

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

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10

## 11 **Abstract**

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

23

## 24 **1. Introduction**

25 Human milk is considered the nutritional gold standard for the newborn as it is tailored for  
26 the needs of the infant. In cases where the infant is not breast-feed it is important to provide  
27 infant formulas (IF) of the highest quality for adequate growth and development and the  
28 composition of these foods are strictly regulated (Codex-Alimentarius, 1987). Delivery of  
29 quality proteins that match the amino acid profile of human milk is to date mostly ensured by  
30 the formulation of bovine protein fractions, namely skim milk combined with whey protein  
31 concentrate from whey, a high quality protein by-product of cheese or casein production.  
32 First stage IF is designed to be the sole food for infants, hence whey protein based formulas  
33 have come to dominate the market place owing to the high nutritional value of whey proteins  
34 as well as their greater similarity to human milk when compared to casein based IF. The  
35 protein ratio of caseins to whey proteins in IF at 40:60, is similar to human milk but different

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36 to bovine milk which has an 80:20 ratio. It is thought that the lower levels of casein in both  
37 first stage IF and breast milk in comparison to bovine milk allows for faster gastric transit due  
38 to the formation of a softer curd during the gastric phase, leading to faster and easier gastro-  
39 intestinal (GI) digestion (Thompkinson & Kharb, 2007). The protein content in IF has also  
40 been lowered to mimic more closely the total protein levels found in human milk and  
41 eliminate excess levels of amino acids thought to cause metabolic stress in the infant (Fomon,  
42 1991; Raiha, 1994). Second stage IF or Follow up Formula (FUF) typically contains a greater  
43 proportion of casein than first stage and mimics the protein profile of late stage breast milk  
44 (Kunz & Lonnerdal, 1992). Skim milk is typically added to infant formula to provide the  
45 casein element of the formula with whey, often in the form of whey protein isolate or other  
46 enriched whey components added to provide the majority of the whey protein (Schuck,  
47 Blanchard, & Zhu, 2013)

48 In contrast to plant based materials the main protein constituents of milk i.e. casein and whey  
49 proteins are readily digested by the human GI system. It would appear that the caseins have a  
50 longer residence time in the gastric phase than the whey proteins and this can be related to the  
51 coagulation of the caseins at their isoelectric point in the stomach by the action of gastric HCl  
52 (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Mulet-Cabero, Rigby,  
53 Brodkorb, & Mackie, 2020). The exact mechanism and degree to which this governs slower  
54 gastric emptying of casein relative to whey protein into the duodenum is not clear. However,  
55 it is known that casein empties from the stomach in the form of degraded peptides whereas  
56 the whey proteins and  $\beta$ -lactoglobulin ( $\beta$ -lg) in particular, enter the duodenum as intact  
57 protein (Mahé, et al., 1996). Processing of milk is also known to affect the gastric curd,  
58 which is formed upon acidification and digestion by pepsin. Raw and pasteurised milk was  
59 shown to form a harder more compressed curd during semi-dynamic (Mulet-Cabero, et al.,

60 2019) as well as dynamic (Ye, Cui, Dalgleish, & Singh, 2016; Ye, et al., 2019) *in vitro*  
61 digestion compared to that of heated UHT milk. This was explained by the denaturation of  
62 the whey proteins and their association with the caseins during heat treatment as well as a re-  
63 distribution of calcium within the casein micelle, which affects the proteolysis by pepsin and  
64 the curd formation.

65 There are two main types of *in vitro* methods, static and dynamic; in static methods the food  
66 is subjected to preset physiological digestion parameters set to emulate the conditions found  
67 in the oral, gastric and intestinal phase. Although more accurate, the dynamic digestion  
68 methods which simulate the gradual addition of digestive fluids as well as continuous gastric  
69 emptying into the intestinal tract are relatively complex, expensive to run, often commercially  
70 operated hence not standardised, and commonly unavailable to the majority of food  
71 researchers. Hence most published digestion studies use simpler static methods. To overcome  
72 the shortcomings such as variations in physiological conditions i.e. enzyme activities,  
73 dilutions and pH, of the different static models present at the time an international group of  
74 experts agreed to a consensus model for adult static *in vitro* digestion (Brodkorb, et al., 2019;  
75 Minekus, et al., 2014) also known as the INFOGEST method. However, no such consensus  
76 exists for different population groups (Levi, et al., 2017). For simulating infant digestion, the  
77 most commonly used static infant *in vitro* method is by Dupont *et al.*, (2010) , which was  
78 further refined and aligned with some aspects of the INFOGEST method by Menard *et al.*  
79 (2018).

80 The objective of this study was to assess the effect of pre-digestion of proteins, i.e. partial  
81 hydrolyses of the proteins in skim milk (SM), Demineralised Whey Protein (DWP) and a  
82 60/40 ratio whey protein/casein mix (60/40IF) representing a model first-stage IF, on their  
83 digestive behaviour *in vitro* and compare this to existing evidence *in vivo*.

84

## 85 **2. Materials and methods**

### 86 **2.1 Materials.**

87 Six powder samples containing varying amounts of casein and whey proteins were provided  
88 by Kerry Group (Naas, Co. Kildare, Ireland). One group consisted of un-hydrolysed Skim  
89 Milk Powder (SMP), De-mineralised Whey Powder (DWP) and a whey dominant infant  
90 formula blend containing 60/40 whey protein to casein ratio (60/40IF). The second group  
91 consisted of the same set of powders, which had been partially hydrolysed (Degree of  
92 hydrolysis (DH): DWPhyd, 12.6%; SMPhyd, 12.2%, 60/40hyd a mixture of DWPhyd and  
93 SMPhyd, figures provided by Kerry Group). Compositional analysis was carried out in-house  
94 by the Moorepark Technical Service (Table 1). The protein content was determined by the  
95 Kjeldahl method (Bradstreet, 1954; Kjeldahl, 1883) using a nitrogen conversion factor (NCF)  
96 of 6.38 (Jones, 1931). The fat content was determined by the Rose Gottlieb method (AOAC,  
97 Arlington, USA, 1980). The moisture and ash were determined by gravimetric oven from  
98 LECO (LECO Instruments, Stockport, United Kingdom).

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

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## 108 **2.2 Methods**

### 109 *2.2.1 In vitro digestion of liquid meals*

110 The food samples which had been re-hydrated overnight on a 2% (w/w) protein basis were  
111 subjected to both gastric and intestinal digestion according to the scheme of Menard *et al.*  
112 (2018). This is the most recent *in vitro* infant digestion model reflecting the latest  
113 physiological data available. For the gastric digestion the samples were collected at time zero,  
114 with no enzyme (G0), after 30 min (G30) and finally after 60 min (G60). The samples for  
115 intestinal digestion were taken after the gastric digestion step was completed for 1h and then  
116 underwent intestinal digestion for either, 15 min (I15), 30 min (I30) or 60 min (I60). The time  
117 points were chosen to reflect the importance of both the gastric and intestinal endpoints and  
118 to allow sufficient time for preparation of the digesta so as to minimise the amount of error in  
119 the replicates. SGF and SIF were prepared and stored at 4°C and all enzyme solutions were  
120 prepared on the day of the trial and stored on ice. The pancreatin added achieved the trypsin  
121 activity of 16 U/mL of intestinal content and covered the required lipase activity of 90 U/mL  
122 of intestinal content and the RGE added achieved the pepsin activity of 268 U/mL of gastric  
123 contents quoted by Menard *et al.* (2018). Porcine bile was added to give a final level of 3.1  
124 mM of bile salts and calcium chloride was added separately to give a final intestinal  
125 concentration of 3 mM. The gastric phase was at pH 5.3 and the intestinal phase was at pH  
126 6.6, which is based on available physiological data. For further justification of the infant  
127 digestion parameters, see Menard *et al.* (2018). All of the solutions were kept at 37°C prior to  
128 digestion. Sample preparation of the gastric samples G0, G30 and G60 involved the  
129 adjustment of the pH to 7.0, followed by snap-freezing in liquid nitrogen. The digestion of

130 the intestinal samples was completed by adding Pefabloc<sup>®</sup> inhibitor to the samples before  
131 snap-freezing in liquid nitrogen.

### 132 2.2.2 Molecular weight distribution

133 Size Exclusion Chromatography-High Performance Liquid Chromatography (SEC-HPLC)  
134 was carried out to estimate the molecular weight distribution of proteins and digesta, using a  
135 TSK G2000 SW<sub>xl</sub> column (600 × 7.5 mm; Tosoh Bioscience GmbH, Stuttgart, Germany), on  
136 a Waters 2695 HPLC with UV/ Visible detector and EMPOWER<sup>®</sup> software. Separation was  
137 achieved by isocratic elution using 0.1% TFA in 30% acetonitrile. 10µl of 0.25% protein  
138 solutions were filtered through a 0.45 µm PES filter prior to injection onto the column. A  
139 series of molecular weight standards (GE Healthcare, Chicago, IL, USA), were ran on the  
140 SEC to create a calibration curve including bovine serum albumin, carbonic anhydrase, β-Ig,  
141 α-lac, aprotinin, insulin chain b, bacitracin, histidine-tyrosine-leucine, phenylalanine and  
142 glycine with molecular weights of 67000, 29000, 18400, 14400, 6500, 3496, 1400, 294, 165  
143 and 75 Da, respectively. Molecular weight intervals were determined according to Gaspard *et*  
144 *al.*(2019).

145

### 146 2.2.3 Estimation of protein digestion

147 The digested protein/peptide content in the *in vitro* samples was determined from the soluble  
148 fraction of a 50:50 v/v mixture of sample and 24% (w/v) trichloroacetic acid (TCA) after  
149 centrifugation at 3,000× g for 30 min using the Kjeldahl method using a NCF of 6.38. The  
150 percentage of protein digestion or digestibility was then calculated according to the scheme  
151 of Rudloff *et al.*(1992) by comparison with the estimated protein in the original digest minus  
152 the amount found in the blank sample, which contained digestive enzyme but no food.

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#### 155 *2.2.4 Protein characterisation*

156 Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) of the protein  
157 products and the *in vitro* digests was carried out using a 4-12% Bis-Tris polyacrylamide gel  
158 (Invitrogen, CA, USA). Samples were prepared under reducing conditions using NUPAGE<sup>®</sup>  
159 sample agent containing dithiothreitol (DTT). Samples were heated to 85°C for 2 min to ensure  
160 unfolding of the proteins. An unstained molecular weight ladder from Invitrogen (Invitrogen,  
161 CA, USA) was also run in two lanes to determine the size of the proteins. The samples were  
162 stained in instant blue stain (Expedeon, Cambs., UK). The gels were de-stained in MilliQ<sup>®</sup>  
163 water before image analysis using an Epson Scanner (Epson-Telford Ltd., Telford, UK).

164

#### 165 *2.2.5 Protein hydrolysis*

166 The levels of the free amine groups in the digests were determined by o-phthaldialdehyde  
167 (OPA) 96-well micro-assay using the method of Spellman *et al.*(2003). A calibration curve  
168 was prepared as described by Mulet-Cabero *et al.* (2019) from a set of L-leucine standard  
169 solutions between 0-10 mM. 10µL of either the standard or sample was then mixed with 200  
170 µL of OPA for 15 min and the resultant absorbance was measured in a microplate reader  
171 (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 340nm.

172

#### 173 *2.2.6 Statistical analysis*



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

176

### 177 **3. Results**

#### 178 **3.1 Visual assessment of digests**

179 SMP, DWP, 60/40IF and their respective hydrolysed products were exposed to *in vitro*  
180 gastric and intestinal digestion based on the most recent static infant method (Ménard, et al.,  
181 2018). The hydrolysed samples DWPhyd and 60/40hyd were largely translucent and  
182 remained so during both the gastric and intestinal phase. The 60/40IF sample was turbid and  
183 white because of the intact casein micelles present in the dispersion but the 60/40hyd sample  
184 was more translucent as the casein present had been pre-hydrolysed. The control DWP and  
185 60/40IF whey protein dominant formulas became largely translucent at the end of the  
186 intestinal phase owing to the action of the intestinal enzymes. Significant differences were  
187 observed during the gastric digestion of SMP compared to SMPhyd. The images in Fig. 1 A  
188 show the SMP dispersion before digestion (in standard laboratory Petri dishes), which has a  
189 milk-white colour due to the presence of 80% casein in micellar form. In contrast to this, the  
190 hydrolysed form of SMP is more translucent (Fig. 1 D) due to the pre-digestion of caseins,  
191 which destabilises the casein micelle and reduces the turbidity. The SMP sample was  
192 observed to curdle after 5 min of gastric digestion due to protein aggregation induced by the  
193 change in pH to 5.3 and the action of pepsin. However, no curdling was observed in the  
194 SMPhyd sample throughout the gastric digestion (Fig. 1 E and F). After 60 min of gastric  
195 digestion the SMP sample contained a greater amount of visibly aggregated material (Fig. 1  
196 C) compared to the equivalent hydrolysed sample (Fig. 1 F). These differences between SMP

197 and SMPhyd disappeared during intestinal digestion due to the rapid action of pancreatic  
198 enzymes, and both digested samples appeared translucent (not shown).

199

200

### 201 **3.2 Protein profile**

202 Digested protein products were analysed by SEC-HPLC, whereby water-soluble proteins and  
203 peptides are separated by size and grouped into size intervals, see Fig. 2. Results can be  
204 correlated to the extent of proteolysis into small and medium peptides. From the SEC data a  
205 number of differences were observed between the control and hydrolysed samples for the  
206 gastric phase. When the molecular weight profiles (Fig. 2 A, B and C) for the samples before  
207 digestion (G0), were compared to the profiles from the gastric samples after 30 min (G30)  
208 and 60 min (G60) of digestion, clear differences were observed. In the control SMP samples  
209 (Fig. 2 A), the proportion of the two largest molecular weight materials (>30 kDa and 20-30  
210 kDa), which are associated to intact proteins and aggregates, decreased significantly ( $p<0.05$ )  
211 as gastric digestion progressed. Conversely, the proportion of the smaller molecular weight  
212 (1-5 kDa and <1 kDa) material increased significantly ( $p<0.05$ ) during gastric digestion. The  
213 molecular weight profiles of SMPhyd were largely unaffected over the same time, except for  
214 some significant ( $p<0.05$ ) differences in the 5-10 kDa material. Overall, there was a  
215 significantly ( $p<0.05$ ) higher proportion of low molecular weight material (<1 kDa) in the  
216 SMPhyd samples in comparison to its corresponding control SMP. The proportion of small  
217 molecular weight material (1-5 kDa and <1 kDa) in both SMPhyd and SMP increased  
218 significantly ( $p<0.05$ ) during intestinal digestion in comparison to the gastric phase, mainly

219 due to the efficient action of the pancreatic proteases including trypsin and chymotrypsin  
220 (Guo, Fox, Flynn, & Kindstedt, 1995; Tunick, et al., 2016).

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

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

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

238 The percentage of <1 kDa molecular weight material in both the DWP and DWPhyd samples  
239 for the gastric phase appeared to be lower than the SMP and SMPhyd samples (Fig. 2 B and  
240 C). This is probably related to the higher percentage of casein present in the SMP, which is  
241 preferentially hydrolysed by pepsin, whereas the major whey proteins  $\beta$ -lg and  $\alpha$ -lactalbumin

242 ( $\alpha$ -lac), are known to resist or delay, but not prohibit the proteolysis by pepsin, mainly due to  
243 the compact native globular structure of the whey proteins (de Oliveira, et al., 2016; de  
244 Oliveira, et al., 2017; Sanchón, et al., 2018; Sullivan, Mok, & Brodkorb, 2013).

### 245 **3.3 Protein identification by SDS-PAGE**

246 The proteolytic action of both the gastric and intestinal enzymes can be clearly seen in the  
247 SDS-PAGE gel electrograms (Fig. 3 A, B and C). There are three bands apparent between  
248 20-30 kDa in the un-digested G0 sample for SMP, corresponding to the three major casein  
249 groups  $\alpha$ ,  $\beta$  and  $\kappa$ -Casein, and two bands for the whey proteins  $\beta$ -lg at ~18kDa  $\alpha$ -lac at  
250 ~14kDa. After 30 minutes of gastric digestion (G30) there is a clear decrease of the casein  
251 bands in comparison with G0, with a further diminution in intact protein observed at G60.  
252 The whey proteins in the SMP remained largely intact throughout gastric digestion. No bands  
253 corresponding to either intact casein or whey proteins were observed in the SMPhyd (Fig. 3  
254 A).

255 The SDS-PAGE of DWP (Fig 3 B) clearly show the bands corresponding to intact  $\alpha$ -lac and  
256  $\beta$ -lg, which remain unchanged during the gastric phase. For the DWPhyd samples faint bands  
257 of both whey proteins, which decrease in intensity during the gastric phase are visible.  
258 However, no whey proteins bands of DWP and DWPhyd are detected during the intestinal  
259 phase.

260 The SDS-PAGE electrograms of 60/40 IF (Fig. 3 C) displayed differences between the casein  
261 bands for G0, G30 and G60. G0 has three bands corresponding to  $\alpha$ ,  $\beta$  and  $\kappa$ -Caseins,  
262 whereas G30 and G60 have only fainter casein bands. The intensity of the bands  
263 corresponding to the whey proteins are not reduced upon gastric digestion. For the 60/40hyd  
264 there are no intact casein bands present and the faint whey protein bands are again reduced

265 during gastric digestion. No bands for casein or whey proteins appear in the intestinal digests  
266 except those of the digestive enzymes.

### 267 **3.4 Estimation of protein digestion**

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

276 The hydrolysed and un-hydrolysed SMP, DWP and 60/40IF samples were prepared and  
277 analysed in triplicate (n=3). As expected, the hydrolysed products SMPhyd, DWPhyd and  
278 60/40hyd (Table 2) have high proportions of TCA-soluble protein material i.e. 87, 49 and  
279 64%, respectively. Un-hydrolysed SMP, DWP and 60/40IF also contain TCA-soluble protein  
280 material i.e. 2.4, 4.7 and 4.2%, respectively. The Kjeldahl nitrogen results include small  
281 amounts of free amino acids and non-protein nitrogen, which amount to approximately 1% in  
282 milk and SMP (Lindmark-Månsson, Fondén, & Pettersson, 2003; McDermott, et al., 2016).  
283 Due to the manufacture of whey proteins, this proportion is expected to be higher in both  
284 DWP and 60/40IF. However, the effect of the initial NPN and free amino acids on the protein  
285 content in the TCA-soluble fraction is reduced upon progression of proteolysis during GI  
286 digestion. The data in Table 2 show that the gastric sample after 60 min (G60) for the SMP  
287 has a greater digestibility at 8.73% than G0 at 2.37%. The same trend was observed for the  
288 both DWP and 60/40IF sample, with digesta at G60 having significantly higher ( $p<0.05$ )

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289 TCA-soluble protein material in comparison to those at G30 and G0. When comparing the  
290 same gastric time points of the ingredients together, significant differences were observed.  
291 The SMPhyd samples had a significantly ( $p<0.05$ ) higher digestibility at G30 and G60 (88.4  
292 and 83.9%, respectively) than the same gastric digestion time points for the DWPhyd sample  
293 (44.1 and 46.2%, respectively). Similar trends were observed for both DWP vs. DWPhyd and  
294 60/40IF vs. 60/40hyd during gastric digestion. It was also noted that based on the TCA-  
295 solubility, hydrolysed protein products were largely unaffected by gastric digestion, except  
296 for some significant ( $p<0.05$ ) difference for SMPhyd at G60.

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

301

### 302 **3.5 Free amine determination by o-phthaldialdehyde assay (OPA)**

303 The OPA assay quantifies the relative amount of free amine groups liberated due to the  
304 cleavage of peptide bonds, and is therefore suitable indicator for the extent of proteolysis.  
305 OPA results are largely in line with all other results presented in this study. All gastric  
306 samples (Fig. 4) both before (G0) and after 30 and 60 min (G30 and G60) digestion had  
307 significantly ( $p<0.05$ ) lower levels of free amine groups than the samples which had  
308 undergone intestinal digestion (I15, I30 and I60). The gastric samples of SMP, DWP and  
309 60/40IF contained significantly ( $p<0.05$ ) lower amounts of free amine groups in comparison  
310 to SMPhyd, DWPhyd and 60/40hyd due to pre-digestion of these samples.

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

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319

#### 320 **4. Discussion**

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

328 From the SEC data it could be seen that the hydrolysed SMP had a greater proportion of  
329 smaller molecular weight material ( $< 1$  kDa) and lower proportion of higher molecular weight  
330 material (20-30 and  $> 30$  kDa) than the non-hydrolysed SMP, throughout the gastric phase.  
331 This was due to the cleavage of both the caseins and whey proteins by the proteolytic action  
332 of commercial enzymes, which had hydrolysed the proteins more effectively than the pepsin  
333 in the gastric phase. This is supported by the SDS-PAGE gels, which show a complete

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334 hydrolysis of the casein and indeed whey protein bands in the SMPhyd samples and to a  
335 lesser extent the partial hydrolysis of the  $\beta$ -lg and  $\alpha$ -lac in both the DWPhyd and 60/40hyd  
336 samples.

337 The intact  $\beta$ -lg and  $\alpha$ -lac of SMP, DWP and 60/40IF (Fig. 3) are not degraded to proteolytic  
338 products by gastric digestion alone as seen in the SDS-PAGE. The finding that the whey  
339 proteins in particular are more resistant to the action of the gastric enzymes concurs with the  
340 earlier findings of infant *in vitro* (de Oliveira, et al., 2016) and adult *in vivo* (Sanchón, et al.,  
341 2018) studies.

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

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

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358 pepsin digestion at a constant pH of 5.3. Under physiological conditions, the pH is gradually  
359 lowered to pH 3-3.5 with increasing pepsin addition. This is likely to result in an even stronger  
360 protein coagulation due to the longer time frame of gelation, compared to the sudden drop in  
361 pH experienced in the static models. This effect could be simulated in dynamic or semi-  
362 dynamic models (de Oliveira, et al., 2015; Mulet-Cabero, Torcello-Gómez, et al., 2020; Mulet,  
363 et al., 2019; Ye, et al., 2019). The coagulation observed for the SMP sample at pH 5.3 was  
364 probably due to two factors namely, the partial proteolysis of the caseins by pepsin, which can  
365 retain activity up to pH 5.5 (Piper & Fenton, 1965) and a concomitant pH induced coagulation  
366 of the SMP as observed in previous studies (Lucey, Teo, Munro, & Singh, 1997; Lucey,  
367 Tamehana, Singh, & Munro, 1998). Huppertz & Lambers (2020) recently suggested that the  
368 micellar calcium phosphate content also influenced the gastric coagulation behaviour of infant  
369 formula *in vitro*. The gastric transit i.e. (i) feeding, (ii) gastric restructuring by pH, enzymatic  
370 hydrolysis and peristalsis and (iii) gastric emptying into the duodenum are key factors in the  
371 overall kinetics of protein digestion. Pepsin is thought to act on milk in a similar manner as  
372 chymosin, which hydrolyses the Phe<sub>105</sub> – Met<sub>106</sub> peptide bond of  $\kappa$ -casein during cheese  
373 manufacture releasing caseinomacropeptide (CMP) and causing the milk to coagulate  
374 (Hooydonk, Olieman, & Hagedoorn, 1984). The gastric coagulation of protein can be  
375 modulated by pre-treatments such as heating, by formulation (Mulet-Cabero, Rigby, et al.,  
376 2020) or by enzymatic hydrolysis of the proteins as demonstrated in this study. Differences in  
377 the physical properties of the coagulum have been shown to affect the kinetics of gastric  
378 emptying, appetite, satiety and feeling of fullness (Mackie, Rafiee, Malcolm, Salt, & van Aken,  
379 2013; Mulet-Cabero, Rigby, et al., 2020); liquid gastric contents are emptied easier and faster.  
380 The ease of digestion for infant formulations is thought to be important for the growing infant  
381 from the viewpoint of both abdominal discomfort as well as nutritional uptake and bio-

382 accessibility (Boirie, et al., 1997; Gan, Bornhorst, Henrick, & German, 2018). Clinical trials  
383 with adults as well as studies using dynamic or semi-dynamic digestion methods seem to point  
384 towards gastric restructuring as a cause for changes in overall digestive kinetics and gastric  
385 emptying in particular. Only a small number of *in vivo* infant studies have correlated the use of  
386 hydrolysed proteins with gastric emptying kinetics. Mihatsch et al. (2001) observed with  
387 preterm infants (n = 15) that hydrolysed protein formula (75 % of the protein was smaller than  
388 1,500 Da) resulted in a significantly ( $p < 0.0022$ ) shorter total gastro-intestinal transit time (9.8  
389 h) compared to standard preterm formula containing intact proteins (19 h). In a later study  
390 Mihatsch et al. (2005) proposed that the pre-hydrolysis of casein reduces the opioid activity of  
391 some peptides released from intact caseins during GI digestion with adult rats thereby  
392 accelerating total GI transit compared to intact caseins. Another study using the adult rat model  
393 (n = 8 to 15) showed that the gastric emptying time measured by x-ray was unaffected by the  
394 pre-hydrolysis of caseins whereas the whey proteins emptied faster than both hydrolysed whey  
395 proteins and caseins (Dalziel, Young, McKenzie, Haggarty, & Roy, 2017). An extensively  
396 hydrolysed formula (88% of the protein smaller than 1,500 Da) showed a significantly ( $p <$   
397  $0.05$ ) faster gastric emptying time in healthy newborns (n = 20, measured by breath  $^{13}\text{C}$ -  
398 octanoic acid) compared to both intact protein formula and partially hydrolysed formula  
399 (Staelens, et al., 2008); however no details on the extent of hydrolysis of the latter product  
400 were provided in the paper. The authors concluded that IF with extensively hydrolysed proteins  
401 may be better tolerated by infants with gastric emptying problems. An international working  
402 group consensus on the recommendation of partially hydrolysed formula concluded that  
403 “partially hydrolysed whey based formula is likely to result in faster gastric emptying than  
404 formula based on intact protein, but the clinical relevance, for instance with respect to gastric  
405 digestion, of this finding has not been demonstrated in term infants” (Vandenplas, et al., 2016).  
406 Median gastric emptying time as measured by real-time ultrasonography in preterm infants

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407 (triple-blind, controlled trial) was significantly ( $p \leq 0.018$ ) faster using extensively hydrolysed  
408 formula compared to control formula (Baldassarre, et al., 2019). Other benefits of hydrolysed  
409 protein formula include the reduction in oesophageal acid exposure in preterm infants  
410 (gestational age  $\leq 33$  week, randomised crossover trial) with feeding intolerance and symptoms  
411 of gastro-oesophageal reflux (Corvaglia, Mariani, Aceti, Galletti, & Faldella, 2013).  
412 Hydrolysed whey proteins in combination with higher concentration sn-2 palmitic acid and  
413 prebiotic oligosaccharides resulted in a strong tendency (but no statistical significance) of  
414 softer stools in constipated infants ( $n = 35$ , randomised crossover trial). Hence, to date there is  
415 no clear consensus on the matter, even though some results point towards a shorter digestion  
416 time, which is generally associated with easier digestion. However, most clinical studies with  
417 hydrolysed protein formulas were conducted using relatively small numbers of infants  
418 compared to larger studies (Alarcon, Tressler, Mulvaney, Lam, & Comer, 2002) correlating  
419 protein re-formulation (e.g. caseins vs. whey proteins enriched formula) with changes in the  
420 gastric emptying, among others ( $n=6,999$  in 17 countries). Many clinical studies also fall short  
421 of correlating the molecular or micro-structural changes with the mechanism and kinetics of GI  
422 digestion. More invasive techniques such as neonatal naso-gastric aspiration (de Oliveira, et al.,  
423 2017) in combination with *in vitro* digestion studies are necessary for any meaningful  
424 correlation to be drawn between the food, digestion and health, though such *in vivo* studies  
425 have clear ethical limitations as regards to risk vs. benefit and fewer and fewer studies are  
426 currently being conducted.

427 For the protein products presented in this study, it is reasonable to assume that hydrolysed  
428 proteins are emptied faster than products containing intact proteins. This might aid their ease of  
429 digestion, based on some of the *in vivo* studies mentioned above, and thus help to reduce some  
430 symptoms of discomfort. More sophisticated digestion models such as the consensus semi-

431 dynamic models (Mulet-Cabero, Egger, et al., 2020) can also be applied to study gastric  
432 coagulation behaviour more accurately (Mulet-Cabero, Torcello-Gómez, et al., 2020). Efforts  
433 within the research community, such as the INFOGEST network, are being made to agree on  
434 an acceptable consensus on digestion methods for population groups such as infants (Levi, et  
435 al., 2017).

436

## 437 **5. Conclusions**

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

450

451

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632

633 **Contributions:**

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

636

637 **Conflict of interest**

638 There is no conflict of interest. The study was financed by Kerry group under a contract  
639 agreement with Teagasc. The authors B.C. and A.B. did not financially benefit from this  
640 contract.

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655 **Table 1:** Average compositional analysis of powders for trials  $\pm$  standard deviation.

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

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659 **Table 2:** Protein content of the TCA-soluble fraction determined by the Kjeldahl method (NCF  
660 6.38) before, during and after *in vitro* digestion: gastric time points G0, G30 and G60 and intestinal  
661 time points I15 and I60 for SMP, DWP and 60/40IF in comparison to corresponding time points for  
662 pre-hydrolysed samples SMPhyd, DWPhyd and 60/40hyd, respectively. Lower case letters denote  
663 significant differences across rows, while upper case letters denote significant differences between  
664 means down columns (n = 3).

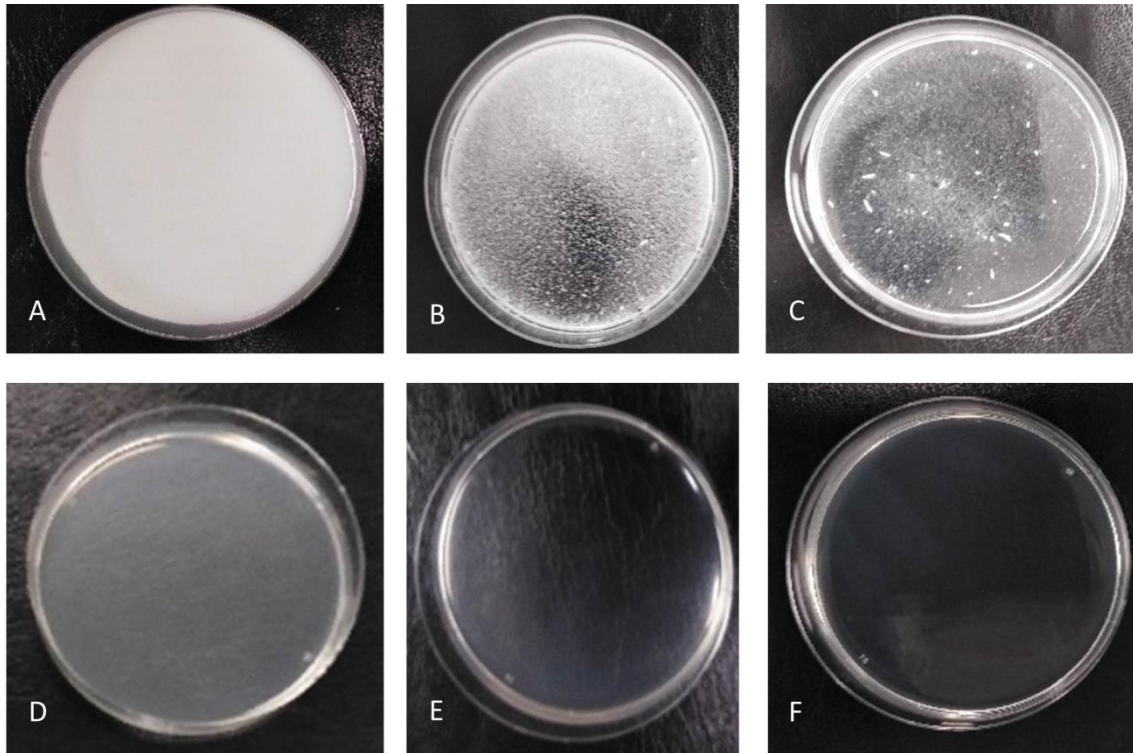
	<b>G0</b>	<b>G30</b>	<b>G60</b>	<b>I15</b>	<b>I30</b>	<b>I60</b>
<b>SMP</b>	2.37 $\pm$ 0.65 <sup>bd</sup>	6.77 $\pm$ 0.62 <sup>ad</sup>	8.73 $\pm$ 1.59 <sup>ad</sup>	82.8 $\pm$ 3.56	85.8 $\pm$ 5.19	91.2 $\pm$ 11.7
<b>SMPhyd</b>	86.7 $\pm$ 2.75 <sup>aA</sup>	88.4 $\pm$ 4.74 <sup>aA</sup>	83.9 $\pm$ 8.53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

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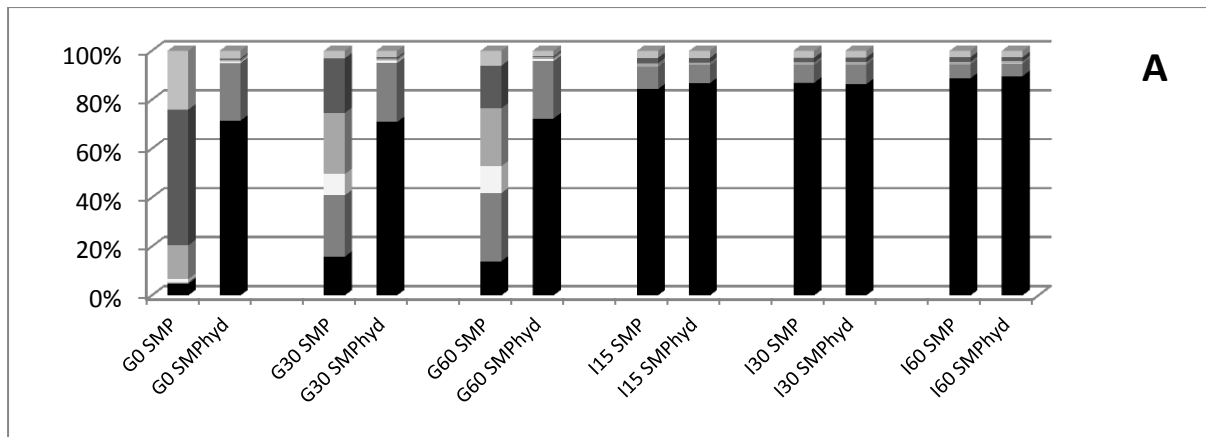
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668 **Fig. 1:** Example images of digestion of SMP and SMPhyd using static *in vitro* digestion:  
669 SMP reconstituted at 2% (w/w) protein before digestion (A), after 5 min gastric digestion (B)  
670 and after 60 min gastric digestion (C); SMPhyd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

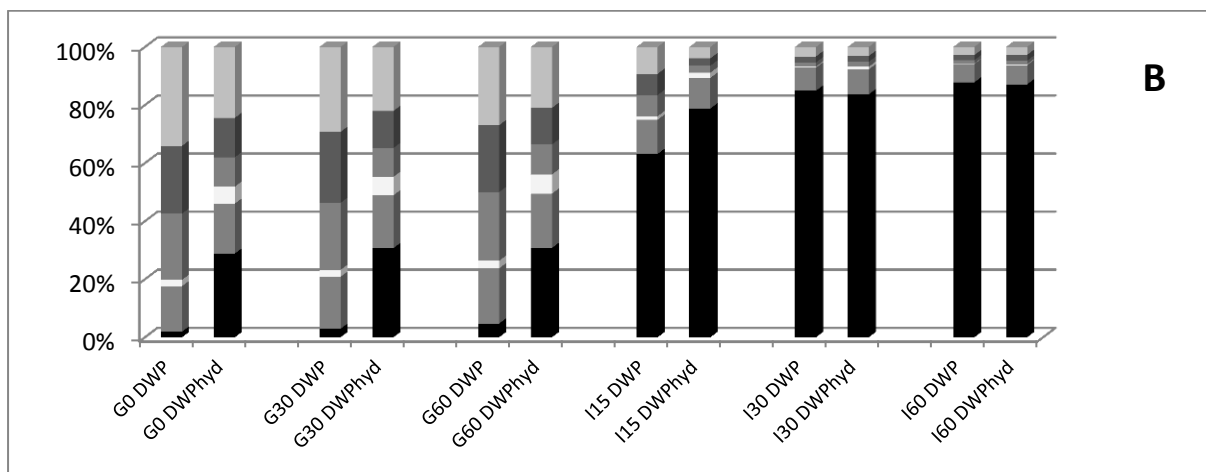
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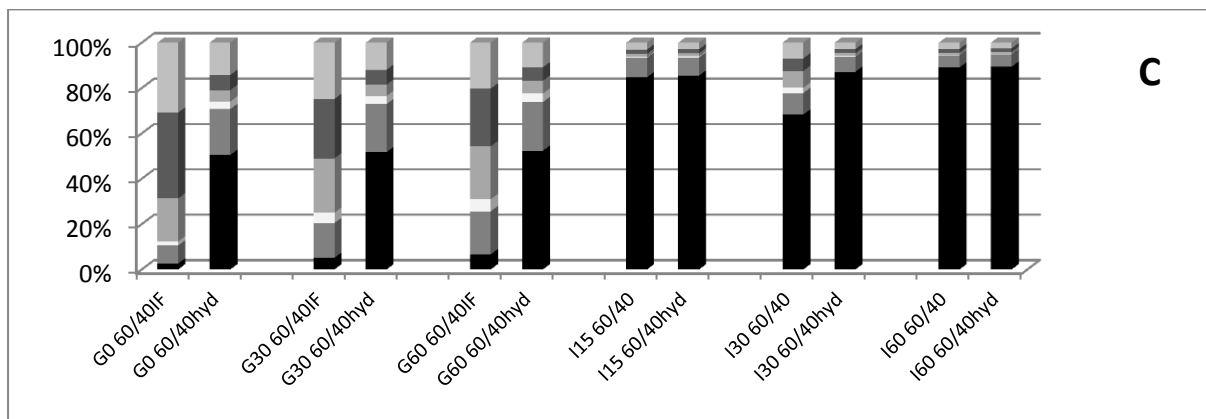
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678 **Fig. 2:** Molecular weight distribution as determined by size exclusion HPLC of the soluble proteins  
 679 and peptides; <1 kDa ( — ), 1-5 kDa ( — ), 5-10 kDa ( — ), 10-20 kDa ( — ), 20-30kDa ( — ), >30  
 680 kDa ( — ) of digesta from *in vitro* static digestion of (A) skim milk (SMP) compared to hydrolysed  
 681 skim milk (SMPhyd), (B) demineralised whey (DWP) compared to hydrolysed demineralised whey  
 682 (DWPhyd) and (C) an infant formula protein blend with a 60/40 whey protein to casein ratio  
 683 (60/40IF) compared to a hydrolysed equivalent (60/40hyd).

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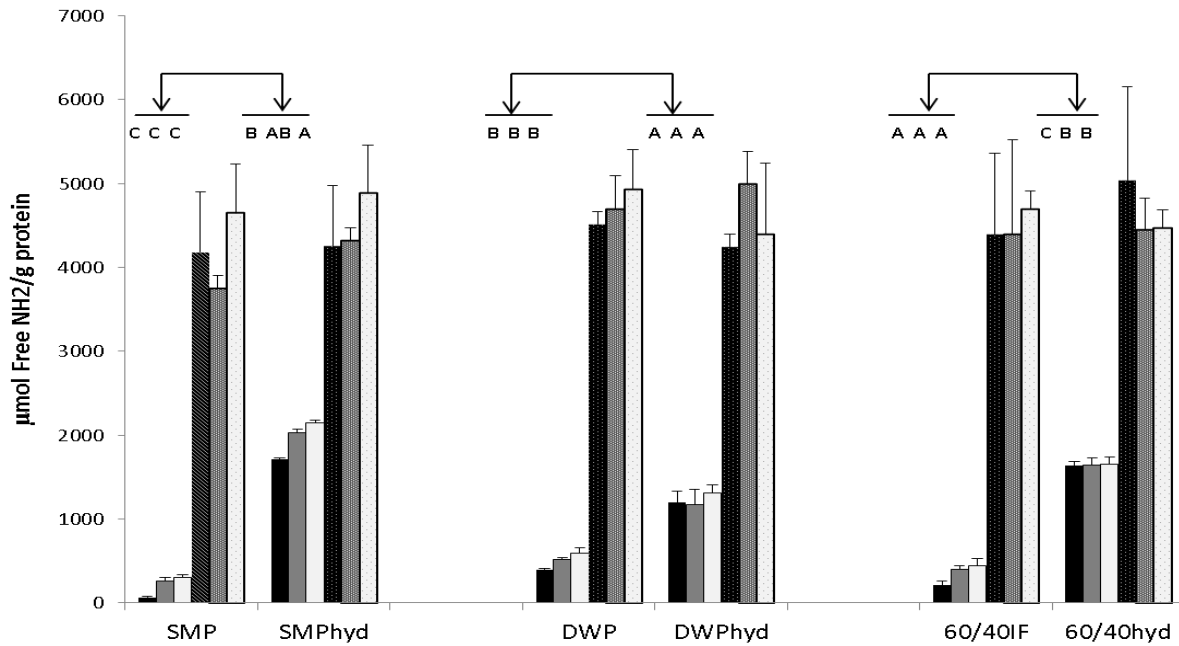




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693 **Fig. 3:** SDS-PAGE protein profiles of static digestion of (A) skim milk (SMP) compared to  
694 hydrolysed skim milk (SMPhyd); (B) demineralised whey (DWP) compared to hydrolysed  
695 demineralised whey (DWPhyd) and (C) an infant formula protein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

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700 **Fig. 4:** Concentration of free amine groups in µmol per g of protein as determined by OPA  
 701 assay: Comparison of digesta of SMP, DWP and 60/40IF to SMPhyd, DWPhyd and  
 702 60/40hyd in the gastric phase at time G0 (■), G30 (▒), G60 (□) and intestinal phase at  
 703 time I15 (■), I30 (▒) and I60 (□). Mean values within a column with different  
 704 uppercase letters (A, B, C) were significantly different (p <0.05), comparison was between  
 705 gastric time points only.

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