Whey Protein Processing Influences Formula-Induced Gut Maturation in Preterm Pigs

Yanqi Li, Mette V. Østergaard, Pingping Jiang, Dereck E. W. Chatterton, Thomas Thymann, Anne S. Kvistgaard, and Per T. Sangild

Abstract

Immaturity of the gut predisposes preterm infants to nutritional challenges potentially leading to clinical complications such as necrotizing enterocolitis. Feeding milk formulas is associated with greater risk than fresh colostrum or milk, probably due to loss of bioactive proteins (e.g., immunoglobulins, lactoferrin, insulin-like growth factor, transforming growth factor-$

\beta$

) during industrial processing (e.g., pasteurization, filtration, spray-drying). We hypothesized that the processing method for whey protein concentrate (WPC) would affect gut maturation in formula-fed preterm pigs used as a model for preterm infants. Fifty-five caesarean-delivered preterm pigs were distributed into 4 groups given 1 of 4 isoenergetic diets: formula containing conventional WPC (filtration, multi-pasteurization, standard spray-drying) (CF); formula containing gently treated WPC (reduced filtration and pasteurization, gentle spray-drying) (GF); formula containing minimally treated WPC (rennet precipitation, reduced filtration, heat treatment $<$40°C, freeze-drying) (MF); and bovine colostrum (used as a positive reference group) (BC).

Relative to CF, GF, and MF pigs, BC pigs had greater villus heights, lactose digestion, and absorption and lower gut permeability ($P<0.05$). MF and BC pigs had greater plasma citrulline concentrations than CF and GF pigs and intestinal interleukin-8 was lower in BC pigs than in the other groups ($P<0.05$). MF pigs had lower concentrations of intestinal claudin-4, cleaved caspase-3, and phosphorylated c-Jun than CF pigs ($P<0.05$). The conventional and gently treated WPCs had similar efficacy in stimulating proliferation of porcine intestinal epithelial cells. We conclude that processing of WPC affects intestinal structure, function, and integrity when included in formulas for preterm pigs. Optimization of WPC processing technology may be important to preserve the bioactivity and nutritional value of formulas for sensitive newborns.


Introduction

Preterm infants are born with an immature intestine that shows reduced motility, digestive and absorptive capacity, an underdeveloped immune system, and altered bacterial colonization (1). This intestinal immaturity makes them more prone to various complications, including feeding intolerance, postnatal growth restriction, infections, and necrotizing enterocolitis (NEC) (2,3).

Clinical complications of NEC are diabetes, acid-base disturbance, electrolyte imbalance, fluid restriction, infections, and necrotizing enterocolitis. Feeding milk formulas is associated with greater risk than fresh colostrum or milk, probably due to loss of bioactive proteins (e.g., immunoglobulins, lactoferrin, insulin-like growth factor, transforming growth factor-$

\beta$

) during industrial processing (e.g., pasteurization, filtration, spray-drying). We hypothesized that the processing method for whey protein concentrate (WPC) would affect gut maturation in formula-fed preterm pigs used as a model for preterm infants. Fifty-five caesarean-delivered preterm pigs were distributed into 4 groups given 1 of 4 isoenergetic diets: formula containing conventional WPC (filtration, multi-pasteurization, standard spray-drying) (CF); formula containing gently treated WPC (reduced filtration and pasteurization, gentle spray-drying) (GF); formula containing minimally treated WPC (rennet precipitation, reduced filtration, heat treatment $<$40°C, freeze-drying) (MF); and bovine colostrum (used as a positive reference group) (BC).

Relative to CF, GF, and MF pigs, BC pigs had greater villus heights, lactose digestion, and absorption and lower gut permeability ($P<0.05$). MF and BC pigs had greater plasma citrulline concentrations than CF and GF pigs and intestinal interleukin-8 was lower in BC pigs than in the other groups ($P<0.05$). MF pigs had lower concentrations of intestinal claudin-4, cleaved caspase-3, and phosphorylated c-Jun than CF pigs ($P<0.05$). The conventional and gently treated WPCs had similar efficacy in stimulating proliferation of porcine intestinal epithelial cells. We conclude that processing of WPC affects intestinal structure, function, and integrity when included in formulas for preterm pigs. Optimization of WPC processing technology may be important to preserve the bioactivity and nutritional value of formulas for sensitive newborns.

ingredient in IF as whey protein concentrate (WPC) (16,17). To produce WPCs, pasteurized milk is precipitated by adding rennet or acid and a soluble whey fraction containing proteins, minerals, and lactose is separated from the precipitated casein. Proteins in the separated whey fraction are further concentrated and made into WPC powder after various processes, including pasteurization, membrane filtration, evaporation, and spray-drying (18). These processes may cause a considerable reduction of many bioactive proteins in WPC and thus in IF (15).

We hypothesized that formulas containing mildly produced WPCs, e.g., reduced filtration and pasteurization steps, gentle spray-drying, or freeze-drying, would improve the effects of formula feeding on gut maturation in preterm pigs. Preterm pigs show a high sensitivity to differences in the quality of the first enteral feeding (19,20). We compared the gut maturational effect of 3 formulas containing conventional WPC, gently treated WPC, and minimally treated WPC. BC was included as a positive reference group based on its consistent maturational effect (19,20). Intestinal morphology, plasma citrulline concentration (a biomarker of mucosal mass and function), brush border enzyme activities, in vivo sugar tests, and nutrient fermentation were analyzed to determine intestinal structure, barrier, digestive, and absorptive functions. Proinflammatory cytokines in intestinal tissue were analyzed to determine mucosal immune responses. In addition, Western blotting was applied to determine the amount of proteins related to proliferation, apoptosis, and tight junctions. Finally, we used a porcine intestinal epithelial cell line to investigate the effect of WPCs on cell proliferation in vitro. In this study, the gut maturational effects were investigated in a state of relatively low NEC risk, secured by using lactose as the major carbohydrate source in the 3 formulas (21).

Materials and Methods

Pigs and experimental design. Fifty-five preterm pigs were delivered from 5 sows by cesarean section at 105 d gestation (Large White \times Danish Landrace \times Duroc, Askelygaard Farm; term = 116 ± 2 d). Surgical preparation with an oro-gastric feeding tube and a vascular catheter for parental nutrition (PN) and passive immunization took place in Denmark.

The nutrient composition of the 3 formulas was adjusted to fit the requirements of pigs by blending the following ingredients that are used for IF manufacture: protein (conventional, gently treated, and minimally treated WPCs), lactose [Variatol 960, Arla Foods Ingredients (AFI)], maltodextrin (Ross Polycose; Abbott Nutrition), lipids (Liquigen and Calogen; Nutricia), and vitamins and minerals (SHS Seravit; Nutricia). The amounts of each ingredient were adjusted to ensure the same macronutrient composition and energy density among the 3 formulas (Table 1). BC (obtained from Assendrup Hovedgaard) was sterilized by γ-irradiation at 5.0 kGy (Sterigenices) and stored at −20°C prior to each feeding. BC was diluted in demineralized water (2:1) to adjust the concentration of protein and energy density to levels that were similar to those in the 3 formulas (Table 1).

The 3 WPCs were produced from pooled bovine milk obtained from Danish dairy cows (Danish Red and Danish Holstein-Friesian) using different precipitation, membrane filtration, pasteurization, and drying methods (Fig. 1). Briefly, conventional WPC was manufactured at AFI from acid whey using a proprietary manufacturing process, including multiple pasteurization steps, standard membrane filtration steps, a special filtration step, and spray-drying (18). Gently treated WPC was also produced at AFI from acid whey under industrial processing conditions with gentle processes, e.g., no special filtration, reduced pasteurization, and low-temperature spray-drying. Minimally treated WPC was produced from sweet whey under laboratory processing conditions in a dairy pilot plant at the University of Copenhagen with minimal processes, e.g., rennet precipitation, no special filtration, processing temperature <40°C, and freeze-drying (Gea Niro). The minimally treated WPC was sterilized by γ-irradiation at 5.0 kGy (Sterigenices) and stored at −20°C. The other 2 WPCs and other ingredients were stored at 4°C. Formulas were prepared by blending WPCs and other ingredients in demineralized water and stored at 4°C (Fig. 1) and aliquots were warmed to 37°C prior to each feeding.

Macronutrient composition and bioactive markers. The macronutrient composition of the formulas was calculated based on the product specification provided by manufacturers. Energy and macronutrient composition of BC and native LF and IgG in WPCs and BC were analyzed by Eurofins Steins Laboratorium. BC, bovine colostrum; CF, formula containing conventional whey protein concentrate; GF, formula containing gently treated whey protein concentrate; LF, lactoferrin; MF, formula containing minimally treated whey protein concentrate; WPC, whey protein concentrate.

Clinical evaluation and sample collection. Pigs were continually monitored and were killed with an intracardiac injection of pentobarbitone sodium (60 mg/kg) if clinical symptoms of NEC appeared or on d 5 for tissue collection according to the procedures previously described (19,23). The mucosal lesion in the stomach, 3 regions of small intestine [proximal (Prox), middle (Mid), and distal (Dist)] and colon were macroscopically evaluated and a lesion score (1–6) was assigned to each region as previously described (19). Pigs with a score of ≥3 in any of the Prox, Mid, Dist, or colon regions were diagnosed as NEC.

Gut structure, digestive capacity, and SCFAs. Paraformaldehyde-fixed tissues from Prox and Dist were processed for measuring of villus heights and crypt depths as previously described (19). To test the

**Table 1** Macronutrient compositions and concentrations of LF and IgG of CF, GF, MF, and BC

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>GF</th>
<th>MF</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>3.84</td>
<td>3.72</td>
<td>3.76</td>
<td>3.56</td>
</tr>
<tr>
<td>Protein, g/L</td>
<td>74</td>
<td>70</td>
<td>70</td>
<td>71</td>
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<tr>
<td>Whey protein, g/L</td>
<td>74</td>
<td>70</td>
<td>70</td>
<td>54</td>
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<td>LF, g/L</td>
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<td>0.66</td>
<td>0.49</td>
<td>0.67</td>
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<td>IgG, g/L</td>
<td>0.2</td>
<td>3.8</td>
<td>4.6</td>
<td>39.3</td>
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<tr>
<td>Carbohydrate, g/L</td>
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<td>42</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>Maltodextrin, g/L</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>–</td>
</tr>
<tr>
<td>Lactose, g/L</td>
<td>26</td>
<td>26</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Fat, g/L</td>
<td>52</td>
<td>50</td>
<td>47</td>
<td>33</td>
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</table>

1 Energy and macronutrient composition of the formulas was calculated based on the product specification provided by manufacturers. Energy and macronutrient composition of BC and native LF and IgG in WPCs and BC were analyzed by Eurofins Steins Laboratorium. BC, bovine colostrum; CF, formula containing conventional whey protein concentrate; GF, formula containing gently treated whey protein concentrate; LF, lactoferrin; MF, formula containing minimally treated whey protein concentrate; WPC, whey protein concentrate.
intestinal permeability, pigs received an oral bolus (15 mL/kg) containing 5% lactulose and 2% mannitol 3–5 h prior to killing. A urine sample was taken at the time of autopsy to measure the concentrations of lactulose and mannitol as previously described (24). The ratio between concentrations of lactulose and mannitol was calculated to determine the intestinal permeability. To determine the intestinal digestive and absorptive capacity, we analyzed the increment of plasma galactose in response to oral boluses of galactose and lactose. On d 3 before full EN, all pigs were given a bolus (15 mL/kg) of 5% galactose, and blood samples were drawn before and 20 min after administration of the bolus. On d 5, prior to killing, all pigs were given a bolus (15 mL/kg) of 10% lactose, and blood samples obtained before and 20 min after administration of the bolus. Concentrations of galactose in plasma were measured by spectrophotometry as previously described (25). The increment of plasma galactose was calculated as the plasma galactose concentration measured at 20 min minus that measured before administration.

To detect brush border enzyme activities, tissue from Prox, Mid, and Dist was homogenized in 1.0% Triton X-100 water solution (10 mL/g wet tissue) using gentleMACS Dissociator (Miltenyi Biotec). After centrifugation (2000 × g, 10 min, 4°C), the supernatant was isolated for determining the activities of lactase, maltase, sucrase, aminopeptidase N, aminopeptidase A, and dipeptidyl peptidase IV by spectrophotometry as modified from a previous method (26). For SCFA concentrations, samples of the stomach or colon contents were analyzed by GC as previously described (27).

Plasma citrulline concentration and tissue cytokines. The detection of plasma citrulline was performed by UPLC-TQD MS (Waters) as previously described with modification (28). Tissue concentrations of IL-1β, IL-8, and TNFα were measured in the Dist intestinal region. The tissue was homogenized as described in the previous section except for the addition of protease inhibitor cocktail (P8340, Sigma-Aldrich). The cytokines were measured in the supernatant by commercial porcine ELISA kits (R&D Systems) according to the manufacturer’s instructions. The concentrations were expressed as picograms per milligram wet tissue.

Western-blotting analysis. Proteins were extracted from the Prox and Dist tissue of 8 healthy CF pigs and 8 healthy MF pigs (29). WPCs were dissolved in Milli-Q water. Determination of protein concentrations and Western blotting were performed as described elsewhere (29). Proliferating cell nuclear antigen (PCNA), cyclin D1, phosphorylated c-Jun, and caspase-3 were determined in Prox and claudin-4 was determined in the Dist. β-Actin was determined in both regions as the reference protein. TGFβ2 and IGF-I were determined in the Milli-Q water reconstituted WPCs. The primary antibodies used were anti-PCNA (DB Biosciences Pharmingen), anti-cyclin D1 (DB), anti-caspase-3 (intact and cleaved, Oncogene), anti-c-Jun (phospho S73, Abcam), anti-claudin-4 (Invitrogen), anti-β-actin (Sigma), anti-TGF-β2 (Santa Cruz Biotechnology), and anti-IGF-I (Santa Cruz). The intensity change of protein bands was estimated using Quantity One (Bio-Rad Laboratories) and presented as the percentage of adjusted volume of total protein intensity.


Statistical analysis. Binary data were evaluated using Pearson Contingency analysis (JMP 9, SAS Institute) for overall diet effect and Fisher’s exact test for subgroup comparisons (Graph Pad Prism 3, Graph Pad Software). The continuous data were evaluated using Linear Mixed Model (JMP 9) with diet/region as the fixed variables and sow/pig as the random variables. Correlations among response variables were tested by Linear Mixed Model using sow as the random variable. Data were transformed before analysis if they were not normally distributed or with

**FIGURE 1** Schematic diagram displaying the production flow of conventional WPC, gently treated WPC, and minimally treated WPC. CF, formula containing conventional WPC; GF, formula containing gently treated WPC; MF, formula containing minimally treated WPC; WPC, whey protein concentrate.
unilateral variance. Tukey’s post hoc test was performed for repeated comparisons (JMP 9). The Kruskal-Wallis test was applied when data were not able to be properly transformed (JMP 9). Binary data are presented as percentage and continuous data are presented as arithmetic means ± SEMs. The critical level of significance was 0.05.

Results

Macronutrient composition and bioactive markers. The macronutrient compositions of the formulas are shown in Table 1. Concentrations of native LF and IgG in the gently and minimally treated WPCs were 10–20 times greater than those in the conventional WPC (Table 1). Concentrations of native LF in BC were similar to those in MF, whereas IgG was 10 times greater than that in GF and MF. Neither IGF-I nor TGFβ2 were detected in the conventional WPC by Western blotting (Fig. 2A, B). Large amounts of IGF-I were observed in the minimally treated WPC, whereas much less was detected in the gently treated WPC (Fig. 2A). TGFβ2 monomer (band 1) and the small latent complex (band 2) were detected in the gently and minimally treated WPCs (Fig. 2B). The abundance of total TGFβ2 (monomer plus small latent complex) in the minimally treated WPC was ~3 times that of the gently treated WPC (P < 0.05) (Fig. 2C).

Clinical outcomes. There were no differences among groups in birth weight, daily weight gain, age at tissue collection, intestinal lesion score, or NEC incidence (Supplemental Table 1). Relative to body weight, the weight of the small intestine tended to be greater in the MF group compared with the CF group (P = 0.06) and a lower kidney weight was observed in the CF group compared with the BC group (P < 0.05) (Table 2). The relative stomach weight was greater in the BC group relative to the GF and MF groups (P < 0.05) and corresponded to a higher stomach lesion score in the BC group than in the GF and MF groups (P < 0.05) (Table 2). The relative weight of heart, lungs, liver, spleen, and colon and relative intestinal length did not differ among groups (data not shown).

Gut structure, digestive capacity, and SCFAs. Villus height in Prox region was lower in the CF group than in the other 3 groups (P < 0.05) (Table 2), whereas villus height in the Dist region did not differ among groups (512 ± 20 μm). The ratio between villus height and crypt depth in Prox was correspondingly reduced in the CF group compared with the other 3 groups (P < 0.05) (Table 2). Intestinal permeability, determined by the mean ratio of lactulose:mannitol concentrations in the urine, was greater in the CF group than in the GF (P = 0.055), MF, and BC groups (P < 0.05) (Fig. 3A).

The increment of plasma galactose tended to be lower in the CF and GF groups compared with the BC group (P = 0.06) on d 3 before full EN (Fig. 3A). An increment in plasma galactose in response to lactose ingestion was detected in 66% pigs (responders) but not in the remaining ones (nonresponders). Among the 66% responders, the magnitude of increment was less in the CF compared with the GF (P < 0.05) and BC groups (P < 0.001) (Fig. 3A). Diet had an overall effect on the proportion of responders (P < 0.01), which was less in the CF group relative to the MF and BC groups (Fig. 4B). Diet did not affect the activities of sucrase, maltase, aminopeptidase N, and dipeptidyl peptidase IV across the 3 intestinal regions (Table 2). Lactase activity was lower in the CF, GF, and MF groups relative to the BC group as analyzed across the 3 regions (P < 0.05) (Table 2). In the Mid, lactase activity was greater in the MF than in the CF group (P < 0.05, data not shown). Aminopeptidase A activity was lower in the CF and GF groups compared with the BC group (P < 0.05), with intermediate values in the MF group (Table 2).

Among the 15 tested SCFAs, acetate, lactate, succinate, butyrate, and caproate were detected in stomach contents. In general, the BC group had greater total SCFA, acetate, butyrate, and lactate in stomach content compared with other groups (Table 2). Formate, acetate, propionate, lactate, succinate, butyrate, and caproate were detected in the colon contents with lactate, acetate, and formate as the predominant forms. SCFAs in the colon contents did not differ significantly among groups (data not shown).

Plasma citrulline concentration and tissue cytokines. The plasma citrulline concentration was greater in the BC and MF groups than in the CF and GF groups (P < 0.05) (Fig. 3B). In addition, plasma citrulline concentration correlated with proximal villus height (r² = 0.38; P < 0.05) and galactose absorption on d 3 (r² = 0.44; P < 0.01). The tissue IL-8 in the Dist was lower in the BC group compared with the CF (P < 0.05), GF (P < 0.05), and MF groups (P = 0.1) (Fig. 3C), whereas IL-1β (3.7 ± 0.5 pg/mg tissue) and TNFα (0.6 ± 0.7 pg/mg tissue) did not differ among groups. Lesion score in the Dist was positively correlated with IL-1β (r² = 0.22; P < 0.01) but not with IL-8. Furthermore, TNFα concentration was positively correlated with intestinal permeability (r² = 0.45; P < 0.05).

Western-blotting analysis. PCNA and cyclin D1 in the Prox did not differ between the CF and MF groups (data not shown), whereas cleaved-caspase-3 (CC-3; P < 0.05; Fig. 5A) and phosphorylated c-Jun (P < 0.01; Fig. 5B) in Prox and claudin-4 (P < 0.05; Fig. 5C) in Dist were lower in the MF group

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compared with the CF group. β-Actin was comparable among all the analyzed pigs (data not shown). Additionally, CC-3 ($r^2 = 0.52; \ P = 0.06$) and phosphorylated c-Jun ($r^2 = 0.69; \ P < 0.01$) were inversely correlated with proximal villous height, whereas claudin-4 was positively correlated with values for intestinal permeability ($r^2 = 0.90; \ P < 0.05$).

**Cell proliferation assay.** Both conventional and gently treated WPCs significantly increased porcine intestinal epithelial cell line c1 proliferation in vitro after incubation for 24 h (data not shown) or 48 h (Supplemental Fig. 1). When comparing the 2 WPCs, their effects on proliferation did not differ at any of the tested concentrations. Relative to the negative control (0 g/L WPCs), the proliferation was increased by both conventional and gently treated WPCs at 1 g/L (up to 100%; $\ P < 0.001$) and by only gently treated WPC at 0.1 g/L ($\ P < 0.05$).

**Discussion**

The different processing conditions of the 3 WPCs changed the amounts of bioactive proteins and these changes were associated with a number of differences in the structural and functional variables in the immature pig intestine. The results document rapid, WPC-dependent gut changes just after preterm birth and show how these compare with the protective effects of an intact bovine colostrum diet. The most important bioactive protein(s) from milk and colostrum remain to be identified, but our results underline the importance of employing mild processing techniques to maintain the bioactivity of milk formulas.

In most cases, the 2 formulas containing the mildly processed WPCs (GF and MF, containing gently or minimally treated WPCs, respectively) were associated with improved intestinal structure and function relative to CF; the formula containing the conventional WPC. Like the BC-fed pigs, the pigs fed GF or MF had increased villus heights, lactose absorptive capacity, and reduced intestinal permeability. However, GF and MF were less effective in improving galactose absorption and did not enhance brush border enzyme activities, nor did they dampen the proinflammatory IL-8 secretion in the Dist tissue relative to BC. This is probably due to much higher amounts of bioactive proteins in BC (15). Lipid and carbohydrate fractions in BC may also exert bioactive effects and these constituents are absent or present in low amounts in IF (e.g., gangliosides, oligosaccharides) (30,31). Similar to the minimally treated WPC, gently treated WPC was also associated with improved intestinal structure and function, suggesting that preservation of bioactivity in WPC can be achieved under industrial processing conditions.

Industrial processes have adverse effects on the amount and activity of bioactive proteins in WPC product. For example, heat treatment during pasteurization and spray-drying significantly reduces the concentration and activity of LF, lysozyme, IgG, IgA, lactoperoxidase, and IGF-I (32–34). Although TGFβ is relatively stable during heat treatment, its distribution in different milk fractions is affected by heat treatment. It has been reported that only 8% of the total TGFβ2 in pasteurized skimmed milk is present in the whey fraction, whereas 37% is found when the milk is not pasteurized (35). Similarly, we found in this study that the WPC produced with a temperature <40°C has a much higher TGFβ2 concentration than the WPC produced after pasteurization. Moreover, some special filtration processes remove the aggregates and higher molecular weight proteins to a considerable extent, including IGF-binding proteins, latent TGFβ complex, LF, and Igs (36). In our study, the mildly processed WPCs were produced with less heat and without a special filtration process. The selected bioactive markers, LF, IgG, TGFβ2, and IGF-I, may therefore be present at much higher concentrations in the 2 mildly processed WPCs relative to the conventional WPC that is processed by more severe heat treatment and a special filtration step. In addition, the concentrations of IGF-I and TGFβ2 were

**TABLE 2** Organ weights, stomach lesion score, stomach SCFAs, mucosal structure, and brush border enzyme activities in the small intestine of preterm pigs.

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>GF</th>
<th>MF</th>
<th>BC</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach weight, g/kg</td>
<td>8.0 ± 0.7$^{ab}$</td>
<td>6.1 ± 0.8$^b$</td>
<td>6.7 ± 0.8$^a$</td>
<td>9.1 ± 0.9$^b$</td>
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<td>Kidney weight, g/kg</td>
<td>9.6 ± 0.4$^b$</td>
<td>10.8 ± 0.4$^{ab}$</td>
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<td>11.5 ± 0.5$^a$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lesion score</td>
<td>2.5 ± 0.8$^{ab}$</td>
<td>1.6 ± 0.4$^b$</td>
<td>1.7 ± 0.5$^b$</td>
<td>3.4 ± 0.6$^b$</td>
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<td>Total SCFAs, μmol/g</td>
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<td>23 ± 6$^b$</td>
<td>39 ± 7$^a$</td>
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<td>Acetate, μmol/g</td>
<td>5 ± 2$^{ab}$</td>
<td>3 ± 1$^b$</td>
<td>5 ± 3$^b$</td>
<td>9 ± 2$^b$</td>
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<td>Butyrate, μmol/g</td>
<td>3 ± 2$^b$</td>
<td>2 ± 1$^b$</td>
<td>2 ± 0$^b$</td>
<td>10 ± 3$^b$</td>
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<td>Lactate, μmol/g</td>
<td>13 ± 4$^{ab}$</td>
<td>6 ± 1$^b$</td>
<td>16 ± 3$^b$</td>
<td>18 ± 2$^b$</td>
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<td>Stomach:</td>
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<tr>
<td>Weight, g/kg</td>
<td>28.1 ± 0.7</td>
<td>27.0 ± 1.4</td>
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<td>28.5 ± 0.9</td>
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<td>Prox villus height, μm</td>
<td>440 ± 47$^b$</td>
<td>582 ± 46$^a$</td>
<td>667 ± 46.0$^a$</td>
<td>625 ± 46$^a$</td>
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<td>Mucosal proportion, %</td>
<td>62.8 ± 1.9</td>
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<td>64.2 ± 1.7</td>
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<td>Sucrase activity, U/g</td>
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<td>2.2 ± 0.1</td>
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<td>1.9 ± 0.1</td>
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<td>Lactate activity, U/g</td>
<td>9.7 ± 0.8$^b$</td>
<td>13.7 ± 3.4$^b$</td>
<td>14.7 ± 2.7$^b$</td>
<td>25.4 ± 6.5$^b$</td>
<td>&lt;0.001</td>
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<td>Aminopeptidase A activity, U/g</td>
<td>2.5 ± 0.8$^b$</td>
<td>3.0 ± 0.4$^b$</td>
<td>3.1 ± 0.7$^{ab}$</td>
<td>3.8 ± 0.7$^b$</td>
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<tr>
<td>Aminopeptidase N activity, U/g</td>
<td>5.9 ± 1.4</td>
<td>5.6 ± 0.2</td>
<td>5.6 ± 1.7</td>
<td>7.0 ± 0.9</td>
<td>0.059</td>
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<tr>
<td>Dipeptidyl peptidase IV activity, U/g</td>
<td>2.3 ± 0.7</td>
<td>1.9 ± 0.2</td>
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</tbody>
</table>

1 Values are means ± SEMs, $n = 13–14/group$ unless otherwise noted. Labeled means in a row with a common letter differ, $P < 0.05$. BC, bovine colostrum; CF, formula containing conventional whey protein concentrate; GF, formula containing gently treated whey protein concentrate; MF, formula containing minimally treated whey protein concentrate; Prox, proximal small intestine; WPC, whey protein concentrate.
2 SCFAs are presented as μmol/g wet weight of stomach content.
3 The activity of brush border enzymes is presented as U/g small intestinal tissue.
higher in the minimally treated WPC than in the gently treated WPC, which indicates that their concentrations are affected by the degree of heat treatment and/or precipitation method. The elevated concentrations of LF, IgG, IGF-I, and TGFβ2 in the mildly processed WPCs may partly explain their greater bioactivity in stimulating gut maturation in preterm pigs when used as the protein source in formulas.

The impact of industrial processing on the amount and activity of bioactive proteins in human milk and dairy products (e.g., WPC) has been extensively studied in vitro, but the implication for infant health is not known. Pasteurization may induce milk allergy, because mice ingesting pasteurized dairy proteins show greater allergic sensitization via enhanced protein uptake through Peyer’s patches (37). Pasteurized human milk may also have reduced trophic and protective effects compared with non-pasteurized mother’s own milk in preterm infants (38,39). The present study is among the first to investigate how industrial processing of WPC influences gut maturation in preterm newborns.

Previous studies using the same preterm pig model showed that feeding a lactose-based formula is associated with reduced NEC incidence and severity relative to a maltodextrin-based diet (21), consistent with the relatively low NEC incidence in this study. Hence, the differences we observed in the intestine were likely caused mainly by differences in diets rather than by the different progression of inflammation and NEC. In the BC-fed pigs, we observed a higher lesion score and wet weight (likely due to tissue edema) in the stomach. Further studies are required to investigate this effect, but it might partly result from the casein-dependent milk clotting in the stomach of colostrum-fed pigs.

Relative to CF feeding, GF, MF, and BC feeding resulted in an increased villus height and villus:crypt ratio in the Prox, indicating a higher proliferation and/or lower apoptosis in these groups. However, we were unable to demonstrate differences between the CF and GF groups using an in vitro proliferation assay or analyzing tissue proliferation markers (PCNA and Cyclin D1). On the contrary, proteins associated with apoptosis (CC-3 and phosphorylated c-Jun (40)) were higher in CF than in MF-fed pigs and inversely correlated with villus height. Therefore, the elevated villus height in the GF- and MF-fed pigs may be caused by a reduced enterocyte apoptosis rather than by increased proliferation. Immaturity may render the intestine sensitive to epithelial apoptosis through proinflammatory responses and production of NO, which in turn predisposes the intestine to complications, such as NEC (41,42). Among others, LF (43), TGFβ2 (44), and IGF-I (45) have been shown to attenuate epithelial apoptosis in challenged...
intestinal cells or animal models. The mildly processed WPCs may in this way facilitate the postnatal maturation and protection of the immature intestine.

Another key characteristic of intestinal immaturity is the increased permeability found in both human and pig preterm neonates (46,47). The BC, GF, and MF diets all decreased the intestinal permeability relative to CF feeding. This may relate to a high amount of bioactive proteins, controlling the mucusol inamnulation and regulating the expression and the function of tight junction proteins (13,48,49). Surprisingly, the amount of claudin-4 was higher in CF pigs and positively correlated with intestinal permeability, suggesting that the synthesis of claudin-4 increases in response to epithelial damage.

The capacity to absorb galactose is a sensitive marker of intestinal function and this capacity was reduced after 2 d minimal feeding of CF and GF relative to the BC feeding. The carrier-mediated galactose transport occurs via the sodium-glucose linked transporter 1 (SGLT-1) that is located mainly in the upper part of the villi in the Prox and Mid (50). The differences observed in galactose transport among groups may relate to a combination of changes in mucusol mass and density of functional transporters (51). Interestingly, the concentrations of plasma citrulline, a marker of mucusol mass (52), were also lower in the CF and GF pigs than in the MF and BC pigs. This suggests that the lowered galactose absorption capacity in the CF- and GF-fed pigs was related to a lower mucusol mass and surface area. In vitro and in vivo studies have shown that supplementation of growth factors such as IGFs and TGFβ increases mucusol mass (53,54) and SGLT-1 expression (55). Thus, the higher amounts of IGF-I and TGFβ in the MF and BC diets may contribute to the greater galactose absorption and plasma citrulline concentrations in these 2 groups. Conversely, the CF-fed pigs had dramatically reduced lactose digestive and absorptive capacity, also supported by the observed differences in tissue lactase activity.

Intestinal complications, impaired gut barrier function, and NEC are often associated with overexpression of proinflammatory cytokines (53,54). We found that the concentration of Dist TNFα was positively correlated with intestinal permeability and that the concentration of IL-1β was positively correlated with NEC severity in the same region, in line with observations in other animal and human studies (56,57). A dampening effect of colostrum on tissue IL-1β and IL-8 concentrations was observed in earlier studies (57,58), whereas in this study, only IL-8 was reduced by the BC diet. Considering that NEC incidence and severity were low in all groups, this may indicate that BC exerts a direct suppression of IL-8 in the immature intestine, whereas IL-1β production is more likely related to the progression of intestinal inflammation.

In the present study, the investigated WPCs were produced differently with regards to casein precipitation method (acid or sweet whey), filtration (with or without a special filtration step), pasteurization (multi, single, or none), and drying method (standard, gentle spray-drying, or freeze drying). Our results reflect the combined effect of these processes on some selected bioactive markers in WPC products and on the structure and function of the immature gut. Consequently, our results do not allow for conclusions regarding the effects of specific production processes. In addition, we cannot exclude that milder production methods may lead to decreased hygiene quality, and the stability of bioactive components during storage and following formula production still remains to be investigated. Regardless, our results show that WPCs produced by different processing methods contain different amounts of bioactive proteins and possess different maturational effects on the immature gut. Mildly processed WPCs, used as the protein source in formulas, improve gut maturation during the critical neonatal period in preterm pigs. Optimization of the processing methods of WPC may therefore raise the quality of IFs used for preterm and term infants.

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Literature Cited


