Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets

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Li Y, Jensen ML, Chatterton DE, Jensen BB, Thymann T, Kvistgaard AS, Sangild PT. Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets. Am J Physiol Gastrointest Liver Physiol 306: G81–G90, 2014. First published October 24, 2013; doi:10.1152/ajpgi.00255.2013.—For preterm neonates, the quality of the first milk is crucial for intestinal maturation and resistance to necrotizing enterocolitis (NEC). Among other factors, milk quality is determined by the stage of lactation and processing. We hypothesized that unprocessed mature bovine milk (BM; raw bovine milk) would have less bioactivity than corresponding bovine colostrum (BC) in a preterm pig model, but have improved bioactivity relative to its homogenized, pasteurized, spray-dried equivalent, whole milk powder (WMP), or a bovine milk protein-based infant formula (IF). For 5 days, newborn preterm pigs received parenteral and enteral nutrition consisting of IF (n = 13), BM (n = 13), or BC (n = 14). In a second study, WMP (n = 15) was compared with IF (n = 10) and BM (n = 9). Compared with pigs fed IF, pigs that were fed BM had significantly improved intestinal structure (mucosal weight, villus height) and function (increased nutrient absorption and enzyme activities, decreased gut permeability, nutrient fermentation, and NEC severity). BC further improved these effects relative to BM (lactase activity, lactose absorption, plasma citrulline, and tissue interleukin-8). WMP induced similar effects as BM, except for lactase activity and lactose absorption. In conclusion, the maturational and protective effects on the immature intestine decreased in the order BC > BM > WMP, but all three intact bovine milk diets were markedly better than IF. The stage of lactation (colostrum vs. mature milk) and milk processing (e.g., homogenization, fractionation, pasteurization, spray-drying) are important factors in determining milk quality during the early postnatal period of preterm neonates.

colostrum; mature milk; processing; preterm; intestinal maturation

PREReTERM NEO NATES HAVE AN IMMATURE intestine that exhibits compromised nutrient absorption, reduced intestinal barrier function, and suboptimal immune responses toward microbes and food antigens (32). Consequently, preterm infants are very sensitive to enteral feeding and more prone to gastrointestinal complications, the most severe one of which is necrotizing enterocolitis (NEC). Early introduction of enteral feeding is beneficial for intestinal maturation and protection against NEC, but the quality of the diet may critically affect these positive effects. For example, the feeding of mother’s milk matures the preterm gut and reduces the risk of NEC relative to bovine milk-based infant formula (IF) and possibly also banked donor milk (22, 29, 34, 39, 40).

Donor milk (DM) is normally obtained at a relatively late stage of lactation, and therefore may not be appropriate as the first diet, when infants are supposed to receive mother’s colostrum. Colostrum is the first milk after parturition (1–3 days) and contains trophic factors and immunoregulatory factors in much higher concentrations than mature milk (e.g., >14 days of lactation) (8). Preterm infants may have a particularly high requirement for colostrum through its supply of bioactive factors for gut growth and immunological protection (53). Among preterm infants who develop NEC, the severity is greater if the first feeding is not colostrum (27). Comparable to DM, bovine milk (BM) used to produce infant formula (IF) is also mature milk, despite inherent differences between mature bovine and human milk (46). Apart from the stage of lactation, processing may also have a potent effect on milk quality. Unlike breast milk, both DM and IF normally undergo various milk processing procedures. Examples of these processes include pasteurization and spray-drying, which are potentially damaging to protein- and peptide-based milk growth and immune factors, including IgA, IgG, IgM, lactoferrin, lysozyme, and lactoperoxidase (11, 14, 15, 20, 26, 33, 45).

Performing randomized clinical trials with preterm infants is difficult, and the use of limited human colostrum for animal studies is equally difficult. We therefore used a well-established pig model of preterm infants (36) to investigate how the stage of lactation and milk processing would affect the bioactivity of BM to mature and protect the immature gut. In these pigs, we have previously shown that bovine colostrum (BC) is very effective in improving gut maturation and NEC resistance in the first week of life relative to IF (4, 28). We hypothesized that mature, raw BM would show reduced protective and maturational effects on the immature gut relative to fresh BC, and that a processed (e.g., homogenized, pasteurized, spray-dried) BM product would further decrease this bioactivity. In study 1, BM was compared with BC and a BM-based powdered IF product. In study 2, BM was compared with a homogenized, pasteurized, and spray-dried BM powder product (whole milk powder, WMP) and a BM-based powdered IF. Intestinal NEC severity, structure, digestive and absorptive functions, and cytokine responses were analyzed.

MATERIAL AND METHODS

Animals, Enteral Nutrition Protocol, and Experimental Design

Seventy-four preterm pigs were delivered from six sows by caesarean birth at 105 days of gestation (Large White × Danish Landrace × Duroc; Askelygaard Farm, Roskilde, Denmark; term = 116 ± 2 days).
The piglets were catheterized with orogastric feeding tubes (6-Fr, Portex; Smiths Medical, St Paul, MN) and vascular catheters (4-Fr, Portex) in an umbilical artery, and reared in temperature-regulated individual incubators with oxygen supply for the first 4–6 h. The pigs were given three doses of maternal serum (a total of 16 ml/kg) during the first 24 h after birth for passive immunization.

All pigs were administered parenteral nutrition (PN) solution through the vascular catheters for the first 2 days (day 1, 4 ml-1·kg-1·h; day 2, 6 ml-1·kg-1·h). The PN solution was based on a Kabiven three-chamber bag product (kindly donated by Fresenius nutrition (EN, 15 ml feeding tubes. Following the PN period, pigs were fed full enteral nutrition (EN, 15 ml-1·kg-1·h) for another 2 days until euthanasia and tissue collection as described previously (9). The studies were approved by the National Committee on Animal Experimentation in Denmark.

**Study 1.** The pigs (n = 40) were stratified according to birth weight and gender into three enteral feeding groups: 1) infant formula (IF, n = 13), 2) mature bovine milk (BM, n = 13; Assendrup Hovedgaard, Haslev, Denmark), pooled milk from 4 days to up to 1 year after parturition, and 3) bovine colostrum (BC, n = 14; Assendrup Hovedgaard, pooled colostrum from 1 to 3 days after parturition). IF was prepared by dissolving the following ingredients in demineralized water: per 1 liter of formula: 3,788 kJ energy; 80 g whey protein isolate (Arla Foods Ingredients, Viby, Denmark); 33 g Ross Polycolese (Abbott Nutrition, Columbus, OH); 12 g Seravit Paediatric (Scientific Hospital Supplies, Nutricia, Allerød, Denmark); 60 g Liqigen medium-chain triglyceride; and 40 g Calogen long-chain triglyceride (Nutricia, Allerød, Denmark). BM and BC were frozen once collected, and BM was freeze-dried into a powder at Gea Niro (Søborg, Denmark) to be freeze-dried for the entire small intestine was divided into three equal regions: proximal (Prox), middle (Mid), and distal (Dist).

Table 1. Nutrient composition of diets

<table>
<thead>
<tr>
<th>Component*</th>
<th>IF</th>
<th>BM</th>
<th>BC</th>
<th>WMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kJ</td>
<td>3,788</td>
<td>3,730</td>
<td>3,560</td>
<td>3,730</td>
</tr>
<tr>
<td>Protein, g</td>
<td>74</td>
<td>49</td>
<td>71</td>
<td>42</td>
</tr>
<tr>
<td>Glutamine, g</td>
<td>16</td>
<td>6</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Proline, g</td>
<td>5.4</td>
<td>4.9</td>
<td>5.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Carbohydrate, g</td>
<td>40</td>
<td>62</td>
<td>23</td>
<td>69</td>
</tr>
<tr>
<td>Maltodextrin, g</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactose, g</td>
<td>4</td>
<td>62</td>
<td>23</td>
<td>69</td>
</tr>
<tr>
<td>Fat, g</td>
<td>50</td>
<td>49</td>
<td>33</td>
<td>49</td>
</tr>
</tbody>
</table>

In Vivo Sugar Digestion and Absorption Test

To test intestinal permeability in vivo, pigs received an oral bolus (15 ml/kg body wt) containing 5% lactulose and 2% mannitol 3–5 h prior to euthanasia. Postmortem urine samples were taken to measure the concentrations of lactulose and mannitol as described previously (5). The lactulose-to-mannitol concentration ratio was calculated as an indicator of intestinal permeability.

**Clinical Evaluation and Tissue Collection**

Pigs were monitored every 3 h and euthanized if severe clinical symptoms of NEC appeared, such as abdominal distension, lethargy, cyanosis, or bloody diarrhea. If no symptoms were observed, pigs were euthanized on day 5 after birth. After anesthesia with a zoletil mixture (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; 1 ml/10 kg im), pigs were euthanized with an intracardiac injection of sodium pentobarbitone (200 ml/kg). Wet weights of heart, lungs, liver, kidneys, spleen, stomach, small intestine, and colon were recorded. The length of the small intestine was measured, and the entire small intestine was divided into three equal regions: proximal (Prox), middle (Mid), and distal (Dist).

Intestinal tissue samples were taken in the middle of each region and immediately snap-frozen in liquid nitrogen and stored at −80°C or fixed using paraformaldehyde solution for further analysis. Stomach and colon contents were also collected and snap-frozen in liquid nitrogen and stored at −80°C for organic acid analysis. Finally, 10-cm segments from the same position of each region were taken and slit along their lengths for determination of circumference and mucosal proportion. The mucosa was isolated by gentle scraping, and its weight proportion was determined on a dry matter basis as described previously (38).

Three regions of small intestine, stomach, and colon were graded using a macroscopic inflammatory lesion score (1, no or minimal focal hyperemic gastroenterocolitis; 2, mild focal gastroenterocolitis; 3, moderate locally extensive gastroenterocolitis; 4, severe focal gastroenterocolitis; 5, severe locally extensive hemorrhagic and necrotic gastroenterocolitis; or 6, severe extensive hemorrhagic and necrotic gastroenterocolitis). Pigs with a score of 3 or more in any of the intestinal regions (Prox, Mid, Dist, or Colon) were regarded as a case of NEC. Lesion severity was expressed as the means of the macroscopic inflammatory scores in the stomach, small intestine (average of Prox, Mid, and Dist), and colon.

**Intestinal Morphology**

Paraformaldehyde-fixed Prox and Dist samples were embedded in paraffin, sectioned (5 μm), mounted on slides, and stained with hematoxylin and eosin. Scanned images were obtained by use of a light microscope (Ortho-plane, Leitz, Germany) and an attached camera. Mean villus height (μm) and crypt depth (μm) were measured on scanned images in 10 representative vertically well-oriented villus-crypt axes by Image J software (version 1.44p, National Institutes of Health, Bethesda, MD). The average of the 10 villus heights and crypt depths in each region was used as the representative value for one pig.

**Intestinal Permeability Test**

To test intestinal permeability in vivo, pigs received an oral bolus (15 ml/kg body wt) containing 5% lactulose and 2% mannitol 3–5 h prior to euthanasia. Postmortem urine samples were taken to measure the concentrations of lactulose and mannitol as described previously (5). The lactulose-to-mannitol concentration ratio was calculated as an indicator of intestinal permeability.

**In Vivo Sugar Digestion and Absorption Test**

To determine the intestinal monosaccharide absorptive function after the MEN period, we measured the concentration of plasma...
galactose increment in response to oral boluses of galactose on day 3 before the initiation of full EN. All pigs were given a bolus (15 mL/kg body wt) of 5% galactose via orogastric feeding tube before being transferred to full EN on day 3. Blood samples were collected into heparinized tubes from the umbilical artery catheter before and 20 min after administration. To determine the combined digestive and absorptive capacity of disaccharide, we measured the plasma levels of galactose increment in response to oral boluses of lactose on day 5 before euthanasia. All pigs were given a bolus (15 mL/kg body wt) of 10% lactose solution, and blood samples were obtained as described for the galactose test. Concentrations of galactose in plasma were measured spectrophotometrically, as described previously (47).

Ex Vivo Brush Border Enzyme Activities

Activities of brush border enzymes, lactate, maltase, sucrase, aminopeptidase N (APN), aminopeptidase A (ApA), and dipeptidyl peptidase IV (DPPIV) were analyzed in homogenates of Prox, Mid, and Dist tissues by spectrophotometry as described previously (35). Enzyme activities were expressed as units per gram of wet tissue. Tissue homogenates were obtained by homogenizing the tissue sample in 1% Triton X-100 water solution (10 mL/g tissue).

Organic Acids

Fifteen organic acids (OAs) were measured in stomach and colon contents by gas chromatography (GC) as described previously (7). Briefly, content sample (1 g) was diluted in a sodium hydroxide solution, and 2-ethylbutyric acid was used as an internal standard. Quantification of citrulline was performed using QuanLynx (Waters).

Statistical Analysis

Group differences of NEC incidence were evaluated by Fishers exact test (Graph Pad Prism 5; Graph Pad Software, La Jolla, CA). Lesion scores were analyzed by a nonparametric Kruskal-Wallis test (JMP 9; SAS Institute, Cary, NC). The effect of diet on continuous response variables and the correlation between two continuous response variables were evaluated by a linear mixed model (JMP 9). Post hoc comparison between groups was performed with Tukey’s correction. Binary data are presented as a percentage (%), and continuous data are presented as arithmetic means ± SE. P < 0.05 was considered significant.

RESULTS

Clinical Outcomes

There were no differences among IF, BM, and BC groups in study 1, nor among IF, BM, and WMP groups in study 2 in birth weight (937 ± 41 and 1,016 ± 36 g, respectively), daily weight gain (−1.8 ± 4.4 and −0.8 ± 3.1 g, respectively), or life time (97.4 ± 1.2 and 97.7 ± 1.2 h, respectively). NEC was observed in one IF-fed pig in study 1, and one WMP-fed pig in study 2 at, respectively, 12 and 29 h after initiation of full enteral feeding, and these animals were immediately euthanized for tissue collection. The remaining pigs were all euthanized on day 5 as planned. Relative to body weight, liver weights were higher in the IF group compared with the BM and BC groups in study 1, and with the BM and WMP groups in study 2 (P < 0.05; Table 2). Relative kidney weight was lower in the IF group relative to that in BC and IF groups in study 1, whereas relative kidney weight was lower in the BM and WMP groups relative to the IF group in study 2 (P < 0.05; Table 2). Relative stomach weight was higher in the BC group.

Table 2. Relative organ weights, small intestinal structure, NEC incidence, and lesion scores

<table>
<thead>
<tr>
<th>Organ</th>
<th>Study 1</th>
<th></th>
<th></th>
<th>Study 2</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>IF</td>
<td>BM</td>
<td>BC</td>
<td>IF</td>
<td>BM</td>
<td>WMP</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>13</td>
<td>14</td>
<td>10</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Liver, g/kg</td>
<td>31.9 ± 0.8 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.0 ± 1.3 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.5 ± 1.0 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.9 ± 1.1 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.9 ± 1.1 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>25.7 ± 0.9 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney, g/kg</td>
<td>11.0 ± 0.4 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5 ± 0.5 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.6 ± 0.5 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.5 ± 0.5 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.7 ± 0.6 &lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.8 ± 0.4 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stomach, g/kg</td>
<td>6.7 ± 0.3 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2 ± 0.5 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.1 ± 0.9 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 0.4</td>
<td>5.9 ± 0.4</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter, %</td>
<td>15.4 ± 0.2 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.0 ± 0.3 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5 ± 0.2 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.7 ± 0.4 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.8 ± 0.4 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.0 ± 0.3 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mucosa proportion, %</td>
<td>62.7 ± 1.8 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.3 ± 1.9 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.2 ± 1.7 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.7 ± 1.8</td>
<td>69.1 ± 1.9</td>
<td>65.7 ± 1.5</td>
</tr>
<tr>
<td>Prox villus height, μm</td>
<td>301 ± 47 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>645 ± 48 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>632 ± 46 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>360 ± 53 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>639 ± 39 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>594 ± 28 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prox villus/crypt ratio</td>
<td>4.7 ± 0.7 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.3 ± 0.9 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.9 ± 1.0 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8 ± 0.9 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.1 ± 0.7 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.7 ± 0.4 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEC incidence, %</td>
<td>54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
<td>14</td>
<td>38</td>
</tr>
<tr>
<td>Stomach lesion score</td>
<td>1.2 ± 0.2 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.1 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4 ± 0.6 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.5</td>
<td>1.6 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Small intestine lesion score</td>
<td>1.7 ± 0.3 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.0 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2 ± 0.1 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Colon lesion score</td>
<td>2.8 ± 0.6</td>
<td>1.3 ± 0.2</td>
<td>2.0 ± 0.4</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.9 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Dry matter was calculated as dry weight divided by wet weight. Mucosal proportion was calculated as mucosal dry weight divided by the dry weight of total small intestine. In each study, means not sharing the same superscript letter are significantly different (P < 0.05). NEC, necrotizing enterocolitis; Prox, proximal small intestine.
compared with that in the IF and BM groups in study 1 (P < 0.05), and this was associated with a higher stomach lesion score in the BC group (P < 0.05; Table 2). The relative weight of other organs, including relative intestinal length, did not differ among treatment groups in either study (data not shown). In study 1, the IF group had a higher NEC incidence (7/13) than the BM group (1/13, P < 0.05) and the BC group (3/14, P = 0.12), with the lesion score being highest in the small intestine (Table 2). In study 2, NEC incidence and severity did not differ significantly among groups (Table 2).

Intestinal Morphology and Permeability

In study 1, a compromised small intestinal structure was observed in the IF group relative to the BC and BM groups, indicated by the presence of shorter villi (P < 0.05), a lower villus-to-crypt ratio (P < 0.05) in the proximal intestine, and a lower mucosal dry weight proportion relative to BM (P < 0.05) or BC (P = 0.06, Table 2). In study 2, markedly shorter proximal villi and villus-to-crypt ratios were observed in the IF group relative to the BM and WMP groups (P < 0.05; Table 2). The BM group had lower dry matter content in the small intestine relative to the BC and IF groups in study 1 (P < 0.05), and this was lower in both the BM (P = 0.05) and WMP (P < 0.05) groups relative to the IF group in study 2 (P < 0.05; Table 2). Intestinal permeability, as assessed by the urinary lactulose-to-mannitol ratio, was higher in IF-fed pigs than BM- and BC-fed pigs in study 1 (P < 0.05; Fig. 1A), and also higher than BM- and WMP-fed pigs in study 2 (BM, P < 0.05; WMP, P = 0.06; Fig. 1D).

Intestinal Digestive and Absorptive Capacity

A plasma galactose increment at 20 min in response to an oral bolus of galactose was used to indicate intestinal monosaccharide absorptive capacity before initiation of full EN. This was reduced in the two IF groups compared with the BC and BM groups in study 1 (P < 0.05; Fig. 2A), and with the BM and WMP groups in study 2 (P < 0.05; Fig. 2C). At the end of the full EN period, the increment of plasma galactose at 20 min in response to an oral bolus of lactose was determined to indicate the combined lactose digestive and absorptive capacity. Fifty-seven percent of the pigs in study 1 and 70% of the pigs in study 2 had detectable plasma galactose at 20 min, defined as responders, whereas those with no detectable plasma galactose at 20 min were defined as nonresponders. The number of responders was affected by diet, and the fewest responders were found in the IF group in both studies (P < 0.05; Fig. 2, B and D). Among responders, BM-fed pigs had lower lactose digestive and absorptive capacity compared with BC-fed pigs.
fed pigs in study 1 (P < 0.05; Fig. 2A), whereas this capacity was further reduced in WMP-fed pigs relative to BM-fed pigs in study 2 (P < 0.05; Fig. 2C).

**Ex Vivo Digestive Enzyme Activities**

In study 1, enzyme activities were analyzed across the three regions and were generally highest in the BC group, intermediate in the BM group, and lowest in the IF group (Fig. 3, A–C and Fig. 4, A–C). Lactase activity differed significantly among groups, especially in Prox and Mid (P < 0.05; Fig. 3C), consistent with results of the in vivo lactose day 5 test. In study 2, maltase (Fig. 3E) and DPP IV (Fig. 4F) activities were lower in the IF group relative to the BM and WMP groups (P < 0.05), whereas a higher distal ApA activity was observed in the IF group relative to the BM and WMP groups (P < 0.05; Fig. 4D). BM-fed pigs had the highest lactase activity compared with both WMP- and IF-fed pigs, and the effect was most pronounced in Prox and Mid (P < 0.05; Fig. 3C), again confirming the results of the in vivo lactose test.

**Organic Acids**

Among the 15 investigated OAs, acetate, lactate, succinate, butyrate, caproate, and octanoate were detected in stomach contents. Formate, acetate, propionate, lactate, succinate, butyrate, and octanoate were detected in colon contents. Octanoate is mainly derived from the lipid fraction of bovine colos- 

trum/milk, and medium-chain triglycerides from Liquigen used in IF rather than from bacterial fermentation. Consequently, octanoate is not shown in Fig. 5 and was omitted from total OA calculation for both stomach and colon contents. In stomach contents, BC-fed pigs had higher acetate and total OAs than BM- and IF-fed pigs in study 1 (P < 0.05; Fig. 5A); whereas in study 2, BM- and WMP-fed pigs had higher lactate, butyrate, and total OAs relative to IF-fed pigs (P < 0.05; Fig. 5C). In colon contents, IF-fed pigs had less formate and more lactate relative to BC-fed pigs in study 1 (P < 0.05; Fig. 5B), whereas more lactate was also observed in IF-fed pigs relative to BM- and WMP-fed pigs in study 2 (P < 0.05; Fig. 5D).

**Plasma Citrulline and Proinflammatory Cytokines**

Concentrations of plasma citrulline were higher in the BC group than those in the IF (65.8 ± 7.2 μmol/L; P < 0.01) and BM groups (81.2 ± 11.6 μmol/L; P < 0.05) in study 1 (Fig. 1B). The concentrations were not different among the three groups in study 2, the pooled value being 83.2 ± 6.2 μmol/L (Fig. 1E). The concentration of IL-8 in Dist was reduced in the BC-fed pigs compared with IF- and BM-fed pigs in study 1 (P < 0.05; Fig. 1C), whereas it did not differ among groups in study 2 (Fig. 1F). Dist IL-1β and TNF-α were not different among groups in study 1 and study 2. Pooled values for IL-1β and TNF-α in study 1 were 4.7 ± 0.9 and 0.5 ± 0.1 pg/mg tissue, respectively; in study 2 they were 7.6 ± 2.6 and 0.9 ± 0.2 pg/mg tissue, respectively. The concentration of Dist IL-1β but not IL-8 correlated positively with Dist NEC severity (r² = 0.40; P < 0.01).

**DISCUSSION**

Milk quality plays a crucial role in response of the immature gut to the first enteral feeding. Nevertheless, it remains unknown how basic determinants such as stage of lactation and milk processing affect gut responses during the first week of life, and thus optimal feeding strategies. Ethical and practical challenges in performing randomized feeding intervention

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**Fig. 2.** Detectable increments of plasma galactose 20 min after administration of oral boluses of galactose solution (relative to before administration) on day 3 (Galactose d 3) or lactose solution on day 5 (Lactose d 5) in study 1 (A) and study 2 (C); number of pigs with (Responder) or without (Nonresponder) detectable plasma galactose increment at Lactose d 5 test in study 1 (B) and study 2 (D). Values are means ± SE: (Galactose d 3, n = 9–14; Lactose d 5, n = 1–12) or number of Responders and Nonresponders (n = 7–13). In each study, means not sharing the same letter within the same region are significantly different in increments of plasma galactose, and groups not sharing the same letter are significantly different in the proportion of Responder (P < 0.05).
studies in preterm infants and the numerous biological, clinical, and technological factors that affect natural milk and infant formulas explain this lack of knowledge. We now show that the bioactivity of raw, mature BM was moderately inferior to that of corresponding BC in supporting maturation of gut structure and function during the first week after preterm birth. WMP (i.e., BM subjected to considerable heat treatment) showed a further slight decline in bioactivity. However, major gut maturational deficits were found in groups fed the most artificially composed diet, IF, which was formulated on the basis of commercially available ingredients for infants. We conclude that stage of lactation and milk processing influence gut responses in preterm neonates.

We have previously identified that the carbohydrate fraction (lactose, maltodextrin) plays a key role in determining formula quality in preterm pigs (49), but also that the quality of the whey protein fraction (which is produced by different processing methods) also has a substantial influence, at least in preterm pigs (19). Interestingly, in this study we also identified lactose digestive and absorptive capacity to be very sensitive to the differences between BC and mature milk, and also between milk powders produced with and without heat treatment. In preterm infants, deficient lactase activity and lactose absorption may have a direct relation with feeding intolerance and NEC (42).

Although BM contains milk-borne trophic and immune-regulatory factors, including lactoferrin, lysozyme, osteopontin, immunoglobulins, lactoperoxidase, IL-10, TGF-β, insulin-like growth factors (IGFs), and epidermal growth factor, these are present in much higher amounts in colostrum (e.g., 10–100 fold for lactoferrin, TGF-β, immunoglobulins, IGFs, and osteopontin) (8, 51). These differences may explain a large part of the specific role of colostrum that is particularly important for immature newborns. Relative to IF and BM, BC feeding increased levels of plasma citrulline, an amino acid that can be used as a marker of small intestinal mucosal mass and function. Plasma citrulline originates mainly from enterocytes with glutamine and proline being its precursors (3), the former providing ~80% of plasma citrulline in humans (10, 52). In neonatal pigs, enteral proline is the main precursor for citrulline synthesis, followed by glutamine, on the basis of in vivo, isotopic kinetic measurements (25). The glutamine content of the IF diet was greater than that in BC, BM, and WMP diets, whereas the proline content was comparable among the four diets (Table 1). This indicates that the different plasma citrulline levels we observed are likely a result of the total mass and/or function of enterocytes rather than the different dietary intake of its precursors. Further support for a separate role for BC is found in the lowered IL-8 protein levels in the distal intestinal tissues, which is consistent with other studies in preterm pigs (43) and intestinal epithelial cells, where BC inhibited the nuclear factor-κB signaling pathway and reduced IL-8 levels (2). In the premature intestine, overexpression of IL-8 is related to later intestinal complications, such as development of NEC (30).

The WMP powder used in the present study is considered to be more severely processed than DM (Holder pasteurization; 62.5°C, 30 min). In one study, the IGF-I content in a commercially available WMP product was not affected relative to its raw milk counterpart (16), whereas immunoglobulins, lacto-
ferrin, lactoperoxidase, and lysozyme were at least partially destroyed during pasteurization and spray-drying (15, 20, 26, 45). Similar to WMP, pasteurization and spray-drying are normally applied as part of IF production processes. Unlike WMP, however, IF production also includes various ingredient separation and modification steps employing whey separation, membrane-filtration, replacing milk fat with vegetable oil, and replacing lactose with maltodextrin (46). These modifications...
result in the loss of bioactive proteins and peptides that are heat stable (e.g., TGF-β and IGF-I) (1), and also loss of bioactive factors in milk fat and carbohydrate fractions. As noted above, using maltodextrin instead of lactose in IF is associated with increased NEC incidence, at least in preterm pigs (49).

Not only the maltodextrin-based IF used in the present study, but also several commercially available preterm IFs (our unpublished results) have been associated with NEC and intestinal deficits in our preterm pig model, relative to intact milk products. These IF-related intestinal deficits may not always lead to the outbreak of NEC in the short term but are likely to increase NEC-related risk factors such as villus atrophy and increased intestinal permeability. Reduced villus height, villusto-crypt ratio, and increased intestinal permeability were observed in IF-fed pigs in both of the present studies, although IF-fed pigs developed less NEC in study 2. When we prolong the duration of our preterm pig model beyond 5 days, an increasing number of pigs develop NEC during the following week (our unpublished data). The increased villus atrophy and permeability may enhance the invasion of bacteria and toxins via paracellular routes, or even translocation to the blood stream, leading to later inflammation, infection, and sepsis during this extended experimental period. The increased liver weight in IF-fed pigs in both studies may also imply hepatic inflammation related to elevated intestinal permeability (13, 17).

Preterm pigs more frequently develop gastric inflammatory necrosis, relative to preterm infants (50). This occurs most often in BC-fed pigs, but it is also observed in BM-, WMP-, and IF-fed pigs.Unlike human infants, piglets produce chymosin, which coagulates casein, and they have a low acid-secretory capacity at birth (37), which increases the risk of gastric food retention, bacterial overgrowth, bacterial fermentation, and subsequent tissue inflammation. The higher incidence of gastric inflammatory necrosis in BC-fed pigs may be related to the high protein/casein content. On the other hand, casein effects are unlikely to fully explain these lesions because BM and WMP diets also contain significant amounts of casein, whereas IF contains no casein.

Galactose transport decreased dramatically after just 2 days of MEN IF feeding, consistent with earlier studies (9, 28). The intestinal uptake of galactose and glucose is carrier-mediated and is mainly determined by the sodium glucose-linked transporter-1 (SGLT-1) protein. SGLT-1 is located along the entire length of the small intestine, but its highest expression occurs in the duodenum and jejunum, and in the upper villus (6). The reduced proximal mucosal mass and villus height in IF-fed pigs may thus contribute to their decreased galactose absorptive function. In IF-fed rat pups, SGLT-1 is upregulated by IGF-I and IGF-II (18), and such a mechanism may also play a role for the BC, BM, and WMP effects in this study. In our study, most of the nonresponders during the lactose test were found in the IF groups, and the combined effect of reduced lactase activity and reduced SGLT-1 activity explains this. Interestingly, pigs delivered at 105 days of gestation in the present study exhibited a substantially lower galactose increment 20 min after ingestion of a lactose solution (200 and 50 μmol/l for pigs fed BC and IF, respectively) compared with pigs delivered close to term at 113 days in a previous study (1,000 and 120 μmol/l, respectively) (47). These results suggest that intestinal lactose digestion capacity depends on both diet and gestational age at delivery.

Plasma galactose increments during the lactose test were generally in good agreement with measured brush border lactase activities ex vivo (i.e., they were lowest in IF- and WMP-fed pigs, intermediate in BM-fed pigs, and highest in BC-fed pigs). This also indicates that dietary lactose does not play a direct role in stimulating lactase activity because the lactose content in WMP and BM diets were similar and far higher than that in the BC diet. Mother’s milk has also been reported to be superior to formula in enhancing lactose absorption in preterm infants (41, 42). Taken together, it appears that lactase activity and glucose-galactose absorption are very sensitive parameters in response to the stage of lactation and milk processing. Other brush-border enzyme activities were also increased by BC feeding, but in contrast to lactase, WMP feeding did not reduce these, relative to BM feeding.

Bacterial fermentation produces OAs from carbohydrate and protein precursors (54). The level of OAs in colon contents is an indicator of the degree of bacterial fermentation of unabsorbed nutrients. In this study, the level of colon lactate in each group is in general agreement with the activity of brush-border lactase and lactose absorption (e.g., greater lactase activity and lactose absorption are associated with less colon lactate). Excessive fermentation of unabsorbed nutrients may in turn promote bacterial overgrowth, and some fermentation products such as acetate have been shown to induce colitis and other mucosal injury in rat models (21, 24). Stomach OAs may relate partly to dietary nutrient composition. For example, a higher butyrate content in the stomach of pigs fed BC, BM, and WMP diets may partly result from the fact that milk fat content contains 3.3% butyrate, whereas very little butyrate is present in the vegetable oils used in the IF diet (46).

Our results provide important information on how the stage of lactation and milk processing influence the bioactivity of milk on gut responses to the first feeds. We conclude that raw, mature BM was slightly inferior to BC in improving the intestinal integrity in preterm pigs, but both are markedly better than IF for intestinal protection and maturation. The slightly reduced bioactivity of WMP in preterm pigs indicates that milk processing, such as pasteurization and spray-drying, may reduce maturational effects of BM, although the main deficits appear to be associated with the multiple processing steps of IF production. These data suggest that providing colostrum during the first weeks of life is of great importance for intestinal health in preterm newborns. The results indicate that milk processing applied to whole milk powder production (e.g., homogenization, pasteurization, and spray-drying) is not as detrimental as milk processing applied to IF production, including modification of milk fractions (e.g., whey proteins, casein, lactose, oligosaccharides, lipids).

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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