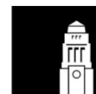

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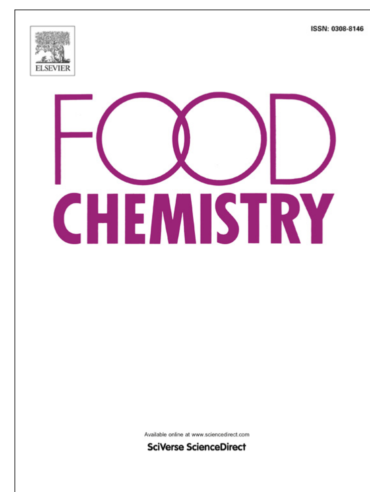

Impact of caseins and whey proteins ratio and lipid content on *in vitro* digestion and *ex vivo* absorption

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1 Impact of caseins and whey proteins ratio and lipid content on *in vitro* digestion and *ex vivo*
2 absorption

3

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21 **Abstract**

22 Caseins and whey proteins are known as 'slow' and 'fast' proteins, respectively, based
23 on their amino acid absorption rate. However, there is limited understanding of the
24 mechanisms controlling their behaviour during gastro-intestinal transit. A protein model
25 system (8% total protein) with varying casein:whey protein ratios (0:100, 20:80, 50:50 and
26 80:20) were subjected to *in vitro* gastro-intestinal digestion using a semi-dynamic gastric
27 model, a static intestinal model and an *ex vivo* absorption model (Ussing chambers). The
28 casein-rich (≥ 50%) samples showed the formation of solid coagula that were persistent
29 throughout gastric digestion, which caused a delay in nutrient emptying, slower digestion
30 and leucine absorption kinetics. In contrast, whey proteins formed more soluble aggregates
31 during the gastric phase, which led to faster gastric emptying, rapid intestinal hydrolysis, and
32 higher and faster leucine absorption. This work shows the key role of the gastric
33 restructuring for the overall digestive mechanism and kinetics of food, in particular proteins.

34 **Key words**

35 dairy proteins; caseins; whey proteins; gastric digestion; semi-dynamic model; digestion
36 kinetics

37

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38 **1. Introduction**

39 Milk proteins are considered a high quality protein source taking into account the
40 essential amino acid score, protein-digestibility corrected amino acid score (Schaafsma,
41 2000) and digestible indispensable amino acid scores (Mathai, Liu, & Stein, 2017).
42 Moreover, they are generally considered a superior source of protein compared to plant
43 proteins since the latter are generally less digestible and deficient in one or more essential
44 amino acids (AAs) and their leucine (Leu) content is 6-8%, compared to 10-13% in dairy
45 proteins (Gorissen & Witard, 2018). The main milk proteins, i.e. caseins and whey proteins,
46 have been reported to show different postprandial protein kinetics in humans, which affect
47 the whole body protein metabolism (Boirie, Dangin, Gachon, Vasson, Maubois, & Beaufrère,
48 1997). In this study, the ingestion of whey proteins resulted in a high, rapid and transient
49 increase in plasma AAs, promoting protein synthesis without supporting protein breakdown.
50 In contrast, caseins induced a low, slow and prolonged aminoacidemia profile, which
51 inhibited body protein breakdown. The Leu balance was positive for the casein drink over 7
52 hours, promoting protein deposition whereas no effect was provided from the whey protein
53 drink. From this study, whey proteins and caseins were labelled as 'fast' and 'slow' proteins,
54 respectively, as analogy to the carbohydrate metabolism. Some studies have also shown
55 that the faster digestion of whey proteins results in an enhancement of muscle protein
56 synthesis responses in elderly men (Dangin, Guillet, Garcia-Rodenas, Gachon,
57 Bouteloup-Demange, Reiffers-Magnani, et al., 2003; Pennings, Boirie, Senden, Gijsen,
58 Kuipers, & van Loon, 2011; West, Burd, Coffey, Baker, Burke, Hawley, et al., 2011). The
59 coingestion of other macronutrients with dairy proteins is another factor to consider that may
60 affect the metabolic responses. Elliot, Cree, Sanford, Wolfe, and Tipton (2006) showed that
61 the uptake of AAs, based on threonine and phenylalanine, was greater for whole milk
62 compared to fat-free milk.

63 The underlying mechanisms of the link between protein structure and metabolic
64 responses are not well understood. The main milk proteins have different physico-chemical

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65 properties, which are governed by their structure. Caseins have a relatively open and flexible
66 conformation, forming ordered structures known as casein micelles and are insoluble at pH
67 4.7. In contrast, whey proteins have a globular, compact structure and are more soluble
68 under acidic conditions. Food is subjected to several digestive conditions within the
69 gastrointestinal (GI) tract and the physico-chemical properties of food will determine the
70 changes in the different compartments. Therefore, it is important to understand the
71 interactions of food structures within the GI tract to underpin the health effects, but this
72 information is still scarce.

73 There are some studies suggesting the gastric phase as the rate-limiting step of some
74 of the metabolic effects observed; however its study has been rarely undertaken. Boirie,
75 Dangin, Gachon, Vasson, Maubois, and Beaufrère (1997) suggested that the slow AA
76 absorption behaviour of casein was due to the coagulation that might occur in the acidic
77 conditions of the human stomach, which could result in longer gastric emptying (GE),
78 delaying the digesta that is delivered into the small intestine, in contrast to the whey proteins
79 that remain soluble and can enter the small intestine rapidly. Investigation of the food
80 behaviour in the human stomach is complex and requires advanced techniques such as
81 magnetic resonance imaging or invasive techniques, which have restrictions in terms of cost
82 and ethics. For that reason, *in vitro* models are often used to investigate the mechanisms
83 controlling nutrient digestion within the GI tract (Dupont, Alric, Blanquet-Diot, Bornhorst,
84 Cueva, Deglaire, et al., 2018). However, static models do not simulate the dynamics of
85 digestion in the stomach, in particular progressive fluid secretion and peristaltic contractions,
86 which might affect structural changes. These gastric dynamics are of relevance, in particular
87 for milk proteins. For example, in contrast to what has been suggested *in vivo*, caseins were
88 reported to be digested rapidly using a static model (Egger, Schlegel, Baumann, Stoffers,
89 Guggisberg, Brügger, et al., 2017). Dynamic models are generally expensive to run and not
90 widely accessible. Therefore, a semi-dynamic model was developed (Ana-Isabel Mulet-
91 Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Ana-Isabel. Mulet-Cabero, Rigby,

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92 Brodkorb, & Mackie, 2017) and used in the present study to simulate the main dynamics of
93 the human stomach including a gradual pH decrease and progressive gastric secretion and
94 emptying. This model has been standardised in Mulet-Cabero, et al (2019) (accepted for
95 publication).

96 We hypothesised that the different rates of absorption of the main milk proteins were
97 governed by behaviour under gastric conditions was tested. Also, the strategy of controlling
98 nutrient uptake by the different rates of nutrient bioaccessibility was studied. This was
99 achieved using formulations differing in the ratio of whey proteins and caseins. The influence
100 of the inclusion of lipids in the protein matrix was also studied. Therefore, the aim of this
101 study was to investigate the influence of native protein structure on gastric behaviour that
102 could potentially result in different physiological responses.

103 2. Materials and methods

104 2.1. Materials

105 Whey protein isolate (WPI), BiPRO, was purchased from Davisco Foods international
106 INC, USA. The protein content was 88.48% (w/w) of dry powder measured by the Kjeldahl
107 method in duplicate (in-house analytical service). Milk protein concentrate (MPC), Solmiko®
108 MPC 80 (80% caseins: 20% whey proteins), was obtained from Glanbia Ingredients, Ireland.
109 The protein content was 79.23% (w/w) of dry powder measured by the Kjeldahl method in
110 duplicate (in-house analytical service). Rapeseed oil was purchased from a local
111 supermarket (Tesco, Ireland). Pepsin from porcine gastric mucosa (P7012), pancreatin from
112 porcine pancreas (P7545) and bile bovine (B3883) were purchased from Sigma Aldrich (St
113 Louise, MO, USA), and their activities were measured according the assays detailed in
114 Brodkorb, Egger, Alminger, Alvito, Assuncao, Ballance, et al. (2019). Pepsin had an activity
115 of 3555.12 units/mg protein, pancreatin had an activity of 6.48 units/mg based on trypsin and
116 bile extract had a concentration of 1.90 mmol/L. Deuterated Leu (5,5,5-D3, 99%), Ile (D10,
117 98%) and Val (D8, 98%) were purchased from Cambridge Isotope Laboratories, Inc. (CK

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118 Isotopes Ltd., Leicestershire, UK). MilliQ® water was used for the preparation of samples
119 and digestion fluids.

120 **2.2. Methods**

121 **2.2.1. Preparation of samples**

122 Table 1 shows the content of protein, lipid and total solids of the studied samples as
123 protein solutions and emulsions. The protein solutions referred to as 0C:100W and 80C:20W
124 were prepared at 8% (w/w) protein. The 0C:100W was prepared by dissolving WPI powder
125 in water using a mixer (IKA Eurostar, mix speed 700 rpm) with a paddle stirrer for 2 hours at
126 room temperature. The sample 80C:20W was prepared by dissolving MPC in water using a
127 mixer (IKA Eurostar, mix speed 700 rpm) with a paddle stirrer for 2 hours at 50 °C (using a
128 water bath). The commercial MPC was produced from skim milk by cascade membrane
129 filtration, which keeps the casein to whey ratio unchanged at 80:20. NaN_3 was added (0.02%
130 in the final solution) as an anti-microbial agent and the solutions were stored overnight at 4
131 °C for rehydration. The 20C:80W sample was prepared by mixing 75% (w/w) of 0C:100W
132 and 25% (w/w) of 80C:20W. The sample 50C:50W was prepared by mixing 37.5% (w/w)
133 0C:100W and 62.5% (w/w) 80C:20W. The pH of the samples was adjusted to pH 7 using
134 NaOH (2 mol/L).

135 Samples with lipid inclusion were prepared starting with the preparation of 0C:100W
136 and 80C:20W at 10% protein (w/w) following the same protocol described previously. Then,
137 the samples 20C:80W and 50C:50W were prepared in the same manner as described
138 before. Each stock protein solution was mixed with 2% rapeseed oil and water in order to
139 achieve a final protein concentration of 8% (w/w). These emulsion samples were named as
140 follows: (0C:100W)2%, (20C:80W)2%, (50C:50W)2% and (80C:20W)2%**.** The protein
141 solution of 50C:50W was also used to obtained emulsions containing 4% and 8% rapeseed
142 oil. The coarse emulsions were prepared using an Ultra-Turrax (T25 digital, IKA, Germany)
143 at 10,000 rpm for 1 min. Then, they were processed using a homogeniser (APV 1000, SPX

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144 Flow Technology, Charlotte, North Carolina, USA) at 200 bar x 3 passes. The pH of the
145 samples was adjusted to pH 7 using NaOH (2 mol/L).

146 ***2.2.2. In vitro* gastric digestion by the semi-dynamic model**

147 The simulation of the adult GI digestion was performed using a semi-dynamic model
148 previously described by Ana-Isabel Mulet-Cabero, Mackie, Wilde, Fenelon, and Brodkorb
149 (2019). The preparation of the digestion fluids, including electrolyte simulated salivary fluid
150 (eSSF), electrolyte simulated gastric fluid (eSGF) and electrolyte simulated intestinal fluid
151 (eSIF) (x1.25 concentrated) and proportions added of digestion fluids were according to
152 Brodkorb, et al. (2019).

153 Since the amount of oral mixture, dependent on the total solids of the sample, was
154 slightly different between the samples due to their composition, the amount of gastric mixture
155 was added accordingly. Table S1 of the supplementary material shows the detailed volumes
156 of both oral and gastric mixture added in the simulated digestion of each sample.

157 The simulation of the emptying was based on the caloric density. A linear GE rate of 2
158 kcal/min, which is considered the average caloric content that is emptied *in vivo* in a
159 regulated manner by the antrum for an average food volume of 500 mL (Hunt, Smith, &
160 Jiang, 1985), was used and scaled down for this reduced-volume system. This implied that
161 the volume and time of each emptying point differed between protein solutions and
162 emulsions due to variations in the caloric content (see Table S2 of the supplementary
163 material).

164 Gastric emptying (GE) was simulated by taking five aliquots, referred to as GE1-5 in
165 the text. Samples were taken from the bottom of the vessel using a 10 mL plastic syringe
166 (BD Plastipak, Ireland), the aperture of which had an inner diameter of 2.5 mm with a plastic
167 tube attached (3.6 mm inner diameter). This was aimed to simulate the gastric sieving of 2
168 mm approximately created by the pylorus (Thomas, 2006). It is important to note that in
169 some cases there was some residue left in the last GE point that could not be taken using

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170 that syringe; this was taken using a spatula and included in the last point. The pH was
171 measured and a sufficient volume of NaOH (2 mol/L) was added to the samples to increase
172 the pH above 7, inhibiting pepsin activity. Finally, samples were snap-frozen in liquid
173 nitrogen and stored at -20 °C until subsequent intestinal digestion. A separate gastric
174 digestion was performed in order to study the gastric restructuring and nutrient delivery. An
175 aliquot (250 µL) of these GE aliquots was used for microscopy. Then, the GE sample was
176 mixed using a homogeniser (T10 basic Ultra-Turrax®, IKA®, Germany) at approximately
177 30,000 rpm for 30 s to obtain a homogenous sample for subsequent analyses.

178 **2.2.3. *In vitro* intestinal digestion and ex *vivo* absorption by Ussing chamber**
179 **technique**

180 The Ussing chamber methodology, which consisted of two chambers separated by a
181 fresh piece of murine intestine that simulated the transfer of nutrients across the intestinal
182 barrier based on the polarity and tightness of epithelia, was applied to study the absorption
183 kinetics of amino acids by *ex vivo* murine small intestinal tissue (He, Yin, Li, Huang, Xie, Wu,
184 et al., 2013).

185 The digesta emptied in the early (i.e. GE1) and late (i.e. GE5) stages of the gastric
186 digestion were selected to assess the behaviour of the samples The Ussing chamber
187 experiment was performed using an *in situ* intestinal digestion in order to simulate the
188 simultaneous processes of protein/peptide hydrolysis and AA absorption, in contrast to the
189 use of pre-digested samples in most of the studies previously presented in the literature
190 (Awati, Rutherfurd, Plugge, Reynolds, Marrant, Kies, et al., 2009).

191 **2.2.3.1. Intestinal tissue samples**

192 All animal protocols were approved by local ethical review committees and conformed
193 to relevant national guidelines (University of Leeds, Leeds, UK). Intestinal tissue sections
194 were obtained from 6 to 8-week-old male/female mice (strain C57BL/6). The animals had
195 free access to water and usual meal any time before the collection. Mice were euthanized by

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196 cervical dislocation. The whole length of the digestive tract was immediately collected,
197 flushed through with ice-cold 10 mmol/L glucose solution and transported in the same
198 medium in an insulating bag.

199 **2.2.3.2. Ussing chamber set up**

200 A 1.5 cm jejunum section was taken and cut longitudinally along the mesenteric
201 attachment. The section was washed with 10 mmol/L glucose solution and most of the
202 muscularis layer was stripped away with fine forceps. The tissue segment was mounted on
203 the slider (P2404, Physiologic Instruments, San Diego, USA), which was inserted in an
204 Ussing chamber system (EM-CSYS-4 with low volume P2400 chamber), separating the
205 chamber in apical and basolateral sides. Up to three segments from each animal were used.
206 The active epithelial surface area of each segment was 0.25 cm^2. The set up of the Ussing
207 chamber and the measurements were performed according to Brighton, Rievaj, Kuhre,
208 Glass, Schoonjans, Holst, et al. (2015). The open-circuit transepithelial potential difference
209 was continuously monitored using a DVC-1000 multichannel voltage clamp unit (World
210 Precision Instruments, New Haven, USA) with a Micro1401-3 data acquisition unit
211 (Cambridge Electronic Design, Cambridge, UK). The recordings were collected using Spike2
212 8.08 software (Cambridge Electronic Design, Cambridge, UK).

213 **2.2.3.3. *In situ* intestinal digestion**

214 The sample placed in the apical side of the Ussing chamber was aimed to simulate an
215 *in situ* intestinal digestion while diffusion took place. For that, an aliquot from gastric
216 digestion was quickly mixed with bile solution, water, $CaCl_2(H_2O)_2$ and pancreatin solution (in
217 eSIF). The proportions used were according to the standardised INFOGEST static protocol
218 for small intestinal digestion (Brodkorb, et al., 2019), in order to achieve 100 U/mL of
219 pancreatin, 0.6 mmol/L $CaCl_2(H_2O)_2$ and 10 mmol/L of bile, in the final digestion mixture. A
220 volume of this mixture was mixed with Ringer solution (10x concentrated) and mannitol (100

221 mmol/L) to achieve a final concentration of 1x Ringer and 10 mmol/L mannitol in the sample
222 that was finally placed in the apical side of the Ussing chamber.

223 **2.2.3.4. Sampling in Ussing Chamber**

224 An aliquot of 100 µL was taken from both apical and basolateral compartments at 5, 30
225 and 60 min and the same volume was replaced with fresh Ringer solution containing 10
226 mmol/L mannitol and glucose accordingly. The aliquots collected were mixed with 100 µL of
227 24% trichloroacetic acid to stop protease activity and stored at -20 °C for further analysis.
228 These samples were analysed for the concentration of the individual BCAAs (i.e. Leu, Ile
229 and Val) as indicated in section 2.2.8.

230 Two Ussing chambers were used simultaneously each experimental day using the
231 tissue sections of one mouse. The two GE points, i.e. GE1 and GE5 of each sample were
232 usually assessed each day.

233 **2.2.4. Confocal laser scanning microscopy**

234 The microstructure of the initial and the emptied aliquots from the semi-dynamic
235 gastric digestion was observed by confocal laser scanning microscopy using a Leica TCS
236 SP5 microscope (Leica Microsystems, Baden-Württemberg, Germany). The images were
237 taken using both 20 x and 63 x oil-immersion objectives and simultaneous dual-channel
238 imaging, He–Ne laser (excitation wavelength at 633 nm) and an Argon laser (excitation
239 wavelength at 488 nm). A dye mixture of Fast green FCF solution (0.1% made with water)
240 and Nile red solution (0.1% made with propanediol) at 1:3 proportion was used. 250 µL of
241 initial/digested sample was gently mixed with 25 µL of mixed dye.

242 **2.2.5. Texture analysis of gastric digesta**

243 Some indication of the consistency with regards to the strength of the coagulum
244 formed during semi-dynamic gastric digestion was assessed using a texture analyser (TA.XT
245 Plus, Stable Micro Systems, surrey, UK) by performing a penetration test. For that, additional

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246 gastric digestions were performed but stopping the run at GE2 time and the vessel of
247 reaction was immediately placed in the texture analyser instrument. The digesta was
248 compressed by a cylindrical stainless-steel probe (6 mm diameter) until the distance of the
249 probe inside the coagulum was 10 mm. The test was run at a speed of 1.0 mm/s and the
250 trigger force was 1 g. Five measurements were made for the same digesta sample and three
251 independent gastric digestions were performed. The value of strength, i.e. the maximum
252 force, in each measurement was obtained from the force-time curve of the texture profile.

253 **2.2.6. Total protein and lipid content analysis**

254 The protein and lipid content of the initial sample and the emptied aliquots was
255 determined using a LECO FP628 Protein analyser (LECO Corp., St Joseph, MI, USA) and
256 CEM Smart Trac System-5 and a SMART Trac Rapid Fat Analyzer (CEM Corp., Matthews,
257 NC, USA), respectively, as described in (Ana-Isabel Mulet-Cabero, Mackie, Wilde, Fenelon,
258 & Brodkorb, 2019). Each measurement was carried out in three independent replicates.

259 **2.2.7. Protein identification in emptied digesta**

260 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which is
261 described in Ana-Isabel Mulet-Cabero, Mackie, Wilde, Fenelon, and Brodkorb (2019) was
262 used to determine protein composition. SDS-PAGE was performed on the initial and
263 digested samples previously diluted (1:100) with water.

264 **2.2.8. Liquid chromatography coupled to tandem mass spectrophotometry for amino**
265 **acid analysis**

266 The branch chain AAs (BCAAs), i.e. Leu, Ile and Val, were detected and quantified by
267 LC-MS/MS on the samples from the Ussing chamber experiments, from both apical and
268 basolateral sides.

269 A mixture of Leu, Ile and Val standard was used for the calibration ranging from 0.31 to
270 10 µmol/L. Mixed isotope labelled internal standards consisted of 5 µmol/L of each

271 deuterated Leu, Ile and Val. 10 μL of this internal standard was added to 50 μL of each
272 concentration standard or sample. After centrifugation (17,403 × g, 4 °C for 10 min), sample
273 was transferred to HPLC vials for LC-MS/MS analysis. Agilent 6490 Triple Quad MS mass
274 spectrometer equipped with an Agilent 1290 HPLC system (Agilent Technologies, Santa
275 Clara, CA, USA) was used. The method for the separation of AAs was adapted from
276 Nemkov, D'Alessandro, and Hansen (2015). The LC flow rate was 0.78 mL/min. The column
277 used for the analysis was Phenomenex Kinetex XB-C18 2.6 μm (150 x 4.6 mm) column. The
278 temperature of the column and auto sampler were maintained at 25 °C and 4 °C,
279 respectively. 1 μL was used for the injection volume. The samples were analysed using
280 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile
281 phase B). The isocratic elution was 5% mobile phase B and 95% mobile phase B. The run
282 time was 6 min.

283 The 6490 MS/MS system was equipped with an electrospray ionization source
284 operated in positive-ion detection mode. Nitrogen gas was used for nebulization,
285 desolvation, and collision. The analytes were monitored in multiple-reaction monitoring
286 (MRM) mode. The MRM precursor, product ions and collision energy were optimized by
287 Agilent optimizer software. The transitions of precursor ions to product ions (*m/z)* and some
288 optimized MS operating parameters of the analyte are described in Table S3 of the
289 supplementary material.

290 The quantification was done by the MassHunter Quantitative B.06 Workstation
291 software (Agilent Technologies, CA, US). Calibration curves were obtained by using
292 authentic standards (Leu, Ile and Val) containing deuterated Leu, Ile and Val mixture as
293 internal standard. The ratio of analyte and internal standard peak area was plotted against
294 the corresponding concentration (0-10 μmol/L) to obtain the calibration curve. In the
295 analysed samples, the peak area ratio (peak area of analyte/peak area of the internal
296 standard) was calculated and applied to the calibration curve to obtain the concentration of
297 each AA.

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298 **2.2.9. Statistical analysis**

299 The results were expressed as mean ± standard deviation of three independent
300 replicates unless otherwise stated. To identify differences in normally distributed results
301 within groups during gastric digestion, one-way ANOVA was applied. Where overall
302 significant interaction was observed ($p < 0.05$), the means of individual formulations were
303 compared using Tukey's post hoc test. Statistical analyses were performed using GraphPad
304 Prism software (Prism 5 for Windows, Version 5.04).

305 **3. Results and discussion**

306 **3.1. Influence of protein formulation and addition of lipids on the gastric behaviour**

307 Figure 1 shows images of the semi-dynamic gastric digestion vessel containing the
308 protein solution samples at the time points of GE1, GE3 and GE5. In general, all the
309 samples presented protein aggregation within the first 10 min of gastric digestion. However,
310 those small aggregates in samples 0C:100W and 20C:80W progressively dissipated and re-
311 solubilised, resulting in a completely clear solution at the end of the gastric digestion. The
312 extent of this aggregation was visually larger in the case of 20C:80W as seen in Figure 1F,
313 and the clear solution was obtained later on in the gastric digestion when compared to the
314 0C:100W sample. In contrast, the initial aggregates formed within the vessel in the samples
315 50C:50W and 80C:20W led to the formation of firm and compact coagula located at the
316 bottom of the vessel, similar to mozzarella cheese in texture, and a clear layer at the top.
317 However, according to visual observations, the formation of that compact coagula in the
318 case of 50C:50W tended to be slightly later and having a more particulate consistency
319 compared with 80C:20W. The total digesta volume of the samples obtained at GE2 time was
320 placed on a petri dish (Figure 1 M-P), illustrating the range of structures obtained in the
321 gastric phase.

322 The distinct behaviour of the milk proteins observed in the present study is in
323 agreement with suggestions in the literature of casein coagulation in the stomach (Boirie,

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324 Dangin, Gachon, Vasson, Maubois, & Beaufrère, 1997; Hall, Millward, Long, & Morgan,
325 2003; Mahé, Roos, Benamouzig, Davin, Luengo, Gagnon, et al., 1996). Similar to our
326 results, Wang, Lin, Ye, Han, and Singh (2018) showed, using the Human Gastric Simulator
327 (HGS), that MPC presented a firm coagulation whereas WPI did not present any aggregation
328 after 220 min of gastric digestion. Moreover, the authors reported that the coagulation of the
329 MPC sample was visible in the first 10 min of digestion corresponding to a pH of
330 approximately 6, in agreement to the present study.

331 The difference in gastric behaviour between the main milk proteins can be attributed to
332 their molecular structure and physico-chemical properties. Caseins are insoluble at pH 4.6
333 (isoelectric point) whereas native whey proteins remain soluble but can aggregate at that pH
334 when heat-denatured or covantly linked to caseins (Alting, de Jongh, Visschers, & Simons,
335 2002). In the present study, the protein aggregation started after 10 min of gastric digestion
336 at pH values of about 6 for both 50C:50W and 80C:20W, which was much higher pH than
337 the isolectric point of caseins. This suggests that the initial coagulation could be driven by
338 the action of pepsin that has been reported to favour the hydrolysis of κ-caseins, cleaving
339 among others the -Phe-105-Met-106 bond in κ-casein, and the other caseins at pH 6.0
340 (Tam & Whitaker, 1972), which will reduce the steric repulsion between casein micelles and
341 destabilise the overall structure of the casein micelle. Also, the samples were added to a
342 basal volume of SGF of low pH, therefore some of the protein could present a lower pH that
343 might not be homogenous in the system and accelerate the process of aggregation.

344 Similar gastric behaviour was observed in the samples with the addition of 2% lipid
345 (Figure S1 of the supplementary material), with the formation of solid coagula in the
346 formulations containing at least 50% of casein content. Samples containing 4% and 8% lipid
347 within the same protein C:W ratio of 1, i.e. formulation 50C:50W (Figure S2 of the
348 supplementary material) also presented similar behaviour. However, it was observed that the
349 inclusion of lipid reduced the firmness of the coagulum, in particular for the sample with the
350 highest lipid content.

351 **3.1.1. Strength and structure of the gastric coagula**

352 Figure 2 shows the strength of the coagula corresponding to GE2 time point in
353 50C:50W, 80C:20W, (50C:50W)2%, (80C:20W)2% and (50C:50W)4%. The sample
354 (50C:50W)8% could not be measured because the strength of the coagula was below the
355 limit of the detection of the instrument. The sample 80C:20W presented the highest value of
356 force accounting for 22.8 g whereas the weakest coagula were obtained in the sample with
357 the highest lipid content tested, i.e. (50C:50W)4%, resulting in an average value of 2.25 g.
358 The results showed that the addition of lipid significantly affected the coagula strength of the
359 milk sample with C:W ratio of 4, i.e. 80C:20W, more than for the samples containing C:W
360 ratio of 1, i.e. 50C:50W, in which the decline of the coagula strength was much less strongly
361 affected. Similarly, Lambers, van den Bosch, and de Jong (2013) showed that the viscosity,
362 measured by an in-line rheometer during the gastric digestion, of caseins was higher than
363 the whey proteins in solution. However, the casein source was from sodium caseinate, which
364 might affect the formation of the solid coagula and differ from the observed coagula using
365 MPC. Indeed, Wang, Lin, Ye, Han, and Singh (2018) reported that the coagulation formed in
366 sodium caseinate was loose and fragmented compared to the dense coagula obtained in the
367 MPC sample, which can be attributed to differences in the casein association.

368 The addition of lipid seemed to weaken the protein aggregates formed, which was
369 particularly visible at microscopic level, for instance in 50C:50W comparing Figure 2c with
370 Figure 2f. The fragmentation of the coagulum was enhanced as more lipid was added,
371 showing a great number of protein particles of different sizes with some free droplets,
372 particularly in (50C:50W)8% (Figure 2f). It seems that lipids hamper the casein interactions,
373 which leads to the formation of a less cohesive protein network. Therefore, it was shown that
374 lipid inclusion weakened the consistency of the coagula but it was only significant in the
375 hardest protein network of C:W ratio of 4.

376 **3.2. Effect of gastric behaviour on nutrient delivery**

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377 Figure 3A shows the protein content at each GE point in the four main formulations with and
378 without the addition of 2% lipid, which can be associated with the proteins that were
379 delivered from the stomach to the small intestine during the simulated gastric digestion. The
380 formulations with higher whey protein contents (C:W ratio of 0 and 0.25) presented a
381 remarkably similar trend, showing the highest protein delivery in GE1 and gradually
382 decreasing during gastric digestion with the lowest amount of protein delivered at GE5,
383 regardless of lipid addition. In contrast, the formulation with the highest casein content (C:W
384 ratio of 4) showed the lowest protein delivery at the early stages of gastric digestion, in
385 particular in GE2, increasing in GE4 and significantly higher in GE5. An intermediate pattern
386 between these two extreme behaviours was found in the formulation with a C:W ratio of 1, in
387 which there was a more constant protein delivery for a longer gastric digestion time, from
388 GE1 to GE4. However, using the same protein ratio, the inclusion of 2% lipid resulted in a
389 lower protein concentration emptied in GE5.

390 The pattern of lipid delivery for the emulsion samples with 2% lipid (Figure 3B) showed
391 similar trends to those observed for protein. There was a progressive decline in lipid
392 concentration during the gastric digestion in the samples with higher whey protein contents
393 (0C:100W)2% and (20C:80W)2%. In contrast, higher lipid delivery was observed in GE5 for
394 (50C:50W)2% and (80C:20W)2% accounting for 4.43 and 4.84% (w/w) lipid, respectively,
395 which suggests the entrapment of lipid in the protein coagulum. This shows the effect of the
396 protein matrix on kinetics of lipid emptying since the lipid delivery was driven by the structure
397 formed in the simulated stomach. It is important to note that the duration of the gastric phase
398 was longer when lipid was included as highlighted in the Table S2 of the supplementary
399 material due to a higher caloric content.

400 The gastric behaviour of the protein formulations impacted the *in vitro* kinetics of
401 protein emptying, simulating the delivery from the stomach to the small intestine. The solid
402 coagulation, in particular in the 80C:20W sample, led to delayed protein delivery through the
403 retention of caseins at GE5 as seen by SDS-PAGE (Figure 4A). The solid coagulum that

404 formed, physically resisted being emptied from the stomach through the tubing, in a manner
405 similar to the way the pylorus prevents the emptying of solids or large particulates, but
406 allowing the liquid phase to be emptied. In contrast, the formation of small aggregates that
407 were suspended in a serum in the formulations with higher whey protein concentrations
408 allowed the liquid phase containing these small aggregates to be emptied, enabling a larger
409 extent of protein delivery at the earlier stage of digestion. This was related to the gradual
410 emptying of intact β-Lg (Figure 4A) and was controlled mainly by the dilution of the gastric
411 contents by the continuous secretions and emptying. Similarly, Wang, Lin, Ye, Han, and
412 Singh (2018) showed that, in the gastric digestion of WPI, β-Lg remained intact during the
413 gastric digestion due to the well-known property of the native β-Lg to resist hydrolysis by
414 pepsin because of its compact globular structure. Moreover, in the same study, the authors
415 detected strong bands of intact caseins from the coagulum particles after 220 min of
416 digestion of MPC, similarly to the present results. Interestingly, the sample 50C:50W
417 presented an intermediate pattern of protein delivery, which can be attributed to the softer
418 coagula formed during the gastric digestion that was more easily emptied. Moreover, the
419 latter sample presented a more constant emptying of both caseins and whey proteins as
420 observed in the electrophoresis gel (Figure 4A), which may be related to a reduced
421 syneresis in that sample.

422 The incorporation of lipids affected the protein network structure and modify their
423 digestion and behaviour within the GI tract. the casein bands seemed to be weaker, in
424 particular in the case of (50C:50W)2% and (80C:20W)2% at GE5, showing a more constant
425 and lower content of caseins during gastric digestion (Figure 4B). Guo, Ye, Lad, Ferrua,
426 Dalgleish, and Singh (2015) studied, in whey protein emulsion gels (hard versus soft), the
427 effect of gastric disintegration using the HGS on lipid bioaccessibility during a simulated
428 intestinal digestion. The size of the gel particles was reduced after 60 min of gastric
429 digestion in both samples but the initial rate of lipolysis of the soft gel was significantly higher
430 than the hard gel, even though the solid content of that digesta was lower. At 240 min, the

431 digesta from the soft gel consisted of individual oil droplets as well as smaller particles,
432 compared to the hard gel in which most of the oil droplets remained within the protein
433 network. The latter study represents an example of the engineering of gels for specific GI
434 functionality, however the same principle can be applied for protein structures formed within
435 the gastric conditions. Therefore, this shows that protein networks can modulate the release
436 of lipids to the intestine and control the subsequent digestion and absorption kinetics.

437 3.3. Effect of *in vitro* gastric behaviour on protein digestion and absorption in the 438 small intestine *ex vivo*

439 Figure 5 shows the free Leu content in the apical and basolateral sides of the Ussing
440 chamber. Leu was selected since it plays a key role in the body protein deposition (Garlick,
441 2005). The rest of BCAAs quantification can be seen in the supplementary material (Table
442 S4-S13). The determination in the apical side represents the Leu content that was digested
443 and became accessible whereas the determination in the basolateral side represents the
444 Leu content that was able to be absorbed and transported across the intestinal wall and thus
445 available to be metabolised and used for physiological functions.

446 In general, the Leu concentration in all the samples in both GE points increased during
447 small intestinal digestion, showing the progressive breakdown of the protein, liberating AAs
448 and increasing their absorption. However, the rate and extent of these processes were
449 different between the samples. In GE1 (Figure 5A), among the protein solution samples in
450 the absence of lipid, the sample 0C:100W showed the most rapid increase in Leu
451 concentration whereas the lowest rate was found in the sample 80C:20W. These patterns
452 were related to the Leu concentration that was absorbed (basolateral concentrations). The
453 highest rate of Leu absorption was observed in 0C:100W whereas the samples 50C:50W
454 and 80C:20W presented the slowest rate with a similar pattern. In contrast, in GE5, there
455 was a rapid rate of Leu accessibility in the samples 50C:50W and 80C:20W, which was
456 reflected in a higher amount of absorbed Leu obtained in the basolateral side. This

457 behaviour could be related to the available protein in GE5 of the samples with higher casein
458 content (Figure 3 A).

459 The patterns in emulsion samples with the inclusion of 2% lipid (Figure 5B and b) were
460 similar to the respective samples without lipid, however, it seemed that the differences
461 between the extremes are reduced. In addition, the samples with different inclusions of lipid
462 using the sample protein C:W ratio of 1 i.e. 50C:50W, (50C:50W)2%, (50C:50W)4% and
463 (50C:50W)8% were also considered in this analysis (Figure 5C for GE1 and Figure 5c for
464 GE5). They presented significant differences in GE5. The sample without lipid (50C:50W)
465 showed more rapid rate and higher concentration of Leu that was accessible, which led to a
466 higher absorbed Leu concentration in contrast to the sample (50C:50W)8%.

467 This *ex vivo* model using intact intestinal tissue segments from an animal provides a
468 better representation of the *in vivo* situation since it provides the morphological and
469 physiological features of the intestinal wall, including the multicellular conglomeration and
470 presence of the mucus layer allowing the simulation of the possible further hydrolysis of
471 some peptides by aminopeptidases located on the brush border membranes. It is important
472 to note that a lower concentration of Leu in the basolateral side was obtained comparing to
473 the apical side, which could be attributed to the reduce area of the mouse tissue in the
474 Ussing chamber experiment resulting in possible saturation in the tissue hampering the AA
475 transport. There are a few studies investigating the absorption of AAs by Ussing chamber
476 but the results are difficult to compare since the experimental set ups are different, including
477 the source of the animal tissue (Grøndahl & Skadhauge, 1997).

478 In general, it was shown quite clearly that the different rates of delivery of Leu from the
479 different samples at different stages of digestion, explained how whey proteins and caseins
480 are responsible for most of the early and late AA delivery, respectively. For protein solutions,
481 the sample 0C:100W presented the highest rate and extent of both Leu accessibility and
482 absorption in the first GE aliquot when compared with the samples 50C:50W and 80C:20W.
483 In contrast, the latter samples presented a higher level of digestion and absorption in GE5,

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484 which might be attributed to the delayed protein delivery to the intestinal phase due to the
485 coagula that remained at the end of the gastric digestion. These results are in agreement
486 with the plasma Leu concentrations after protein ingestion obtained by Boirie, Dangin,
487 Gachon, Vasson, Maubois, and Beaufrère (1997), in which they described whey proteins
488 and casein as 'fast' and 'slow' proteins respectively, a concept previously adopted for dietary
489 carbohydrates due to the evidence of the link between their rate of digestion and absorption,
490 and metabolic response. Boirie, Dangin, Gachon, Vasson, Maubois, and Beaufrère (1997)
491 showed that there was a rapid increase in plasma Leu levels after the ingestion of a whey
492 protein drink when compared to that of caseins, which showed a more attenuated pattern
493 over time. Similarly, in the present study, the rate and extent of Leu absorption at early
494 stages of digestion was higher in the sample 0C:100W whereas the sample with higher
495 content of casein presented low levels of Leu absorption at the beginning but a substantial
496 increase in the later stage of the digestion, that could have been prolonged if the gastric
497 digestion had lasted longer. Interestingly, the sample 50C:50W, showing a solid coagulation
498 with weaker coagula consistency, could present an intermediate metabolic effect since the
499 kinetics of protein delivery and Leu absorption were overall showing middle levels. It is
500 important to note that the sample containing 100% casein was not included in this study
501 however a similar behaviour to the sample containing 80% can be expected. Lacroix, Bos,
502 Léonil, Airinei, Luengo, Daré, et al. (2006) showed that a milk protein drink containing 20:80
503 whey protein:casein ratio presented no significant difference in the dietary nitrogen utilization
504 when compared to 100% casein drink, which might be due to the profound effect of solid
505 coagulation in the stomach from a casein content level higher than 80%.

506 The distinct absorption pattern could be attributed to a higher level of Leu present in
507 whey proteins compared to caseins (Gorissen & Witard, 2018). In the current study the
508 protein content was matched but they differed in the Leu content due to the nature of the
509 proteins. Nevertheless, this did not affect the distinct absorption pattern of the milk proteins
510 as studied by Boirie, Dangin, Gachon, Vasson, Maubois, and Beaufrère (1997) *in vivo*, in

511 which both casein and whey protein drinks were matched in Leu content as well, showing
512 that that Leu content was not the limiting factor for the protein synthesis. Moreover, the
513 patterns for the other measured BCAAs reflected that of Leu pattern (Table S4-S13 of
514 supplementary material) showing the relevance of the kinetics of digestion. Indeed, the
515 independence of the protein digestion rate on modulating postprandial deposition of protein
516 was also confirmed by Dangin, Boirie, Garcia-Rodenas, Gachon, Fauquant, Callier, et al.
517 (2001). In addition, the effect of the protein digestion rates on the protein metabolism
518 seemed to be age-dependent (Dangin, et al., 2003; Pennings, Boirie, Senden, Gijsen,
519 Kuipers, & van Loon, 2011). Pennings, Boirie, Senden, Gijsen, Kuipers, and van Loon
520 (2011) showed that whey proteins resulted in a more effective enhancement in protein
521 retention than casein in the elderly.

522 The inclusion of higher levels of lipid did not affect the Leu absorption pattern at the
523 early stage but it lowered the Leu absorption in the latest stage, which could be attributed to
524 the lower protein delivery at the end of gastric digestion due to the softer coagula formed.
525 These findings are in contrast with that of Gaudichon, Mahé, Benamouzig, Luengo, Fouillet,
526 Daré, et al. (1999), showing no difference in postprandial protein utilization when milk protein
527 was supplemented with milk fat compared to the milk protein alone. This contrasts with Elliot,
528 Cree, Sanford, Wolfe, and Tipton (2006), in which the *in vivo* ingestion of whole milk was
529 suggested to increase the utilization of AAs for protein synthesis when compared to fat-free
530 isocolaric milk. There is little information about the influence of other macronutrients in the
531 postprandial nitrogen utilization to draw any conclusion hence more research is needed.

532 **3.4. Method limitations**

533 A semi-dynamic *in vitro* model was used in this study. which reproduces some of the
534 main dynamic processes in the human stomach, including the gradual pH decrease (Figure
535 S3 of the supplementary material). The pH is a crucial factor affecting the structure, charge
536 and interaction of proteins, and enzyme activity and therefore affecting the kinetics of protein
537 degradation. Emptying rate is the other main essential parameter in the gastric phase. In the

538 semi-dynamic model, the rate of GE was based on the caloric content of the sample. This
539 GE approach implied that the emptying time of the samples was the same regardless the
540 behaviour of the proteins in the simulated stomach. However, this may not be totally
541 accurate since casein might present longer times of gastric digestion due to the formation of
542 the solid coagula that remains longer in the stomach to be broken down and emptied, which
543 may influence the extent of the protein hydrolysis. In humans, Boutrou, Gaudichon, Dupont,
544 Jardin, Airinei, Marsset-Baglieri, et al. (2013) found that, after casein ingestion, the delivery
545 of dietary protein in the jejunum was progressive for 6 hours and in the form of medium size-
546 peptides (750-1,050 Da) whereas the ingestion of whey protein induced the release of
547 larger-size peptides (1,050-1,800 Da) and was completed after 3 hours. The regulation of
548 GE is a complex process which depends on factors including gut hormones and properties
549 of food, e.g. viscosity, consistency, volume, particle size and caloric density. However a
550 simple *in vitro* model cannot take into account all of these factors so the present semi-
551 dynamic model considered the caloric density as the main factor in regulating the rate of GE;
552 a high caloric density inducing a slower/longer emptying.

553 Gastric motility also plays an important role in food disintegration, which is
554 characterised by two types of motion. There is a slow motor activity at the upper part of the
555 stomach, fundus and body, by which gastric contents are pushed into the antrum. Therefore,
556 the body part mainly acts as a storage place with negligible mixing. In contrast, the strongest
557 fluid motions are found in the antral part and are responsible for the major food grinding and
558 mixing with gastric fluids (Ferrua & Singh, 2010). However, the simulation of these
559 mechanical forces is rather difficult due to their complexity in amplitude, frequency and
560 intensity. The present model does not allow an accurate simulation of the gastric motility in
561 particular the mechanical forces of the antrum. However, the weak mixing applied allows the
562 mixing of the fluids added with the food and possible structure formed during digestion. For
563 instance, studies have shown phase separation in the stomach, highlighting the low mixing
564 in the body (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013; Marciani, Wickham, Singh,

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565 Bush, Pick, Cox, et al., 2007) contradicting the idea of intragastric homogenisation of the
566 food.

567 Despite the simplification of the gastric processes, the results showed distinct
568 digestion kinetics of the different milk proteins highlighting the concept of 'slow' and 'fast'
569 proteins observed in vivo as described above.

570 **4. Conclusions**

571 This study has proposed underlying mechanisms behind the denoted 'fast' and 'slow'
572 digested protein for whey proteins and caseins, respectively. The main milk proteins
573 presented different digestive behaviour and AA availabilities, which are factors for defining
574 protein quality, and the gastric phase of digestion was shown to be the rate limiting step. The
575 solid coagulation of the casein-rich samples contributed to the delay in nutrient delivery from
576 the gastric phase and thus overall digestion and AAs absorption kinetics. In contrast, whey
577 proteins formed small aggregates during gastric digestion that led to a gradual decrease of
578 nutrient delivery and a higher Leu absorption in early stages of GI digestion. The modulation
579 of the solid coagula could be obtained by addition of whey proteins and lipid, which altered
580 the kinetics of digestion. The differences in AA absorption kinetics, as modulated through
581 gastric behaviour, can be associated to different physiological effects. Therefore, this
582 methodological approach is a powerful tool to understand the mechanisms underlying the
583 physiological impact of foods, in order to design foods with different rates of nutrient
584 digestion addressed to the nutritional and health needs of different populations. There is
585 evidence showing that a slower pattern of protein digestion leads to a better postprandial
586 utilization of dietary nitrogen, improving AA retention. It was also shown the rapid
587 appearance of high plasma AA concentrations from the 'fast' protein such as whey proteins
588 induced greater deamination rates by the liver, which decreases the AA concentration in
589 plasma.

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590 In general, the understanding of the gastric phase and how it modulates the gastric
591 behaviour for instance of protein formulation needs further investigation using appropriate
592 models since it offers great potential to design foods that can exert physiological effects such
593 as satiety, glycaemic control, lipemia control and improve GI complications such as reflux
594 and aspiration pneumonia.

595

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604 **Conflicts of interest**

605 The authors are not aware of any affiliations, memberships, funding or financial holdings that
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732 **Tables**

733 **Table 1.** Compositional description of the studied samples.

	Protein composition[1]				
Sample	Caseins % (w/w)	Whey proteins % (w/w)	Added lipid % (w/w)	Total solids[2] %	Mean particle diameter $d_{4,3}$ (μm)[3]
0C:100W	0	8	0	8.58 ± 0.08	nm
20C: 80W	1.8	6.4	0	8.96 ± 0.08	nm
50C:50W	4	4	0	9.47 ± 0.04	nm
80C: 20W	6.4	1.8	0	9.83 ± 0.06	nm
(0C:100W)2%	0	8	2	10.45 ± 0.02	0.73 ± 0.08
(20C: 80W)2%	1.8	6.4	2	10.68 ± 0.06	0.53 ± 0.03
(50C:50W)2%	4	4	2	11.19 ± 0.04	0.34 ± 0.02
(80C: 20W)2%	6.4	1.8	2	11.71 ± 0.03	0.29 ± 0.04
(50C:50W)4%	4	4	4	12.98 ± 0.01	0.47 ± 0.06
(50C:50W)8%	4	4	8	16.67 ± 0.05	0.62 ± 0.04

734

735 [1]The content of whey proteins and caseins was based on the content of 80% caseins and 20% whey
736 proteins in the MPC that was used. [2]The total solid content was measured using CEM Smart Trac
737 System-5 (CEM Corp., Matthews, N.C., U.S.A.). Values are the mean ± standard deviation of two
738 independent duplicates. [3]The droplet size was measured using static light scattering with a laser
739 diffraction unit (Mastersizer, Malvern Instruments Ltd, Worcestershire, UK). The optical parameters
740 chosen were a particle and dispersant (water) refractive index of 1.47 and 1.33, respectively. The
741 absorbance value of the lipid globules was 0.001. Values are the mean ± standard deviation of at
742 least three independent duplicates. nm: not measured.

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744 **Figure Captions**

745

746 **Figure 1.** Gastric behaviour of the protein solution samples displayed in the vessel of the gastric
747 model at 16, 48 and 80 min, corresponding to the GE1, GE3 and GE5 time points, respectively. The
748 images correspond to the behaviour immediately before emptying. Figures from M to P correspond to
749 the gastric behaviour displayed in a petri dish at 32 min (GE2 time point). A white arrow in Figures E, I
750 and J indicates the filling level in the vessel as it is difficult to distinguish.

751 **Figure 2.** Strength, based on the force (g), of the whole coagula obtained at GE2 time point of the
752 samples in which solid structures were formed during gastric digestion. The samples are based on the
753 protein ratio (C:W) of 1 (i.e. 50C:50W, (50C:50W)2% and (50C:50W)4%) and 4 (i.e. 80C:20W and
754 (80C:20W)2%). Note that the force of sample (50C:50W)8% was out of the detection limit of the
755 instrument. Each data point is the mean of five measurements in an independent replicate, having
756 three replicates for each sample. Letters in the graph correspond to the pictures showing the gastric
757 digesta at GE2 time point displayed in a petri dish, together with an example of confocal microscopy
758 image. Green shows the proteins and red shows the lipids. The scale bar corresponds to 100 μm.

759 **Figure 3.** (A) Protein content (w/w, %) of the gastric emptying points (GE1-GE5) of the protein
760 solution in line and emulsions samples in symbols and (B) lipid content (w/w, %) of the emulsions with
761 2% lipid. Values are presented as means ± SD (n=3). The values were corrected by the different
762 gastric dilution in each point. Significant difference in nutrient content between samples in each GE
763 point was determined by one-way ANOVA, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$
764 (****).

765 **Figure 4.** SDS-PAGE (under reducing conditions) of (A) protein solution samples, (B) emulsion
766 samples with 2% lipid and (C) samples with the C:W ratio of 1 (i.e. 50C:50W) with 0%, 2%, 4% and
767 8% lipid. The emptied aliquots at the corresponding GE points (GE1-GE5) were analysed together
768 with the initial sample (I), referred to before digestion and a molecular weight marker. The samples
769 are labelled in the figure accordingly. Samples were diluted (1:100) with water. (MFGM: milk fat
770 globule membrane, β-Lg: β-Lactoglobulin, α-La: α-Lactalbumin).

771 **Figure 5.** Concentration of Leu (μg/mL) of the (A, a) protein solution samples, (B, b)
772 emulsions with 2% lipid and (C, c) comparison of the different lipid inclusion (0, 2, 4 and 8%)
773 in the same protein composition matrix, C:W ratio of 1, during the small intestinal digestion of
774 the digesta related to GE1 (upper case) and GE5 (lower case), in both apical and basolateral
775 sides in solid and broken line, respectively, using Ussing chamber methodology. Values are
776 presented as means ± SD of two independent determinations. Significant difference in Leu
777 content between samples in each GE point was determined by one-way ANOVA, $p \leq 0.05$
778 (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****), black relates to the apical side axes
779 and red r

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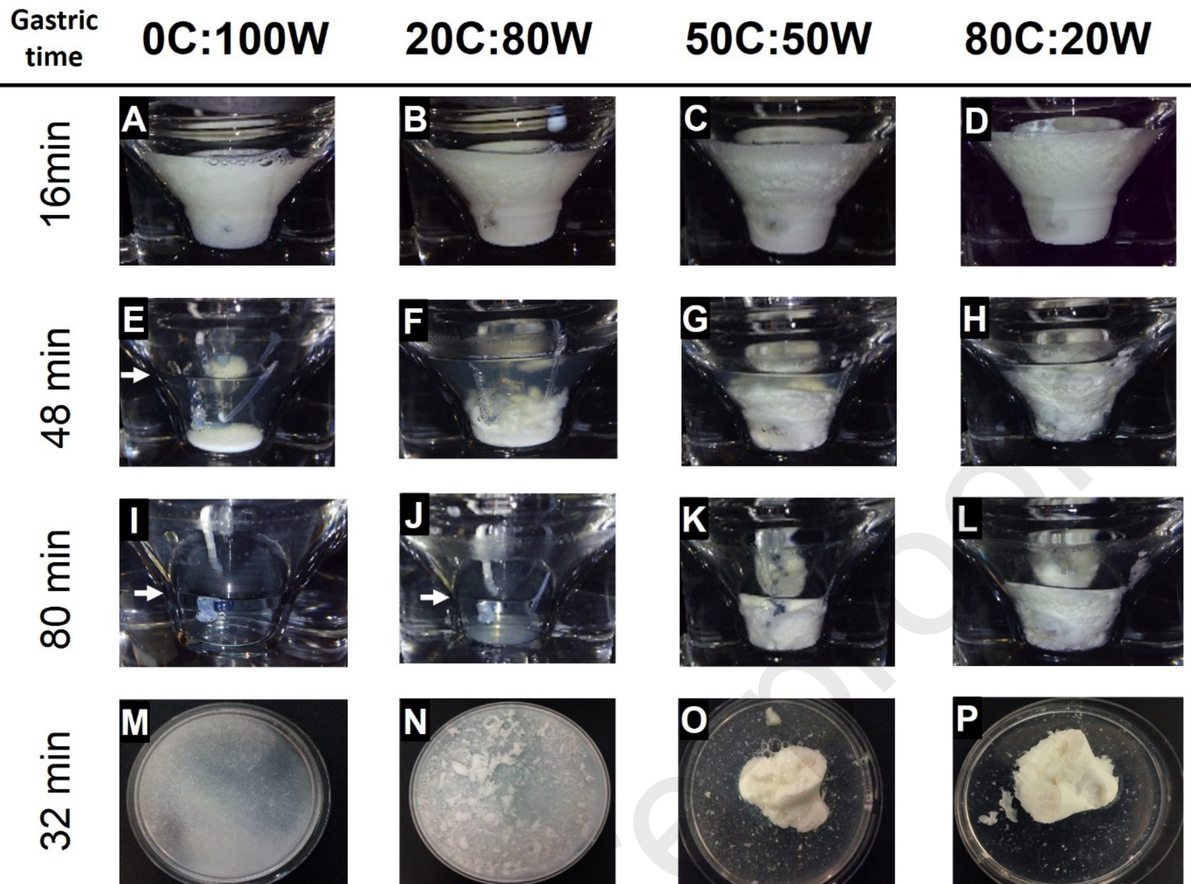

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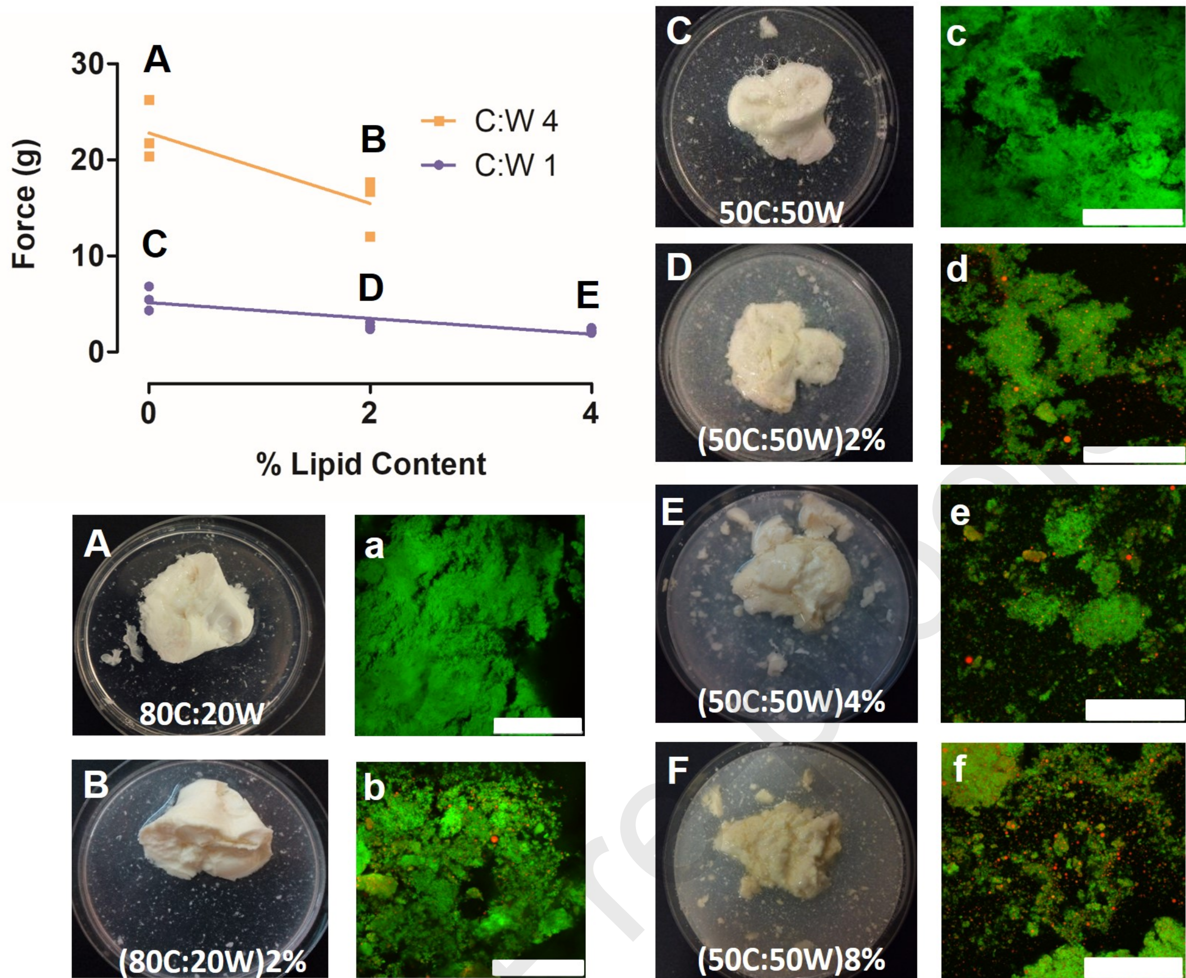

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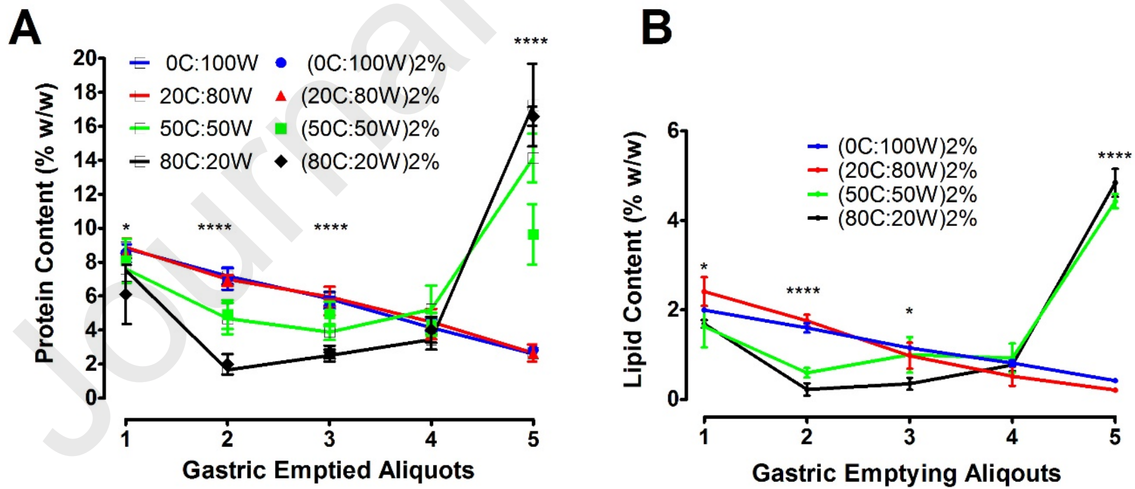

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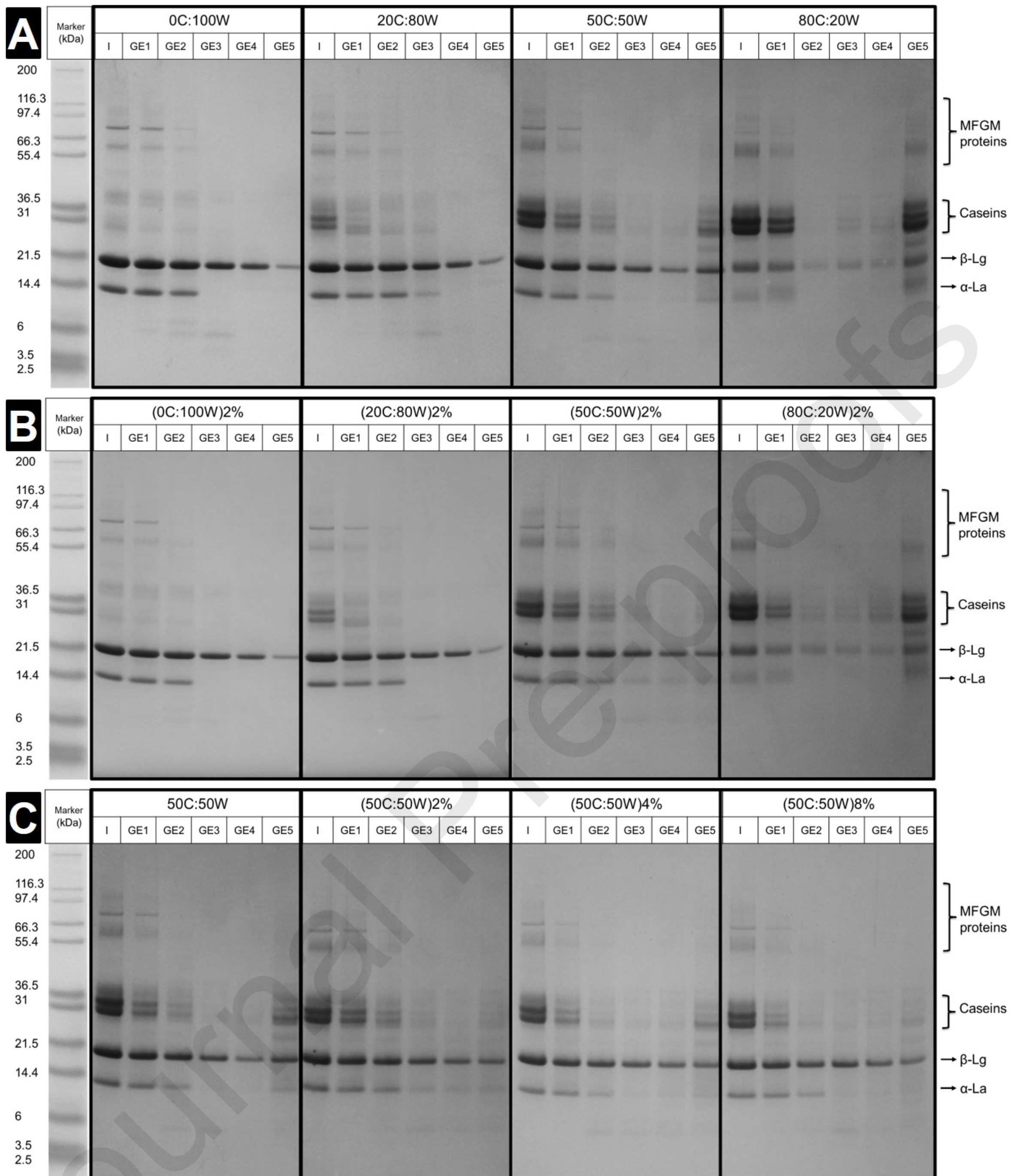

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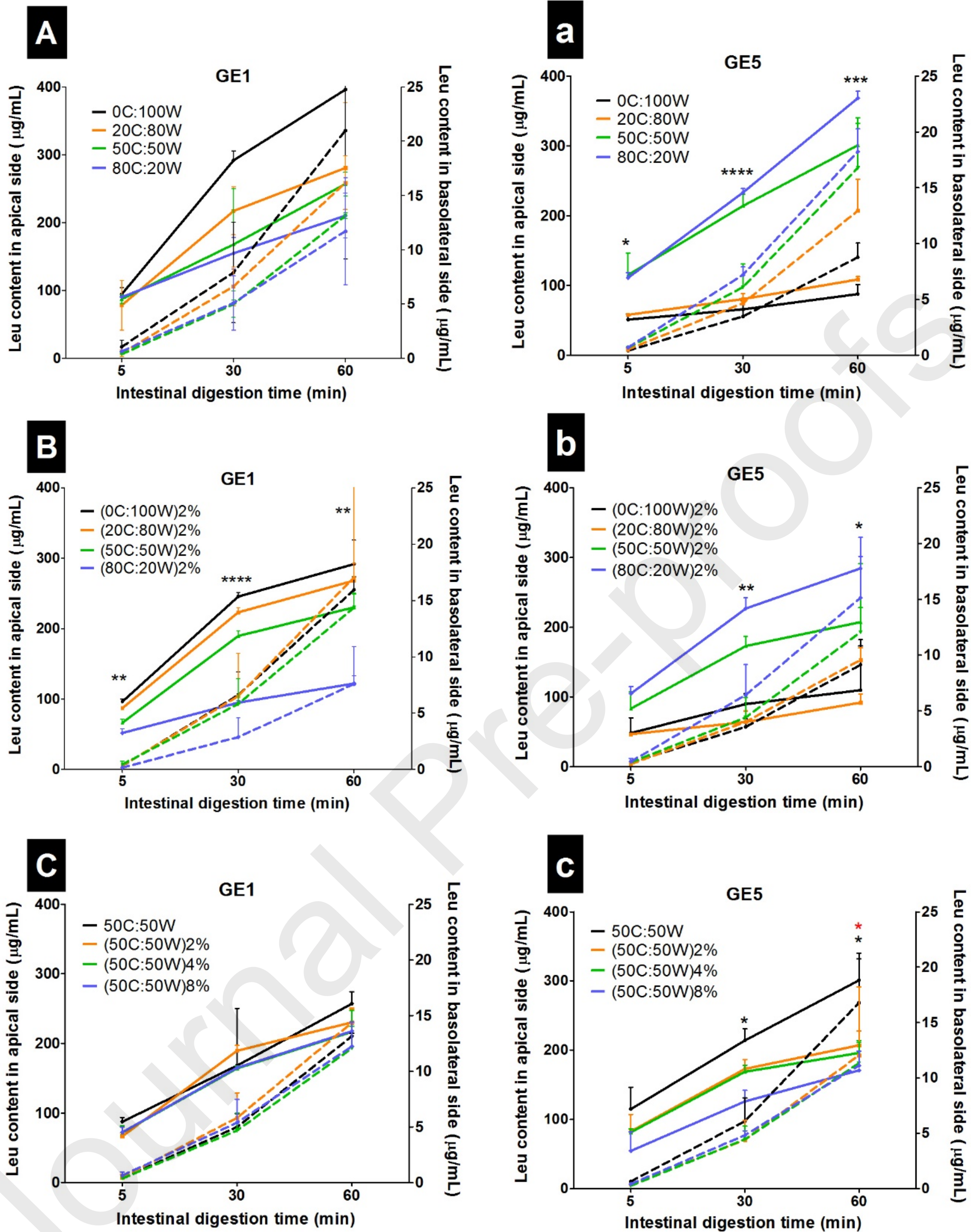

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785 **Conflicts of interest**

786 The authors are not aware of any affiliations, memberships, funding or financial holdings that
787 might be perceived as affecting the objectivity of this work.

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791 **Author Contributions**

792 A.I.M.C. carried out the experimental work and wrote the manuscript. A.T.G. set up
793 and assisted the *ex vivo* absorption model experiments. S.S. developed the method and
794 assisted with the analysis of the amino acids. A.R.M, P.J.W and A.B. designed the
795 experiments together with A.I.M.C.; A.R.M, P.J.W and A.B. corrected the manuscript and
796 supervised the study.

797

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799

800 • Caseins and whey mixtures were digested differently using a semi-dynamic
801 model

802 • Caseins (≥ 50%) formed solid coagula and whey proteins were more soluble

803 • The different gastric behaviour was the main factor controlling digestion rates

804 • Caseins delayed but whey proteins accelerated bioaccessibility and absorption

805 • Foods with different nutrient digestion rates can address specific populations
806 needs

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