Provision of Amniotic Fluid During Parenteral Nutrition Increases Weight Gain With Limited Effects on Gut Structure, Function, Immunity, and Microbiology in Newborn Preterm Pigs

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Abstract
Background: Small enteral boluses with human milk may reduce the risk of subsequent feeding intolerance and necrotizing enterocolitis in preterm infants receiving parenteral nutrition (PN). We hypothesized that feeding amniotic fluid, the natural enteral diet of the mammalian fetus, will have similar effects and improve growth and gastrointestinal (GI) maturation in preterm neonates receiving PN, prior to the transition to milk feeding. Materials and Methods: Twenty-seven pigs, delivered by cesarean section at ~90% of gestation, were provided with PN and also fed boluses with amniotic fluid (AF; n = 13, 24–72 mL/kg/d) or no oral supplements (nil per os [NPO]; n = 14) until day 5 when blood, tissue, and fecal samples were collected for analyses. Results: Body weight gain was 2.7-fold higher in AF vs NPO pigs. AF pigs showed slower gastric emptying, reduced meal-induced release of gastric inhibitory peptide and glucagon-like peptide 2, changed gut microbiota, and reduced intestinal permeability. There were no effects on GI weight, percentage mucosa, villus height, plasma citrulline, hexose absorptive capacity, and digestive enzymes. Intestinal interleukin (IL)–1β levels and expression of IL1B and IL8 were increased in AF pigs, while blood biochemistry and amino acid levels were minimally affected. Conclusion: Enteral boluses of AF were well tolerated in the first 5 days of life in preterm pigs receiving PN. Enteral provision of AF before the initiation of milk feeding may stimulate body growth and improve hydration in preterm infants receiving PN. Furthermore, it may improve GI motility and integrity, although most markers of GI maturation remain unchanged. (JPEN J Parenter Enteral Nutr. XXXX;xx:xx-xx)

Keywords
amniotic fluid; minimal enteral nutrition; preterm birth; immature intestine

Clinical Relevancy Statement
Infants born prematurely are born with an immature gastrointestinal (GI) tract and therefore often rely on parenteral nutrition (PN) to achieve acceptable growth and development in the first days or weeks after birth. As soon as mother’s milk is available, gradually increasing minimal enteral nutrition (MEN) of breast milk may be provided to stimulate growth and GI maturation, as well as improve the resistance against necrotizing enterocolitis (NEC). Since lactation is often delayed or inadequate after preterm birth, alternative sources of MEN are required. We tested if amniotic fluid, the natural enteral diet of the mammalian fetus, could improve growth and GI maturation in a clinically and physiologically relevant pig model of the preterm infant. Collectively, our data indicate good tolerability and increased body weight gain when amniotic fluid is fed for a few days as an enteral supplement to PN for preterm neonates. It may also promote GI integrity and motility, probably via maturation of gut peptide release, although most parameters of GI maturation remain unaffected. Further detailed studies on the safety and efficacy of providing amniotic fluid as the first enteral diet for preterm neonates are required before this regimen can be tested for preterm infants without access to mother’s milk during the first week after birth.

Introduction
Infants born prematurely are born with an immature gastrointestinal (GI) tract and therefore often rely on parenteral nutrition (PN) to achieve acceptable growth and development in the first days or weeks after birth. However, prolonged use of PN is associated with increased risk of infections, metabolic complications related to the liver, and complications related to the GI tract. The latter are caused by villous atrophy and increased intestinal permeability that may lead to an increased risk of feeding intolerance and necrotizing enterocolitis (NEC) during the subsequent transition to full enteral feeding. This consequently leads to extended lengths of hospitalization, long-term health problems, and increased costs of healthcare. Minimal
enteral nutrition (MEN) with human breast milk in supplement to PN is provided to reduce the risk of feeding intolerance and NEC during the transition to full enteral feeding in preterm infants. However, the optimal feeding strategy in terms of the timing of initiation and advancement of enteral feeds remains uncertain. In addition, since lactation is often delayed or inadequate after preterm birth, and at the same time donor human milk is often not available, alternative sources of MEN are required.

Amniotic fluid is the natural source of enteral nutrition (EN) for the fetus and provides up to 10%–15% of the fetal nitrogen and energy requirements, as well as supports somatic growth during fetal development in mammals. Fetal swallowing of amniotic fluid also stimulates GI growth and maturation through growth factors and regulates mucosal immunity in the prenatal period via its content of immunomodulatory factors and antimicrobial peptides. The prenatal effects of amniotic fluid are comparable to the postnatal effects of colostrum and mature milk, which also provide resistance to NEC in neonatal animal models of NEC such as pigs and rodents.

We have previously shown that MEN with porcine amniotic fluid during 2 days of PN and with continued supply during the transition to full enteral formula feeding reduced the sensitivity to NEC in preterm pigs. This protective effect was mediated through modulation of intestinal gene expression related to inflammation and immunity, as well as modifications of the intestinal microbial composition. Furthermore, we have shown that MEN with porcine amniotic fluid reduced the intestinal levels of proinflammatory cytokines, but it failed to reduce the sensitivity to NEC-like lesions when amniotic fluid was withdrawn before the transition to full EN. In rodent models of neonatal NEC, postnatal amniotic fluid reduced the sensitivity to feeding-related intestinal NEC-like lesions. Enteral supplements with murine amniotic fluid reduced the severity of NEC lesions and expression of genes related to inflammation in the small intestine of 10-day-old mice. The effects ascribed were related to epidermal growth factor–mediated inhibition of Toll-like receptor 4–coupled signaling pathways. Similarly, a reduced sensitivity of NEC-like lesions was demonstrated in neonatal rat pups after enteral amniotic fluid supplements. This effect was mainly attributed to hepatocyte growth factor that enhanced intestinal epithelial cell survival, proliferation, and migration in vitro. These results in animal models emphasize the important role of amniotic fluid in perinatal gut development and immunity, as well as show that amniotic fluid may constitute an alternative enteral supplement for preterm neonates receiving PN. However, important questions regarding the tolerance toward and efficacy of enteral feeding with amniotic fluid remain unanswered.

In the present study, we used a clinically and physiologically relevant pig model of the newborn, preterm infant to specifically test the tolerability and the effects of enteral supplements with amniotic fluid (AF) during a period of PN in the immediate postnatal period and thus prior to the risk of feeding intolerance and development of NEC induced by enteral milk feeding. This AF regimen matches the conditions for preterm infants in the neonatal intensive care unit (NICU) when lactation is delayed and other relevant sources of EN are unavailable. We hypothesized that an increasing enteral intake of amniotic fluid up to a clinically relevant volume of 72 mL/kg/d within 5 days after birth would be well tolerated as assessed by clinical indices and blood biochemical parameters. Furthermore, we hypothesized that amniotic fluid supplements would improve GI growth and functions, preserve intestinal structure, reduce intestinal inflammation, and modulate the fecal microbiota during a period of PN just after preterm birth but prior to enteral milk feeding. Because no enteral fluid (or nil per os [NPO]) is a clinically relevant alternative to MEN, and because our previous studies showed effects of amniotic fluid independent of administration of control fluid, we compared preterm pigs receiving PN fed with small enteral boluses of porcine AF with controls given the same amounts of PN but with no enteral feeding.

**Methods**

**Collection of AF**

Porcine AF was collected during cesarean section in sows (Danish Landrace X Large White) at ~90% of gestation.
Between 100 and 500 mL amniotic fluid was collected from each sow. For aseptic collection and to avoid blood admixture, a needle was inserted into the exposed amnion of each individual pig and amniotic fluid was collected by suction, centrifuged (2000 × g, 10 minutes, 4°C) to remove meconium, and stored at −80°C before use. AF from multiple cesarean sections was pooled to establish 3 pools of amniotic fluid, 1 pool used for each litter of pigs. The total concentration of protein in the pooled AF samples was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Slangerup, Denmark) according to the manufacturer’s protocol. The protein concentration ranged between 660 and 1040 mg/L.

Animal Experimental Procedures

All animal procedures were approved by the Danish National Committee on Animal Experimentation. Briefly, a total of 27 pigs (Danish Landrace X Large White X Duroc) were delivered by cesarean section from 3 sows at days 105–107 of gestation (term, days 115–117) and were immediately transferred to heated (37–38°C) and oxygenated (0.5–2 L/min) incubators. Each pig was fitted with an orogastric feeding tube (6F Portex; Smiths Medical, Kent, UK) to supply MEN and solutions for later oral challenge tests. A vascular catheter (4F Portex) was inserted into the transected umbilical artery for provision of PN and for blood sampling. To provide passive immunity, each pig received intra-arterial infusions of maternal porcine plasma 0, 12, and 20 hours after birth (4, 5, and 7 mL/kg body weight, respectively). Pigs from each litter were stratified according to birth weight and sex and randomly allocated to the 2 treatment groups of the experiment (see below).

Nutrition Protocol

The nutrition protocol and in vivo tests are presented in Figure 1. All pigs were started on PN within 6 hours after delivery at a rate of 4 mL/h/kg for 24 hours, 5 mL/kg/h for the subsequent 24 hours, and 6 mL/kg/h for the last 48–58 hours of the study period. The parenteral solution was based on commercially available products (Kabiven and Vamin; Fresenius Kabi, Bad Homburg, Germany) with a macronutrient composition adjusted for the nutrition requirements of preterm pigs as described previously (mean daily intake of energy, 96.7 kcal/kg/d; protein, 5.8 g/kg/d; carbohydrate, 9.7 g/kg/d; and fat, 3.9 g/kg/d).21 Vitamins and minerals (Soluvit, Vitalipid, and Tracel, all Fresenius Kabi) were supplemented according to the manufacturer’s instructions. Pigs received no supplements orally (NPO group; n = 14) or small enteral boluses of porcine AF (AF group; n = 13) starting at 3 mL/kg/3 h for the first 24 hours, 6 mL/kg/3 h for the subsequent 24 hours, and ending at 9 mL/kg/3 h for the final 48–58 hours. While there is no universal recommendation for volume intake of parenteral nutrition (PN) and EN for preterm neonates, this dosing strategy is compatible with the time and feeding advancement rates often used for moderately preterm infants.22 Pigs were weighed once daily and parenteral and enteral feeding volumes adjusted according to increases in body weight.

Clinical Observations

Throughout the study period, pigs were evaluated daily and given a clinical score from 1–4. Score 1: responsive to stimuli and active behavior, warm extremities, pink skin, rhythmic and calm respiration, soft abdomen. Score 2: as score 1 but inactive behavior and/or weakness. Score 3: limited responsiveness to stimuli and/or weak with inactive behavior, cold extremities and/or pale skin, labored respiration, and/or distended abdomen. Score 4: weak and lethargic, unresponsive to stimuli, cold extremities and gray skin, cyanosis and labored respiration, apnea and gasping, and/or distended abdomen. A fecal scoring was also given daily with assessment of fecal consistency and volume: meconium (M), no stool (score 0), solid stool (score 1), mild diarrhea (score 2), or severe diarrhea (score 3).

Blood Biochemistry

Blood pH, hematocrit, glucose, and lactate were quantified on days 2 and 4 of the study using the GEM Premier 3000 (Instrumentation Laboratory, Brussels, Belgium). Blood samples were drawn on day 4 and plasma diluted 1:20 before cortisol quantification using a competitive immunoassay (Cortisol Parameter Assay Kit; R&D Systems, Abingdon, England) and
measured spectrophotometrically according to the manufacturer’s instructions. Plasma free amino acids and blood biochemistry, including total protein, serum albumin, creatinine, serum urea nitrogen, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase, were determined at the time of euthanasia on day 5. Plasma free amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) of their phenylisothiocyanate derivatives as described previously. Blood chemistry was measured using the Advia 1800 Chemistry System (Siemens, Erlangen, Germany).

Functional GI Tests In Vivo

To assess intestinal hexose absorptive capacity on day 4, a bolus of 8 mL/kg of a 10% galactose solution was given orally, blood samples were collected after 20 minutes and centrifuged (2500 × g, 4°C, 10 minutes), and plasma was stored at −80°C. For galactose, deproteinized samples of plasma were added to NAD on a microtiter plate, and galactose dehydrogenase (Boehringer Mannheim, Darmstadt, Germany) was measured as the amount of NADH produced by oxidation of D-galactose, catalyzed by galactose 1-dehydrogenase in the presence of NAD, to D-galacturonic acid, H+, and NADH and quantified spectrophotometrically at 340 nm. For mannitol, a bolus of an 8-mL/kg solution with 10% lactulose and 10% mannitol was given orally 3 hours before urine collection using a syringe to perform a bladder puncture immediately after the pig was euthanized. Urine lactulose was quantified by oxidation of lactulose in a 3-step process and in the presence of NAD to generate NADPH, which was quantified at 340 nm using a spectrophotometer. To quantify urine mannitol, conversion of mannitol using mannitol dehydrogenase and NAD generated fructose and NADH, the latter of which was measured spectrophotometrically at 340 nm. For both assays, the generation of NADPH and NADH was proportional to the initial concentration of lactulose and mannitol, respectively.

The GI responses to the first bolus of enteral milk formula were tested in vivo on day 5. A bolus of 16 mL/kg of a 10% galactose solution was given by stomach tube, preceded by a 20-minute control period (Multigent; Abbott Laboratories, Abbott Park, IL). Briefly, 5 µL plasma was incubated with aryl acylamidase to hydrolyze acetaminophen to p-aminophenol and acetate. Subsequently, p-aminophenol reacted with 8-hydroxyquinoline-5-sulfonic acid to produce 5-(4-aminophenol)-8-quinolon, which was formed in amounts proportional to the initial acetaminophen concentration and was quantified spectrophotometrically at 615 nm. The passage length of chromiumoxide was assessed at the time of euthanasia as the most distal GI segment (stomach, proximal, middle or distal small intestine, colon, or rectum) where the chromiumoxide marker was visually detectable.

NEC Evaluation and Tissue Collection

All pigs were euthanized on day 5, with alternating tissue collections for pigs from the 2 different treatment groups. The 5 regions of the GI tract (stomach, proximal, middle, and distal small intestine, and colon) were evaluated for macroscopic pathologic NEC lesions. Each GI region was assigned a NEC severity score from 1 (a normal region without lesions) to 6 (a severely affected region with pneumatosis intestinalis and extensive hemorrhagic and necrosis). Pigs with a minimum score of 3 in any of the 5 GI regions were defined as having NEC. Samples from the proximal, middle, and distal small intestine and the colon were snap frozen and stored at −80°C to evaluate enzymatic activities as well as gene expression and protein levels of inflammatory mediators. Other samples were fixed in formalin for later histologic evaluation.

After removal of all internal organs and decapitation, 1 liter of pigs (n = 4) was subjected to dual-energy X-ray absorptiometry (DEXA) (Lunar prodigy; GE Healthcare, Diegem, Belgium). Fat mass, lean mass, and bone mineral were determined to estimate body composition in the remaining carcass. The pigs were placed in a prone position during the scanning process, and values of fat and lean mass percentages plus bone mineral density were estimated using software provided with the DEXA scanner.

Mucosal Structure and Brush-Border Enzyme Activities

Mucosal weight proportions were quantified after scraping off the mucosa from a 10-cm segment from all 3 small intestinal regions and determining the proportion of the dry weight of the mucosa relative to the total dry weight of the respective segment. To evaluate mucosal morphology, fixed samples from the 3 regions of the small intestine and colon were embedded in paraffin, sectioned, and stained with hematoxylin and eosin before measuring villus height and crypt depth.
Tissue from the 3 small intestinal regions were homogenized in 1.0% Triton X-100 (10 mL/g tissue) at 0°C. Homogenates were assayed for disaccharidase activities using substrates for each individual hydrolase activity (lactase [EC 3.2.1.108], sucrase [EC 3.2.1.10], and maltase [EC 3.2.1.20 and EC 3.2.1.48]) using 0.01 M lactose, 0.01 M sucrose, and 0.0112 M maltose as substrates (all from Sigma-Aldrich, Brondby, Denmark), respectively. The liberated glucose was subsequently quantified in 2 steps through oxidation by glucose oxidase and subsequent oxidation of O-dianisidine by the generated hydrogen peroxide in the presence of peroxidase activity. The resulting oxidized O-dianisidine was measured spectrophotometrically at 450 nm. One unit (U) of enzymatic activity was equivalent to hydrolysis of 1 µmol substrate per minute at 37°C, and enzyme activities were expressed per gram of wet intestine.

**Intestinal Gene Expression**

Frozen distal small intestinal tissue samples were transferred to gentleMACS M tubes (MACS; Miltenyi Biotec, Lund, Sweden) and homogenized in QIAzol Lysis Reagent (Qiagen, Ballerup, Denmark) using the gentleMACS Dissociator (MACS; Miltenyi Biotec). Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen), including DNA digestion using the RNase-Free DNase set (Qiagen) according to the manufacturer’s protocol. Quantity and quality of the extracted total RNA were evaluated as previously described. First-strand complementary DNA (cDNA) synthesis was performed with 500 ng total RNA per sample using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s protocol. For assay validation, 2 cDNA synthesis reactions were prepared per sample (including non–reverse transcriptase controls).

Primer assays were designed using Primer3 (http://frodo.wi.mit.edu/) and synthesized at TAG Copenhagen (Copenhagen, Denmark) or Sigma-Aldrich. Gene symbols and names of all genes of interest analyzed are found in Table 1, while primer sequences of genes of interest and reference genes are found in Supplemental Table S1. Preamplification was performed using TaqMan PreAmp Master Mix as previously described. Quantitative polymerase chain reaction (PCR) was performed in 96.96 Dynamic Array Integrated Fluidic Circuits (Fluidigm, South San Francisco, CA) following the protocol described previously. Amplification efficiencies, dynamic range, and specificity were estimated based on 4 separate dilution series of pooled cDNA, and melting curve profiles were generated to confirm a single PCR product for each reaction.

Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm) was used to acquire quantification cycles, which were exported to GenEx5 (MultiD, Göteborg, Sweden) for data preprocessing as previously described. Using GeNorm and NormFinder, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), and TATA-box binding protein (TBP) were identified as the most stably expressed reference genes out of 7 candidates. Their geometric mean was used to normalize all samples in GenEx5. For each primer assay, the relative expression level was set to 1 in the sample with the lowest level of expression, and all other samples were scaled accordingly.

**Small Intestinal Cytokine and Tight Junction Protein Levels**

To evaluate intestinal inflammation, tissue samples from the distal small intestine were homogenized in 1.0% Triton X-100 containing protease inhibitor cocktail (Sigma-Aldrich) at 0°C. Concentrations of proinflammatory cytokines interleukin (IL)–1β, IL-6, IL-8, and interferon (IFN)–γ were quantified using Porcine IL-1β, IL-6, IL-8, and IFN-γ DuoSet ELISA Development kits (R&D Systems) according to the manufacturer’s protocol. Concentrations were expressed per gram of wet intestine.

The relative levels of tight junction proteins were determined by Western blot. Briefly, equal amounts of protein were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Expealon, San Diego, CA) and transferred to polyvinylidene fluoride (PVDF) membranes in the

<table>
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<tr>
<th>Gene Symbol</th>
<th>Name</th>
<th>Result</th>
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<tr>
<td>AOAH</td>
<td>Acyloxyacyl hydrolase</td>
<td>NS</td>
</tr>
<tr>
<td>2M</td>
<td>β2 microglobulin</td>
<td>NS</td>
</tr>
<tr>
<td>CASP1</td>
<td>Caspase 1</td>
<td>NS</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine 2</td>
<td>NS</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
<td>NS</td>
</tr>
<tr>
<td>CXCL10</td>
<td>C-X-C motif chemokine 10</td>
<td>NS</td>
</tr>
<tr>
<td>CXCL12</td>
<td>C-X-C motif chemokine 12</td>
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<tr>
<td>CXCL14</td>
<td>C-X-C motif chemokine 14</td>
<td>NS</td>
</tr>
<tr>
<td>HMBG1</td>
<td>High mobility group box 1</td>
<td>NS</td>
</tr>
<tr>
<td>HSPA14</td>
<td>Heat shock 70-kDa protein 14</td>
<td>NS</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α</td>
<td>NS</td>
</tr>
<tr>
<td>IL1B</td>
<td>Interleukin 1β</td>
<td>AF &gt; NPO</td>
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<tr>
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<td>Interleukin 8</td>
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<td>Inter-α trypsin inhibitor heavy chain 4</td>
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</tr>
<tr>
<td>LBP</td>
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<td>Trefoil factor 3</td>
<td>NS</td>
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<td>TGFβ</td>
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</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
<td>NS</td>
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XCell II Blot Module (Invitrogen, Carlsbad, CA). Protein levels were determined using primary antibodies against occludin (Santa Cruz, Dallas, TX), claudin-1, and zona occludens (ZO) 1 (both Abcam, Cambridge, UK) and corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) and ImageQuant LAS 4000 digital imaging system and quantified using the ImageQuant TL 7.0 software (both GE Healthcare, Tokyo, Japan).

**Gut Microbiota Characterization**

The fecal bacterial microbiota composition was determined using tag-encoded 16S ribosomal RNA (rRNA) gene MiSeq-based (Illumina, San Diego, CA) high-throughput sequencing on DNA isolated from a mix of fecal content from the entire length of the colon. Total DNA was extracted from colonic fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer’s instructions, including an initial bead-beating step using FastPrep (QBiogene; MP Biomedicals, Ilkirch, France).32The V3 and V4 regions of the 16S rRNA gene were amplified using primers compatible with the Nextera Index Kit (Illumina) (NXt_V3-V4_F 5'-TCGTCGCGCAGGTCAATGTGATATAAGAGACAG GTGGGCTCGGAGATGTGTATAAGAGACAG GGACTACNNGGGTATCTAAT-3′; adapters in bold). PCR reactions containing 12 µL AccuPrime SuperMix II (Life Technologies, Carlsbad, CA), 0.5 µL each primer (10 µM), 5 µL genomic DNA (~10 ng/µL), and nuclease-free water to a total volume of 20 µL were run on a SureCycler 8800 (Agilent Technologies, Santa Clara, CA). Cycling conditions applied were as follows: denaturation at 95°C for 2 minutes, 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 68°C for 40 seconds, followed by final elongation at 68°C for 5 minutes. To incorporate primers with adapters and indexes, PCR reactions contained 12 µL Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 2 µL corresponding P5 and P7 primer (Nextera Index Kit; Illumina), 2 µL PCR product, and nuclease-free water for a total volume of 25 µL. Cycling conditions applied were as follows: 98°C for 1 minute, 12 cycles of 98°C for 10 seconds, 55°C for 20 seconds and 72°C for 20 seconds, and elongation at 72°C for 5 minutes. The amplified fragments with adapters and tags were purified using AMPure XP beads (Beckman Coulter, Brea, CA). Prior to library pooling, clean constructs were quantified using a Qubit fluorometer (Invitrogen) and mixed in approximately equal concentrations to ensure even representation of reads per sample followed by 250-bp pair-ended MiSeq (Illumina) sequencing performed according to the instructions of the manufacturer.

The raw data set (NCBI accession number SRP040471) containing pair-ended reads with corresponding quality scores were trimmed using the CLC Genomic Workbench (CLC bio, Arhus, Denmark). Trimming settings were set to low-quality limit of 0.01, with no ambiguous nucleotides allowed, and trimming off the primer sequences. Merging overlapped reads was performed using the “Merge overlapping pairs” tool using default settings. The Quantitative Insights Into Microbial Ecology (QIME) tool (version 1.8.0) was used for further analysis.33 Purging the data set from chimeric reads was performed using USEARCH,34 while the usearch61 method was used for operational taxonomic units (OTU) selection.34 The Greengenes (version 12.10) 16S rRNA gene database was used as reference.35

Principal coordinate analysis (PCoA) plots were generated with the jackknifed β diversity workflow based on 10 distance metrics calculated using 10 subsampled OTU tables. The –e value (number of sequences taken for each jackknifed subset) was set to 85% of the sequence number within the second most indigent sample. Samples whose number of reads was below 48,000 were removed from this step. Analysis of similarities (ANOSIM) was used to evaluate group differences using weighted and unweighted unifrac distance metrics that were generated based on rarefied (48,000 reads per sample) OTU tables. The relative distribution of the genera registered was calculated for unified and summarized at the genus-level OTU tables. The α diversity measures expressed with an observed species (sequence similarity 97% OTUs) value were computed for rarefied OTU tables (48,000 reads per sample) using the α rarefaction workflow, leaving out 1 sample due to the low number of reads (below 48,000). Differences in α diversity were determined using a t test–based approach employing the nonparametric (Monte Carlo) method (999 permutations) implemented in the compare α diversity workflow.

**Statistical Analysis**

Data on group sex ratios and the incidences of NEC and diarrhea were compared using the Fisher exact test. NEC severity scores were analyzed using the nonparametric Mann-Whitney test, and linear correlation analyses were conducted in GraphPad Prism (version 6.02; GraphPad Software, La Jolla, CA). Gene expression data were log2 transformed, and an unpaired 2-tailed t test was used for statistical evaluation in GenEx5. Data are presented as relative expression and considered significant if \( P < .05\) and fold-change >2.0. Other data were analyzed using R (version 2.15.0; R Foundation, Vienna, Austria). Body weight gain was analyzed as repeated measurements using the lme function, while other end points were modeled using the lmer function, including “litter” as a random variable and “treatment group” and/or “birth weight” as a fixed variable. Model residuals and fitted values were tested for normality. In cases of nonnormality, data were log10 transformed before modeling in lmer. NEC severity scores were presented as medians with ranges, whereas all other data were presented as means ± SEM. Resulting \( P\) values were evaluated at a 5% significance level.
Results

Clinical Observations, Body Weight, and Blood Biochemistry

Despite the absence of clinical NEC symptoms, mild NEC-like lesions were observed in the GI tract from a few pigs at necropsy on day 5. The NEC incidence did not differ between treatment groups (AF 1/13 vs NPO 2/14). NEC-like lesions were found in the colon of all pigs affected with NEC (median, 3; range, 3–5) but were absent in the stomach and middle small intestine of all animals. Assessment of diarrhea did not differ between treatment groups (AF 3/13 vs NPO 1/14).

After stratification and allocation of pigs into treatment groups on day 1, the sex ratio (18/9 female/male across groups) and mean birth weight (1037 ± 54 g across groups) did not differ between treatment groups. All pigs lost weight during the first 24 hours of the study, but less weight loss was observed for AF than NPO pigs (−53 ± 5 vs −85 ± 6 g; P < .05; Figure 2A). AF pigs gained more weight throughout the remainder of the study period (P < .05; Figure 2A), which resulted in a 2.7-fold higher total relative body weight gain in AF vs NPO pigs (P < .001; Figure 2B). For body composition, there was a tendency to reduced fat mass (12.9% ± 0.7% vs 12.9% ± 0.7%; P = .15; Figure 2C) and increased lean mass percentage (89.4% ± 1.2% vs 87.0% ± 0.7%; P = .13; Figure 2D) in AF pigs. The bone mineral density did not differ between groups (0.13 ± 0.01 g/cm² across groups; Figure 2E).

Blood pH, hematocrit, glucose, and lactate did not differ between treatment groups on days 2 and 4, but values decreased for glucose (5.0 ± 0.8 vs 3.7 ± 0.6 mM; P < .05), lactate (1.6 ± 0.1 vs 0.8 ± 0.1 mM; P < .001), and hematocrit (28.8% ± 0.6% vs 21.0% ± 0.7%; P < .001) from days 2–4 in both groups (Suppl. Table S2). Likewise, ALAT, ASAT, alkaline phosphatase, serum urea nitrogen, and cortisol did not differ between treatment groups. Blood creatinine and total plasma protein levels were lower in AF compared with NPO pigs (P < .05; Suppl. Table S3), with a similar tendency for plasma albumin levels (P = .06). Finally, of the 21 free amino acids determined in plasma at euthanasia, only asparagine levels were different between groups, with a similar tendency for plasma albumin levels (P = .02; Figure 2C). The urine lactulose/mannitol ratio, a marker of intestinal permeability, was reduced in AF pigs (0.07 ± 0.02 vs 0.09 ± 0.03; P < .05; Figure 5A). The protein levels of ZO-1 were reduced in AF pigs (0.07 ± 0.02 vs 0.09 ± 0.03; P < .05; Figure 5B), whereas no differences were observed in claudin-1 and occludin levels.

Organ Weight and Intestinal Mucosa Structure and Function

The relative weight of the stomach (mean across groups 6.8 ± 0.2 g/kg), small intestine (mean 24.2 ± 0.6 g/kg), and colon (9.4 ± 0.4 g/kg) did not differ between treatment groups. The relative weight of the lungs was higher in AF pigs (37.3 ± 1.8 vs 31.0 ± 2.1 g/kg; P < .05), while the relative weight of the heart (mean across groups 10.0 ± 0.3 g/kg), kidneys (10.4 ± 0.3 g/kg), liver (30.9 ± 0.8 g/kg), and spleen (2.21 ± 0.09 g/kg) did not differ between groups.

No differences in the dry mucosa percentage (data not shown) or in villus height were observed between groups in any of the 3 small intestinal regions (Figure 6A). Crypt depth differed between groups (AF 72 ± 4 vs NPO 59 ± 2 µm; P < .05) in the proximal small intestine, while a similar tendency was observed in the middle small intestine (AF 65 ± 4 vs NPO 55 ± 4 µm; P = .09; Figure 6B). The protein levels of X-linked inhibitor of apoptosis protein (XIAP), a marker of cell proliferation, were ~25% lower in the distal small intestine of AF vs NPO pigs (P < .01; Figure 6C). Plasma citrulline concentration, used as a marker of functional enterocyte mass, was quantified 1 hour after the administration of an oral bolus of standard infant formula on day 5, but no differences were observed between groups (64.5 ± 4.5 µM across groups; Figure 6D). Correlation analyses identified a moderate positive correlation between citrulline and proximal villus height (r² = .27, P < 0.01). Finally, sucrase activity was lower in AF vs NPO pigs in the proximal small intestine (0.22 ± 0.04 vs 0.36 ± 0.04 U/g
tissue; $P < .05$; Table 2). The brush-border enzyme activity was highest for lactase. Neither lactase nor maltase activities differed between groups.

**Intestinal Inflammation**

Gene expression levels in the distal small intestine were quantified for 21 genes related to mucosal inflammation and immunity (Table 1). The expression of *IL1B* and *IL8* was higher in AF pigs (2- to 3-fold higher; $P < .01$ for both; Figure 7A). The expression of *CXCL10* and *LBP* tended to be higher in AF pigs ($P = .06$ and .07, respectively), while none of the remaining analyzed genes differed between groups. The concentration of proinflammatory cytokines IL-1β, IL-6, IL-8, and IFN-γ was quantified in the distal small intestine. IL-1β levels were higher in AF vs NPO pigs (7.0 ± 1.2 vs 4.7 ± 1.2 pg/mg

![Figure 2](image_url)

**Figure 2.** (A) Daily and (B) total body weight gain, (C) body fat, and (D) lean mass percentages, plus (E) bone mineral density in preterm pigs after 5 days of parenteral nutrition with or without minimal enteral nutrition with amniotic fluid. Data presented are mean ± SEM, $n = 13–14$ (A, B) and $n = 4$ (C–E). *$P < .05$, ***$P < .001$: significant difference between treatment groups. AF, amniotic fluid; NPO, nil per os.
tissue; $P < .05$), whereas IL-6, IL-8, and IFN-γ concentrations did not differ between groups (mean across groups, 6.1 ± 0.6, 56.1 ± 4.2, and 3.6 ± 0.3 pg/mg, respectively; Figure 7B).

**Gut Microbiota Composition**

Sequences free from chimeric reads yielded a total of 2,346,983, providing an average of 106,681 sequences per sample (minimum, 34,137; maximum, 177,160; SD, 40,228) with a mean (SD) sequence length of 432 (14) bp. All reads were classified to 5 phyla in addition to an unclassified phylum. The most abundant phyla were Proteobacteria and Firmicutes, while other phyla detected were Actinobacteria, Cyanobacteria, and Bacteroidetes (Suppl. Table S5). A total of 108 genera clusters were identified, with 6 of these representing more than 90% of all reads (Suppl. Table S6). The α diversity analysis showed no difference between treatments (AF vs NPO), but when comparing the 3 litters, 1 litter differed from the 2 others ($P = .003$ and .012). Similarly, PCoA and ANOSIM analysis revealed no differences according to treatment

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**Figure 3.** (A) Chromium oxide passage length 60 minutes after an oral bolus of chromium oxide-containing infant formula (day 5), $n = 13–14$. (B) Plasma acetaminophen and (C) gastric residual volume 60 minutes after an oral bolus of infant formula (day 5). Data presented are mean ± SEM, $n = 13$. *$P < .05$: significant difference between treatment groups. AF, amniotic fluid; NPO, nil per os. (D) Correlation between gastric residual volume and plasma acetaminophen across groups. Solid line is fitted by linear regression and 95% confidence interval is marked with gray fill. The correlation is significant ($r^2 = 0.30$, $P < .01$), $n = 26$. 

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Figure 4. Plasma (A) GIP and (B) GLP-2 at baseline (days 2 and 4) and 60 minutes after an oral bolus of infant formula (day 5). Data presented are mean ± SEM, n = 5–13. **P < .01: significant difference in increment between treatment groups on day 5. & P < .001: significant difference between treatment groups on day 4. AF, amniotic fluid; GIP, gastric inhibitory peptide; GLP-2, glucagon-like peptide 2; NPO, nil per os.

Figure 5. (A) Intestinal permeability assessed by lactulose/mannitol test on day 5 and (B) levels of tight junction proteins quantified by Western blot in the distal small intestine. Data presented are mean ± SEM, n = 6–14. *P < .05: significant difference in data between treatment groups (for [A], comparison between groups conducted on log_{10}-transformed data). AF, amniotic fluid; NPO, nil per os; ZO, zonula occludens.

groups, but clear clustering was observed according to litters of origin (data not shown). To test whether the litter effect suppressed the influence of treatment, PCoA and ANOSIM analysis was repeated for the 2 litters presenting no clear clustering according to litter. Here treatment induced a relatively small but significant separation of the microbiota compositions.
Figure 6. (A) Villus height and (B) crypt depth in 3 small intestinal regions and (C) distal small intestinal XIAP levels. (D) Plasma citrulline 60 minutes after an oral bolus of infant formula on day 5. Data presented are mean ± SEM, n = 12–14. *P < .05, **P < .01: significant difference between treatment groups. AF, amniotic fluid; NPO, nil per os; XIAP, X-linked inhibitor of apoptosis protein.

(weighted dissimilarity matrix, $R = 0.264$, $P = .002$). This effect was not confirmed for the third litter, probably due to low sample size (n = 7). Subsequently, data were reanalyzed aiming to control for the litter effect by identifying OTUs accounting for significant differences between litters by using analysis of variance, followed by filtering out OTUs with significantly different abundance among litters (2.6% of OTUs). Subsequent PCoA and ANOSIM analyses, including all 3 litters, again showed a modest but significant separation in response to treatment (AF vs NPO, $R = 0.202$, $P = .02$).
Table 2. Brush-Border Enzyme Activities in the Small Intestinal Regions in Preterm Pigs After 5 Days of Parenteral Nutrition With or Without Minimal Enteral Nutrition With Amniotic Fluid.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Small Intestinal Region</th>
<th>NPO</th>
<th>AF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactase, U/g</td>
<td>Proximal</td>
<td>40.3 ± 5.3</td>
<td>34.3 ± 6.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>25.6 ± 3.6</td>
<td>18.9 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>6.5 ± 1.2</td>
<td>8.8 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Maltase, U/g</td>
<td>Proximal</td>
<td>2.21 ± 0.30</td>
<td>3.23 ± 0.54</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>1.67 ± 0.17</td>
<td>2.08 ± 0.28</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0.83 ± 0.09</td>
<td>1.12 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Sucrase, U/g</td>
<td>Proximal</td>
<td>0.36 ± 0.04</td>
<td>0.22 ± 0.04</td>
<td>&lt;.05</td>
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<tr>
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<td>0.35 ± 0.05</td>
<td>0.40 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0.21 ± 0.05</td>
<td>0.25 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 12–14. AF, amniotic fluid; NPO, nil per os; NS, no significant difference between treatment groups.

Figure 7. (A) Gene expression determined by quantitative polymerase chain reaction and (B) concentrations of proinflammatory cytokines quantified by enzyme-linked immunosorbent assay in the distal small intestine. Data presented are mean ± SEM, n = 8–13. *P < .05, **P < .01: significant difference between treatment groups. AF, amniotic fluid; CXCL, C-X-C motif chemokine; IFN, interferon; IL, interleukin; LBP, lipopolysaccharide-binding protein; NPO, nil per os.

Discussion

Enteral supplements with AF reduce the incidence and severity of NEC-like lesions in neonatal animal models of NEC such as mice, rats, and pigs. Still, the true potential of AF, as an enteral supplement to reduce the risk of feeding intolerance and NEC during the transition to full enteral feeding in preterm infants receiving PN, remains to be clarified. Important questions regarding the optimal time and amount of AF feeding in relation to the transition to enteral milk feeding are not answered. Here, we investigated the effects of enteral AF specifically during a postnatal period of PN using a clinically and physiologically relevant pig model of preterm infants. We tested if increasing doses of porcine AF over the first 5 days after preterm birth would affect clinical indices and improve GI growth, structure, function, immunology, and microbiology relative to preterm pigs receiving no enteral supplement. We demonstrated that AF was well tolerated in preterm PN-fed pigs, as indicated by clinical observations and blood biochemistry values. Enteral AF enhanced body weight gain, probably in part via increased hydration, in addition to the reduced rate of gastric emptying, gut hormone release, and intestinal permeability. AF induced a modest change in the gut microbiota and increased expression of a few proinflammatory mediators, while other parameters of GI maturation remained unchanged. We conclude that enteral AF increases body weight gain in PN-fed preterm pigs, together with modest and variable effects on markers of gut structure, function, immunity, and microbial colonization.
Optimal nutrition support is vital for preterm infants as deficient postnatal growth is associated with increased risk of neurodevelopmental impairment. Achieving acceptable growth rates in preterm infants is often difficult due to the metabolic complications and the immature GI functions that make rapid advancement of milk feeding challenging. Optimal strategies to facilitate the difficult transition from PN are crucial. We observed a 2.7-fold increase in body weight gain in preterm PN-fed pigs when these were fed supplemental AF compared with no oral feeding. This is consistent with the growth-promoting effects of both porcine and human amniotic fluid observed in preterm pigs after 2 days on PN and with the growth restriction observed after surgical esophageal ligation and disruption of AF ingestion in fetal pigs, sheep, and rabbits. DEXA scanning showed tendencies to a reduced fat mass and increased lean body mass in pigs fed AF. In addition, as total plasma protein and creatinine were decreased and relative lung weight increased in pigs fed AF, our data indicate that the body weight gain may have been partly caused by additional hydration. On the other hand, increased weight gain after AF supplementation was also observed in our earlier studies when control preterm pigs received an equal volume of control fluid. Thus, the marked weight gain effects observed in this study could result partly from direct growth-stimulating effects of growth factors in AF, as the additional supply of total protein via AF was limited. Ingestion of AF may also supply extra sodium that may affect intracellular and extracellular fluid compartments in the first few days of life. Regardless, the mechanism of the observed growth stimulation by AF warrants further investigation because inadequate postnatal growth and fluid balance dysregulation, even with adequate nutrition and fluid intake, is a large problem in the care of preterm infants.

The rate of gastric emptying after the first bolus feeding with milk formula was reduced in pigs previously fed AF, which was also indicated by the reduced meal-induced GIP and GLP-2 increment. Reduced passage of luminal substrates for enterocyte citrulline synthesis after the formula test meal (mainly from glutamine) may also explain the tendency to reduced plasma citrulline levels in AF pigs at the end of the experiment. While enteral feeding intolerance is a big problem for both preterm pigs and infants, a prolonged retention of the first milk feeding in the stomach may also reflect improved regulation of gastric motility and maturation of the enteric nervous system, thereby preventing an uncontrolled flow of milk substrates into the immature intestine. While this hypothesis remains to be tested in detail, rapid food passage and excessive influx of undigested milk to the distal GI tract are consistently related to NEC in preterm pigs. A coordinated delay in gastric emptying may prevent this and enhance the time available for nutrient digestion and absorption. Delayed gastric emptying may also provide enhanced time for bacterial elimination by gastric acid, thereby reducing the risk of bacterial overgrowth and NEC. However, newborn preterm pigs have a depressed capacity for gastric acid secretion, indicated in this study also by the relatively high pH (pH ~5) of the gastric content 60 minutes after the final meal test.

Enteral AF induced a slight improvement of the intestinal barrier function in vivo, while the levels of tight junction proteins tended to be reduced. During adaptation, increased permeability could likely induce a physiological drive for higher expression of tight junction proteins. The latter finding may reflect reduced needs to synthesize new tight junction proteins in the AF group, although only a few of the proteins related to permeability have been measured, and similar effects have been observed in other studies where permeability is improved. The integrity of the intestinal barrier is important for controlling the compartmentalization of microbial products to the lumen of the GI tract. Translocation of bacteria and production and accumulation of gas in the intestinal wall (ie, pneumatosis intestinalis), as seen in some cases of NEC, emphasize the importance of the intestinal epithelial barrier and bacterial colonization in the pathogenesis of NEC. In this study, gut microbiota analyses showed relatively small separation between the groups (low R values), indicating that AF feeding might modulate the gut microbiota to a limited extent (eg, by providing luminal substrates for growth of specific bacteria, like human milk prebiotics).

In contrast to body weight gain and gastric emptying, AF had minimal effects on parameters of GI structure and digestive function. AF did, however, enhance crypt depth in the proximal small intestine, probably reflecting a proliferative effect to the crypt epithelium, consistent with the reduced villus heights and crypt depths observed in neonates with congenital intestinal obstruction. AF did not stimulate overall GI and mucosal growth, villus height, and plasma citrulline levels (a biomarker of functional enterocyte mass) and had limited effects on brush-border enzymatic activities and hexose absorption. The relatively short period of enteral supplementation in this study (5 days) compared with the longer duration of esophageal ligation experiments (≥15 days) in fetal pigs and sheep may explain in part the more subtle effects on GI maturation in this study. It is also possible that the physiologic effects of AF differ between the fetal state and the postnatal state of preterm neonates. Both the growth-promoting and the immunomodulatory properties of AF may work differently in the sterile intrauterine environment relative to the nonsterile extraterine environment. Furthermore, relative to milk diets, the concentration of proteins, and thereby bioactive factors, is quite dilute in AF (eg, ~0.6–1 g protein/L). Even when providing concentrated AF before and after the introduction of enteral milk feeding in an earlier study on preterm pigs, the effects on intestinal structure and function were minimal.

Clinical observations suggest that ~90% of all cases of NEC occur in preterm infants who receive enteral nutrients from human milk or infant formula. In our classic formula-induced NEC model in preterm pigs, where the pigs are subjected to a 5-day period of parenteral and enteral feeding, NEC incidences of 40%–80% are common. In this study, we investigated
effects of AF before the transition to milk feeding, and therefore clinical NEC symptoms and intestinal NEC lesions were few or absent. A relatively healthy state of the intestine was supported by the observed low levels of proinflammatory cytokines in the intestine that were comparable to levels observed in healthy preterm pigs fed bovine colostrum or human milk\textsuperscript{14} and lower than in NEC-affected formula-fed pigs.\textsuperscript{48}

AF increased the intestinal expression of IL1β and IL8 and also of CXCL10 (a chemokine known from intestinal inflammatory disorders)\textsuperscript{49} and LPB (LPS binding protein). Cytokines, chemokines, and LBP are known components of AF, even in healthy pregnancies.\textsuperscript{50} A tendency for AF to increase the expression of some inflammatory mediators (eg, CXCL1, CXCL2, CXCL5, CCL2, CCL4, CCL5, IFN-γ, iNOS) has also been reported for healthy neonatal rats supplemented with AF.\textsuperscript{20} While increased expression of such molecules would normally be considered detrimental, it is also possible that this response is part of a physiologic mechanism to prime the mucosal barrier and improve immunity, as indicated by the NEC studies on formula-fed rats supplemented with AF.\textsuperscript{20} Studies in pigs support that a moderate elevation of IL-8 levels induces epithelial repair while excessive IL-8 levels induce inflammatory damage in the developing intestine.\textsuperscript{51} Similarly, a modest increase in intestinal expression of LBP after AF feeding may increase tolerance to Gram-negative bacteria. Most of the measured proinflammatory mediators remained unaffected by AF feeding (eg, IL-18, HMGB1, CD36, CCL2), supporting blood chemistry and amino acid data in that the AF feeding was well tolerated in preterm pigs receiving PN.

Several guidelines for feeding preterm infants exist,\textsuperscript{22} but none incorporates all the different subgroups of preterm infants or the possibility of different regimens for different diets. In this study, we focused on investigating the tolerability and intestinal effects of enteral AF just before introduction of milk to preterm pigs, used as a model for moderately preterm infants. The feeding regimen used (time, volume of AF) was similar to that often used for preterm infants fed enterally with the mother’s milk or donor human milk within the first week after birth. The clinical observations and blood biochemical parameters indicate that this AF regimen was well tolerated, increased body growth, and induced modest effects on some parameters. This also indicates that this AF regimen was well tolerated, supporting blood chemistry and amino acid data in that the AF feeding was well tolerated in preterm pigs receiving PN.

Acknowledgments
We thank Jane Povlsen, Elin Skytte, and Kristina Møller from Department of Nutrition, Exercise and Sports, University of Copenhagen, and Karin Tarp from National Veterinary Institute, Technical University of Denmark, for technical support with animal procedures and laboratory analyses. We also thank Douglas G. Burrin from Children’s Nutrition Research Center, Huston, Texas, for support with amino acid analyses. Finally, we thank Christian Ritz from Department of Nutrition, Exercise and Sports, University of Copenhagen for statistical counseling.

References


