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The authors B.C. and A.B. designed the study, compiled all results and wrote the manuscript. B.C. carried out all experimental work including data and statistical analysis.

**The effect of pre-treatment of protein ingredients for infant formula on their in vitro
gastro-intestinal behaviour**

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ABSTRACT

Three milk products, skim milk powder (SMP), demineralised whey powder (DWP) and a whey dominant infant formula (60/40IF) and their corresponding partially hydrolysed products (SMPhyd, DWPhyd and 60/40hyd, respectively) were subjected to static infant in vitro gastro-intestinal (GI) digestion and their digesta were subsequently analysed for protein breakdown. The pre-hydrolysis of proteins provided a head-start in the gastric digestion process compared with the intact proteins, resulting in a higher proportion of small peptides (<1 kDa), a higher degree of hydrolysis and lower observable protein coagulation or curd formation in the gastric phase of the casein dominant systems in particular, which may lead to an earlier onset of gastric emptying in vivo. Little or no differences were detected during the intestinal phase. Hence pre-hydrolysis of proteins may be used as a strategy to lower gastric transit times which may ease the gastric digestion of infant formulations.

1. Introduction

Human milk is considered the nutritional gold standard for the newborn as it is tailored for the needs of the infant. In cases where the infant is not breast-fed it is important to provide infant formulas (IFs) of the highest quality for adequate growth and development and the composition of these foods are strictly regulated (Codex-Alimentarius, 1987). Delivery of quality proteins that match the amino acid profile of human milk is, to date, mostly ensured by the formulation of bovine protein fractions, namely skim milk combined with whey protein concentrate from whey, a high quality protein by-product of cheese or casein production. First stage IF is designed to be the sole food for infants, hence whey protein based formulas have come to dominate the marketplace owing to the high nutritional value of whey proteins as well as their greater similarity to human milk when compared with casein based IF. The protein ratio of caseins to whey proteins in IF at 40:60, is similar to human milk, but different from bovine milk that has an 80:20 ratio. It is thought that the lower levels of casein in both first stage IF and breast milk in comparison with bovine milk allows for faster gastric transit due to the formation of a softer curd during the gastric phase, leading to faster and easier gastro-intestinal (GI) digestion (Thompson & Kharb, 2007). The protein content in IF has also been lowered to mimic more closely the total protein levels found in human milk and eliminate excess levels of amino acids thought to cause metabolic stress in the infant (Fomon, 1991; Raiha, 1994). Second stage IF or Follow up Formula (FUF) typically contains a greater proportion of casein than first stage and mimics the protein profile of late stage breast milk (Kunz & Lonnerdal, 1992). Skim milk is typically added to IF to provide the casein element of the formula with whey, often in the form of whey protein isolate or other enriched whey components added to provide the majority of the whey protein (Schuck, Blanchard, & Zhu, 2013).

In contrast to plant based materials, the main protein constituents of milk, i.e., casein and whey proteins, are readily digested by the human GI system. It would appear that the caseins have a longer residence time in the gastric phase than the whey proteins and this can be related to the coagulation of the caseins at their isoelectric point in the stomach by the action of gastric HCl (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2020b). The exact mechanism and degree to which this governs slower gastric emptying of casein relative to whey protein into the duodenum is not clear. However, it is known that casein empties from the stomach in the form of degraded peptides, whereas the whey proteins, and β -lactoglobulin (β -lg) in particular, enter the duodenum as intact protein (Mahé et al., 1996). Processing of milk is also known to affect the gastric curd, which is formed upon acidification and digestion by pepsin. Raw and pasteurised milk was shown to form a harder, more compressed curd during semi-dynamic (Mulet-Cabero et al., 2019) as well as dynamic (Ye, Cui, Dalgleish, & Singh, 2016; Ye et al., 2019) in vitro digestion compared with that of heated UHT milk. This was explained by the denaturation of the whey proteins and their association with the caseins during heat treatment as well as a re-distribution of calcium within the casein micelle, which affects the proteolysis by pepsin and the curd formation.

There are two main types of in vitro methods, static and dynamic; in static methods the food is subjected to preset physiological digestion parameters set to emulate the conditions found in the oral, gastric and intestinal phase. Although more accurate, the dynamic digestion methods that simulate the gradual addition of digestive fluids as well as continuous gastric emptying into the intestinal tract are relatively complex, expensive to run, often commercially operated hence not standardised, and commonly unavailable to the majority of food researchers. Hence most published digestion studies use simpler static methods. To overcome the shortcomings such as variations in physiological conditions, i.e.,

enzyme activities, dilutions and pH, of the different static models present at the time an international group of experts agreed to a consensus model for adult static in vitro digestion (Brodkorb et al., 2019; Minekus et al., 2014) also known as the INFOGEST method. However, no such consensus exists for different population groups (Levi et al., 2017). For simulating infant digestion, the most commonly used static infant in vitro method is by Dupont et al., (2010) , which was further refined and aligned with some aspects of the INFOGEST method by Menard et al. (2018).

The objective of this study was to assess the effect of pre-digestion of proteins, i.e. partial hydrolyses of the proteins in skim milk powder (SMP), demineralised whey protein (DWP) and a 60/40 ratio whey protein/casein mix (60/40IF) representing a model first-stage IF, on their digestive behaviour in vitro and compare this with existing evidence in vivo.

2. Materials and methods

2.1. Materials

Six powder samples containing varying amounts of casein and whey proteins were provided by Kerry Group (Naas, Co. Kildare, Ireland). One group consisted of unhydrolysed SMP, DWP and a whey dominant infant formula blend containing 60/40 whey protein to casein ratio (60/40IF). The second group consisted of the same set of powders that had been partially hydrolysed [degree of hydrolysis (DH): DWPhyd, 12.6%; SMPhyd, 12.2%; 0/40hyd a mixture of DWPhyd and SMPhyd (figures provided by Kerry Group)]. Compositional analysis was carried out in-house by the Moorepark Technical Service (Table 1). The protein content was determined by the Kjeldahl method (Bradstreet, 1954; Kjeldahl, 1883) using a nitrogen conversion factor (NCF) of 6.38 (Jones, 1931). The fat content was determined by

the Rose Gottlieb method (AOAC, Arlington, USA, 1980). The moisture and ash were determined by gravimetric oven from LECO (LECO Instruments, Stockport, UK).

Each of the samples was prepared to 2% (w/w) protein and rehydrated overnight at 4 °C before being stirred for 2 h at ambient temperature prior to digestion. Three independent sets of protein solutions were prepared for the experiments ($n = 3$). All salts for the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared using analytical grade chemicals supplied by Sigma-Aldrich (Sigma Chemical Co. St. Louis, MO, USA). Rabbit Gastric Extract (RGE) was supplied by Lipolytech (Marseille, France). Pancreatin (P-7545, SLBV6830) and bile extract (B8631, 031MO106V) and all other reagents were sourced from Sigma-Aldrich (Sigma Chemical Co.) unless stated otherwise.

2.2. Methods

2.2.1. *In vitro* digestion of liquid meals

The food samples which had been re-hydrated overnight on a 2% (w/w) protein basis were subjected to both gastric and intestinal digestion according to the scheme of Menard et al. (2018). This is the most recent *in vitro* infant digestion model reflecting the latest physiological data available. For the gastric digestion the samples were collected at time zero, with no enzyme (G0), after 30 min (G30) and finally after 60 min (G60). The samples for intestinal digestion were taken after the gastric digestion step was completed for 1 h and then underwent intestinal digestion for either, 15 min (I15), 30 min (I30) or 60 min (I60). The time points were chosen to reflect the importance of both the gastric and intestinal endpoints and to allow sufficient time for preparation of the digesta so as to minimise the amount of error in the replicates. SGF and SIF were prepared and stored at 4 °C and all enzyme solutions were prepared on the day of the trial and stored on ice. The pancreatin added achieved the trypsin

activity of 16 U mL⁻¹ of intestinal content and covered the required lipase activity of 90 U mL⁻¹ of intestinal content and the RGE added achieved the pepsin activity of 268 U mL⁻¹ of gastric contents quoted by Menard et al. (2018). Porcine bile was added to give a final level of 3.1 mM of bile salts and calcium chloride was added separately to give a final intestinal concentration of 3 mM. The gastric phase was at pH 5.3 and the intestinal phase was at pH 6.6, which is based on available physiological data. For further justification of the infant digestion parameters, see Menard et al. (2018). All of the solutions were kept at 37 °C prior to digestion. Sample preparation of the gastric samples G0, G30 and G60 involved the adjustment of the pH to 7.0, followed by snap-freezing in liquid nitrogen. The digestion of the intestinal samples was completed by adding Pefabloc[®] inhibitor to the samples before snap-freezing in liquid nitrogen.

2.2.2. Molecular mass distribution

Size exclusion chromatography-high performance liquid chromatography (SEC-HPLC) was carried out to estimate the molecular mass distribution of proteins and digesta, using a TSK G2000 SW_{XL} column (600 × 7.5 mm; Tosoh Bioscience GmbH, Stuttgart, Germany), on a Waters 2695 HPLC with UV/ Visible detector and EMPOWER[®] software. Separation was achieved by isocratic elution using 0.1% TFA in 30% acetonitrile. Ten microlitres of 0.25% protein solutions were filtered through a 0.45 µm PES filter prior to injection onto the column. A series of molecular weight standards (GE Healthcare, Chicago, IL, USA), were ran on the SEC to create a calibration curve including bovine serum albumin, carbonic anhydrase, β-Ig, α-lactalbumin (α-lac), aprotinin, insulin chain b, bacitracin, histidine-tyrosine-leucine, phenylalanine and glycine with molecular masses of 67,000, 29,000, 18,400, 14,400, 6500, 3496, 1400, 294, 165 and 75 Da, respectively. Molecular mass intervals were determined according to Gaspard and Brodkorb (2019).

2.2.3. Estimation of protein digestion

The digested protein/peptide content in the in vitro samples was determined from the soluble fraction of a 50:50 (v/v) mixture of sample and 24% (w/v) trichloroacetic acid (TCA) after centrifugation at $3000 \times g$ for 30 min using the Kjeldahl method using a NCF of 6.38. The percentage of protein digestion or digestibility was then calculated according to the scheme of Rudloff and Lönnerdal (1992) by comparison with the estimated protein in the original digest minus the amount found in the blank sample, which contained digestive enzyme but no food.

2.2.4. Protein characterisation

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) of the protein products and the in vitro digests was carried out using a 4–12% bis-Tris polyacrylamide gel (Invitrogen, CA, USA). Samples were prepared under reducing conditions using NUPAGE[®] sample agent containing dithiothreitol (DTT). Samples were heated to 85 °C for 2 min to ensure unfolding of the proteins. An unstained molecular weight ladder (Invitrogen) was also run in two lanes to determine the size of the proteins. The samples were stained in instant blue stain (Expedeon, Cambridge, UK). The gels were de-stained in MilliQ[®] water before image analysis using an Epson Scanner (Epson-Telford Ltd., Telford, UK).

2.2.5. Protein hydrolysis

The levels of the free amine groups in the digests were determined by o-phthaldialdehyde (OPA) 96-well micro-assay using the method of Spellman, McEvoy, Cuinn, and FitzGerald (2003). A calibration curve was prepared as described by Mulet-Cabero et al.

(2019) from a set of L-leucine standard solutions between 0 and 10 mM. Ten microlitres of either the standard or sample was then mixed with 200 μ L of OPA for 15 min and the resultant absorbance was measured in a microplate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 340nm.

2.3. Statistical analysis

All statistical analysis was carried out using Minitab software (Minitab Inc., PA, USA) with comparison of the means using Tukey's model ($n = 3$).

3. Results

3.1. Visual assessment of digests

SMP, DWP, 60/40IF and their respective hydrolysed products were exposed to in vitro gastric and intestinal digestion based on the most recent static infant method (Ménard et al., 2018). The hydrolysed samples DWPhyd and 60/40hyd were largely translucent and remained so during both the gastric and intestinal phase. The 60/40IF sample was turbid and white because of the intact casein micelles present in the dispersion, but the 60/40hyd sample was more translucent as the casein present had been pre-hydrolysed. The control DWP and 60/40IF whey protein dominant formulas became largely translucent at the end of the intestinal phase owing to the action of the intestinal enzymes. Significant differences were observed during the gastric digestion of SMP compared with SMPhyd. The image in Fig. 1A show the SMP dispersion before digestion (in standard laboratory Petri dishes), which has a milk-white colour due to the presence of 80% casein in micellar form. In contrast to this, the

hydrolysed form of SMP is more translucent (Fig. 1D) due to the pre-digestion of caseins, which destabilises the casein micelle and reduces the turbidity. The SMP sample was observed to curdle after 5 min of gastric digestion due to protein aggregation induced by the change in pH to 5.3 and the action of pepsin. However, no curdling was observed in the SMPhyd sample throughout the gastric digestion (Fig. 1E,F). After 60 min of gastric digestion the SMP sample contained a greater amount of visibly aggregated material (Fig. 1C) compared with the equivalent hydrolysed sample (Fig. 1F). These differences between SMP and SMPhyd disappeared during intestinal digestion due to the rapid action of pancreatic enzymes, and both digested samples appeared translucent (not shown).

3.2. Protein profile

Digested protein products were analysed by SEC-HPLC, whereby water-soluble proteins and peptides are separated by size and grouped into size intervals, see Fig. 2. Results can be correlated to the extent of proteolysis into small and medium peptides. From the SEC data a number of differences were observed between the control and hydrolysed samples for the gastric phase. When the molecular mass profiles (Fig. 2A–C) for the samples before digestion (G0), were compared with the profiles from the gastric samples after 30 min (G30) and 60 min (G60) of digestion, clear differences were observed. In the control SMP samples (Fig. 2A), the proportion of the two largest molecular mass materials (>30 kDa and 20–30 kDa), which are associated to intact proteins and aggregates, decreased significantly ($p < 0.05$) as gastric digestion progressed. Conversely, the proportion of the smaller molecular mass (1–5 kDa and <1 kDa) material increased significantly ($p < 0.05$) during gastric digestion. The molecular mass profiles of SMPhyd were largely unaffected over the same time, except for some significant ($p < 0.05$) differences in the 5–10 kDa material. Overall,

there was a significantly ($p < 0.05$) higher proportion of low molecular mass material (<1 kDa) in the SMPhyd samples in comparison with its corresponding control SMP. The proportion of small molecular mass material (1–5 kDa and <1 kDa) in both SMPhyd and SMP increased significantly ($p < 0.05$) during intestinal digestion in comparison with the gastric phase, mainly due to the efficient action of the pancreatic proteases including trypsin and chymotrypsin (Guo, Fox, Flynn, & Kindstedt, 1995; Tunick et al., 2016).

The whey proteins in the control DWP samples (Fig. 2B) largely resisted gastric proteolysis and no changes were observed in their molecular mass profiles. In DWPhyd samples the proportion of the largest material (>30 kDa) decreased significantly ($p < 0.05$) during the gastric phase. Comparing DWP and DWPhyd, there was significantly ($p < 0.05$) more low molecular mass material (<1.0 kDa) present in the pre-hydrolysed DWPhyd throughout the gastric phase. There was a significantly ($p < 0.05$) greater proportion of small (<1 kDa) molecular mass material in both the hydrolysed and control DWP samples after intestinal digestion (I15, I30 and I60) in comparison with the gastric samples (G30 and G60) as well as G0.

A small but significant ($p < 0.05$) decrease in the proportion of the high (>30 kDa) molecular mass material in the 60/40hyd (Fig. 2C) after both 30 and 60 min gastric digestion, was observed when compared with the undigested sample (G0). There was no significant increase or decrease in the proportion of the different molecular mass materials after 30 and 60 min gastric digestion of the 60/40IF when compared with the sample prior to digestion (G0).

There was a significantly ($p < 0.05$) greater proportion of small (<1 kDa) molecular mass material in both 60/40IF and 60/40hyd after intestinal digestion (I15, I30 and I60) in comparison with their respective gastric samples (G30, G60 and G0).

The percentage of <1 kDa molecular mass material in both the DWP and DWPhyd samples for the gastric phase appeared to be lower than the SMP and SMPhyd samples (Fig. 2B,C). This is probably related to the higher percentage of casein present in the SMP, which is preferentially hydrolysed by pepsin, whereas the major whey proteins β -lg and α -lac, are known to resist or delay, but not prohibit the proteolysis by pepsin, mainly due to the compact native globular structure of the whey proteins (de Oliveira et al., 2016, 2017; Sanchón et al., 2018; Sullivan, Mok, & Brodkorb, 2013).

3.3. Protein identification by SDS-PAGE

The proteolytic action of both the gastric and intestinal enzymes can be clearly seen in the SDS-PAGE gel electrograms (Fig. 3A–C). There are three bands apparent between 20 and 30 kDa in the un-digested G0 sample for SMP, corresponding to the three major casein groups α -, β - and κ -casein, and two bands for the whey proteins β -lg at ~18kDa α -lac at ~14kDa. After 30 min of gastric digestion (G30) there was a clear decrease of the casein bands in comparison with G0, with a further diminution in intact protein observed at G60. The whey proteins in the SMP remained largely intact throughout gastric digestion. No bands corresponding to either intact casein or whey proteins were observed in the SMPhyd (Fig. 3A).

The SDS-PAGE of DWP (Fig 3B) clearly show the bands corresponding to intact α -lac and β -lg, which remained unchanged during the gastric phase. For the DWPhyd samples faint bands of both whey proteins, which decreased in intensity during the gastric phase are visible. However, no whey proteins bands of DWP and DWPhyd were detected during the intestinal phase.

The SDS-PAGE electrograms of 60/40 IF (Fig. 3C) displayed differences between the casein bands for G0, G30 and G60. G0 had three bands corresponding to α -, β - and κ -caseins, whereas G30 and G60 had only fainter casein bands. The intensities of the bands corresponding to the whey proteins were not reduced upon gastric digestion. For the 60/40hyd there were no intact casein bands present and the faint whey protein bands were again reduced during gastric digestion. No bands for casein or whey proteins appeared in the intestinal digests except those of the digestive enzymes.

3.4. Estimation of protein digestion

The estimation of the protein digestion or percentage of digestibility was based on the quantification of TCA-soluble protein/peptide material (Calsamiglia & Stern, 1995; Rudloff & Lönnerdal, 1992), i.e., the TCA-soluble fraction in digested samples compared with the estimated protein in the original samples using the Kjeldahl method and a NCF of 6.38. The protein material coming from the enzyme extracts (pepsin and pancreatin) in blank experiments were deducted from quantified proteins in the digested samples. Only small peptides and amino acids remain soluble in 12% TCA (Yvon, Chabanet, & Pélissier, 1989) hence its use as an estimation of bioaccessible peptides and amino acids.

The hydrolysed and un-hydrolysed SMP, DWP and 60/40IF samples were prepared and analysed in triplicate ($n = 3$). As expected, the hydrolysed products SMPhyd, DWPhyd and 60/40hyd (Table 2) have high proportions of TCA-soluble protein material, i.e., 87, 49 and 64%, respectively. Un-hydrolysed SMP, DWP and 60/40IF also contain TCA-soluble protein material, i.e., 2.4, 4.7 and 4.2%, respectively. The Kjeldahl nitrogen results include small amounts of free amino acids and non-protein nitrogen, which amount to approximately 1% in milk and SMP (Lindmark-Månsson, Fondén, & Pettersson, 2003; McDermott et al.,

2016). Due to the manufacture of whey proteins, this proportion is expected to be higher in both DWP and 60/40IF. However, the effect of the initial NPN and free amino acids on the protein content in the TCA-soluble fraction is reduced upon progression of proteolysis during GI digestion. The data in Table 2 show that the gastric sample after 60 min (G60) for the SMP had a greater digestibility at 8.73% than G0 at 2.37%. The same trend was observed for the both DWP and 60/40IF sample, with digesta at G60 having significantly higher ($p < 0.05$) TCA-soluble protein material in comparison with those at G30 and G0. When comparing the same gastric time points of the ingredients together, significant differences were observed. The SMPhyd samples had a significantly ($p < 0.05$) higher digestibility at G30 and G60 (88.4 and 83.9%, respectively) than the same gastric digestion time points for the DWPhyd sample (44.1 and 46.2%, respectively). Similar trends were observed for both DWP versus DWPhyd and 60/40IF versus 60/40hyd during gastric digestion. It was also noted that based on the TCA-solubility, hydrolysed protein products were largely unaffected by gastric digestion, except for some significant ($p < 0.05$) difference for SMPhyd at G60.

When looking at the intestinal digestions at the I15, I30 and I60 time points (Table 2) there did not appear to be significant differences in digestibility for the hydrolysed or non-hydrolysed samples, i.e., the large differences observed for the gastric phase were negated in the intestinal digestion phase.

3.5. Free amine determination by *o*-phthaldialdehyde assay (OPA)

The OPA assay quantifies the relative amount of free amine groups liberated due to the cleavage of peptide bonds, and is therefore suitable indicator for the extent of proteolysis. OPA results were largely in line with all other results presented in this study. All gastric samples (Fig. 4) both before (G0) and after 30 and 60 min (G30 and G60) digestion had

significantly ($p < 0.05$) lower levels of free amine groups than the samples that had undergone intestinal digestion (I15, I30 and I60). The gastric samples of SMP, DWP and 60/40IF contained significantly ($p < 0.05$) lower amounts of free amine groups in comparison with SMPhyd, DWPhyd and 60/40hyd due to pre-digestion of these samples.

There was a significantly lower amount ($p < 0.05$) of free amine groups in G0 of both the SMP and SMPhyd than after both 30 and 60 min gastric digestion when the gastric time points were compared together. This trend was repeated for the DWP and 60/40IF gastric samples. The intestinal samples (I15, I30 and I60) were not significantly different from one another confirming the high degree of proteolysis during the intestinal phase compared with the gastric phase.

4. Discussion

The results of the study undertaken here show that pre-hydrolysing proteins appeared to aid the speed of gastric digestion of both the protein ingredients (SMPhyd, DWPhyd) as well as the resulting first stage infant protein formula (60/40hyd) when compared with their non-hydrolysed counterparts. This is simply due to the hydrolysed proteins having a head-start in proteolysis during gastric digestion. After intestinal digestion the differences in digestibility, based on the TCA-soluble Kjeldahl protein nitrogen and free amine group analysis, disappeared completely.

From the SEC data it could be seen that the hydrolysed SMP had a greater proportion of smaller molecular mass material (<1 kDa) and lower proportion of higher molecular mass material (20–30 and > 30 kDa) than the non-hydrolysed SMP, throughout the gastric phase. This was due to the cleavage of both the caseins and whey proteins by the proteolytic action of commercial enzymes, which had hydrolysed the proteins more effectively than the pepsin

in the gastric phase. This is supported by the SDS-PAGE gels, which showed a complete hydrolysis of the casein and indeed whey protein bands in the SMPhyd samples and to a lesser extent the partial hydrolysis of the β -lg and α -lac in both the DWPhyd and 60/40hyd samples.

The intact β -lg and α -lac of SMP, DWP and 60/40IF (Fig. 3) were not degraded to proteolytic products by gastric digestion alone as seen in the SDS-PAGE. The finding that the whey proteins in particular were more resistant to the action of the gastric enzymes concurs with the earlier findings of infant in vitro (de Oliveira et al., 2016) and adult in vivo (Sanchón et al., 2018) studies.

It is interesting to note that the remaining intact whey proteins in DWPhyd are hydrolysed by pepsin due to the commonly used industrial practice of heat-inactivation of the commercial enzymes after hydrolysis, which causes irreversible denaturation and aggregation of the whey proteins β -lg and α -lac. Unfolding and in particular aggregation of whey proteins has been shown to increase enzyme accessibility, which can accelerate its degradation (O'Loughlin, Murray, Kelly, FitzGerald, & Brodkorb, 2012). The SDS-PAGE and SEC data for the intestinal digestion showed a complete degradation of the SMP, DWP and IMF samples irrespective of whether they had been pre-hydrolysed or not. This is not surprising as pancreatic enzymes, trypsin and chymotrypsin in particular, are strong proteolytic enzymes (Kim et al., 2007).

Hydrolysed proteins in nutritional infant products are generally used for the purpose of avoiding or reducing the allergenicity of intact proteins (Alles, Scholtens, & Bindels, 2004; Zeiger, Heller, Mellon, O'Connor, & Hamburger, 1986) and the degradation of allergenic epitopes (Chobert, 2012). The current study presents evidence in vitro that the hydrolysis of protein products can reduce or prevent protein coagulation in the gastric phase, in particular for SMP but also 60/40IF. The coagulation observed in the static in vitro digestions is a result

of pepsin digestion at a constant pH of 5.3. Under physiological conditions, the pH is gradually lowered to pH 3–3.5 with increasing pepsin addition. This is likely to result in an even stronger protein coagulation due to the longer time frame of gelation, compared with the sudden drop in pH experienced in the static models. This effect could be simulated in dynamic or semi-dynamic models (de Oliveira et al., 2015; Mulet-Cabero et al., 2019, 2020c; Ye et al., 2019). The coagulation observed for the SMP sample at pH 5.3 was probably due to two factors, namely the partial proteolysis of the caseins by pepsin, which can retain activity up to pH 5.5 (Piper & Fenton, 1965) and a concomitant pH induced coagulation of the SMP as observed in previous studies (Lucey, Teo, Munro, & Singh, 1997; Lucey, Tamehana, Singh, & Munro, 1998). Huppertz and Lambers (2020) recently suggested that the micellar calcium phosphate content also influenced the gastric coagulation behaviour of infant formula in vitro. The gastric transit, i.e., (i) feeding, (ii) gastric restructuring by pH, enzymatic hydrolysis and peristalsis and (iii) gastric emptying into the duodenum are key factors in the overall kinetics of protein digestion. Pepsin is thought to act on milk in a similar manner as chymosin, which hydrolyses the Phe₁₀₅–Met₁₀₆ peptide bond of κ -casein during cheese manufacture releasing caseinomacropeptide (CMP) and causing the milk to coagulate (Hooydonk, Olieman, & Hagedoorn, 1984). The gastric coagulation of protein can be modulated by pre-treatments such as heating, by formulation (Mulet-Cabero et al., 2020b) or by enzymatic hydrolysis of the proteins as demonstrated in this study. Differences in the physical properties of the coagulum have been shown to affect the kinetics of gastric emptying, appetite, satiety and feeling of fullness (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Mulet-Cabero et al., 2020b); liquid gastric contents are emptied easier and faster.

The ease of digestion for infant formulations is thought to be important for the growing infant from the viewpoint of both abdominal discomfort as well as nutritional uptake

and bio-accessibility (Boirie et al., 1997; Gan, Bornhorst, Henrick, & German, 2018). Clinical trials with adults as well as studies using dynamic or semi-dynamic digestion methods seem to point towards gastric restructuring as a cause for changes in overall digestive kinetics and gastric emptying in particular.

Only a small number of in vivo infant studies have correlated the use of hydrolysed proteins with gastric emptying kinetics. Mihatsch, Högel, and Pohlandt (2001) observed with preterm infants ($n = 15$) that hydrolysed protein formula (75% of the protein was smaller than 1,500 Da) resulted in a significantly ($p < 0.0022$) shorter total gastro-intestinal transit time (9.8 h) compared with standard preterm formula containing intact proteins (19 h). In a later study, Mihatsch, Franz, Kuhnt, Högel, and Pohlandt (2005) proposed that the pre-hydrolysis of casein reduces the opioid activity of some peptides released from intact caseins during GI digestion with adult rats thereby accelerating total GI transit compared with intact caseins. Another study using the adult rat model ($n = 8-15$) showed that the gastric emptying time measured by x-ray was unaffected by the pre-hydrolysis of caseins whereas the whey proteins emptied faster than both hydrolysed whey proteins and caseins (Dalziel, Young, McKenzie, Haggarty, & Roy, 2017). An extensively hydrolysed formula (88% of the protein smaller than 1,500 Da) showed a significantly ($p < 0.05$) faster gastric emptying time in healthy newborns ($n = 20$, measured by breath ^{13}C -octanoic acid) compared with both intact protein formula and partially hydrolysed formula (Staelens et al., 2008); however no details on the extent of hydrolysis of the latter product were provided in the paper. The authors concluded that IF with extensively hydrolysed proteins may be better tolerated by infants with gastric emptying problems.

An international working group consensus on the recommendation of partially hydrolysed formula concluded that “partially hydrolysed whey based formula is likely to result in faster gastric emptying than formula based on intact protein, but the clinical

relevance, for instance with respect to gastric digestion, of this finding has not been demonstrated in term infants” (Vandenplas et al., 2016).

Median gastric emptying time as measured by real-time ultrasonography in preterm infants (triple-blind, controlled trial) was significantly ($p \leq 0.018$) faster using extensively hydrolysed formula compared with control formula (Baldassarre et al., 2019). Other benefits of hydrolysed protein formula include the reduction in oesophageal acid exposure in preterm infants (gestational age ≤ 33 week, randomised crossover trial) with feeding intolerance and symptoms of gastro-oesophageal reflux (Corvaglia, Mariani, Aceti, Galletti, & Faldella, 2013). Hydrolysed whey proteins in combination with higher concentration sn-2 palmitic acid and prebiotic oligosaccharides resulted in a strong tendency (but no statistical significance) of softer stools in constipated infants ($n = 35$, randomised crossover trial). Hence, to date there is no clear consensus on the matter, even though some results point towards a shorter digestion time, which is generally associated with easier digestion. However, most clinical studies with hydrolysed protein formulas were conducted using relatively small numbers of infants compared with larger studies (Alarcon, Tressler, Mulvaney, Lam, & Comer, 2002) correlating protein re-formulation (e.g., caseins versus whey proteins enriched formula) with changes in the gastric emptying, among others ($n = 6999$ in 17 countries). Many clinical studies also fall short of correlating the molecular or micro-structural changes with the mechanism and kinetics of GI digestion. More invasive techniques such as neonatal nasogastric aspiration (de Oliveira et al., 2017) in combination with in vitro digestion studies are necessary for any meaningful correlation to be drawn between the food, digestion and health, though such in vivo studies have clear ethical limitations as regards to risk versus benefit and fewer and fewer studies are currently being conducted.

For the protein products presented in this study, it is reasonable to assume that hydrolysed proteins are emptied faster than products containing intact proteins. This might

aid their ease of digestion, based on some of the in vivo studies mentioned above, and thus help to reduce some symptoms of discomfort. More sophisticated digestion models such as the consensus semi-dynamic models (Mulet-Cabero et al., 2020a) can also be applied to study gastric coagulation behaviour more accurately (Mulet-Cabero et al., 2020c). Efforts within the research community, such as the INFOGEST network, are being made to agree on an acceptable consensus on digestion methods for population groups such as infants (Levi et al., 2017).

5. Conclusions

Infant in vitro digestion of protein ingredients and model IF provide a good insight into the mechanism of protein digestion. The results of the study showed that pre-treatment such as partial hydrolysis of proteins can accelerate the gastric digestion of proteins in ingredients and model infant protein formulations compared with the equivalent non-hydrolysed, samples containing whole proteins. This is particularly true when comparing the protein and peptide pattern at the end of the gastric phase, where pre-treated proteins already exhibited a higher degree of proteolysis even prior to digestion. The final digestion product after intestinal digestion seemed largely unaffected by pre-treatment. From the SDS-PAGE data it also appeared that the gastric enzymes acted faster on α -lac than β -lg, which correlates well with available in vivo data. The information of this study could be used to help design formula, which would have lower GI transit times and help design easier to digest formula for infants where breastfeeding is not an option.

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Figure legends

Fig. 1. Example images of digestion of SMP and SMPhyd using static in vitro digestion: SMP reconstituted at 2% (w/w) protein before digestion (A), after 5 min gastric digestion (B) and after 60 min gastric digestion (C); SMPhyd reconstituted at 2% (w/w) protein before digestion (D), after 5 min gastric digestion (E) and after 60 min gastric digestion (F).

Fig. 2. Molecular mass distribution as determined by size exclusion HPLC of the soluble proteins and peptides; <1 kDa (—), 1–5 kDa (—), 5–10 kDa (▤), 10–20 kDa (—), 20–30 kDa (—), >30 kDa (—) of digesta from in vitro static digestion of (A) skim milk (SMP) compared with hydrolysed skim milk (SMPhyd), (B) demineralised whey (DWP) compared with hydrolysed demineralised whey (DWPhyd) and (C) an infant formula protein blend with a 60/40 whey protein to casein ratio (60/40IF) compared with a hydrolysed equivalent (60/40hyd).

Fig. 3. SDS-PAGE protein profiles of static digestion of (A) skim milk (SMP) compared with hydrolysed skim milk (SMPhyd); (B) demineralised whey (DWP) compared with hydrolysed demineralised whey (DWPhyd) and (C) an infant formula protein blend with a 60/40 whey protein to casein ratio (60/40IF) compared with the hydrolysed equivalent (60/40hyd).

Fig. 4: Concentration of free amine groups in $\mu\text{mol per g}$ of protein as determined by OPA assay: Comparison of digesta of SMP, DWP and 60/40IF to SMPhyd, DWPhyd and 60/40hyd in the gastric phase at time G0 (■), G30 (▤), G60 (▤) and intestinal phase at time I15 (■), I30 (▤) and I60 (▤). Mean values within a column with different

uppercase letters (A, B, C) were significantly different ($p < 0.05$), comparison was between gastric time points only.

Table 1Average compositional analysis of powders for trials. ^a

Powder	Protein %	Fat %	Lactose %	Moisture %	Ash %
SMP	35.9 ± 0.09	0.71 ± 0.07	51.3 ± 0.03	4.40 ± 0.02	7.74 ± 0.01
SMPhyd	35.3 ± 0.09	0.76 ± 0.21	57.0 ± 0.28	2.55 ± 0.24	4.31 ± 0.58
DWP	12.2 ± 0.02	0.80 ± 0.02	84.3 ± 10.1	2.06 ± 0.03	0.65 ± 0.08
DWPhyd	12.9 ± 0.01	0.93 ± 0.13	82.7 ± 0.25	2.09 ± 0.02	1.35 ± 0.09
60/40IF	19.2 ± 0.21	0.99 ± 0.04	74.1 ± 0.17	2.21 ± 0.04	3.47 ± 0.01
60/40hyd	18.5 ± 0.23	0.83 ± 0.03	75.4 ± 0.16	2.80 ± 0.03	2.53 ± 0.05

^a Powders are: SMP, skim milk powder; DWP, demineralised whey powder; 60/40IF, whey dominant infant formula; SMPhyd, DWPhyd and 60/40hyd, the corresponding partially hydrolysed products. Data are shown ± standard deviation.

Table 2Protein content of the TCA-soluble fraction determined before, during and after in vitro digestion. ^a

Powder	G0	G30	G60	I15	I30	I60
SMP	2.37±0.65 ^{bD}	6.77±0.62 ^{aD}	8.73±1.59 ^{aD}	82.8±3.56	85.8±5.19	91.2±11.7
SMPhyd	86.7±2.75 ^{aA}	88.4±4.74 ^{aA}	83.9±8.53 ^{bA}	96.9±2.91	95.6±6.55	92.2±2.69
DWP	4.66±1.01 ^{bD}	6.42±0.48 ^{bD}	9.04±0.90 ^{aD}	87.2±0.36	89.9±7.15	93.2±5.12
DWPhyd	49.4±8.73 ^{aC}	44.1±2.30 ^{aC}	46.2±2.35 ^{aC}	85.5±3.72	97.7±9.59	87.1±10.5
60/40IF	4.22±0.13 ^{cD}	7.41±0.45 ^{bD}	8.89±0.53 ^{aD}	84.5±5.77	82.9±9.12	88.1±4.12
60/40hyd	64.3±2.26 ^{aB}	63.6±3.04 ^{aB}	66.5±5.40 ^{aB}	83.3±2.34	88.0±6.69	82.2±9.46

^a TCA-soluble fraction determined was by the Kjeldahl method (nitrogen conversion factor 6.38) at gastric time points G0, G30 and G60 and intestinal time points I15 and I60 for skim milk powder (SMP), demineralised whey powder (DWP), whey dominant infant formula (60/40IF) and the corresponding partially hydrolysed products (SMPhyd, DWPhyd and 60/40hyd). Lower case letters denote significant differences ($p < 0.05$) across rows, while upper case letters denote significant differences between means down columns ($n = 3$).

Figure 1

Figure 2

Figure 3

Figure 4