Casein addition to a whey-based formula has limited effects on gut function in preterm pigs

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ABSTRACT: Preterm infants are susceptible to necrotizing enterocolitis (NEC). Using preterm pigs, we determined whether a whey–casein-based formula would be superior to a formula based on whey protein alone. Twenty cesarean-derived preterm pigs (92% gestation) were given total parenteral nutrition for 36 h followed by 30 h of enteral feeding with whey [protein fraction of milk formula based on whey (WHEY); n = 11] or casein and/or whey [protein fraction of milk formula based on a combination of casein and whey (CASEIN); n = 9]-based formulas. Sugar absorptive function was investigated at 6 and 30 h after initiation of enteral feeding using bolus feedings with galactose and mannitol. Pigs were killed after the last in vivo sugar absorption test and evaluated for NEC and the mid intestine was used for ex vivo measurements of hexose absorption. Microbiota profile and short chain fatty acid (SCFA) levels were studied in gut contents. Severity of NEC lesions was similar between diet groups but galactose absorption was markedly higher in CASEIN than in WHEY (P < 0.01) although only 6 h after the start of the enteral feeding period. There were no differences in ex vivo 14C-D-glucose uptake, digestive enzymes, microbiota profile, or SCFA concentration. Casein may transiently stimulate intestinal sugar absorption but has limited effects on gut structure, microbiota, and NEC in preterm pigs.

Key words: casein, microbiota, necrotizing enterocolitis, piglets, preterm, whey

INTRODUCTION

Risk factors for development of necrotizing enterocolitis (NEC) in preterm infants include formula feeding and bacterial colonization of the intestine. Whereas the nature of the carbohydrate fraction has been shown to influence NEC development in preterm pigs (Thymann et al., 2009), the role of the protein fraction is less well understood. Casein is coagulated by gastric acid and proteolytic enzymes and may thereby alter food passage and bacterial load in the gut (Lahov and Regelson, 1996). Whey proteins also display modulating effects on the gut microbiota (van Hooijdonk et al., 2000) and are used as the predominant protein in milk formula for preterm infants. However, mature human breast milk contains both casein and whey protein and reduces incidence of NEC. Gastric function is compromised in preterm infants (Udall, 1990) but the extent to which casein coagulation is affected is unclear. Uncoagulated casein may show pro-inflammatory intestinal effects (Miller et al., 1990) but whether inclusion of casein has protective or accelerating effects on NEC development in preterm newborns is unknown. Our aim was therefore to study how addition of casein influences NEC development and gut function in a preterm pig model.

MATERIALS AND METHODS

Twenty pigs (Large White × Danish Landrace × Duroc; Askelygaard, Roskilde, Denmark) from 2 sows were delivered by caesarean section at 105 to 106 d gestation (90–92% gestation) and fitted with vascular and esophageal catheters and immunized with maternal plasma. All pigs were given total parenteral nutrition at 4 to 6 mL/kg/h for 36 h and then divided into 2 groups being tube fed at 15 ml/kg/3 h for an additional 30 h. Dietary carbohydrate, fat, and protein levels were identical but the casein:whey ratios differed (Table

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Table 1. Diet composition in macronutrient content per liter of milk formula

<table>
<thead>
<tr>
<th></th>
<th>CASEIN^2</th>
<th>WHEY^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kJ</td>
<td>4620</td>
<td>4634</td>
</tr>
<tr>
<td>Protein, g</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Casein:whey ratio</td>
<td>60:40</td>
<td>0:100</td>
</tr>
<tr>
<td>Maltodextrin, g</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Lactose, g</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Fat, g</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

^1 Ingredients used: Seravit, Liquigen MCT, and Calogen LCT (Nutricia, Allerød, Denmark); Variolac, Lacprodan alpha 15, and Miprodan (ARLA Foods Ingredient, Viby, Denmark); and Polycose (Abbott Nutrition, Colombus, OH).

^2 CASEIN = protein fraction of milk formula based on a combination of casein and whey.

^3 WHEY = protein fraction of milk formula based on whey.

To assess hexose absorptive function, each pig was given an oral bolus (15 mL/kg) of a 5% galactose and 2% mannitol solution at 6 and 30 h after initiation of enteral nutrition and blood was collected at intervals to measure plasma levels of galactose and mannitol (Thymann et al., 2009). After the in vivo test at 30 h, all pigs were killed and intestinal and gut content samples were collected (stomach, proximal, middle, and distal intestine and colon). Content from the distal small intestine was used for analyses of the gut microbiota (see later) and short chain fatty acid (SCFA) levels. Necrotizing enterocolitis-like pathologic lesions were assessed with a score between 1 and 6 (1 = no or minimal focal hyperemia, 2 = mild focal hyperemia, 3 = moderate locally extensive hyperemia, 4 = severe focal hyperemia, 5 = severe locally extensive hemorrhage and necrosis, and 6 = severe extensive hemorrhage and necrosis). All animal procedures were done in accordance with a protocol approved by The Danish Experimental Animal Inspectorate.

Tissue from the middle small intestine was used to study glucose absorptive function ex vivo (Thymann et al., 2009). Digestive enzyme activities for lactase, sucrase, maltase, dipeptidyl-peptidase IV, and aminopeptidases A and N were measured in snap-frozen samples of the proximal, middle, and distal small intestine (Sangild et al., 1995). Luminal contents from the distal small intestine were diluted and cultured under aerobic and anaerobic conditions for colony forming units (cfu/g content). The microbiota profile associated with the distal small intestinal mucosa was characterized by terminal RFLP as described previously (Thymann et al., 2009). The possible bacterial species represented by terminal restriction fragments (T-RF) were identified using a reference list of virtual digests obtained from the in silico Microbial Community Analysis III virtual digests (http://mica.ibest.uidaho.edu/digest.php).

All data were analyzed using the MIXED procedure of SAS (software package version 9.1; SAS Institute, Cary, NC). Probability levels below 0.05 were considered significant.

RESULTS

Following 6 h of enteral nutrition, plasma galactose was markedly higher in protein fraction of milk formula based on a combination of casein and whey (CASEIN) vs. protein fraction of milk formula based on whey (WHEY) pigs following the galactose bolus (P < 0.01; Figure 1). After 30 h of enteral nutrition, this difference disappeared, and there was no diet effect on NEC incidence and severity, digestive enzyme activities, or ex vivo glucose absorption (Figure 1; Table 2). Microbial composition in the distal small intestinal tissues showed a relatively low

![Figure 1](image1.png)

**Figure 1.** Plasma levels of galactose (μmol/L) and mannitol (mg/L) in vivo following bolus test at 6 (upper panels) and 30 h (lower panels) after initiation of enteral nutrition. Different letters indicate significant difference between means at each time point, P < 0.01.

![Figure 2](image2.png)

**Figure 2.** Individual band intensity of terminal restriction fragments (T-RF) relative to total band intensity, and possible identification of bacterial species of dominating T-RF based on the in silico Microbial Community Analysis virtual digests obtained using the Ribosomal Database Project II, release 9.51, update 51, bacterial SSU 16S rRNA (http://mica.ibest.uidaho.edu/digest.php) in which the used primers and restriction enzyme were inserted. 209 = Leuconostoc spp. (Pseudomonas spp.); 218 = Enterococcus faecalis or Enterococcus faecium; 234 = Clostridium spp.; 237 = Enterobacteriaceae bacterium, Klebsiella, Salmonella enterica, Escherichia coli, or Bifidobacterium; 550 = Streptococcus; 569 = Vibrio spp.; 571 = Salmonella enterica; 583 = Lactobacillus rennani; 597 = Lactobacillus brevis, or Lactobacillus acidophilus; 611 = Weisella cibaria. CASEIN = protein fraction of milk formula based on a combination of casein and whey; WHEY = protein fraction of milk formula based on whey.
Table 2. Clinical and functional endpoints as means ± SEM

<table>
<thead>
<tr>
<th>CASEIN³</th>
<th>WHEY²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>NEC¹ incidence</td>
<td>6/9 (67%)</td>
<td>10/11 (91%)</td>
</tr>
<tr>
<td>NEC score</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Anaerobic bacteria, cfu/g</td>
<td>$4.54 \times 10^9 \pm 2.35 \times 10^9$</td>
<td>$1.66 \times 10^{10} \pm 1.01 \times 10^{10}$</td>
</tr>
<tr>
<td>Aerobic bacteria, cfu/g</td>
<td>$3.38 \times 10^9 \pm 2.52 \times 10^9$</td>
<td>$1.82 \times 10^{10} \pm 1.05 \times 10^{10}$</td>
</tr>
<tr>
<td>Glucose abs⁵, pmol/g/min</td>
<td>33 ± 6</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Lactase⁶, U/g</td>
<td>7.34 ± 1.05</td>
<td>9.70 ± 1.18</td>
</tr>
</tbody>
</table>

¹CASEIN = protein fraction of milk formula based on a combination of casein and whey.
²WHEY = protein fraction of milk formula based on whey.
³NEC = necrotizing enterocolitis.
⁴NS = not significant.
⁵abs = absorption.
⁶Average across proximal, middle, and distal regions of the small intestine.

number of T-RF (totally 44 different T-RF were found), and each pig was dominated by only 11 to 13 fragments (Figure 2). Diversity and band intensity (relative to total band intensity) were similar between CASEIN and WHEY pigs, and the most dominant fragments were identified as Clostridium, Streptococcus, and Enterococcus (Figure 2). Bacterial fermentation as indicated by the SCFA levels in stomach and colon contents was similar between diets. The dominant SCFA were octanoic acid (stomach) and lactic acid (colon), with a significantly lower concentration of lactic acid in the CASEIN group ($P < 0.05$).

**DISCUSSION**

Our data suggest that casein transiently improves intestinal function in the transition from parenteral to enteral nutrition. However, most endpoints following 30 h of enteral nutrition were similar between CASEIN and WHEY; factors other than casein are important for protection against NEC when these lesions commonly develop at 24 to 48 h after initiation of formula feeding. It is possible that the high level of maltodextrin, known to predispose to NEC in preterm pigs (Thymann et al., 2009), has masked a potential limited beneficial effect of casein. Undigested maltodextrin is presumably fermented by resident bacteria leading to excess production of gas and SCFA. Downstream clinical effects may be lactic acidosis and reduced gut motility. We speculate that a compromised gastric function leading to limited clotting of milk casein reduce the possible beneficial effects of casein on intestinal motility in preterm pigs. It remains that both the beneficial and detrimental effects of milk casein are poorly studied, especially in compromised newborns such as those needing optimal nutrition following preterm birth.

**LITERATURE CITED**


