In vitro gastric digestion of an experimental infant formula containing both intact and hydrolyzed milk proteins

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ABSTRACT

Milk protein hydrolysates may have several benefits for digestion and digestion-related complications in infants, whereas intact milk proteins have been demonstrated to provide functionality beyond their nutritional value. In this study, in vitro digestion of an experimental infant formula containing both intact milk proteins and a milk protein hydrolysate was determined. Relative to an intact milk protein control formula, the experimental formula displayed a higher initial protein digestion during simulated gastric digestion as illustrated by a larger proportion of smaller peptides and higher level of available amino groups during digestion. Gastric protein coagulation was not affected by the hydrolysate addition. Further in vivo studies should demonstrate whether partial replacement of the protein source by a hydrolysate and observed differences in in vitro protein digestion result in overall altered protein digestion and absorption kinetics or affect functional gastrointestinal disorders as has been demonstrated for full hydrolysate formula.

Key words: infant formula, protein digestion, aggregation, coagulation

INTRODUCTION

Human milk is the gold standard for feeding newborn infants, as it is tailored for the nutritional needs of the neonate and provides protection against environmental challenges during the early stages of development. The human milk proteome, generally classified into casein, whey, and milk fat globule membrane proteins, is complex and multifunctional, providing functionality beyond its nutritional value (Lönnerdal, 2016; Dingess et al., 2021). Furthermore, as part of the classically defined NPN [i.e., trichloroacetic acid (TCA) soluble] fraction of human milk, free AA and peptides naturally occur in human milk (Atkinson and Lönnerdal, 2019). Although, to date, the functionality of the human milk peptidome is still largely unknown, several studies discuss the potential of bioactive peptides, mainly based on sequence similarity to known bioactive milk protein sequences or predictions of activity based on sequence and proposed peptide structures (Dallas et al., 2013; Dingess et al., 2017; Cai et al., 2021).

Within infant formula, peptides (as milk protein hydrolysates) are mainly applied for the prevention (partially hydrolyzed protein formula) or dietary management (extensively hydrolyzed protein formula) of cow milk allergy and the dietary management of functional gastrointestinal disorders (partially hydrolyzed formula) (Koletzko et al., 2012; Vandenplas et al., 2014; Vandenplas and Salvatore, 2016).

Compared with their intact counterparts, milk protein hydrolysates may have several physiological advantages for digestion, mainly related to a higher initial digestibility or effects on milk protein coagulation and gastric emptying that may affect digestion-related outcomes (Lambers et al., 2013; Meyer et al., 2015; Corrigan and Brodkorb, 2020). In vivo, hydrolysate formula displayed a faster gastric emptying with possible benefits regarding the dietary management of gastrointestinal disorders in infants, although results of different studies have been conflicting and outcomes likely depend on the formula composition, including the specific hydrolysate type and other optional ingredients that affect overall gastric emptying of the formula (reviewed in Meyer et al., 2015). Although the physiological complexity of gastrointestinal digestion and absorption is difficult to simulate, in vitro digestion models can, at least to a certain extent, provide meaningful insights (Dupont et al., 2019).

In vitro, hydrolysate formula provided a head start in simulated gastric digestion as compared with intact milk protein formula, resulting in a higher proportion of smaller peptides, a higher overall degree of hydrolysis, and reduced gastric protein coagulation (Lambers et al., 2013; Corrigan and Brodkorb, 2020). Milk protein coagulation in the stomach is mostly a result of pepsin-mediated hydrolysis of casein micelles that, as with cheesemaking, coagulate upon initial hydro-

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lysis of caseins that provide steric stabilization (Roy et al., 2020; Huppertz and Chia, 2021). As a result, gastric emptying of nutrients may be delayed because of delayed digestion or alter emulsion stability and coherent phase separation affecting gastric emptying (Marciani et al., 2007). Physiologically, differences in gastric protein coagulation among the individual milk proteins have been associated with a controlled release of protein from the stomach into duodenum to facilitate a sustained gastrointestinal absorption of amino acids into the bloodstream and overall optimal utilization of protein from milk (Boirie et al., 1997; Soop et al., 2012).

Although the digestion characteristics of milk protein hydrolysates have been described previously, to date no study has investigated the effect of a blend of intact proteins and peptides, which may be preferred over complete hydrolysate formula given functionalities of intact milk proteins beyond their nutritional value or a better similarity to human milk, which naturally contains a proportion of peptides (Atkinson and Lonnerdal, 2019). The current study therefore determined in vitro digestion of an experimental infant formula containing a blend of intact proteins and a hydrolysate to study potential digestive differences with regular intact milk protein formula.

## MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

### Formula

All formulas were formulated to meet the nutritional requirements of infants (Table 1) and processed at industrial scale into powdered formula to meet the microbial safety standards for infant formula. The formulas were produced through a process of wet mixing and dry mixing. All liquid dairy ingredients, vegetable fat, minerals, and vitamins were processed in the wet blending process, which included pasteurization, evaporation, homogenization, and spray drying. Probiotics and the hydrolysate were dry blended into the base powder. The experimental formula (Frisolac Comfort Multio, FrieslandCampina) contained a protein source that consisted of 46% intact whey proteins (from milk and demineralized whey, FrieslandCampina), 19% partially hydrolyzed whey (degree of hydrolysis 8%, FrieslandCampina) and 35% casein (from milk). Both formulas contained lactose, a fat blend consisting of vegetable oils and milk fat (35% milkfat in the experimental formula, 50% in the control formula), and fish oil and single cell oils as a source for PUFA. Additionally, both formulas contained added oligosaccharides including galactooligosaccharides and 2-′-fucosyllactose. Furthermore, the experimental formula contained inulin and locust bean gum fiber and probiotics (Bifidobacterium animalis ssp. Lactis BB-12 and Streptococcus thermophilus).

Specifically to investigate effects of the hydrolysate addition on top of the control protein source (as part of the blend at equal protein concentration) on gastric protein coagulation, a fat-free model infant formula was prepared by mixing pasteurized skim milk and demineralized whey (both from FrieslandCampina) to obtain a whey protein–to-casein ratio of 60:40, representing that of a typical stage 1 infant formula as described previously (Huppertz and Lambers, 2020). The fat-free model infant formula of the experimental formula containing the hydrolysate was prepared by dissolving the partially hydrolyzed whey in a similar intact protein-to-hydrolysate ratio as with the complete experimental formula. Subsequently, milk permeate (obtained from ultrafiltration of pasteurized skim milk using a 10 kDa membrane) was added to the mixtures to standardize protein content to 1.5% (wt/vol). Additionally, a fat-free model infant formula (termed experimental absolute) was prepared by adding the hydrolysate in addition to the control protein source, specifically to investigate the effect of the hydrolysate at a similar intact casein concentration as the control but therefore having a slightly higher overall protein concentration, 1.8% (wt/vol), than control.

### Simulated Gastric Digestion

Gastric digestion was performed using a semidynamic digestion model simulating infant gastric conditions. Digestion was performed on a DASbox system (Eppendorf) adopting mixing conditions (including a 3-D printed stirrer head operated at 15 rpm) according to the American Society for Nutritional Sciences (2012) guidelines.

#### Table 1. Macronutrient content of the formulas

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control formula</th>
<th>Experimental formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/100 mL)</td>
<td>65.8</td>
<td>63.0</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>11.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>5.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Minerals (g)</td>
<td>0.49</td>
<td>0.48</td>
</tr>
</tbody>
</table>

1All nutrients are expressed per 100 kcal, except for the energy content, which is per 100 mL.
Protein Analyses

Size-exclusion chromatography was performed on a Shimadzu UPLC system equipped with an Acquity Protein BEH SEC 200 Å, 2.5 µm, 7.8 × 30 mm guard and 2.5 µm, 7.8 × 300 mm analytical column (Waters) and a UV detector (20°C). All chemicals were obtained from Sigma-Aldrich. Digesta (1.5 mg of protein/mL in a buffer of 0.1 M potassium phosphate and 0.15 M sodium chloride pH 6.8) were eluted isocratic (0.3 mL/min) using the same buffer as used to dissolve the proteins and detected at 214 nm. A calibration curve was prepared using standards with a molecular weight range from 1,300 to 600,000 Da (thyroglobulin, BSA, α-LA, aprotinin, and vitamin B12, all from Sigma-Aldrich).

Available amino groups in digesta were determined using o-phthalaldehyde/N-acetyl cysteine reagent as described previously (Hernández et al., 1991). Directly following digestion, TCA precipitation using 0.45 mM TCA was performed to inactivate pepsin and precipitate all intact proteins, thereby focusing the analyses on digested proteins. After centrifugation (10 min, 18,500 × g at room temperature), 40 µL of sample was mixed with 140 µL of 100 mM sodium borate (pH 9.5), 10 µL of 50 mM N-acetyl cysteine, and 10 µL of 50 mM o-phthalaldehyde in ethanol. The presence of alkylisoindoles formed by the reaction of free amino groups with o-phthalaldehyde was measured by the absorbance at 340 nm. To calculate the amount of free amino groups, a calibration curve was measured using leucine as a reference compound. Finally, to compare free amino groups during simulated digestion, corrections were made for differences by secreted SGF at the different pH sampling points by correcting available NH₂ groups for total protein.

Statistical Analysis

Statistical comparison between control and experimental formulas (n = 8 independent replicates) or model fat-free infant formulas (n = 3 independent replicates) during digestion was done using multivariate analyses of variance with Tukey post-hoc tests. The significance threshold for all analyses was set at \( P = 0.05 \). Data are expressed as means ± standard error of the mean unless stated otherwise.

RESULTS AND DISCUSSION

Control and experimental whole infant formulas were subjected to simulated gastric digestion to determine the effect of the hydrolysate addition in the experimental formula on gastric protein digestion. Gastric acidification is, among others, determined by the buffering capacity of the formula and may affect protein digestion because of possible effects on pepsin protease activity. Buffering capacity of infant formula is affected by the protein source (including degree of hydrolysis) and minerals, and to reliably compare differences in gastric protein digestion of milk formula attributable to different protein sources, buffering capacity and coherent gastric acidification dynamics should thus be comparable. Therefore, gastric acidification was determined over time (Figure 1A) before subsequent analyses of protein digestion. Because of the downscaled and simulated in vivo conditions to adopt to the volume of the DASbox units, the coherent small start volume of SGF in the digestion model, and the gradual addition of formula and SGF volumes during digestion (simulating in vivo conditions of bottle feeding and gastric acid and digestive enzyme secretion by the stomach), pH could not be measured for the first 30 to 40 min until sufficient volume was contained in the gastric compartment. Overall, no significant differences were observed between the control and experimental formulas, suggesting that potential differences in digestion of the formula cannot be attributed to differences in gastric acid buffering capacity. Moreover, gastric acidification dynamics (i.e., time to pH) were in line with previously established in vivo patterns of gastric digestion of human milk and infant formula in infants (reviewed in...
Bourlieu et al., 2014), thereby further illustrating the validity of the model to simulate in vivo gastric digestive conditions of infants.

Protein digestion of the formulas after simulated gastric digestion was determined by different methods. Descriptive size-exclusion chromatography measurements (Figure 1B) revealed that the proportion of smaller protein/peptide fragments in both formulas increased during simulated digestion, illustrative for pepsin-mediated protein hydrolysis over time. The experimental formula contained a larger proportion of smaller protein/peptide fragments (i.e., <10,000 Da) during digestion, which is likely the result of the different protein sources of control and experimental formulas and inclusion of a hydrolysate in the experimental formula. Although, relative to the early phase of digestion (i.e., at higher gastric pH; pH 6.0), the difference between the fraction of smaller fragments (i.e., <10,000 Da) in control and experimental formula is smaller at the end of the simulated digestion (i.e., eventual lower gastric pH; pH 3.5), the experimental formula still contained a higher fraction of smaller peptides at the end of the stomach digestion. The origin of these smaller fragments (whether they originate from hydrolysis of larger peptides or intact milk proteins) cannot be determined. Nonetheless, this descriptive analysis illustrates that the digestive difference of the experimental formula because of the hydrolysate addition remained at the end of gastric digestion. In line with the size-exclusion chromatography observations, more quantitative available amino group measurements (Figure 1C) revealed a higher level of amino groups in the experimental formula. Although a trend was evident at each time point, statistical differences were only obtained at the earlier phase of gastric digestion (i.e., pH 6 and pH 5.5). Overall, these results are in line with previous studies comparing intact protein formulas and hydrolysate formulas (Corrigan and Brodkorb, 2020) and may thus suggest a similar initial higher digestibility for formulas containing a blend of intact proteins and hydrolysate as compared with intact protein formulas. Speculatively, this may result in a head start of digestion of the experimental formula and a coherent change in digestion and absorption kinetics in vivo because of the presence of a larger proportion of smaller peptides and a possible faster digestion and absorption of these smaller peptides in the intestine. However, the latter, as well as possible effects on other protein digestion-related outcomes such as gastric emptying and functional gastrointestinal disorders, as has been demonstrated for hydrolysate formulas, remains to be established in vivo.

With respect to the composition of human milk naturally containing a proportion of hydrolyzed proteins, the observed effect of the combination of both intact proteins and peptides may be interesting, as this may suggest that the small proportion of peptides natively present in milk (Dallas et al., 2013) may contribute to the overall digestion kinetics of human milk. The latter, however, remains to be studied, as it is currently unclear whether peptides natively present in human milk are digested and absorbed faster than intact human milk proteins and whether the presence of these peptides indeed results in a measurable effect given the relatively low proportion of peptides in human milk as compared with intact human milk proteins (Atkinson and Lonnerdal, 2019). Moreover, from a milk protein functionality perspective, intact bovine milk proteins may be more beneficial for infants than fully hydrolyzed proteins given their postulated bioactivities beyond their nutritional value as demonstrated for, for example, immunoglobulins, lactoferrin, osteopontin, tropic factors, and milk fat globule membrane proteins (Ward and German, 2004; Lönnerdal, 2016). Thus, at least to a certain extent, formulas containing a combination of intact milk proteins and milk protein hydrolysates may have the potential to benefit from the bioactivity of intact milk proteins and the digestive benefit of hydrolysates. This, however, remains to be studied further in vivo.

Protein coagulation in the gastric compartment is an important factor determining the overall rate of digestion and absorption of milk products (Boirie et al., 1997). Milk protein coagulation is mainly driven by pepsin-mediated coagulation of casein and affected by several factors including protein-to-fat ratios, casein mineralization, and characteristics related to milk processing such as homogenization and heat treatment (Roy et al., 2020; Huppertz and Chia, 2021).

To investigate a possible effect of peptides on gastric protein coagulation, a fat-free model formula containing a comparable protein source as the complete control and experimental formula were prepared. Another version of the experimental formula was prepared in which the hydrolysate was added in addition to the control protein source. The latter was tested because of a possible effect of the small difference in casein content between the control and experimental formulas on casein coagulation and to be able to directly study the effect of peptides on the coagulation of the milk proteins. A possible limitation with using the fat-free model formula is that possible effects of the fat source on protein coagulation are neglected. However, because the fat source as well as total concentration were similar in both experimental and control formulas and the successful previous application of fat-free model infant formula to study alterations in the protein source (Huppertz and Lambers, 2020), a fat-free model formula was selected.
Figure 1. Protein digestion of formulas during simulated gastric digestion. Formulas were submitted to simulated gastric digestion and (A) gastric acidification dynamics of control (closed circles) and experimental (open circles) formulas were determined (n = 6). Protein digestion was evaluated by determining (B) the overall molecular weight distribution of control (CF) and experimental (EF) formulas and (C) the amount of available nitrogen groups (corrected for total protein) of control (closed bars) and experimental (open bars) over time (i.e., sampled at pH 6.0 to 3.5 during gastric digestion, n = 8). Error bars indicate SEM. Significant differences (P < 0.05) between control and experimental formulas are indicated by an asterisk.
All fat-free model formulas were subjected to simulated gastric digestion and, initially, gastric acidification was determined over time. As with the full formula, the fat-free model formula displayed comparable gastric acidification dynamics (Figure 2A) in line with previously established in vivo conditions (Bourlieu et al., 2014). Subsequently, protein coagulation was determined at several time points (i.e., pH 6.0 to 3.5) during digestion. Visual inspection of the coagulates at the start of digestion (neutral pH) up to the end of digestion (acidic pH) (Figure 2B) suggested that the amount of coagulates decreased, although at the end of the simulated digestion coagulates could still be observed. These observations are in line with other studies investigating milk proteins or formulations thereof (Li-Chan, 1987; Mulet-Cabero et al., 2017, 2019; Huppertz and Lambers, 2020) and illustrate that, although likely at a slower rate than soluble proteins, coagulates are digested within the stomach by the concerted action of pepsin-mediated hydrolysis and antral mixing. In addition, the fact that protein coagulation was already observed at neutral pH further illustrates that pepsin-mediated hydrolysis, rather than acidification, is the main driver for casein coagulation. This is probably the result of pepsin-induced hydrolysis of κ-casein leading to coagulation of para-casein micelles as discussed previously (Huppertz and Lambers, 2020; Yang et al., 2022). No obvious differences were observed between the different model formulas. In line with the visual inspection, wet-weight measurements of the coagulates decreased during digestion (Figure 2C) and no significant differences were identified between the formulas. Overall, these results thus suggest that the observed differences in protein digestion could not be attributed to differences in gastric protein coagulation, although further work, preferably assessing coagulation and coherant digestive kinetics in vivo, should be performed to conclusively determine the effects of added peptides on milk protein coagulation.

CONCLUSIONS

Simulated gastric digestion of an experimental infant formula in which a proportion of the protein source was replaced by a hydrolysate revealed a higher initial protein digestion than an intact milk protein formula,
Figure 2. Protein coagulation of model formulas during simulated gastric digestion. A fat-free model infant formula was submitted to simulated gastric digestion (n = 3) and (A) gastric acidification dynamics of control (closed black circles), experimental (closed gray circles), and experimental absolute (open circles) were determined. Protein coagulation was determined by (B) visual inspection in a 10 × 14.5 cm Petri dish (representative images of n = 3) and (C) wet-weight measurements of the coagulate in control (closed black bars), experimental (closed gray bars), and experimental absolute (open bars) formulas. Error bars indicate SEM.
as illustrated by a larger proportion of smaller peptides and a higher level of available amino groups during the early phase of simulated gastric digestion. Gastric protein coagulation was not affected by the hydrolysate addition in model infant formula and the coherent small reduction in casein concentration. Future clinical studies in infants should demonstrate whether partial replacement of the protein source by a hydrolysate and observed differences in in vitro protein digestion result in overall gastric protein coagulation or altered protein digestion and absorption kinetics or affect functional gastrointestinal disorders as has been demonstrated for full hydrolysate infant formulas.

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REFERENCES


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