 12 and 36 h after birth (during TPN period) and at 60 h (24 h of enteral feeding)

<table>
<thead>
<tr>
<th></th>
<th>12 h TPN</th>
<th>36 h TPN</th>
<th>24 h Enteral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>VD</td>
<td>CS</td>
</tr>
<tr>
<td><strong>Cortisol, nM</strong></td>
<td>500±41</td>
<td>689±40*</td>
<td>371±42</td>
</tr>
<tr>
<td><strong>Acidity, pH</strong></td>
<td>7.48±0.02</td>
<td>7.49±0.01</td>
<td>7.47±0.01</td>
</tr>
<tr>
<td><strong>PO2, mmHg</strong></td>
<td>98.3±9.2</td>
<td>83.5±7.2</td>
<td>76.1±7.4</td>
</tr>
<tr>
<td><strong>PCO2, mmHg</strong></td>
<td>40.5±2.4</td>
<td>31.7±2.0*</td>
<td>44.2±2.0*</td>
</tr>
<tr>
<td><strong>O2sat, %</strong></td>
<td>100.4±1.0</td>
<td>101.8±0.9</td>
<td>99.3±0.9</td>
</tr>
<tr>
<td><strong>Hb, g/dl</strong></td>
<td>7.54±0.40</td>
<td>8.52±0.36</td>
<td>7.44±0.36</td>
</tr>
<tr>
<td><strong>Na</strong>, mmol/l</td>
<td>136.1±3.0</td>
<td>137.2±2.5</td>
<td>143.8±2.5</td>
</tr>
<tr>
<td><strong>K</strong>, mmol/l</td>
<td>4.46±0.22</td>
<td>5.13±0.16*</td>
<td>4.62±0.17</td>
</tr>
<tr>
<td><strong>Ca</strong>, mmol/l</td>
<td>1.00±0.07</td>
<td>1.22±0.06*</td>
<td>1.34±0.06</td>
</tr>
<tr>
<td><strong>Cl</strong>, mmol/l</td>
<td>98.9±2.3</td>
<td>107.1±1.9*</td>
<td>105.6±2.0</td>
</tr>
<tr>
<td><strong>Glut</strong>, mmol/l</td>
<td>4.41±0.93</td>
<td>6.54±0.70</td>
<td>5.59±0.72</td>
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<tr>
<td><strong>Lactate, mmol/l</strong></td>
<td>2.37±0.28</td>
<td>1.79±0.21</td>
<td>1.32±0.22</td>
</tr>
</tbody>
</table>

Blood chemistry values are given as means ± SE; n = 6–10. *Cortisol 24 h enteral value taken at time of final tissue collection. TPN, total parenteral nutrition; CS, cesarean section; VD, vaginal delivery. *Within each time period, mean values are significantly different (P < 0.05).
most abundant T-RFs. Comparison of delivery mode revealed the T-RF at 207 bp for VD pigs was elevated compared with CS pigs (P < 0.05; Fig. 5A). Likewise, a comparison of diet groups showed two T-RFs (233 and 583 bp) that were significantly higher in FORM compared with COL pigs (Fig. 5B). On the basis of available 16S rRNA databases, T-RFs 233 and 583 bp are predominantly identified as *Clostridium perfringens* and different *Streptococcus* spp, respectively, while the identity of T-RF 207 bp remains uncertain.

Gastric concentrations of acetic and butyric acid were similar for CS and VD pigs, whereas propionic acid was not detected in any group (data not presented). Lactic acid was elevated in COL pigs (59.3 ± 8.0 mmol/kg contents) compared with FORM pigs (32.8 ± 6.9, P < 0.05). Interestingly, four of the five pigs with NEC lesions in the stomach had higher total SCFA in the contents (205.2 ± 48.4 vs. 60.3 ± 9.0, P < 0.05) and all were formula fed.

The pH of stomach contents was not influenced by mode of delivery. However, formula feeding resulted in stomach pH values that were higher compared with those measured in colostrum-fed pigs (4.1 ± 0.1 vs. 3.7 ± 0.1, P < 0.05).

**Gut characteristics of NEC and healthy pigs.** Statistical analysis across diets and modes of delivery showed that physiological and microbiological differences existed between healthy pigs (n = 20) and those diagnosed with NEC (n = 12). Compared with healthy pigs, the gut of NEC pigs were characterized by significantly shorter villi (−58%), decreased lactase and ApN activities (30–35%), decreased mucosal mass (−9%), and increased stomach pH (+17%; all P < 0.05). Maltase and ApA activities of NEC pigs tended to be lower (−22%; P < 0.12) compared with healthy pigs, while no differences were detected for villus width, crypt depth, SI weight, and gastric SCFA (acetic and butyric, lactic). Differences in identifiable bacterial populations in the distal intestinal mucosa were detected by the conventional and molecular microbiological analyses. Densities of *Clostridia* were higher in NEC pigs compared with healthy pigs (+1.5 log CFU/g mucosa; P < 0.05). This was corroborated by the 300% greater relative intensity of T-RF 233 bp, the T-RF corresponding to *Clostridium perfringens*, in NEC compared with healthy pigs (P < 0.001).

**DISCUSSION**

The sensitivity of the preterm intestine to diet and bacteria-induced dysfunction is elevated compared with term neonates (23). Preterm neonates receiving TPN are particularly predisposed to mucosal atrophy and intestinal dysfunction, resulting in increased susceptibility to gut disorders, such as NEC, when enteral nutrients are provided. In the current study, we demonstrated that newborn preterm VD pigs have improved intestinal digestive and absorptive functions compared with CS pigs. However, the initial VD-induced acceleration of gut maturation was not obvious following enteral nutrition and bacterial colonization, and these initial benefits were most diminished in neonates fed formula.

Infants born preterm are more susceptible to hypothermia and hypoxia (44). Blood chemistry values during the perinatal period indicate delivery mode is another determinant of lung, liver, and kidney functions of human infants (2, 46) and preterm pigs (present study). Hypothermia and hypoxia cause a redirection of blood away from the intestine and colon to more vital organs, such as the heart, liver, brain, and adrenal glands (2). The resulting hypoxia/reoxygenation-induced ischemia/reperfusion of the intestine has been related to an increased prevalence of gut dysfunction (1) and is the basis of several animal models of NEC (6, 13, 21). In the present study, despite CS pigs experiencing a period of hypothermia and impaired respiratory function, the incidence and severity of NEC did not differ between CS and VD pigs. Thus, the delivery mode may influence postnatal metabolic parameters, although it is not a principle determinant of NEC.

Postnatal administration of exogenous corticosteroids to neonatal animals has been used to accelerate maturation of the intestine and other organs (35), and antenatal administration of glucocorticoids reduces the incidence of NEC in preterm hu-

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**Fig. 3.** Bacterial counts pooled across diets for distal small intestine mucosa of preterm pigs born vaginally or by cesarean section. Culturing media selective for enterobacteria and coliform bacteria (MacConkey), clostridium (TSC), lactic acid bacteria (MRS), lactobacilli (ROGOSA), enterococci (SLA), and total anaerobes (RFGC) (LS means ± SE). *Significantly different (P < 0.05).

**Fig. 4.** T-RFLP electrophoresis gel of distal intestinal mucosal samples from preterm pigs born vaginally or by cesarean section. Each band represents a single bacterial species or a group of different bacterial species characteristic of the used primers and restriction enzyme. Identification of bacteria corresponding to the bands is done at the MiCA homepage (http://mica.ibest.uidaho.edu/digest.php) using the RDPII database (Release 9, Update 37, Bacterial SSU 16S rRNA).
man infants (40). The higher cortisol levels of the newborn preterm VD appeared to accelerate gut maturation. Specifically, the gut characteristics of the VD pigs differed more than expected from those of the CS pigs based on the 30- to 36-h longer gestation and approached those of newborn term CS pigs (42). The findings suggest that VD accelerates maturation of some gut parameters in newborn preterm pigs, potentially via an associated cortisol surge. The possibility that a prior CS may have contributed to the accelerated maturation of VD pigs was considered; however, comparison of VD piglets born with and without prior CS (experiment 2) showed no differences between litters. Interestingly, despite persistently higher cortisol levels, accelerated gut maturation, and reduced hypothermia and hypoxia, the VD pigs did not have a lower incidence and severity of NEC compared with CS pigs. Gut growth and brush-border enzyme values in 4-day-old VD pigs did not indicate accelerated maturation, but rather diminished intestinal responses relative to CS pigs. Thus, despite mode of delivery not being a primary contributor to NEC, the combination of delivery mode, enteral feeding, and bacterial colonization may be an important determinant of intestinal function in the preterm neonate.

The bacterial assemblages in the gut do play a central role in NEC. This is evident from the lack of NEC in preterm gnotobiotic pigs fed formula (42). The patterns of bacterial colonization are particularly important for preterm neonates because of their increased sensitivity to bacterial colonization (9, 29), with aberrant bacterial growth causing severe gut dysfunction and disease (15, 37). Delivery mode and diet are determinants of initial colonization and development of the bacterial assemblages in the infant gut (17, 18). Exemplifying this are the different densities of bacterial colonization, stability, and diversity among CS and VD neonates (15, 17, 37, 48). VD infants and the preterm pigs are exposed at birth to an abundant and complex mixture of maternal (vaginal and fecal) and environmental bacteria, whereas CS neonates acquire bacteria slower and from the environment only. Correspondingly, the CS preterm pigs harbored assemblages of bacteria that were lower in density and diversity than those of VD pigs. The delayed colonization, as well as the less diverse and unstable assemblages typical of CS infants, is thought to increase susceptibility to pathogen overgrowth and disease (11, 47); however, how bacterial diversity affects preterm intestines highly sensitive to bacterial colonization remains poorly defined. Yet, CS alone did not increase the incidence of NEC among the preterm pigs. This may be related to the general similarities among the CS and VD pigs for the dominant bacterial T-RFs, as the only related differences were the higher band intensity of the unknown T-RF 207 bp in the VD pigs and the increased proportion of Clostridia in the stomach of CS pigs. The most notable diet-related differences in bacterial assemblages were the greater intensities for the T-RF 233 and 583-bp bands in the distal SI of the FORM pigs. The T-RF 233-bp band corresponds with Clostridium perfringens, which has been implicated as a causative agent of NEC (19), and the greater band intensity is consistent with the higher clostridial densities associated with formula feeding (25). The 583-bp band includes several Streptococcus species, with some considered as potential pathogens (38), whereas others have probiotic properties (9). Although the present findings do not demonstrate a causative relationship between Clostridium perfringens and NEC development, they do suggest that the formula use in the present study resulted in assemblages of gut bacteria that could lead to NEC (34).

Preterm neonates have a propensity to react to luminal antigens with an exaggerated inflammatory response, and the

Table 3. Average number of terminal restriction fragments and Dice coefficients for pairwise comparisons of T-RFs from distal small intestinal mucosa in pigs from the same or different treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Number of T-RFs</th>
<th>CS-FORM</th>
<th>CS-COL</th>
<th>VD-FORM</th>
<th>VD-COL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-FORM</td>
<td>6.1 ± 0.9a</td>
<td>86.8 ± 0.9a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS-COL</td>
<td>4.2 ± 1.1a</td>
<td>89.1 ± 0.8a</td>
<td>92.0 ± 1.7c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VD-FORM</td>
<td>12.8 ± 3.3a</td>
<td>81.7 ± 1.1a</td>
<td>82.5 ± 1.5a</td>
<td>79.2 ± 1.8a</td>
<td></td>
</tr>
<tr>
<td>VD-COL</td>
<td>11.0 ± 3.2a</td>
<td>81.4 ± 1.3a</td>
<td>83.0 ± 2.0a</td>
<td>80.8 ± 1.5a</td>
<td>79.1 ± 2.6a</td>
</tr>
</tbody>
</table>

T-RFs, terminal restriction fragments; S<sub>D</sub>, Dice coefficients; FORM, formula; COL, colostrum. a,b,cValues for number of T-RFs or S<sub>D</sub> not sharing a common superscript letter are significantly different (P < 0.05).

Fig. 5. Relative fluorescent intensity of dominant terminal restriction fragments (T-RFs) determined T-RFLP analysis of distal small intestine tissue of preterm pigs (LS means ± SE). Samples are pooled across delivery (A) or diet (B). The band represented by TR-F 207 bp was not be identified, while TR-F 233 bp and TR-F 583 bp represent Clostridium perfringens and a group of Streptococcus species, respectively. *Significantly different (P < 0.05).
increased expression of proinflammatory cytokines has been implicated in the development of NEC (7, 8). Corresponding with this link, increases in the densities of bacteria and concentrations of bacterial products in the preterm intestine are considered to play a role in causing mucosal damage (15). The reduced NEC incidence, lower concentrations of proinflammatory cytokines, and less gut dysfunction of CS and VD pigs fed colostrum after 36 h of TPN compared with those fed formula indicate that diet type is more important than delivery mode as a determinant of gut development and health. This can be attributed to the protective factors, including bacteria, that are present in colostrum but absent in formula (15). Hence, pigs, like infants (45), respond to formula with elevated levels of IFN-γ and IL-6, and this may contribute to intestinal inflammation and precede NEC.

There appears to be an interesting and previously unrecognized interaction between delivery mode and inflammatory responses. Specifically, levels of IL-6 were elevated in VD-FORM compared with CS-FORM pigs. This is intriguing since higher glucocorticoid levels, such as in the VD pigs, have traditionally been considered to downregulate the production of proinflammatory cytokines (12, 45). However, elevated, glucocorticoid levels just before an inflammatory challenge (i.e., start of feeding formula and bacterial colonization) can enhance immune responses (16). Thus, it is possible that the higher glucocorticoid levels combined with the increased density and diversity of colonizing bacteria in the VD pigs, increased the inflammatory responses when formula was introduced to the pigs.

In conclusion, VD and the associated elevation of glucocorticoids accelerate the maturation of the gut and other organs. Yet, it may increase the inflammatory responses of the gut of preterm infants fed formula, further predisposing neonates to gut dysfunction and disease. Any influence of delivery mode is obscured when colostrum is fed. Since colostrum is not available to many preterm infants, there is a need to better understand the interactions among delivery mode, diet, gut maturation, the assemblages of gut bacteria, inflammatory responses, and the relationship with NEC. Nonetheless, the present study provides clinically relevant data that may aid in the difficult choice of obstetrical procedures in relation to preterm birth.

**Perspectives and Significance**

Conclusive clinical evidence about the effects of delivery mode on early colonization and gut function in the preterm infant is limited due to effects of confounding variables such as antibiotic use, diet regime, hospital environment, infant genetic diversity and obstetrical procedures. This study used a clinically relevant animal model, under tightly controlled conditions to examine the differential effects of induced vaginal delivery and elective caesarean section. The results of the study suggest that the preterm gut is very responsive to delivery mode, and it suggests a possible connection between the stress of early colonization and increased inflammatory response, particularly in vaginally delivered neonates. Further studies examining the connection between delivery mode, metabolic stress, and the inflammation response to early colonization and feeding are required to identify whether a specific mode of delivery should be considered a predisposing risk factor for intestinal dysfunction in preterm infants. Furthermore, it has been suggested that exposure to stress during the initial bacterial colonization may result in an altered bacterial profile, potentially predisposing the newborn to the later development of disease in later life (16). Therefore, it is important to investigate whether there is a difference in stress-induced changes to the early bacterial colonization and whether these changes affect preterm intestinal function. Finally, studies on early colonization and gut responses after elective caesarean section and caesarean delivery following induced or spontaneous labor may provide further clinically relevant information for the obstetricians, given the difficult task of deciding how to deliver preterm infants.

**REFERENCES**

BIRTH, GUT COLONIZATION, AND NEC


