Fermented Infant Formula Increases Ileal Protein Digestibility and Reduces Ileal Proteolytic Activity Compared with Standard and Hydrolyzed Infant Formula in Piglets1–3

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Abstract

Background: An infant formula that contained milk fermented by the bacteria Bifidobacterium breve and Streptococcus thermophilus (Lactofidus) was reported to alleviate functional digestive symptoms in infants. It was hypothesized that improved protein digestibility of the fermented infant formula could contribute to this effect.

Objective: The aim of this study was to evaluate the protein digestibility of a specific fermented (FF), standard (SF), and an extensively hydrolyzed protein (HF) formula.

Methods: Four-week-old piglets (n = 7) were fitted with a T-cannula at the terminal ileum and received each formula in a Latin square design. FF, SF, and HF contained 11.7%, 9.3%, and 11.9% (w/w) crude protein; 1.5%, 5.4%, and 5.6% (w/w) fiber; and had a casein/whey ratio of 60:40, 50:50, and 0:100 per kilogram of powder, respectively. Ileal digesta were collected and analyzed for amino acids and proteolytic activity.

Results: FF had a significantly higher apparent ileal crude protein digestibility (92.1% ± 1.0%) than SF and HF (84.4% ± 1.0% and 83.9% ± 0.9%, respectively). FF also had a significantly higher dry matter digestibility than SF and HF. The ileal crude protein flow of FF was significantly lower than that of SF and HF. The ileal flow of FF of total proteolytic activity was significantly lower than that of SF but not significantly different from that of HF (412 ± 163 kU/8 h vs. 1530 ± 163 and 703 ± 156 kU/8 h, respectively).

Conclusions: The FF in piglets had a significantly higher apparent ileal crude protein digestibility than the SF and the HF and displayed lower ileal proteolytic activity than SF. Both effects may contribute to the alleviation of functional gastrointestinal symptoms reported in infants fed fermented infant milk formula.


Keywords: functional gastrointestinal disorder, protein digestibility, infantile colic, fermented infant formula, protein hydrolyzate

Introduction

Adequate protein digestion and absorption are essential to meet the amino acid (AA) requirements of infants for growth, maintenance, and development. The digestive system and its digestive capacity are not fully mature and further develop during infancy (1, 2). For instance, the postprandial gastric pH of infants is higher than that of adults (3). Because efficient protein denaturation and the enzymatic activity of the gastric protease pepsin depend critically on the stomach’s low intraluminal pH (3–5), the higher postprandial pH in infants could, therefore, hamper dietary protein digestion and absorption.

In case of incomplete digestion, proteins will reach the colon where their fermentation may generate potentially harmful compounds (e.g., ammonia, phenols, indols, nitrosamines), gases (hydrogen sulfide), and microbial proteases (6, 7). The latter ones were linked, for example, to colonic pain and impaired barrier function (8, 9).

Several studies have shown that production processes can affect infant milk protein digestibility and, ultimately, the metabolic status of the infant (10, 11). In addition, several studies have reported beneficial gastrointestinal effects of fermented or acidified infant formulas (12–15). As such, it was

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1 Supported by Nutricia Research.
2 Author disclosures: E Abrahamse, S Huybers, MS Alles, IB Renes, J Knol, H Bouritius, and T Ludwig are employees of Nutricia Research.
3 Supplemental Table 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
4 These authors contributed equally to this work.
5 Abbreviations used: AA, amino acid; CP, crude protein; DM, dry matter; DMI, dry matter intake; FF, fermented formula; HF, hydrolyzed formula; PAR, protease-activated receptor; PI, protein intake; SF, standard formula.
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reported that a starch-thickened formula that contained a specific milk ferment of *Bifidobacterium breve* and *Streptococcus thermophilus* reduced flatulence intensity and alleviated gut discomfort in infants (16). The fermentation process acidifies the product, which may, for example, improve protein digestibility via an enhanced gastric pepsin activity. Hence, we hypothesized an improved protein digestibility of this fermented formula (FF).

Another approach to improve protein absorption is prescription of products that contain only extensively hydrolyzed protein, which may be done when infants have malabsorption-related chronic diarrhea (17). These products are usually based on extensively hydrolyzed whey protein, and ~85% of the peptides have a molecular weight <1500 Da and could therefore be considered as predigested. Hence, we expected that an extensively hydrolyzed protein-based formula (HF) would result in the highest possible protein digestibility.

The objective of the present study was thus to investigate protein digestibility of an infant FF in comparison with a standard formula (SF) and an extensively HF in young piglets. For this purpose, the piglets were fitted with an ileal cannula as a model to study dietary protein digestion in infants. Infant and piglet anatomy and digestive physiology were reported to be highly comparable (18, 19).

**Methods**

**Piglets.** Eight age- and weight-matched (Landrace × York) male piglets (mean weight: 4.9 ± 0.16 kg) were selected 14 d after birth from 3 different litters. Six piglets were required for the study, and 2 additional piglets were included in the procedures in case of drop outs. The piglets were housed in 2 groups of 4 at the facilities of the Animal Sciences Group in Lelystad in a temperature-controlled room at 28°C. At 3 wk of age, all 8 piglets were fitted with a T-cannula at the terminal ileum after being deprived of food overnight. After the procedure, the piglets were housed individually and were allowed to recover for 10 d before the start of the digestion experiment (Figure 1). One piglet died shortly after insertion of the cannula because of an obstruction of the gut. Six piglets were included in the digestion experiment, of which 1 piglet developed leakage of the cannula. This piglet had to be replaced by the last piglet that was not included initially in the digestion experiment. This piglet received 2 out of 3 formulas. The piglet that had to be replaced completed 1 formula.

The digestive system of 3-wk-old piglets was described as an approximation of the digestive system of 3-mo-old infants (20). The piglets used in this study were for technical reasons older than this, that is, 30–40 d. It was demonstrated that 30- to 40-d-old piglets fed a milk-based formula are a good model to study the suckling piglet, which resembles the digestive system of infants that are younger than 6 mo (21). The ileal cannula is a necessity to enable the determination of ileal digestibility. Ileal digestibility is a more relevant outcome measure than fecal digestibility, because AAs that appear in the colon are most probably lost for body protein synthesis. In addition, it allows direct measurement of protein flow into the colon (22).

**Chemical analyses.** The chemical analyses of freeze-dried digesta samples and formula powders for DM, chromium oxide, and crude protein (CP) were performed at CCL Nutricontrol. DM was determined at 80°C by using gravimetry. Chromium oxide was analyzed by inductively coupled plasma mass spectrometry. CP was analyzed with the Kjeldahl method (total nitrogen × 6.25).

AAs were analyzed after hydrolysis in 6 mol/L HCl. Hydrolysis was performed under vacuum to preserve methionine. Quantification of AAs was done with ultrafast liquid chromatography by using a pre-column derivatization and fluorimetric detection. Fluorenylmethoxycarbonyl was used for proline, and α-phenylaldehyde/3-mercaptopyrropropionic acid was used for derivatization of all other AAs. Tryptophan was not determined.

**Analysis of proteolytic enzyme activities.** The activity of pancreatic enzymes was analyzed in the collected ileal digesta. Total proteolytic activity was determined fluorometrically with a standard assay kit according to the manufacturer’s instructions (E6638; Invitrogen). Porcine pancreatin (4× USP; P1750 Sigma) was used as a reference. Proteolytic activity was further characterized with the specific serine protease inhibitor AEBSF (10 mmol/L). Specific trypsin activity was determined with 0.25 mmol/L Nα-Benzoyl-l-arginine ethyl ester (B4500; Sigma) as a substrate. Absorbance change was monitored at 233 nm for 10 min at 25°C. Bovine trypsin (T9201; Sigma) was used as a standard. Specific chymotrypsin activity was determined with 1.18 mmol/L Nα- Benzoyl-l-tyrosine ethyl ester (B6123; Sigma) as a substrate. Absorbance change was monitored at 256 nm for 10 min at 25°C. Bovine chymotrypsin (C3142; Sigma) was used as standard. Specific elastase
activity was analyzed with 4.4 mmol/L SucAla3-PNA (S4760; Sigma) as substrate and porcine elastase (E7885; Sigma) as standard. Absorbance change was monitored at 410 nm for 10 min at 25°C. Enzyme activity was calculated and expressed in 3 ways: as units per gram of wet digesta, as units per 8 h, and as units per gram of protein intake (PI).

**Calculations.** Equations used to calculation flow (Equation 1) and digestibility (Equation 2) of X (X = AAs, CP, etc.) are as follows:

\[
\text{Ileal flow of X [g/8h]} = \frac{\text{Cr}_{2}\text{O}_3 \text{diet [g/kg DM]}}{\text{Cr}_{2}\text{O}_3 \text{digesta [g/kg DM]}}
\]

\[
\text{Apparent ileal X digestibility [%]} = \frac{\text{X intake [g/d]} - \text{X ileal flow [g/8h]}}{\text{X intake [g/d]}} \times 100
\]

**Statistical analysis.** Statistical analyzes were performed with IBM SPSS 19 (SPSS Inc.). Variables were checked for Gaussian distribution with the Shapiro-Wilk test. Levene’s test for equality of variance was used to estimate homogeneity of variances. Effect of treatment was tested with univariate ANOVA (GLM procedure, type III, including intercept, estimate homogeneity of variances. Effect of treatment was tested with observations (see also Piglets section). Values in text are means threshold for significance. The statistics are based on 7 piglets, yielding 6 observations (see also Piglets section). Values in text are means ± SEMs.

**Results**

**Formula intake.** The mean weight of the piglets at the end of the experiment was 9.8 ± 0.3 kg. DM intake (DMI) of FF was significantly lower than that of SF, whereas DMI of HF was intermediate (384 ± 4.2 vs. 399 ± 4.2 g/d and 388 ± 4.0 g/d, respectively). The intervention formulas varied in composition, consequently the intake of individual dietary components varied per formula. The mean intake of protein was significantly higher in the FF-fed group than in the SF-fed group. The PI in the HF-fed group was also significantly higher than in the SF-fed group (FF: 45.0 ± 0.36 g/d; SF: 37.0 ± 0.36 g/d; HF: 46.2 ± 0.34 g/d). The intake of fiber, however, was significantly lower in the piglets fed FF (5.7 ± 0.7 g/d) than in the piglets fed SF (21.6 ± 0.7 g/d) or HF (21.7 ± 0.6 g/d).

**Ileal digesta.** The total ileal digesta, calculated by using ileal flow as determined with the indigestible marker, were significantly lower in the FF group than in the other 2 groups. The total ileal digesta in the HF group were also significantly lower than in the SF group. (Table 1, Figure 2A, B).

<table>
<thead>
<tr>
<th>Formula</th>
<th>FF</th>
<th>SF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.97 ± 0.06</td>
<td>7.93 ± 0.06</td>
<td>7.86 ± 0.06</td>
</tr>
<tr>
<td>Osmolality, mOsmol/kg</td>
<td>304 ± 2.0³</td>
<td>304 ± 2.0³</td>
<td>311 ± 2.0³</td>
</tr>
<tr>
<td>Dry matter, g/kg DMI</td>
<td>38 ± 6.9⁴</td>
<td>134 ± 6.9⁴</td>
<td>107 ± 6.9⁴</td>
</tr>
<tr>
<td>Crude protein, g/kg DMI</td>
<td>27.1 ± 7.63⁴</td>
<td>64.1 ± 7.63³</td>
<td>69.2 ± 7.33³</td>
</tr>
<tr>
<td>Crude protein, g/PI</td>
<td>0.08 ± 0.01³</td>
<td>0.16 ± 0.01³</td>
<td>0.16 ± 0.01³</td>
</tr>
</tbody>
</table>

1 Values are means ± SEMs, n = 6. Labeled means in a row without a common letter differ, P < 0.05. DMI, dry matter intake; FF, fermented formula; HF, hydrolyzed formula; PI, protein intake; SF, standard formula.

The pH of the ileal digesta was identical for all formulas. In addition, ileal digesta osmolality of piglets fed FF and SF was within comparable ranges, although it was significantly higher in HF-fed piglets than in FF-fed piglets.

**Figure 2** Protein digestion of a specific FF, an SF, and an extensively HF. Ileal digesta were collected for 8 h via a T-cannula at the terminal ileum in infant formula-fed piglets for (A) total ileal digesta, (B) ileal protein, (C) apparent ileal CP digestibility, and (D) total ileal protease activity. Values are means ± SEMs, n = 6. Labeled means without a common letter differ, P < 0.05. CP, crude protein; FF, fermented formula; HF, hydrolyzed formula; SF, standard formula.
Ileal protein and AA digestibility. The apparent digestibility of CP and total AA were also calculated with ileal flow as determined with the indigestible marker. The CP and total AA digestibility of FF was significantly higher than that of the other formulas (Table 2, Figure 2C). The same was applicable for the digestibility of all individual AAs, except lysine, which was only higher than that found for SF. The glycine digestibility was low in all formulas and differed most notably between the formulas. The CP and total AA digestibility of SF and HF were similar; however, digestibility of several individual AAs of SF was significantly higher than that of HF.

Ileal enzymatic activities. The activities of pancreatic enzymes in ileal digesta were analyzed. The total proteolytic activity in ileal digesta was reduced by 93% after treatment with the specific chymotrypsin inhibitor AEBSF (data not shown). This indicates that almost all ileal activity is derived from serine proteases (i.e., digestive enzymes). Total proteolytic (Figure 2D, Table 3) and specific trypsin, chymotrypsin, and elastase activities (U/g h) were significantly higher in ileal digesta of piglets fed SF than in piglets fed FF and piglets fed HF, with the exception of chymotrypsin activity (U/g h) which was not significantly different between SF- and HF-fed piglets. The different enzymatic activity amounts found in the digesta of FF-fed piglets (U/g h) were comparable with that found in HF-fed piglets, with the exception of elastase which was significantly higher in the HF-fed group. All the different enzymatic activity amounts found in the digesta, expressed per gram of PI, were significantly lower in piglets fed FF than in piglets fed SF. The enzymatic activity levels per gram of PI found in the digesta of FF-fed piglets were comparable with those of HF-fed piglets.

Discussion

In the present study, we could confirm that significantly lower amounts of protein were detected in ileal digesta of FF-fed piglets than in the ileal digesta of the piglets fed SF or HF. The hypothesis that extensively HF has a higher CP or AA digestibility than SF had to be rejected. We found comparable CP and AA digestibility and CP flow per gram of PI between HF- and SF-fed piglets. The total proteolytic and trypsin activity flow per gram of PI, however, was lower in the HF-fed group than in the SF-fed group. Per gram of PI, HF was also easier to digest than SF, although this did not result in a lower amount of protein delivered to the colon. Dietary peptides (i.e., in the form of protein hydrolyzates) have been reported to increase the secretion of endogenous proteins in pigs, humans, and other species (23–25).

In addition to the differences in serine protease amounts found at the end of the ileum, which suggest different rates of pancreatic secretion, we also found significantly different glycine digestibility for the different formulas. Glycine is present in high amounts in bile, as part of the conjugated bile salts. During the sample preparation for HPLC the acid hydrolysis removes the glycine from the bile salts, leaving bile acids and glycine. The lower glycine digestibility of SF and HF might therefore point toward higher endogenous (bile) secretion.

One aspect of the difference in protein composition between the formulas is the casein/whhey ratio. Casein and whey were demonstrated to be digested at different rates, possibly through differences in gastric emptying. However, the total digestibility of both is considered high (26). Therefore, it is unlikely that differences in casein/whhey ratio are responsible for the observed differences in digestibility.

TABLE 2 Mean apparent AA digestibility as measured in piglets equipped with an ileal T-cannula after being fed for 2 d with a specific FF, an SF, or an extensively HF

<table>
<thead>
<tr>
<th></th>
<th>FF</th>
<th>SF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AA, %</td>
<td>94.3 ± 0.8a</td>
<td>89.3 ± 0.6b</td>
<td>88.5 ± 0.7b</td>
</tr>
<tr>
<td>Alanine</td>
<td>92.1 ± 1.0a</td>
<td>86.5 ± 1.0b</td>
<td>87.9 ± 1.0b</td>
</tr>
<tr>
<td>Arginine</td>
<td>94.5 ± 1.2a</td>
<td>90.1 ± 1.2a</td>
<td>88.8 ± 1.1b</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>94.5 ± 0.7a</td>
<td>89.5 ± 0.7b</td>
<td>89.1 ± 0.6b</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>96.6 ± 0.4a</td>
<td>93.7 ± 0.4a</td>
<td>90.7 ± 0.3a</td>
</tr>
<tr>
<td>Glycine</td>
<td>79.8 ± 4.6a</td>
<td>52.9 ± 4.6b</td>
<td>62.4 ± 4.4b</td>
</tr>
<tr>
<td>Histidine</td>
<td>95.0 ± 0.8a</td>
<td>91.3 ± 0.8a</td>
<td>86.4 ± 0.7a</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>96.1 ± 0.4a</td>
<td>92.8 ± 0.4a</td>
<td>90.0 ± 0.4a</td>
</tr>
<tr>
<td>Leucine</td>
<td>96.8 ± 0.4b</td>
<td>93.9 ± 0.4b</td>
<td>92.3 ± 0.4b</td>
</tr>
<tr>
<td>Lysine</td>
<td>96.7 ± 0.7a</td>
<td>94.1 ± 0.7b</td>
<td>95.1 ± 0.6b</td>
</tr>
<tr>
<td>Methionine</td>
<td>96.8 ± 0.3a</td>
<td>93.8 ± 0.3b</td>
<td>87.5 ± 0.3b</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>96.0 ± 0.6a</td>
<td>91.9 ± 0.6b</td>
<td>89.6 ± 0.5b</td>
</tr>
<tr>
<td>Proline</td>
<td>95.0 ± 0.7a</td>
<td>90.0 ± 0.7b</td>
<td>86.2 ± 0.6b</td>
</tr>
<tr>
<td>Serine</td>
<td>92.9 ± 0.6a</td>
<td>88.2 ± 0.6b</td>
<td>82.9 ± 0.8b</td>
</tr>
<tr>
<td>Threonine</td>
<td>89.0 ± 1.3a</td>
<td>80.8 ± 1.3b</td>
<td>83.3 ± 1.2b</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>96.2 ± 0.4a</td>
<td>93.3 ± 0.4b</td>
<td>90.5 ± 0.4b</td>
</tr>
<tr>
<td>Valine</td>
<td>95.4 ± 0.6a</td>
<td>91.2 ± 0.6b</td>
<td>88.4 ± 0.5b</td>
</tr>
</tbody>
</table>

1 Values are means ± SEMs, n = 6. Labeled means in a row without a common letter differ, P < 0.05. AA, amino acid; FF, fermented formula; HF, hydrolyzed formula; SF, standard formula.
2 Sum of 16 individual AAs.
It must be considered that the complete formulas that were used in this study differ also in aspects not related to protein, such as dietary fiber quantity and quality. Some specific fibers, such as pectin or grain hulls, were reported to increase ideal nitrogen losses (i.e., because of increases in digesta viscosity, their water-holding capacity, or mechanical abrasiveness) (27–30). The fermentation during production of the FF yields transgalacto-oligosaccharides, whereas the SF and HF contain supplemented short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. On the basis of their relatively short-chain length and physicochemical properties, it must be considered that these oligosaccharides are unlikely to yield any of the aforementioned effects (31–33). In addition, despite equal intake of identical sources of fermentable fiber, ileal total proteolytic enzyme activity was significantly lower in HF-fed piglets than in SF-fed piglets. The ileal total proteolytic enzyme activity was furthermore similar between FF and HF, despite differences in dietary fiber intake. The observed effect of FF on ileal total protease activity can thus not be fully explained by differences in fiber quality and quantity between the formulas.

Although the effect of proteases and undigested protein on colonic physiology could not be investigated in this study because of the cannulation of the piglets, ample evidence from literature points toward the negative impact of both. Endogenous proteins, including proteases, have a low bioavailability in the small intestine, but they can be easily metabolized by the gut microbiota. The endogenous proteins that flow into the colon are broken down by microbiota, producing metabolites and gases such as ammonia and hydrogen sulfide. Exposure of the colon to these metabolites is linked to gut discomfort via a direct negative effect on epithelial proliferation, metabolism, gas-induced flatulence, and bloating (7). In this way, the reduced release of endogenous proteases could be linked to beneficial effects of the FF.

In addition, serine proteases (i.e., trypsin, chymotrypsin, and elastase), mast cells (i.e., tryptase), and microbial proteases can directly activate protease-activated receptors (PARs), such as PAR-2. PAR-2 activation was shown to increase pain perception, reduce epithelial barrier function, and may trigger and sustain inflammation (9, 34). We speculate that the reduced colonic delivery of endogenous proteases and reduced stimulation of microbial protease production from undigested protein could be linked to the beneficial effects of the FF. This is an interesting lead that will require further investigation.

In summary, the FF has a higher apparent protein digestibility than the SF or HE. It must be considered that the formulas differed in characteristics other than the fermentation process, which will have to be investigated in future studies. The obtained results point, however, toward a mechanistic pathway that involves a different physiologic response to the FF. This response might involve a diminished secretion of endogenous proteins, a related reduction in exposure of the gastrointestinal tract to undigested proteins, including (serine) proteases. We hypothesize that this reduced exposure may contribute to the observed beneficial effects of FF on gastrointestinal discomfort. The basic mechanism of higher apparent protein digestibility of the FF formula yet remains to be elucidated.

Acknowledgments
We thank Sietse-Jan Koopmans, Ruud Dekker, and Jan van der Meulen from the Animal Sciences Group Lelystad for execution of the piglet trial; Yu Lu and Gerrit de Vrij for their analytical support; and Bert van de Heijning for critical review of the manuscript. EA, SH, MSA, JK, HB, and TL designed the research; EA and SH conducted the research; EA, SH, and IBR analyzed the data; EA, SH, IBR, and TL wrote the paper; TL had primary responsibility for the final content. All authors read and approved the final manuscript.

References


