Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets

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Running head: human milk, bovine colostrum and necrotizing enterocolitis.

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**ABSTRACT:** Preterm birth and formula feeding predispose to necrotizing enterocolitis (NEC) in infants. As mother’s milk is often absent following preterm delivery, infant formula (IF) or human donor milk (HM) are frequently used as alternatives. We have previously shown that porcine and bovine colostrum (BC) provide similar NEC-protection in preterm piglets relative to IF. We hypothesized that HM exerts similar effects and that this effect is partly species-independent. Preterm piglets (n=40) received 2 days of total parenteral nutrition, followed by a rapid transition to full enteral feeding (15mL/kg/2h) for two days using BC (n=13), HM (n=13) or IF (n=14). Intestinal passage time and hexose absorption were tested *in vivo*. Body and organ weights were recorded on day five and macroscopic NEC lesions in the gastrointestinal tract were assessed. Intestinal samples were collected for determination of histomorphology, histopathology, tissue IL-6 and IL-8, organic acids, bacterial adherence by fluorescence in situ hybridization score and digestive enzyme activities. Relative to IF, pigs from BC and HM showed longer intestinal passage time, higher weight gain, hexose absorptive capacity, mucosal proportion, enzyme activities, and lower NEC incidence, organic acids concentration, IL-8 concentration and reduced histopathology lesions. Tissue IL-6 concentration and bacterial adherence score were lower for HM, relative to both BC and IF groups. We conclude that BC and HM are both superior to IF in stimulating gut structure, function and NEC resistance in preterm piglets. BC may be a relevant alternative to HM when mother’s milk is unavailable during the first week after preterm birth.

**Keywords:** donor milk, intestine, NEC, premature.

**INTRODUCTION**
Preterm birth and enteral nutrition are associated with necrotizing enterocolitis (NEC), a disease characterized by extensive inflammation and necrosis mainly in the distal small intestine and proximal colon. While etiology and pathogenesis remain unclear, several studies have shown marked beneficial effects of breast feeding low birth weight and preterm infants (18; 19; 25). Unfortunately, mothers of preterm infants often experience insufficient milk production, and supplementation with pasteurized human donor milk (HM) or infant formula (IF) are often used alternatives (34).

Mother’s milk is a complex diet. It contains bioactive factors, e.g. immunoglobulin, lactoferrin, lysozyme, oligosaccharides, nucleotides, growth factors, enzymes, antioxidants and intact cells. Mother’s milk provides highly digestible nutrients, passive immunization and supports a normal development of the gastrointestinal tract. Moreover, mother’s milk may also modulate the innate immune response and stimulate intestinal bacterial colonization in a favorable direction (3). HM may display some of the same characteristics as mother’s own milk. However, HM is collected during the mature phase of lactation, thus leading to a quite different nutrient composition of HM, relative to the first expressed milk (colostrum). The concentrations of energy, protein, minerals and bioactive factors such as oligosaccharides (10), will typically be insufficient to support the higher demands per kg body weight of preterm infants (11), leading to fortification of HM in many units. HM is pasteurized to prevent transmission of infections (e.g. human immunodeficiency virus and cytomegalovirus) but this heat treatment may also decrease the levels of some important bioactive and immune factors (3; 12). IF can be composed to meet the high energy and protein requirements of preterm infants, but it lacks the important immune- and bioactive components found in mother’s milk and HM. In addition, several studies have documented that IF increases the risk and severity of NEC in preterm infants, compared with mother’s milk and HM (20; 21).
Bovine colostrum (BC) is rich in energy, protein and immune- and bioactive components (38). BC has been shown to prevent induced villous atrophy in mice (31), and stimulate mucosal healing in patients suffering from inflammatory gut diseases (17). BC also protects the intestinal mucosa from bacterial adhesion (29) and reduces diarrhea in infants (35). It has recently been shown that BC inhibits nuclear factor κB-mediated pro-inflammatory cytokine expression in intestinal epithelial cells (1), which may be clinically relevant as nuclear factor-κB has a pivotal role in the development of NEC (7). BC is a commercially available co-product of the dairy industry, and current farming methods allow for production of large volumes of BC for clinical use (13; 31).

Using a well-established animal model we have previously shown that BC provides effects similar to porcine colostrum (mother’s own milk) regarding prevention of NEC in preterm piglets (4). While colostrum contains compounds that are species specific, it also contains compounds that are effective across species, and may exert protective effects against NEC. Given these considerations, a key question is whether BC is a clinically relevant alternative to mother’s milk and HM for preterm neonates when these diets are not available. This would require an assessment of the degree of species-specificity as this is essential before BC can be considered for human neonates. We hypothesized that HM and BC both decrease the incidence and severity of NEC, compared with infant formula in a preterm piglet model of NEC (4). To further support the effects of different diets, intestinal analyses included a series of structural, functional, microbiological and immunological indices.

MATERIALS AND METHODS

Animals: Forty preterm piglets (Danish Landrace x Large White x Duroc) were delivered by caesarean section on day 105 (92% of gestation) from three sows. Anesthesia was
induced with Zoletil mixture (1 mL/15 kg i.m of: 125 mg tiletamine and 125 mg zolazepam, 6.25 mL xylazine 20 mg/mL, 1.25 ml ketamine 100 mg/ml, 2.5 mL butorphanol 10 mg/mL). The sows were placed in latero-lateral recumbency and after aseptic preparation, local infiltration anesthesia was applied (lidocain 2%). An incision was made in the flank and the fetuses were obtained through incisions in the large uterine curvature. The umbilical cord of each piglet was ligated and transected, and a blood sample from the sow was obtained and used later for passive immunization of the piglets. Sows were subsequently euthanized with sodium pentobarbitone (60 mg/kg).

The piglets were transferred to infant incubators with regulated temperature (37-38°C) and oxygen supply (0.5-2 L/min to achieve > 92% arterial blood saturation) over the first 12 hours. While still anesthetized from the caesarean section (supplemental Zoletil mixture 1 mL/40 kg given when needed), each piglet was fitted with a vascular catheter (Infant feeding tube, 4F; Portex, Kent, United Kingdom) inserted into the dorsal aorta via the transected umbilical cord. This was used for parenteral nutrition (PN) and blood sampling. The cord was ligated with a soft cotton thread close to the skin to avoid bleeding, and the catheters were secured with sutures to the cord and skin. Finally an oro-gastric feeding tube (6F Portex) was passed through the cheek and secured to prevent damage from chewing. Following catheterization, all piglets were immunized systemically with arterial administration of plasma isolated aseptically (4,000 g, 4°C, 10 min) from their own mother at 4, 12 and 20 hours after delivery at 4, 5 and 7 ml/kg respectively. All procedures were approved by The Animal Experiments Inspectorate in Denmark (protocol number 2004/561-910).

**Nutrient solutions and feeding protocol:** All piglets were initially provided total parenteral nutrition (TPN) via the umbilical catheter using a TPN solution (Kabiven and Vamin 18 g N/L, Fresenius Kabi, Uppsala, Sweden) modified as previously described (3). Glucose and fat were withdrawn to reach a final nutrient concentration of total energy 3,138 kJ/L; protein 45 g/L, glucose 72 g/L and lipids 31 g/L. The TPN was initiated within 4 hours after birth and infused at 4
6 mL/kg/h on day one, increasing to 6 mL/kg/h on the second day using syringe infusion pumps (Infusomat Secura; Braun). In the morning on day three, parenteral nutrition was reduced every second hour (from 6 mL/kg/h to 4 mL/kg/h to 2 mL/kg/h and 0) while at the same time points enteral nutrition was increased (from 0 to 5 mL/kg/2 h to 10 mL/kg/2 h to 15 mL/kg 2h). Hence a full transition from TPN to TEN was achieved in 6 hours. At this stage the piglets were stratified according to birth weight and sex, and allocated into three treatment groups fed either human donor milk (HM, n=13), bovine colostrum (BC, n=13), or infant formula (IF, n=14). In this study, we used a rapid advancement of feeding volumes to levels similar to those in preterm infants receiving full enteral feeds (150-180 mL/kg/d) because we aimed to challenge the capacity of the immature intestine to tolerate enteral feeding. HM (Holder pasteurized, 62.5°C for 30 min) was obtained from Women’s Milk Central, Hvidovre Hospital, Denmark (3,108 kJ/L, protein 13 g/L, fat 44 g/L). BC was obtained from Gjorslev Gods, Denmark, and γ-radiated to ensure sterility without harming proteins and other bioactive components. To match energy contents of human donor milk, three parts bovine colostrum and five parts tap water were mixed (measured contents before dilution of the bovine colostrum: 5,334 kJ/L, protein 103 g/L and 55 g/L fat, Department of Medical Gastroenterology Section, Rigshospitalet, Copenhagen, Denmark, and after dilution: 3,200 kJ/L, protein 62 g/L and fat 33 g/L). IF was made from three commercially available products used for feeding infants 0-2 years of age and mixed to be iso-caloric with the two other diets (Pepdite 2-0 and Liquigen-MCT, SHS International, Liverpool, UK and Variolac, Arla Foods Ingredients, Aarhus, Denmark to a final concentration of 3,066 kJ/L, protein 13 g/L and 44 g/L fat). After full transition from parenteral to enteral nutrition, all three enteral diets were given at 15 mL/kg/2 h until euthanasia and tissue collection on day five.

Clinical observations and in vivo tests: Piglets were observed minimum every second hour and any signs of discomfort or weakness (unwilling to stand, cold extremities, distended
abdomen, dehydration, pale skin color) was recorded. To assess total gastrointestinal transit time, a color marker (chromium-oxide, 0.6%) was added to the first full enteral nutrition bolus (six hours after initiation of enteral feeding). First defecation with visible contents of green non-absorbable chromium-oxide was recorded as intestinal passage time.

To assess intestinal hexose absorptive capacity, a 15 mL/kg bolus of a 5% galactose solution was given 6 and 48 h after initiation of enteral nutrition. As galactose and glucose use the same Na+/glucose linked transporter (SGLT-1), it is a reliable estimate of hexose absorptive capacity. Blood samples were collected at 0 and 20 minutes and centrifuged (2,500 g, 4°C, 10 min) and plasma was kept at -20°C until later analyses of galactose concentration as previously described (36).

Gastric emptying was assessed using an acetaminophen release assay as acetaminophen is entirely absorbed in the duodenum and can be detected in blood. Acetaminophen was given together with the second galactose bolus 48 h after initiation of enteral nutrition, as a single bolus at 160 mg/kg through the oro-gastric tube. Blood samples were drawn at 0, 20, 40, 60 and 120 minutes. Plasma levels of acetaminophen were determined in duplicates using a commercially available acetaminophen Kit (Multigent 2K99-20, Abbot Laboratories, Abbott Park, IL 60064, USA).

Finally, in vivo intestinal permeability was assessed using a dual sugar absorption test with mannitol and lactulose as previously described (4). Mannitol is absorbed mainly via the transcellular route, whereas lactulose is a marker of paracellular translocation, and the ratio of lactulose-to-mannitol in urine is an indicator of gut permeability (15). A bolus (15 mL/kg) of lactulose and mannitol (both 5% in Millipore water) was given via the oro-gastric tube 3-5 hours before euthanasia on day five. A urine sample was collected and concentrations of lactulose and mannitol were assayed as described previously (5).
**NEC evaluation:** On day five the piglets were weighed and euthanized (sodium-pentobarbitone 200 mg/kg administrated intra cardiac, after anesthesia with Zoletil mixture 1 mL/10 kg). The abdomen was opened and the liver, pancreas and spleen were taken out and placed on ice. The small intestine, from the pyloric sphincter to the ileo-cecal junction, was rapidly separated from the mesentery by cutting along the mesenteric border and placed on an ice cold metal plate. The total length of the small intestine was measured and then divided into three regions of equal length; designated proximal, middle and distal small intestine. The stomach, proximal, middle and distal small intestine and the colon were then evaluated by at least two trained observers for macroscopic pathological changes, indicative of NEC. The lesions were graded as:

1 = absence of lesions, 2 = local hyperemia, 3 = hyperemia, extensive edema and local hemorrhage, 4 = extensive hemorrhage, 5 = local necrosis and pneumatosis intestinalis, 6 = extensive transmural necrosis and pneumatosis intestinalis. An animal was considered as suffering from NEC when having a score of minimum 3 in at least one region.

**Organ weight and tissue collection:** First, the three regions (proximal, middle and distal) of the small intestine were carefully emptied and weighed separately. From the middle of each region 2 cm pieces were put into cryo tubes and snap frozen in liquid nitrogen and kept at -80°C for later analysis. Secondly, the distal region was again divided into three segments and from the middle of each segment a one cm piece was divided into two donut-like pieces and fixed in 4% paraformaldehyde for later assessment of histomorphometry, histopathology and bacterial adherence. Thirdly, a 10-cm piece from each small intestinal region was removed and slit along its length so the mucosa was exposed. The mucosa was gently scraped off with a plastic slide and the proportion of mucosa was determined on wet basis and subsequently on dry matter basis after drying both the mucosa and the underlying tissues (50°C for 72 hours). The luminal contents from the stomach and colon were collected for later measurements of organic acids (OA’s). The weight
of the empty stomach and colon were measured and the colon was then split in two halves and a slice was snap frozen in liquid nitrogen and kept at -80°C for later analysis. A second slice of the remaining colon was fixed in 4% paraformaldehyde and later embedded in paraffin for histopathology assessment. Finally, the wet weights were recorded for heart, lungs, liver, spleen and kidneys.

**Histomorphology and histopathology:** Paraformaldehyde fixed samples from the distal intestinal region and the colon were embedded in paraffin, sectioned (5µm), mounted on slides and stained with hematoxylin and eosin. According to sampling, six distal intestinal cross sections from each piglet were evaluated and crypt depth (µm) and villous height (µm) in twelve representative and well-oriented villous-crypt columns were measured, using an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) and the Image J 1.44 processing and analysis software program (http://imagej.nih.gov/ij/).

Histopathological lesions were evaluated for the distal small intestine and colon. The evaluation was done by a blinded observer, and criteria included 1) hyperemia (tissue appearing more red due to more red blood cells than seen in healthy tissue), 2) intravascular stasis (vessels full of red blood cells), 3) necrotic cells (condensed or fragmented nuclei), 4) sub-epithelial edema, 5) edema in the lamina propria, and 6) villous/epithelial sloughing. The presence of each criteria was evaluated; present or not present, and accumulated into an overall histopathologic score (six parameters on six tissue donuts for the distal small intestine, max score = 36 and six parameters for colon, max score = 6).

**Brush border enzyme activities and tissue IL-6 and IL-8:** Samples from the proximal, middle and distal small intestine and the colon were extracted in 1.0% Triton X-100 and homogenized. Homogenates were assayed for activity of disaccharidases (lactase, maltase, sucrase) and peptidases (aminopeptidase A [ApA], aminopeptidase N [ApN] and dipeptidylpeptidase IV
using lactose, maltose, sucrose, L-alanin-4-nitroanilide, glycyl-l-proline-4-nitro-anilide and α-L-glutamic acid 4-nitroanilide respectively, according to a previously described protocol (33).

The concentrations of IL-6 and IL-8 were determined on tissue samples from the distal small intestine. Homogenates, extracted in 1.0% Triton X-100, were added a 5.0% protease inhibitor P2714 (Sigma-Aldrich, St. Louis, USA). The homogenates were centrifuged (14,000 g, 4°C, 10 min) and the concentration of IL-6 and IL-8 was determined using porcine ELISA kits (DuoSet ELISA Development kits; R&D Systems, Abingdon, UK). The results were expressed per gram of wet intestine.

Microbiology: Bacterial microcolonies were visualized with fluorescent in situ hybridization (FISH) on 3 µm paraffin embedded cross sections of distal small intestine, using a 16S rRNA targeting Cy3 labeled oligonucleotide probe, EUB338, specific for the Bacterial Domain (5’-GCTGCCTCCCGTAGGAGT-3’, (22) and an GenePix 4200AL scanner (Molecular Devices, Sunnyvale, CA). A FISH-score was given according to bacterial abundance and localization on each of six tissue sections; 1 = no visible bacteria, 2 = few microcolonies, 3 = abundant bacteria located in the mucosal periphery, 4 = extensive colonization and bacteria located deep between the villi or 5 = translocation of bacteria. The scores from each of the 6 sections were accumulated into an overall score ranging from 6 (no adherent bacteria) to 30 (most severe). As for histopathology the evaluation was done by a blinded observer.

Finally, the concentration of organic acids (OA) in stomach and colon contents were measured as previously described (14). Briefly, the luminal contents were diluted in a sodium hydroxide solution containing 2-ethylbutyric acid as internal standard. The diluted samples were extracted with HCl and diethyl ether. Following centrifugation (3,000 g, 10 min), the ether layer was isolated and added to the reagent N-methyl-N-t-butyldimethylsilyl trifluoroacetamid. The
reaction mixture was incubated at 80°C for 20 min followed by a further incubation at room temperature for 48 h. Finally, the samples were injected into a gas chromatograph and the chromatograms were analyzed using HP GC ChemStation software (Technical Lab Service, ON, Canada).

**Statistical analyses:** All data were analyzed using STATA version 12. Weakness, euthanasia before end of study, and NEC incidence were evaluated using multilevel mixed effects logistic regression, with ‘treatment’ and ‘sex’ as fixed parameters and ‘sow’ and ‘pig’ as random effects. NEC severity, histopathology and FISH scores were evaluated with an ordered logistic regression model and robust standard errors. All continues variables were modeled using a linear mixed model with ‘treatment’ and ‘sex’ as fixed variables and ‘sow’ and ‘pig’ as random variables, if model requirements were met, otherwise robust standard errors were used. Data are presented as means ± SEM and P-values lower than 0.05 were considered significant.

**RESULTS**

**Clinical observations and in vivo tests:** Six of thirteen (46%) pigs in the HM group had episodes of weakness and one piglet had to be euthanized and sampled before day five. For the BC group these numbers were 5/13 (38%) and 1/13 (8%) respectively, while for the IF group the numbers were 13/14 (93%) and 9 (64%) respectively. For both parameters, HM and BC were better than IF (P < 0.01).

Intestinal passage time was longer for HM and BC relative to IF (49.7 ± 8.6 and 52.5 ± 9.2 vs. 24.9 ± 6.3 h respectively; P < 0.01), while the gastric emptying test showed no difference in plasma acetaminophen concentration indicating a similar release of the marker between the groups at the time of the test (data not shown).
For the initial galactose test (6 hours after initiation of enteral nutrition), plasma galactose concentration, as a marker of hexose absorptive capacity, was higher for HM and BC, compared with IF. The second galactose test (48 hours after initiation of enteral nutrition), showed higher levels for BC relative to IF (1326 ± 272 vs. 445 ± 156 μmol/L; P < 0.05), while HM only tended to be higher (999 ± 251 μmol/L), than IF (P = 0.08; Figure 1A). When comparing the concentration at the two different time points for each individual treatment group, no differences were observed. Finally, the lactulose-mannitol ratio in urine, as a marker of intestinal permeability, showed similar values for HM and BC, whereas sufficient number of urine samples from IF piglets were absent (data not shown).

**NEC incidence and severity:** The incidence of NEC was lower for HM and BC relative to IF (7/13 = 54% for both HM and BC vs. 13/14 = 93%; P < 0.05, Figure 1B). NEC severity scores for each separate region are shown in Figure 1C. Overall NEC severity for the HM and BC was lower relative to IF (1.45 ± 0.10 and 1.74 ± 0.25 vs. 2.81 ± 0.35 respectively; P < 0.01). Exceptions from this included the stomach where severity was lower for HM compared with BC and IF. For the mid and distal small intestinal region and colon the severity was lower for HM and BC compared with IF (P < 0.05).

**Body and organ weight, histomorphology and histopathology:** HM and BC showed similar body weight gain (14.0 ± 3.0 g/kg/d across groups), whereas IF lost weight (-8.0 ± 5.0 g/kg/d) during the experiment (both P < 0.01, Figure 1D). Body weights and organ weights relative to body weight are shown in Table 1.

The dry weight mucosal proportion of the small intestine was higher for HM and BC compared with IF (Figure 1E). Crypt depths for HM, BC and IF were 70 ± 4, 93 ± 10 and 71 ± 8 μm respectively and not found to be significantly different. Whereas villous height for HM, BC and IF were 527 ± 32, 482 ± 64 and 442 ± 60 μm respectively and not significantly different,
histopathologic evaluation of the distal small intestine and colon showed evidence of tissue injury in the IF tissue relative to HM and BC (e.g. villous sloughing, Figure 1F).

**Brush border enzymes and IL-6 and IL-8:** For all brush-border enzymes (three disaccharidases and three peptidases) across all small intestinal regions, the HM and BC groups had similar hydrolytic activities, except that BC had higher activity for lactase, maltase, sucrase and ApN in the mid region, whereas HM had higher DPP IV activity in the proximal region. In general HM and BC showed higher brush border enzyme activities than IF. Exceptions included maltase and sucrase activity that were either similar between the groups or elevated in IF especially in the mid and distal part of the intestine (Figure 2).

The concentration of IL-6 in the distal small intestine was markedly lower in HM relative to both BC and IF (1.15 ± 0.26 vs. 7.41 ± 6.23 and 8.28 ± 3.17 µg/g respectively; P < 0.05). The concentration of IL-8 was, however, lower for both HM and BC relative to IF (54.1 ± 5.6 and 52.7 ± 8.2 vs. 76.5 ± 9.0 µg/g respectively; P < 0.05).

**Microbiology and fermentation:** Adherence of bacteria to the intestinal epithelia quantified by FISH according to scoring was found to be lower for HM relative to BC (11 ± 1 vs. 16 ± 2; P < 0.05), and a similar tendency was found when comparing HM with IF (11 ± 1 vs. 18 ± 3; P = 0.08). The concentration of organic acids (OA’s) found in the stomach and colon is shown in Figure 3. In general total OA’s were higher in the stomach for BC and IF compared with HM. In the colon, the total concentration of OA was lower for both HM and BC relative to IF.

**DISCUSSION**

With our well-established animal model of NEC (4) we found similar reduction of NEC incidence in the HM and BC diet groups, compared with the IF group. This conclusion was supported by all clinical observations, the in vivo hexose absorption test, as well as most of the
structural and functional indices. Both HM and BC piglets gained body weight, whereas IF piglets lost body weight. Additionally, more piglets belonging to the IF diet group had episodes of weakness during the study, and more of these piglets had to be euthanized before the pre-planned end of the experiment. The NEC incidences for both the IF and BC groups were in the upper range of the NEC incidences found in some of our earlier studies (23) using slightly lower enteral feeding volumes. In one study (6), NEC was completely absent when bovine colostrum was first fed as minimal enteral nutrition for a few days (24-36 ml/kg/d before later transition to 120 mL/kg/d). It is well known that aggressive enteral feeding predisposes to NEC, but spontaneous NEC development in preterm piglets also shows considerable variation among studies, just like in human clinics. Regardless, the results of this study represent effects of different milk diets on the preterm neonate in a state of relatively rapid transition to full enteral feeding.

Results from the in vivo gastric emptying test suggested a similar release of fluid from the stomach to the duodenum, whereas it may be less indicative of retention of solids. More importantly however, intestinal transit time was lower in IF compared with HM and BC, indicating less time to digest and absorb nutrients, contributing to diarrhea in the IF group. Remarkably, the diarrhea symptoms were observed already after 6 hours of IF feeding.

Increased hexose absorption capacity, higher mucosal proportion and a general increase in digestive enzyme activity, all indicate better intestinal function in HM and BC piglets, compared with IF piglets and they support the clinical and pathological findings. The intestinal absorption of hexose occurs via the Na+/glucose co-transporter expressed in the brush-border membrane and is coupled to Na+ transport (40). The proinflammatory cytokine TNF-α, inactivates the Na+/K+ ATPase (26). Thus, decreased hexose absorption capacity indirectly indicates increased level of inflammation in the IF group. The lower level of IL-8 in the HM and BC groups further supports the notion that these two diets exert a protective effect against inflammation. One piglet
from the BC group showed an extremely high concentration of IL-6. Post hoc data analysis without this outlier showed that the remaining piglets from the BC group had IL-6 levels similar to HM, and lower levels than in IF. That particular piglet showed clinical signs of NEC and suffered also from peritonitis. Whereas an in vitro cell study has shown BC to cause increased levels of IL-6 and IL-8 (5), this was not reflected in the present in vivo piglet model, which is of greater clinical relevance.

The proinflammatory condition in the IF group was accompanied by reduced lactase and peptidase activities. In contrast, sucrase activity was highest in IF, compared with HM and BC. This finding may indicate high mitotic activity in crypt cells that show high sucrase activity. Normally, as enterocytes mature and migrate from the crypts toward the tip of the villous, they show higher activity of e.g. lactase (8; 24). Our observation indicate that an intestine, compromised by feeding an artificial milk formula, shows histological signs of villous sloughing and concomitant reduced lactase activity. Simultaneously however, regenerative crypt cell activity may take place to compensate and regenerate the villous structure.

We have previously shown that carbohydrate maldigestion is an important risk factor for NEC development. With the premature piglet model, lactose has shown high digestibility and partially protective effects against NEC, while maltodextrin is poorly digested and elicits a NEC response (37). Both the BC and HM diets contain lactose as their principal carbohydrate, while IF contains mainly maltodextrin. Moreover, the presence of immune- and bioactive components in both BC and HM is thought to reduce the inflammatory response and possibly select a beneficial microbial composition in the gut, even if product treatment (spray-drying, pasteurization, γ-radiation) may have damaged some of this milk bioactivity (2; 39). These milk factors include some components of the innate immune system, such as lactoferrin, lysozyme, lactoperoxidase and complement system. A number of cytokines are also found in BC and HM, including interleukins, tumor necrosis factor, and chemokines. In addition to these, BC and HM contain a number of
growth factors, such as insulin-like growth factors, transforming growth factors and epidermal growth factor and they are rich in antimicrobial factors (16; 32). HM is rich in oligosaccharides (human milk oligosaccharides, HMO) and these small carbohydrate molecules are known to possess decoy effects where they bind to pathogens hindering their attachment to the intestinal epithelia and hence, colonization (28). This could partly explain the lower FISH score in HM, compared with the BC and IF groups.

The histological and gut functional indices all support the notion that HM and BC provide similar protective effects, relative to IF, which induces more NEC. Maldigestion may be a key element in the pathogenesis and this is also supported by the increased concentration of OAs in the colon of the IF group. Non-digested nutrients from formula reach the colon where they can serve as substrates in bacterial fermentation. High concentration of small organic acids has been shown to be cytotoxic and cause epithelial cell death in an \textit{in vitro} model (30). Increased histopathology score and higher relative weight for the colon are indicative of inflammation and edema associated with IF feeding, relative to HM and BC. The increased concentration of OA’s produced by an expanding bacterial flora might cause extensive inflammation and hereby compromising a healthy and balanced intestinal integrity and function. The bacterial overgrowth triggering mucosal inflammation may initially increase intestinal peristalsis (diarrhea), followed by decreased intestinal peristalsis and later complete paralysis and stasis of luminal content. Intestinal stasis results in accumulation of nutrients in the stomach, and food intolerance is often seen in infants suspected for NEC (27). Accumulation of nutrients in the stomach together with excessive microbial gas production in the distal intestine and colon causes dilation and distended abdomen. This compromises the blood flow to the gastrointestinal tract, inducing hypoxia and later necrosis (6; 27).
The identified stomach lesions in some of the IF pigs appeared macroscopically to reflect circulatory disturbances. They appeared different from the lesions found in some of the BC pigs, where the lesions were more ulcerative although the nature of these lesions deserves further study. Stomach lesions in the BC group relates also to high levels of OAs in stomach contents, as shown previously (3). Specifically for the IF group, the high concentration of octanoic acid in stomach contents originates from fermentation of medium chain triglycerides supplied via the formula Liquigen MCT product. In the BC group, a large proportion of the NEC cases were due to lesions isolated to the stomach (NEC incidences with/without stomach lesions were 54% versus 31%). The gastric lesions were associated with the formation of a semi-solid curd, although this cannot be established as the cause of the lesions. Piglets display higher capacity to coagulate casein, relative to infants due to their high chymosin activity (9). This, together with impaired stomach acid secretion allowing significant bacterial growth, may explain why gastric lesions are relatively common in preterm pigs.

**PERSPECTIVES AND SIGNIFICANCE**

As the number of very low birth weight infants continues to rise, and as obstetrical procedures and neonatal care improve, the population at risk of NEC increases. Premature infants given their own mother’s expressed breast milk have a lower incidence and severity of NEC, relative to infants fed formula (18), but own mother’s milk is often unavailable or insufficient to meet the high nutrient demands in the premature infants. While infant formula may fulfill the nutritional demands, it lacks the immune- and bioactive components found in mother’s milk. Both mother’s and donor milk can be fortified to increase protein and mineral intake, but due to social, ethical, economical and ethnic reasons, it is difficult to ensure a continuous supply of human banked milk at many hospitals around the world. Bovine colostrum, or fractions of it, may be a plausible alternative when given at
the right time, and in optimal doses, avoiding gut overload and metabolic complications. It is rich in
growth-promoting factors and bioactive components, tailored toward neonates. It is designed to
serve as a link between a cow, and the developing immune system of her sensitive newborn calf
(16), and this may benefit preterm infants. We have previously shown similar efficacy of bovine
and porcine colostrum to prevent NEC in preterm pigs (4), and we now show that bovine colostrum
and human banked milk provide similar intestinal protection within the first week after birth. A
logical next step would be to test the effects of bovine colostrum, fed in slowly increasing volumes
over the first week of life to preterm infants, and compare results with feeding formula, human
banked milk or total parenteral nutrition. However, more basic research is needed to support the use
of alternatives to mother’s own milk as the first enteral diet. Aspects regarding the optimal feeding,
handling, storage and preparation (e.g. spray-drying, sterilization) of products are important. When
mother’s milk is not available, it is important to define the optimal feeding mode, quality, safety
and efficacy of the first enteral diet. This may have both short and long term health consequences,
especially for the weakest preterm infants.

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The authors have nothing to disclose.


FIGURE LEGENDS

**Figure 1.** A) *In vivo* hexose absorption test. B) Incidence of necrotizing enterocolitis (NEC). C) Severity of necrotizing enterocolitis (NEC). D) Relative daily weight gain. E) Mucosal proportion in the small intestine. F) Histopathology (necrosis, congestion, hyperemia, subepithelial edema, edema lamina propria and villous sloughing/loss of epithelia) of tissue in the distal small intestine and colon. For all; in piglets fed either human milk (HM, n = 13), bovine colostrum (BC, n = 13) or infant formula (IF, n = 14). Means ± SEM. Different superscript letters indicate significant differences, P < 0.05.

**Figure 2.** Activity of brush border enzymes in tissue from the small intestine and colon in piglets fed either human milk (HM, n = 13), bovine colostrum (BC, n = 13) or infant formula (IF, n = 14). Different letters indicate significant differences, P < 0.05.

**Figure 3.** Representative cross sections of distal small intestine from piglets fed either human milk, n = 13 (A), bovine colostrums, n = 13 (B) or infant formula, n = 14 (C). Fluorescent in situ hybridization using a Cy3 labeled oligonucleotide probe targeting the Bacterial Domain (red spots marked with a white arrowhead). Fluorescence are seen scarcely (few microcolonies), abundant in the mucosal periphery and deep and abundantly between the villi respectively.

Concentration of organic acids (OA) in the stomach (D) and the colon (E) of piglets fed either human milk (HM, n = 13), bovine colostrum (BC, n = 13) or infant formula (IF, n = 14). Means ± SEM. Different superscript letters indicate significant differences, P < 0.05.
**Table 1.** Birth weight, weight at euthanasia and relative organ weights

<table>
<thead>
<tr>
<th></th>
<th>HM</th>
<th>BC</th>
<th>IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>1149 ± 79</td>
<td>1074 ± 84</td>
<td>1161 ± 72</td>
</tr>
<tr>
<td>Kill weight (g)</td>
<td>1221 ± 87</td>
<td>1139 ± 91</td>
<td>1129 ± 93</td>
</tr>
<tr>
<td>Heart (g/kg)</td>
<td>7.38 ± 0.47</td>
<td>8.19 ± 0.91</td>
<td>7.55 ± 0.49</td>
</tr>
<tr>
<td>Stomach (g/kg)</td>
<td>5.76 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.39 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.35 ± 0.72&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (g/kg)</td>
<td>37.5 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.1 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.9 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen (g/kg)</td>
<td>2.02 ± 0.18</td>
<td>2.16 ± 0.09</td>
<td>1.86 ± 0.15</td>
</tr>
<tr>
<td>Kidneys (g/kg)</td>
<td>8.31 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.33 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.98 ± 0.36&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Length SI (cm/kg)</td>
<td>276 ± 20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>304 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>257 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total weight SI (g/kg)</td>
<td>26.9 ± 0.8</td>
<td>29.5 ± 1.3</td>
<td>29.4 ± 2.6</td>
</tr>
<tr>
<td>Colon (g/kg)</td>
<td>8.35 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.38 ± 0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.53 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values presented are means ± SEM. Means with different superscript letters within a row are significantly different (*P* < 0.05). HM: human donor’s milk (n = 13), BC: bovine colostrum (n = 13), IF: infant formula (n = 14), and SI: small intestine.
Figure 1

A

Galactose (mmol/L)

B

NEC incidence (%)

C

NEC severity (score)

D

Weight gain (g/kg/d)

E

Mucosal proportion (%)

F

Histopathology (score)
Figure 2

A. Lactase

B. Maltase

C. Sucrase

D. ApA

E. ApN

F. DPP IV
Figure 3

A  B  C

250 µm

Organic acids (mmol/kg)

D

HM
BC
IF

Organic acids (mmol/kg)

E

Formic acid  Acetic acid  Propionic acid  Butyric acid  Lactic acid  Succinic acid  Octanoic acid  Total OA

Legend:
- a
- b
- c

Significance levels:
- a
- b
- c

Note: The images and graphs depict the distribution and concentration of various organic acids in different samples, with significance levels indicated by letters.