

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

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21 **Highlights**

- 22 • Caseins and whey proteins mixtures were digested differently using a semi-dynamic
23 model
- 24 • Caseins ($\geq 50\%$) formed solid coagula and whey proteins were more soluble under *in*
25 *vitro* gastric conditions
- 26 • The different gastric behaviour was the main factor controlling digestion rates
- 27 • Caseins delayed, and whey proteins accelerated nutrient bioaccessibility and
28 absorption rate
- 29 • Foods with different nutrient digestion rates can address specific populations needs

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31 **Abstract**

32 Caseins and whey proteins are known as 'slow' and 'fast' proteins, respectively, based
33 on their amino acid absorption rate. However, there is limited understanding of the
34 mechanisms controlling their behaviour during gastro-intestinal transit. A protein model
35 system (8% total protein) with varying casein:whey protein ratios (0:100, 20:80, 50:50 and
36 80:20) were subjected to *in vitro* gastro-intestinal digestion using a semi-dynamic gastric
37 model, a static intestinal model and an *ex vivo* absorption model (Ussing chambers). The
38 casein-rich ($\geq 50\%$) samples showed the formation of solid coagula that were persistent
39 throughout gastric digestion, which caused a delay in nutrient emptying, slower digestion
40 and leucine absorption kinetics. In contrast, whey proteins formed more soluble aggregates
41 during the gastric phase, which led to faster gastric emptying, rapid intestinal hydrolysis, and
42 higher and faster leucine absorption. This work shows the key role of the gastric
43 restructuring for the overall digestive mechanism and kinetics of food, in particular proteins.

44 **Key words**

45 dairy proteins; caseins; whey proteins; gastric digestion; semi-dynamic model; digestion
46 kinetics

47

48 1. Introduction

49 Milk proteins are considered a high quality protein source taking into account the
50 essential amino acid score, protein-digestibility corrected amino acid score (Schaafsma,
51 2000) and digestible indispensable amino acid scores (Mathai *et al.*, 2017). Moreover, they
52 are generally considered a superior source of protein compared to plant proteins since the
53 latter are generally less digestible and deficient in one or more essential amino acids (AAs)
54 and their leucine (Leu) content is 6-8%, compared to 10-13% in dairy proteins (Gorissen *et*
55 *al.*, 2018). The main milk proteins, i.e. caseins and whey proteins, have been reported to
56 show different postprandial protein kinetics in humans, which affect the whole body protein
57 metabolism (Boirie *et al.*, 1997). In this study, the ingestion of whey proteins resulted in a
58 high, rapid and transient increase in plasma AAs, promoting protein synthesis without
59 supporting protein breakdown. In contrast, caseins induced a low, slow and prolonged
60 aminoacidemia profile, which inhibited body protein breakdown. The Leu balance was
61 positive for the casein drink over 7 hours, promoting protein deposition whereas no effect
62 was provided from the whey protein drink. From this study, whey proteins and caseins were
63 labelled as 'fast' and 'slow' proteins, respectively, as analogy to the carbohydrate
64 metabolism. Some studies have also shown that the faster digestion of whey proteins results
65 in an enhancement of muscle protein synthesis responses in elderly men (Dangin *et al.*,
66 2003; Pennings *et al.*, 2011; West *et al.*, 2011). The coingestion of other macronutrients with
67 dairy proteins is another factor to consider that may affect the metabolic responses. Elliot *et*
68 *al.* (2006) showed that the uptake of AAs, based on threonine and phenylalanine, was
69 greater for whole milk compared to fat-free milk.

70 The underlying mechanisms of the link between protein structure and metabolic
71 responses are not well understood. The main milk proteins have different physico-chemical
72 properties, which are governed by their structure. Caseins have a relatively open and flexible
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73 conformation, forming ordered structures known as casein micelles and are insoluble at pH
74 4.7. In contrast, whey proteins have a globular, compact structure and are more soluble
75 under acidic conditions. Food is subjected to several digestive conditions within the
76 gastrointestinal (GI) tract and the physico-chemical properties of food will determine the
77 changes in the different compartments. Therefore, it is important to understand the
78 interactions of food structures within the GI tract to underpin the health effects, but this
79 information is still scarce.

80 There are some studies suggesting the gastric phase as the rate-limiting step of some
81 of the metabolic effects observed; however its study has been rarely undertaken. Boirie *et al.*
82 (1997) suggested that the slow AA absorption behaviour of casein was due to the
83 coagulation that might occur in the acidic conditions of the human stomach, which could
84 result in longer gastric emptying (GE), delaying the digesta that is delivered into the small
85 intestine, in contrast to the whey proteins that remain soluble and can enter the small
86 intestine rapidly. Investigation of the food behaviour in the human stomach is complex and
87 requires advanced techniques such as magnetic resonance imaging or invasive techniques,
88 which have restrictions in terms of cost and ethics. For that reason, *in vitro* models are often
89 used to investigate the mechanisms controlling nutrient digestion within the GI tract (Dupont
90 *et al.*, 2018). However, static models do not simulate the dynamics of digestion in the
91 stomach, in particular progressive fluid secretion and peristaltic contractions, which might
92 affect structural changes. These gastric dynamics are of relevance, in particular for milk
93 proteins. For example, in contrast to what has been suggested *in vivo*, caseins were
94 reported to be digested rapidly using a static model (Egger *et al.*, 2017). Dynamic models
95 are generally expensive to run and not widely accessible. Therefore, a semi-dynamic model
96 was developed (Mulet-Cabero *et al.*, 2017; Mulet-Cabero *et al.*, 2019) and used in the
97 present study to simulate the main dynamics of the human stomach including a gradual pH

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98 decrease and progressive gastric secretion and emptying. This model has been
99 standardised in Mulet-Cabero, et al (2019) (accepted for publication).

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

107 **2. Materials and methods**

108 **2.1. Materials**

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

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121 from Cambridge Isotope Laboratories, Inc. (CK Isotopes Ltd., Leicestershire, UK). MilliQ®
122 water was used for the preparation of samples and digestion fluids.

123 **2.2. Methods**

124 **2.2.1. Preparation of samples**

125 Table 1 shows the content of protein, lipid and total solids of the studied samples as
126 protein solutions and emulsions. The protein solutions referred to as 0C:100W and 80C:20W
127 were prepared at 8% (w/w) protein. The 0C:100W was prepared by dissolving WPI powder
128 in water using a mixer (IKA Eurostar, mix speed 700 rpm) with a paddle stirrer for 2 hours at
129 room temperature. The sample 80C:20W was prepared by dissolving MPC in water using a
130 mixer (IKA Eurostar, mix speed 700 rpm) with a paddle stirrer for 2 hours at 50 °C (using a
131 water bath). The commercial MPC was produced from skim milk by cascade membrane
132 filtration, which keeps the casein to whey ratio unchanged at 80:20. NaN₃ was added (0.02%
133 in the final solution) as an anti-microbial agent and the solutions were stored overnight at 4
134 °C for rehydration. The 20C:80W sample was prepared by mixing 75% (w/w) of 0C:100W
135 and 25% (w/w) of 80C:20W. The sample 50C:50W was prepared by mixing 37.5% (w/w)
136 0C:100W and 62.5% (w/w) 80C:20W. The pH of the samples was adjusted to pH 7 using
137 NaOH (2 mol/L).

138 Samples with lipid inclusion were prepared starting with the preparation of 0C:100W
139 and 80C:20W at 10% protein (w/w) following the same protocol described previously. Then,
140 the samples 20C:80W and 50C:50W were prepared in the same manner as described
141 before. Each stock protein solution was mixed with 2% rapeseed oil and water in order to
142 achieve a final protein concentration of 8% (w/w). These emulsion samples were named as
143 follows: (0C:100W)2%, (20C:80W)2%, (50C:50W)2% and (80C:20W)2%. The protein
144 solution of 50C:50W was also used to obtained emulsions containing 4% and 8% rapeseed

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145 oil. The coarse emulsions were prepared using an Ultra-Turrax (T25 digital, IKA, Germany)
146 at 10,000 rpm for 1 min. Then, they were processed using a homogeniser (APV 1000, SPX
147 Flow Technology, Charlotte, North Carolina, USA) at 200 bar x 3 passes. The pH of the
148 samples was adjusted to pH 7 using NaOH (2 mol/L).

149 **2.2.2. *In vitro* gastric digestion by the semi-dynamic model**

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

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

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

165 Gastric emptying (GE) was simulated by taking five aliquots, referred to as GE1-5 in
166 the text. Samples were taken from the bottom of the vessel using a 10 mL plastic syringe
167 (BD Plastipak, Ireland), the aperture of which had an inner diameter of 2.5 mm with a plastic
168 tube attached (3.6 mm inner diameter). This was aimed to simulate the gastric sieving of 2
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169 mm approximately created by the pylorus (Thomas, 2006). It is important to note that in
170 some cases there was some residue left in the last GE point that could not be taken using
171 that syringe; this was taken using a spatula and included in the last point. The pH was
172 measured and a sufficient volume of NaOH (2 mol/L) was added to the samples to increase
173 the pH above 7, inhibiting pepsin activity. Finally, samples were snap-frozen in liquid
174 nitrogen and stored at -20 °C until subsequent intestinal digestion. A separate gastric
175 digestion was performed in order to study the gastric restructuring and nutrient delivery. An
176 aliquot (250 µL) of these GE aliquots was used for microscopy. Then, the GE sample was
177 mixed using a homogeniser (T10 basic Ultra-Turrax®, IKA®, Germany) at approximately
178 30,000 rpm for 30 s to obtain a homogenous sample for subsequent analyses.

179 **2.2.3. *In vitro* intestinal digestion and *ex vivo* absorption by Ussing chamber** 180 **technique**

181 The Ussing chamber methodology, which consisted of two chambers separated by a
182 fresh piece of murine intestine that simulated the transfer of nutrients across the intestinal
183 barrier based on the polarity and tightness of epithelia, was applied to study the absorption
184 kinetics of amino acids by *ex vivo* murine small intestinal tissue (He *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 *et al.*, 2009).

191 **2.2.3.1. Intestinal tissue samples**

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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
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 cm². The set up of the Ussing
207 chamber and the measurements were performed according to Brighton *et al.* (2015). The
208 open-circuit transepithelial potential difference was continuously monitored using a DVC-
209 1000 multichannel voltage clamp unit (World Precision Instruments, New Haven, USA) with
210 a Micro1401-3 data acquisition unit (Cambridge Electronic Design, Cambridge, UK). The
211 recordings were collected using Spike2 8.08 software (Cambridge Electronic Design,
212 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

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216 digestion was quickly mixed with bile solution, water, $\text{CaCl}_2(\text{H}_2\text{O})_2$ 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 $\text{CaCl}_2(\text{H}_2\text{O})_2$ 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 $-20\text{ }^\circ\text{C}$ 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)

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240 and Nile red solution (0.1% made with propanediol) at 1:3 proportion was used. 250 μ L of
241 initial/digested sample was gently mixed with 25 μ L 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
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 (Mulet-Cabero *et al.*, 2019). Each measurement was
258 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 Mulet-Cabero *et al.* (2019) was used to determine protein composition. SDS-

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262 PAGE was performed on the initial and digested samples previously diluted (1:100) with
263 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 x 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 *et al.* (2015). The LC flow rate was 0.78 mL/min. The column used for the analysis
277 was Phenomenex Kinetex XB-C18 2.6 µm (150 x 4.6 mm) column. The temperature of the
278 column and auto sampler were maintained at 25 °C and 4 °C, respectively. 1 µL was used
279 for the injection volume. The samples were analysed using 0.1% formic acid in water
280 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The isocratic elution
281 was 5% mobile phase B and 95% mobile phase B. The run time was 6 min.

282 The 6490 MS/MS system was equipped with an electrospray ionization source
283 operated in positive-ion detection mode. Nitrogen gas was used for nebulization,
284 desolvation, and collision. The analytes were monitored in multiple-reaction monitoring
285 (MRM) mode. The MRM precursor, product ions and collision energy were optimized by

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286 Agilent optimizer software. The transitions of precursor ions to product ions (m/z) and some
287 optimized MS operating parameters of the analyte are described in Table S3 of the
288 supplementary material.

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

297 **2.2.9. Statistical analysis**

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

304 **3. Results and discussion**

305 **3.1. Influence of protein formulation and addition of lipids on the gastric behaviour**

306 Figure 1 shows images of the semi-dynamic gastric digestion vessel containing the
307 protein solution samples at the time points of GE1, GE3 and GE5. In general, all the
308 samples presented protein aggregation within the first 10 min of gastric digestion. However,

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

321 The distinct behaviour of the milk proteins observed in the present study is in
322 agreement with suggestions in the literature of casein coagulation in the stomach (Boirie *et*
323 *al.*, 1997; Hall *et al.*, 2003; Mahé *et al.*, 1996). Similar to our results, Wang *et al.* (2018)
324 showed, using the Human Gastric Simulator (HGS), that MPC presented a firm coagulation
325 whereas WPI did not present any aggregation after 220 min of gastric digestion. Moreover,
326 the authors reported that the coagulation of the MPC sample was visible in the first 10 min of
327 digestion corresponding to a pH of approximately 6, in agreement to the present study.

328 The difference in gastric behaviour between the main milk proteins can be attributed to
329 their molecular structure and physico-chemical properties. Caseins are insoluble at pH 4.6
330 (isoelectric point) whereas native whey proteins remain soluble but can aggregate at that pH
331 when heat-denatured or covalently linked to caseins (Alting *et al.*, 2002). In the present study,
332 the protein aggregation started after 10 min of gastric digestion at pH values of about 6 for
333 both 50C:50W and 80C:20W, which was much higher pH than the isoelectric point of caseins.

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334 This suggests that the initial coagulation could be driven by the action of pepsin that has
335 been reported to favour the hydrolysis of κ -caseins, cleaving among others the -Phe-105-
336 Met-106 bond in κ -casein, and the other caseins at pH 6.0 (Tam *et al.*, 1972), which will
337 reduce the steric repulsion between casein micelles and destabilise the overall structure of
338 the casein micelle. Also, the samples were added to a basal volume of SGF of low pH,
339 therefore some of the protein could present a lower pH that might not be homogenous in the
340 system and accelerate the process of aggregation.

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

348 **3.1.1. Strength and structure of the gastric coagula**

349 Figure 2 shows the strength of the coagula corresponding to GE2 time point in
350 50C:50W, 80C:20W, (50C:50W)2%, (80C:20W)2% and (50C:50W)4%. The sample
351 (50C:50W)8% could not be measured because the strength of the coagula was below the
352 limit of the detection of the instrument. The sample 80C:20W presented the highest value of
353 force accounting for 22.8 g whereas the weakest coagula were obtained in the sample with
354 the highest lipid content tested, i.e. (50C:50W)4%, resulting in an average value of 2.25 g.
355 The results showed that the addition of lipid significantly affected the coagula strength of the
356 milk sample with C:W ratio of 4, i.e. 80C:20W, more than for the samples containing C:W
357 ratio of 1, i.e. 50C:50W, in which the decline of the coagula strength was much less strongly

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358 affected. Similarly, Lambers *et al.* (2013) showed that the viscosity, measured by an in-line
359 rheometer during the gastric digestion, of caseins was higher than the whey proteins in
360 solution. However, the casein source was from sodium caseinate, which might affect the
361 formation of the solid coagula and differ from the observed coagula using MPC. Indeed,
362 Wang *et al.* (2018) reported that the coagulation formed in sodium caseinate was loose and
363 fragmented compared to the dense coagula obtained in the MPC sample, which can be
364 attributed to differences in the casein association.

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

373 **3.2. Effect of gastric behaviour on nutrient delivery**

374 Figure 3A shows the protein content at each GE point in the four main formulations with and
375 without the addition of 2% lipid, which can be associated with the proteins that were
376 delivered from the stomach to the small intestine during the simulated gastric digestion. The
377 formulations with higher whey protein contents (C:W ratio of 0 and 0.25) presented a
378 remarkably similar trend, showing the highest protein delivery in GE1 and gradually
379 decreasing during gastric digestion with the lowest amount of protein delivered at GE5,
380 regardless of lipid addition. In contrast, the formulation with the highest casein content (C:W
381 ratio of 4) showed the lowest protein delivery at the early stages of gastric digestion, in

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382 particular in GE2, increasing in GE4 and significantly higher in GE5. An intermediate pattern
383 between these two extreme behaviours was found in the formulation with a C:W ratio of 1, in
384 which there was a more constant protein delivery for a longer gastric digestion time, from
385 GE1 to GE4. However, using the same protein ratio, the inclusion of 2% lipid resulted in a
386 lower protein concentration emptied in GE5.

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

397 The gastric behaviour of the protein formulations impacted the *in vitro* kinetics of
398 protein emptying, simulating the delivery from the stomach to the small intestine. The solid
399 coagulation, in particular in the 80C:20W sample, led to delayed protein delivery through the
400 retention of caseins at GE5 as seen by SDS-PAGE (Figure 4A). The solid coagulum that
401 formed, physically resisted being emptied from the stomach through the tubing, in a manner
402 similar to the way the pylorus prevents the emptying of solids or large particulates, but
403 allowing the liquid phase to be emptied. In contrast, the formation of small aggregates that
404 were suspended in a serum in the formulations with higher whey protein concentrations
405 allowed the liquid phase containing these small aggregates to be emptied, enabling a larger
406 extent of protein delivery at the earlier stage of digestion. This was related to the gradual

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407 emptying of intact β -Lg (Figure 4A) and was controlled mainly by the dilution of the gastric
408 contents by the continuous secretions and emptying. Similarly, Wang *et al.* (2018) showed
409 that, in the gastric digestion of WPI, β -Lg remained intact during the gastric digestion due to
410 the well-known property of the native β -Lg to resist hydrolysis by pepsin because of its
411 compact globular structure. Moreover, in the same study, the authors detected strong bands
412 of intact caseins from the coagulum particles after 220 min of digestion of MPC, similarly to
413 the present results. Interestingly, the sample 50C:50W presented an intermediate pattern of
414 protein delivery, which can be attributed to the softer coagula formed during the gastric
415 digestion that was more easily emptied. Moreover, the latter sample presented a more
416 constant emptying of both caseins and whey proteins as observed in the electrophoresis gel
417 (Figure 4A), which may be related to a reduced syneresis in that sample.

418 The incorporation of lipids affected the protein network structure and modify their
419 digestion and behaviour within the GI tract. the casein bands seemed to be weaker, in
420 particular in the case of (50C:50W)2% and (80C:20W)2% at GE5, showing a more constant
421 and lower content of caseins during gastric digestion (Figure 4B). Guo *et al.* (2015) studied,
422 in whey protein emulsion gels (hard versus soft), the effect of gastric disintegration using the
423 HGS on lipid bioaccessibility during a simulated intestinal digestion. The size of the gel
424 particles was reduced after 60 min of gastric digestion in both samples but the initial rate of
425 lipolysis of the soft gel was significantly higher than the hard gel, even though the solid
426 content of that digesta was lower. At 240 min, the digesta from the soft gel consisted of
427 individual oil droplets as well as smaller particles, compared to the hard gel in which most of
428 the oil droplets remained within the protein network. The latter study represents an example
429 of the engineering of gels for specific GI functionality, however the same principle can be
430 applied for protein structures formed within the gastric conditions. Therefore, this shows that

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431 protein networks can modulate the release of lipids to the intestine and control the
432 subsequent digestion and absorption kinetics.

433 **3.3. Effect of *in vitro* gastric behaviour on protein digestion and absorption in the** 434 **small intestine *ex vivo***

435 Figure 5 shows the free Leu content in the apical and basolateral sides of the Ussing
436 chamber. Leu was selected since it plays a key role in the body protein deposition (Garlick,
437 2005). The rest of BCAAs quantification can be seen in the supplementary material (Table
438 S4-S13). The determination in the apical side represents the Leu content that was digested
439 and became accessible whereas the determination in the basolateral side represents the
440 Leu content that was able to be absorbed and transported across the intestinal wall and thus
441 available to be metabolised and used for physiological functions.

442 In general, the Leu concentration in all the samples in both GE points increased during
443 small intestinal digestion, showing the progressive breakdown of the protein, liberating AAs
444 and increasing their absorption. However, the rate and extent of these processes were
445 different between the samples. In GE1 (Figure 5A), among the protein solution samples in
446 the absence of lipid, the sample 0C:100W showed the most rapid increase in Leu
447 concentration whereas the lowest rate was found in the sample 80C:20W. These patterns
448 were related to the Leu concentration that was absorbed (basolateral concentrations). The
449 highest rate of Leu absorption was observed in 0C:100W whereas the samples 50C:50W
450 and 80C:20W presented the slowest rate with a similar pattern. In contrast, in GE5, there
451 was a rapid rate of Leu accessibility in the samples 50C:50W and 80C:20W, which was
452 reflected in a higher amount of absorbed Leu obtained in the basolateral side. This
453 behaviour could be related to the available protein in GE5 of the samples with higher casein
454 content (Figure 3 A).

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

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

474 In general, it was shown quite clearly that the different rates of delivery of Leu from the
475 different samples at different stages of digestion, explained how whey proteins and caseins
476 are responsible for most of the early and late AA delivery, respectively. For protein solutions,
477 the sample 0C:100W presented the highest rate and extent of both Leu accessibility and
478 absorption in the first GE aliquot when compared with the samples 50C:50W and 80C:20W.
479 In contrast, the latter samples presented a higher level of digestion and absorption in GE5,
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480 which might be attributed to the delayed protein delivery to the intestinal phase due to the
481 coagula that remained at the end of the gastric digestion. These results are in agreement
482 with the plasma Leu concentrations after protein ingestion obtained by Boirie *et al.* (1997), in
483 which they described whey proteins and casein as 'fast' and 'slow' proteins respectively, a
484 concept previously adopted for dietary carbohydrates due to the evidence of the link
485 between their rate of digestion and absorption, and metabolic response. Boirie *et al.* (1997)
486 showed that there was a rapid increase in plasma Leu levels after the ingestion of a whey
487 protein drink when compared to that of caseins, which showed a more attenuated pattern
488 over time. Similarly, in the present study, the rate and extent of Leu absorption at early
489 stages of digestion was higher in the sample 0C:100W whereas the sample with higher
490 content of casein presented low levels of Leu absorption at the beginning but a substantial
491 increase in the later stage of the digestion, that could have been prolonged if the gastric
492 digestion had lasted longer. Interestingly, the sample 50C:50W, showing a solid coagulation
493 with weaker coagula consistency, could present an intermediate metabolic effect since the
494 kinetics of protein delivery and Leu absorption were overall showing middle levels. It is
495 important to note that the sample containing 100% casein was not included in this study
496 however a similar behaviour to the sample containing 80% can be expected. Lacroix *et al.*
497 (2006) showed that a milk protein drink containing 20:80 whey protein:casein ratio presented
498 no significant difference in the dietary nitrogen utilization when compared to 100% casein
499 drink, which might be due to the profound effect of solid coagulation in the stomach from a
500 casein content level higher than 80%.

501 The distinct absorption pattern could be attributed to a higher level of Leu present in
502 whey proteins compared to caseins (Gorissen *et al.*, 2018). In the current study the protein
503 content was matched but they differed in the Leu content due to the nature of the proteins.
504 Nevertheless, this did not affect the distinct absorption pattern of the milk proteins as studied

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505 by Boirie *et al.* (1997) *in vivo*, in which both casein and whey protein drinks were matched in
506 Leu content as well, showing that that Leu content was not the limiting factor for the protein
507 synthesis. Moreover, the patterns for the other measured BCAAs reflected that of Leu
508 pattern (Table S4-S13 of supplementary material) showing the relevance of the kinetics of
509 digestion. Indeed, the independence of the protein digestion rate on modulating postprandial
510 deposition of protein was also confirmed by Dangin *et al.* (2001).

511 The inclusion of higher levels of lipid did not affect the Leu absorption pattern at the
512 early stage but it lowered the Leu absorption in the latest stage, which could be attributed to
513 the lower protein delivery at the end of gastric digestion due to the softer coagula formed.
514 These findings are in contrast with that of Gaudichon *et al.* (1999), showing no difference in
515 postprandial protein utilization when milk protein was supplemented with milk fat compared
516 to the milk protein alone. This contrasts with Elliot *et al.* (2006), in which the *in vivo* ingestion
517 of whole milk was suggested to increase the utilization of AAs for protein synthesis when
518 compared to fat-free isocaloric milk. There is little information about the influence of other
519 macronutrients in the postprandial nitrogen utilization to draw any conclusion hence more
520 research is needed.

521 **3.4. Method limitations**

522 A semi-dynamic *in vitro* model was used in this study. which reproduces some of the
523 main dynamic processes in the human stomach, including the gradual pH decrease (Figure
524 S3 of the supplementary material). The pH is a crucial factor affecting the structure, charge
525 and interaction of proteins, and enzyme activity and therefore affecting the kinetics of protein
526 degradation. Emptying rate is the other main essential parameter in the gastric phase. In the
527 semi-dynamic model, the rate of GE was based on the caloric content of the sample. This
528 GE approach implied that the emptying time of the samples was the same regardless the

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529 behaviour of the proteins in the simulated stomach. However, this may not be totally
530 accurate since casein might present longer times of gastric digestion due to the formation of
531 the solid coagula that remains longer in the stomach to be broken down and emptied, which
532 may influence the extent of the protein hydrolysis. In humans, Boutrou et al. (2013) found
533 that, after casein ingestion, the delivery of dietary protein in the jejunum was progressive for
534 6 hours and in the form of medium size-peptides (750-1,050 Da) whereas the ingestion of
535 whey protein induced the release of larger-size peptides (1,050-1,800 Da) and was
536 completed after 3 hours. The regulation of GE is a complex process which depends on
537 factors including gut hormones and properties of food, e.g. viscosity, consistency, volume,
538 particle size and caloric density. However a simple *in vitro* model cannot take into account all
539 of these factors so the present semi-dynamic model considered the caloric density as the
540 main factor in regulating the rate of GE; a high caloric density inducing a slower/longer
541 emptying.

542 Gastric motility also plays an important role in food disintegration, which is
543 characterised by two types of motion. There is a slow motor activity at the upper part of the
544 stomach, fundus and body, by which gastric contents are pushed into the antrum. Therefore,
545 the body part mainly acts as a storage place with negligible mixing. In contrast, the strongest
546 fluid motions are found in the antral part and are responsible for the major food grinding and
547 mixing with gastric fluids (Ferrua *et al.*, 2010). However, the simulation of these mechanical
548 forces is rather difficult due to their complexity in amplitude, frequency and intensity. The
549 present model does not allow an accurate simulation of the gastric motility in particular the
550 mechanical forces of the antrum. However, the weak mixing applied allows the mixing of the
551 fluids added with the food and possible structure formed during digestion. For instance,
552 studies have shown phase separation in the stomach, highlighting the low mixing in the body

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553 (Mackie et al., 2013; Marciani et al., 2007) contradicting the idea of intragastric
554 homogenisation of the food.

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

558 **4. Conclusions**

559 This study has proposed underlying mechanisms behind the denoted 'fast' and 'slow'
560 digested protein for whey proteins and caseins, respectively. The main milk proteins
561 presented different digestive behaviour and AA availabilities, which are factors for defining
562 protein quality, and the gastric phase of digestion was shown to be the rate limiting step. The
563 solid coagulation of the casein-rich samples contributed to the delay in nutrient delivery from
564 the gastric phase and thus overall digestion and AAs absorption kinetics. In contrast, whey
565 proteins formed small aggregates during gastric digestion that led to a gradual decrease of
566 nutrient delivery and a higher Leu absorption in early stages of GI digestion. The modulation
567 of the solid coagula could be obtained by addition of whey proteins and lipid, which altered
568 the kinetics of digestion. The differences in AA absorption kinetics, as modulated through
569 gastric behaviour, can be associated to different physiological effects. Therefore, this
570 methodological approach is a powerful tool to understand the mechanisms underlying the
571 physiological impact of foods, in order to design foods with different rates of nutrient
572 digestion addressed to the nutritional and health needs of different populations. There is
573 evidence showing that a slower pattern of protein digestion leads to a better postprandial
574 utilization of dietary nitrogen, improving AA retention (Boirie *et al.*, 1997; Bos *et al.*, 2003).
575 Lacroix *et al.* (2006) showed that the rapid appearance of high plasma AA concentrations
576 from the 'fast' protein such as whey proteins induced greater deamination rates by the liver,

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577 which decreases the AA concentration in plasma. However, the effect of the protein
578 digestion rates on the protein metabolism seemed to be age-dependent (Dangin *et al.*, 2003;
579 Pennings *et al.*, 2011). Pennings *et al.* (2011) showed that whey proteins resulted in a more
580 effective enhancement in protein retention than casein in the elderly.

581 In general, the understanding of the gastric phase and how it modulates the gastric
582 behaviour for instance of protein formulation needs further investigation using appropriate
583 models since it offers great potential to design foods that can exert several physiological
584 effects such as satiety (Hall *et al.*, 2003), glycaemic control (Gannon *et al.*, 2010), lipemia
585 control (Mariotti *et al.*, 2015) and improve GI complications such as reflux and aspiration
586 pneumonia (van den Braak *et al.*, 2013).

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595 **Conflicts of interest**

596 The authors are not aware of any affiliations, memberships, funding or financial holdings that
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739 **Table 1.** Compositional description of the studied samples.

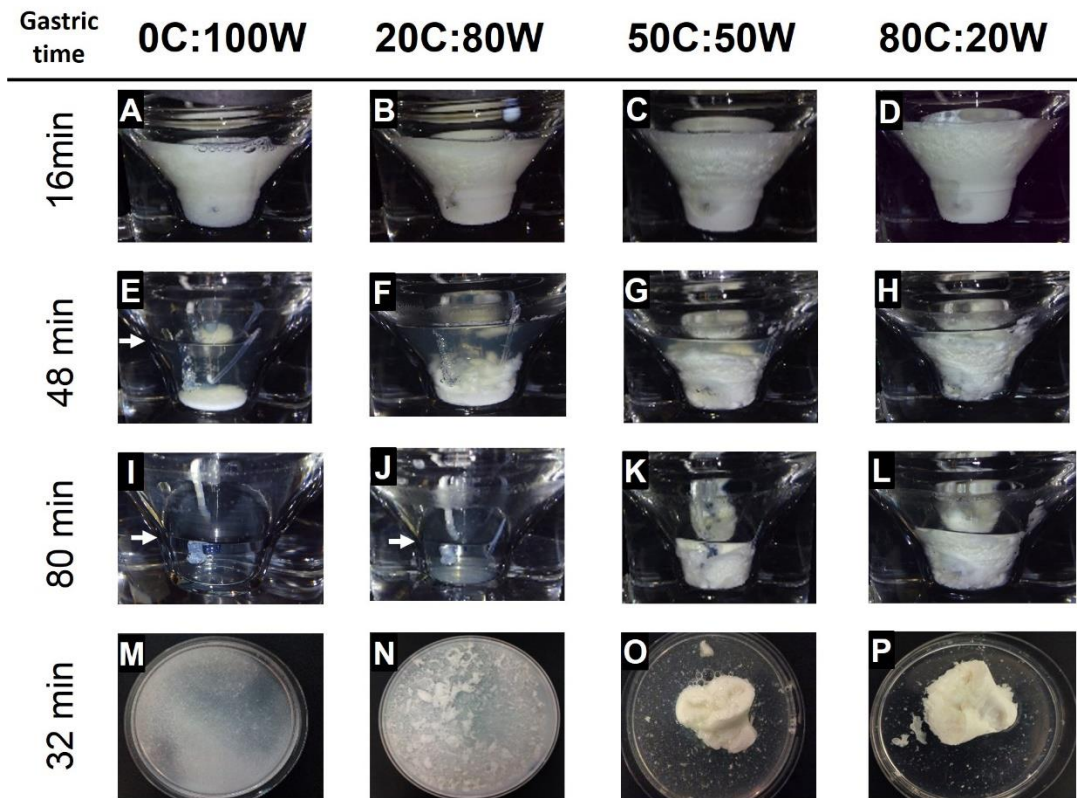
Sample	Protein composition ¹		Added lipid % (w/w)	Total solids ² %	Mean particle diameter d _{4,3} (µm) ³
	Caseins % (w/w)	Whey proteins % (w/w)			
0C:100W	0	8	0	8.58 ± 0.08	nm
20C: 80W	1.8	6.4	0	8.96 ± 0.08	nm
50C:50W	4	4	0	9.47 ± 0.04	nm
80C: 20W	6.4	1.8	0	9.83 ± 0.06	nm
(0C:100W)2%	0	8	2	10.45 ± 0.02	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

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

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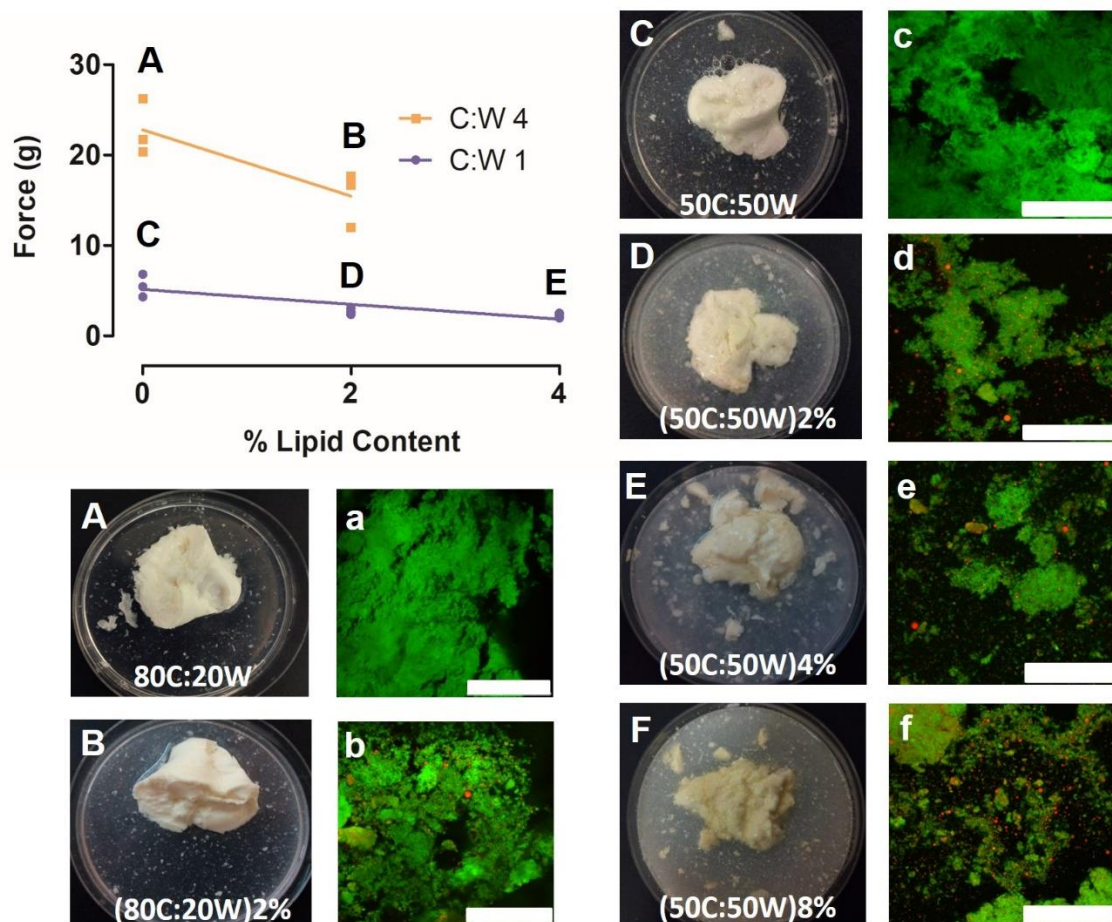


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752 **Figure 1.** Gastric behaviour of the protein solution samples displayed in the vessel of the gastric
 753 model at 16, 48 and 80 min, corresponding to the GE1, GE3 and GE5 time points, respectively. The
 754 images correspond to the behaviour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

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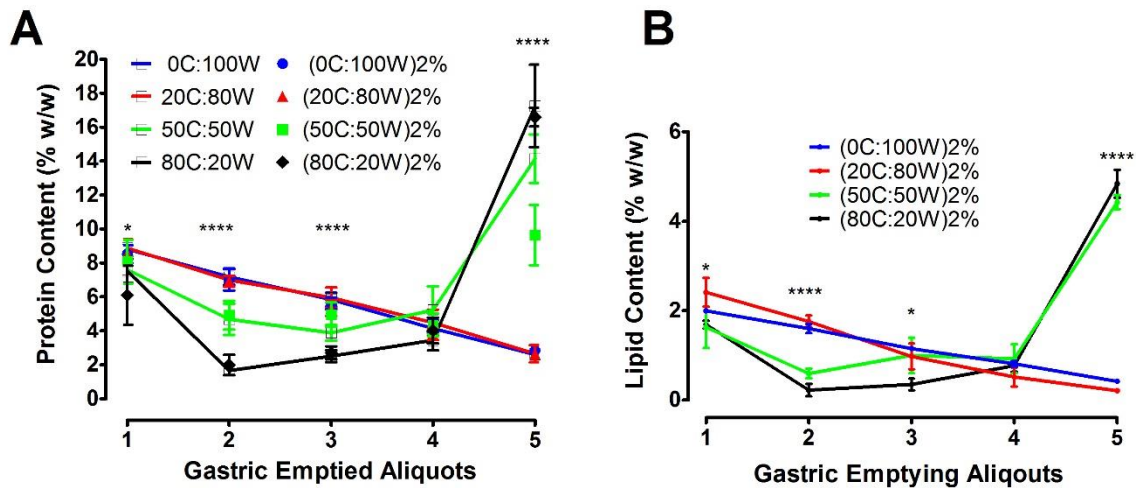
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759 **Figure 2.** Strength, based on the force (g), of the whole coagula obtained at GE2 time point of the
 760 samples in which solid structures were formed during gastric digestion. The samples are based on the
 761 protein ratio (C:W) of 1 (i.e. 50C:50W, (50C:50W)2% and (50C:50W)4%) and 4 (i.e. 80C:20W and
 762 (80C:20W)2%). Note that the force of sample (50C:50W)8% was out of the detection limit of the
 763 instrument. Each data point is the mean of five measurements in an independent replicate, having
 764 three replicates for each sample. Letters in the graph correspond to the pictures showing the gastric
 765 digesta at GE2 time point displayed in a petri dish, together with an example of confocal microscopy
 766 image. Green shows the proteins and red shows the lipids. The scale bar corresponds to 100 μ m.

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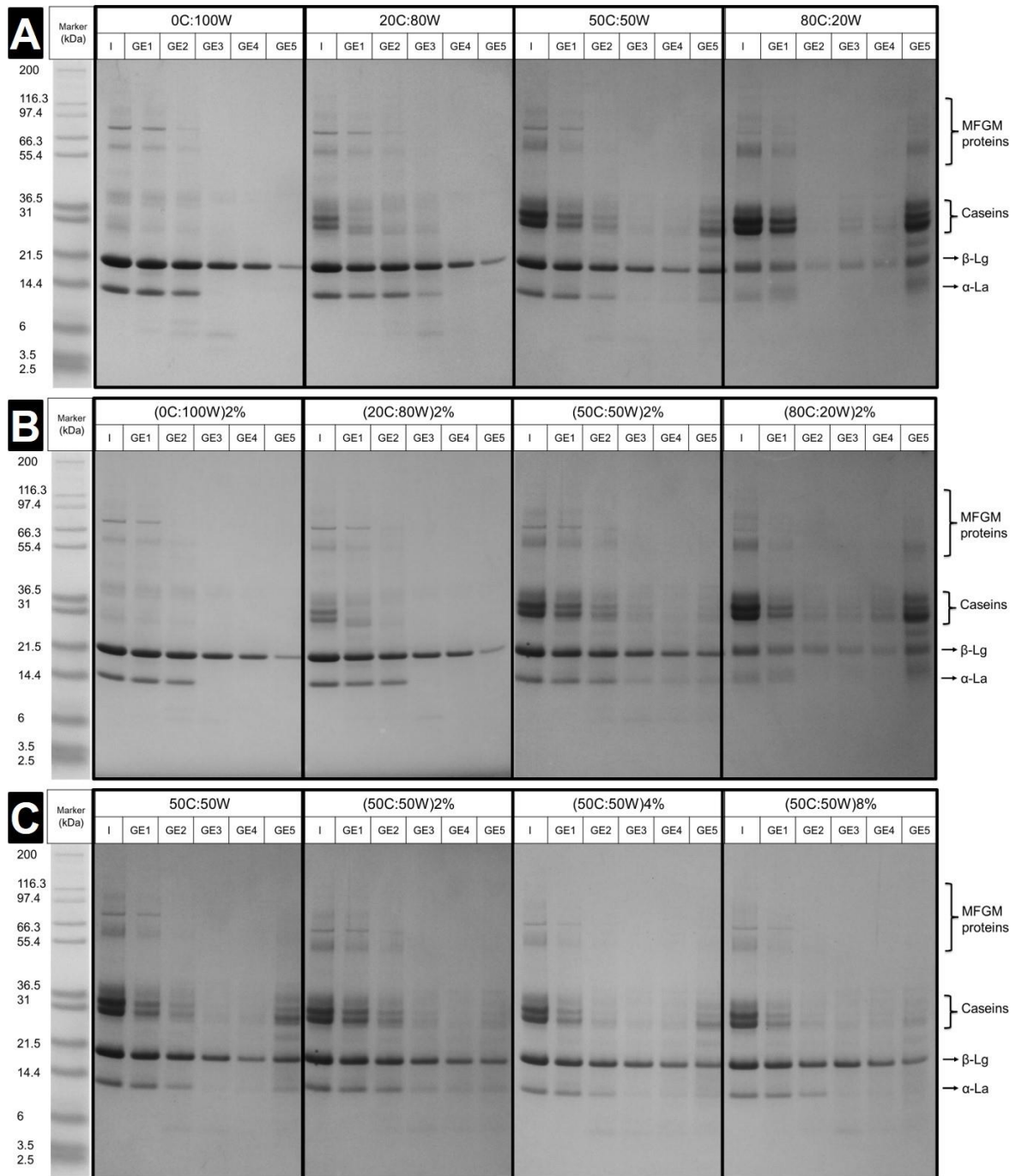
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769 **Figure 3.** (A) Protein content (w/w, %) of the gastric emptying points (GE1-GE5) of the protein
 770 solution in line and emulsions samples in symbols and (B) lipid content (w/w, %) of the emulsions with
 771 2% lipid. Values are presented as means \pm SD (n=3). The values were corrected by the different
 772 gastric dilution in each point. Significant difference in nutrient content between samples in each GE
 773 point was determined by one-way ANOVA, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$
 774 (****).

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Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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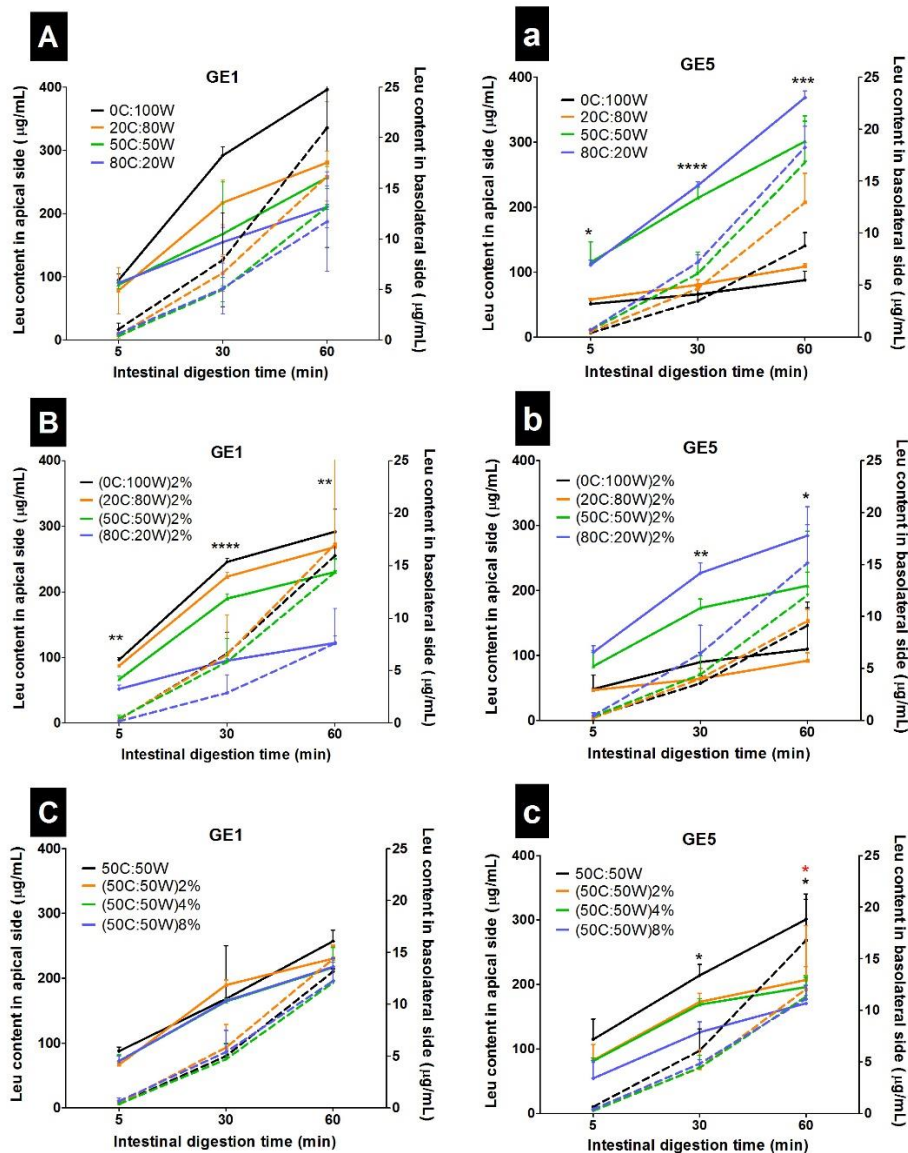


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777 **Figure 4.** SDS-PAGE (under reducing conditions) of (A) protein solution samples, (B) emulsion
 778 samples with 2% lipid and (C) samples with the C:W ratio of 1 (i.e. 50C:50W) with 0%, 2%, 4% and
 779 8% lipid. The emptied aliquots at the corresponding GE points (GE1-GE5) were analysed together
 780 with the initial sample (I), referred to before digestion and a molecular weight marker. The samples
 781 are labelled in the figure accordingly. Samples were diluted (1:100) with water. (MFGM: milk fat
 782 globule membrane, β -Lg: β -Lactoglobulin, α -La: α -Lactalbumin).

Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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784 **Figure 5.** Concentration of Leu ($\mu\text{g/mL}$) of the (A, a) protein solution samples, (B, b)
 785 emulsions with 2% lipid and (C, c) comparison of the different lipid inclusion (0, 2, 4 and 8%)
 786 in the same protein composition matrix, C:W ratio of 1, during the small intestinal digestion of
 787 the digesta related to GE1 (upper case) and GE5 (lower case), in both apical and basolateral
 788 sides in solid and broken line, respectively, using Ussing chamber methodology. Values are
 789 presented as means \pm SD of two independent determinations. Significant difference in Leu
 790 content between samples in each GE point was determined by one-way ANOVA, $p \leq 0.05$
 791 (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****), black relates to the apical side axes
 792 and red relates to the basolateral side axes.

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794 **Supplementary material**795 **Tables**796 **Table S1.** Total volume of both oral and gastric mixture added in the simulated digestion of each
797 sample.

Samples	Total Oral Mixture (mL) ^a	Gastric Fluids Secretions		
		Total Gastric Mixture (mL) ^b	Delivery Rate of Simulated Gastric Electrolyte Mixture (mL/min) ^c	Delivery Rate of pepsin solution (μL/min) ^d
0C:100W	1.72	21.72	0.24	10.0
20C: 80W	1.79	21.79	0.24	10.0
50C:50W	1.89	21.89	0.24	10.0
80C: 20W	1.97	21.97	0.24	10.0
(0C:100W)2%	2.09	22.09	0.15	6.40
(20C: 80W)2%	2.14	22.14	0.15	6.40
(50C:50W)2%	2.24	22.24	0.15	6.40
(80C: 20W)2%	2.34	22.34	0.15	6.40
(50C:50W)4%	2.60	22.60	0.12	4.70
(50C:50W)8%	3.33	23.33	0.08	3.08

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799 ^aThe total oral mixture referred to a mixture of 79.9% eSSF, 19.6% MilliQ[®] water and 0.5%
800 CaCl₂(H₂O)₂ (0.3 mol/L). ^bThe total gastric mixture contained 80% eSGF, 7.78% MilliQ[®] water and
801 0.04% CaCl₂(H₂O)₂ (0.3 mol/L), 8.7% HCl (1.5 mol/L) and 3.48% pepsin solution. ^c The simulated
802 gastric electrolyte mixture represented the 90% of the total gastric mixture of the constituents of
803 eSGF, MilliQ[®] water, CaCl₂(H₂O)₂ (0.3 mol/L) and HCl (1.5 mol/L). This was delivered through a
804 dosing device of an automatic titrator, which was programmed according to the total time of digestion
805 and the remaining 10% was firstly added in the reaction vessel to simulate the basal state. ^dThe
806 volume of 0.8 mL of pepsin solution was added in the gastric digestion of each sample, which was
807 delivered during the corresponding total gastric digestion time.

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809 **Table S2.** Time (min) and volume (mL) at which gastric emptying (GE) was applied in the samples,
 810 based on their caloric content. Five emptying points were used.

Sample	Gastric emptying time (min)					Volume of Gastric Emptying (mL)				
	GE1	GE2	GE3	GE4	GE5	GE1	GE2	GE3	GE4	GE5
0C:100W	16	32	48	68	80	8.69	8.69	8.69	8.69	8.69
20C: 80W	16	32	48	68	80	8.72	8.72	8.72	8.72	8.72
50C:50W	16	32	48	68	80	8.76	8.76	8.76	8.76	8.76
80C: 20W	16	32	48	68	80	8.79	8.79	8.79	8.79	8.79
(0C:100W)2%	25	50	75	100	125	8.84	8.84	8.84	8.84	8.84
(20C: 80W)2%	25	50	75	100	125	8.86	8.86	8.86	8.86	8.86
(50C:50W)2%	25	50	75	100	125	8.90	8.90	8.90	8.90	8.90
(80C: 20W)2%	25	50	75	100	125	8.94	8.94	8.94	8.94	8.94
(50C:50W)4%	34	68	102	136	170	9.04	9.04	9.04	9.04	9.04
(50C:50W)8%	52	104	156	208	260	9.33	9.33	9.33	9.33	9.33

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823 **Table S3.** Operating parameters of the method for determination of the branch chain amino
 824 acids (Leucine, Isoleucine and Valine) by triple quadrupole LC-MS/MS. The source parameters were:
 825 gas temperature of 200 °C with a gas flow of 16 L/min, a sheath gas temperature of 300 °C with a
 826 sheath gas flow of 11 L/min, a nebuliser pressure of 50 psi and capillary voltage of 3500 V for positive
 827 polarity, Nozzle Voltage 1000 V. The iFunnel parameters were high pressure radio frequency of 150 V
 828 and low-pressure radio frequency of 60 V. The LC eluent flow was sprayed into the mass
 829 spectrometer interface without splitting. Identification was achieved based on retention time of
 830 authentic amino acid standards and product ions.

Compound Name	Precursor Ion	Product Ion	Dwell Time (ms)	Fragmentor (V)	Collision Energy	Cell Accelerator (V)	Polarity
D3-Leu	135.20	89	100	380	10	4	Positive
Leu	132.11	86	100	380	10	4	Positive
Leu	132.11	43	100	380	30	4	Positive
D10-Ile	142.24	96.1	100	380	14	4	Positive
Ile	132.11	86	100	380	10	4	Positive
Ile	132.11	56	100	380	50	4	Positive
Ile	132.11	44	100	380	26	4	Positive
D8-Val	126.21	80.1	100	380	14	4	Positive
Val	118.11	72	100	380	14	4	Positive
Val	118.11	58.1	100	380	34	4	Positive

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Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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839 **Table S4.** BCAAs (Leu, Ile and Val) concentration of the 0C:100W sample in the apical and
 840 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 841 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		0C:100W			
G1		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	94.62 \pm 9.81	20.27 \pm 2.77	26.39 \pm 2.88	141.28 \pm 15.45
	30 min	291.89 \pm 14.01	74.47 \pm 10.96	94.15 \pm 2.57	460.51 \pm 0.48
	60 min	396.04 \pm 56.55	126.70 \pm 14.50	140.10 \pm 18.68	662.84 \pm 89.73
	Total concentration	782.56 \pm 80.36	221.44 \pm 6.31	260.63 \pm 18.99	1264.62 \pm 105.66
Basolateral	5 min	0.64 \pm 0.61	0.33 \pm 0.33	0.47 \pm 0.40	1.43 \pm 1.35
	30 min	4.64 \pm 4.62	2.06 \pm 2.10	2.71 \pm 2.53	9.42 \pm 9.25
	60 min	12.62 \pm 11.80	6.74 \pm 4.81	7.73 \pm 6.99	27.10 \pm 23.60
	Total concentration	17.90 \pm 17.04	9.13 \pm 7.24	10.91 \pm 9.92	37.95 \pm 34.21
		G5			
Apical	5 min	51.19 \pm 8.27	16.33 \pm 2.18	20.25 \pm 3.48	87.77 \pm 13.93
	30 min	65.79 \pm 12.78	26.50 \pm 7.39	27.57 \pm 7.27	119.86 \pm 27.43
	60 min	87.59 \pm 13.96	43.84 \pm 1.80	42.07 \pm 5.33	173.51 \pm 21.09
	Total concentration	204.57 \pm 18.47	86.68 \pm 7.01	89.89 \pm 9.12	381.13 \pm 34.59
Basolateral	5 min	0.41 \pm 0.11	0.18 \pm 0.02	0.28 \pm 0.06	0.87 \pm 0.19
	30 min	3.48 \pm 0.60	1.90 \pm 0.28	2.33 \pm 0.03	7.70 \pm 0.91
	60 min	8.77 \pm 1.27	5.57 \pm 2.66	5.67 \pm 0.70	20.01 \pm 4.63
	Total concentration	12.66 \pm 1.98	7.64 \pm 2.96	8.28 \pm 0.79	28.58 \pm 5.74

842

843 **Table S5.** BCAAs (Leu, Ile and Val) concentration of the 20C:80W sample in the apical and
 844 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 845 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		20C:80W			
G1		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	78.21 \pm 36.49	14.79 \pm 8.60	20.50 \pm 11.45	113.50 \pm 56.55
	30 min	217.43 \pm 35.44	55.91 \pm 9.45	67.81 \pm 14.36	341.14 \pm 59.26
	60 min	280.83 \pm 95.81	82.73 \pm 25.60	95.53 \pm 30.08	459.09 \pm 151.49
	Total concentration	576.46 \pm 167.74	153.43 \pm 43.66	183.84 \pm 55.90	913.73 \pm 267.29
Basolateral	5 min	0.49 \pm 0.32	0.19 \pm 0.13	0.28 \pm 0.13	0.97 \pm 0.59
	30 min	6.63 \pm 1.79	3.34 \pm 1.01	3.26 \pm 0.20	13.24 \pm 0.98
	60 min	16.17 \pm 2.46	8.70 \pm 2.79	8.80 \pm 0.21	33.67 \pm 0.12
	Total concentration	23.30 \pm 4.58	12.24 \pm 3.67	12.33 \pm 0.54	47.87 \pm 1.45
		G5			
Apical	5 min	57.87 \pm 0.90	20.17 \pm 1.35	25.13 \pm 2.57	103.17 \pm 4.82
	30 min	80.55 \pm 7.80	36.40 \pm 5.50	39.84 \pm 6.06	156.78 \pm 19.36
	60 min	108.71 \pm 3.90	53.30 \pm 1.02	57.03 \pm 5.88	219.04 \pm 3.00
	Total concentration	247.12 \pm 4.80	109.88 \pm 7.86	121.99 \pm 14.51	478.99 \pm 27.18
Basolateral	5 min	0.52 \pm 0.16	0.30 \pm 0.07	0.36 \pm 0.06	1.18 \pm 0.30
	30 min	4.66 \pm 0.19	2.11 \pm 0.29	3.01 \pm 0.51	9.78 \pm 0.99
	60 min	12.95 \pm 2.80	8.21 \pm 0.94	8.50 \pm 2.44	29.65 \pm 4.30
	Total concentration	18.13 \pm 2.83	10.61 \pm 0.72	11.87 \pm 2.88	40.61 \pm 5.00

846

Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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847 **Table S6.** BCAAs (Leu, Ile and Val) concentration of the 50C:50W sample in the apical and
 848 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 849 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

G1		50C:50W			
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	87.58 \pm 6.26	18.33 \pm 2.58	24.82 \pm 1.12	130.73 \pm 9.96
	30 min	168.28 \pm 82.02	47.66 \pm 17.63	57.82 \pm 24.37	273.75 \pm 124.02
	60 min	257.15 \pm 17.46	87.38 \pm 16.78	101.28 \pm 19.77	445.82 \pm 54.01
	Total concentration	513.01 \pm 70.81	153.37 \pm 3.44	183.92 \pm 5.72	850.30 \pm 79.97
Basolateral	5 min	0.40 \pm 0.09	0.18 \pm 0.06	0.29 \pm 0.09	0.88 \pm 0.23
	30 min	4.99 \pm 1.22	2.23 \pm 0.21	3.02 \pm 0.14	10.24 \pm 1.57
	60 min	13.15 \pm 0.27	6.27 \pm 0.62	8.71 \pm 0.68	28.13 \pm 1.03
	Total concentration	18.53 \pm 1.40	8.68 \pm 0.46	12.03 \pm 0.62	39.25 \pm 0.31
G5					
Apical	5 min	115.10 \pm 31.52	19.10 \pm 6.47	25.50 \pm 6.20	159.70 \pm 44.20
	30 min	214.06 \pm 16.98	50.09 \pm 15.24	68.76 \pm 12.33	332.92 \pm 44.55
	60 min	301.24 \pm 31.02	86.83 \pm 30.58	105.71 \pm 29.08	493.77 \pm 90.69
	Total concentration	630.40 \pm 16.48	156.02 \pm 39.35	199.97 \pm 35.20	986.39 \pm 91.04
Basolateral	5 min	0.65 \pm 0.03	0.26 \pm 0.08	0.40 \pm 0.13	1.31 \pm 0.19
	30 min	6.09 \pm 2.09	2.33 \pm 0.14	3.35 \pm 0.16	11.77 \pm 2.39
	60 min	16.78 \pm 4.50	6.53 \pm 0.93	9.93 \pm 0.07	33.25 \pm 3.64
	Total concentration	23.52 \pm 6.62	9.12 \pm 0.87	13.69 \pm 0.10	46.33 \pm 5.84

850

851 **Table S7.** BCAAs (Leu, Ile and Val) concentration of the 80C:20W sample in the apical and
 852 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 853 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

G1		80C:20W			
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	90.44 \pm 1.88	17.19 \pm 4.44	21.04 \pm 0.92	128.68 \pm 3.48
	30 min	155.09 \pm 23.22	42.86 \pm 10.29	53.63 \pm 11.54	251.57 \pm 45.05
	60 min	210.30 \pm 33.22	65.83 \pm 11.35	82.58 \pm 15.56	358.71 \pm 60.13
	Total concentration	455.83 \pm 54.56	125.88 \pm 26.08	157.25 \pm 28.02	738.95 \pm 108.67
Basolateral	5 min	0.66 \pm 0.05	0.42 \pm 0.18	0.48 \pm 0.08	1.57 \pm 0.31
	30 min	5.11 \pm 2.50	2.66 \pm 0.03	2.93 \pm 1.14	10.70 \pm 3.66
	60 min	11.72 \pm 4.93	5.01 \pm 2.28	7.48 \pm 3.28	24.21 \pm 10.49
	Total concentration	17.49 \pm 7.38	8.09 \pm 2.13	10.89 \pm 4.34	36.47 \pm 13.84
G5					
Apical	5 min	111.09 \pm 7.53	12.26 \pm 0.40	18.37 \pm 1.91	141.73 \pm 9.05
	30 min	233.24 \pm 5.73	45.99 \pm 6.83	63.77 \pm 2.07	343.01 \pm 10.49
	60 min	368.72 \pm 9.88	81.82 \pm 3.97	114.58 \pm 0.03	565.12 \pm 13.88
	Total concentration	713.06 \pm 23.13	140.08 \pm 10.41	196.72 \pm 0.13	1049.86 \pm 33.41
Basolateral	5 min	0.69 \pm 0.08	0.32 \pm 0.04	0.44 \pm 0.08	1.45 \pm 0.20
	30 min	7.20 \pm 0.73	2.97 \pm 0.60	4.14 \pm 0.77	14.31 \pm 2.10
	60 min	18.25 \pm 2.01	7.42 \pm 1.30	10.59 \pm 2.07	36.26 \pm 5.39
	Total concentration	26.14 \pm 2.82	10.72 \pm 1.94	15.16 \pm 2.91	52.01 \pm 7.68

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Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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855 **Table S8.** BCAAs (Leu, Ile and Val) concentration of the (0C:100W)2% sample in the apical and
 856 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 857 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(0C:100W)2%				
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)	
G1	Intestinal digestion time					
	Apical	5 min	96.49 \pm 3.85	23.83 \pm 2.24	28.46 \pm 3.73	148.77 \pm 9.82
		30 min	245.65 \pm 5.81	63.12 \pm 1.06	81.07 \pm 1.64	389.85 \pm 3.10
		60 min	291.74 \pm 33.99	93.24 \pm 19.87	104.93 \pm 17.88	489.90 \pm 71.74
		Total concentration	633.88 \pm 24.33	180.19 \pm 18.70	214.45 \pm 15.79	1028.52 \pm 58.82
	Basolateral	5 min	0.32 \pm 0.06	0.15 \pm 0.02	0.22 \pm 0.02	0.69 \pm 0.10
		30 min	6.62 \pm 2.04	4.16 \pm 2.43	3.74 \pm 0.90	14.53 \pm 5.37
60 min		15.94 \pm 1.18	7.18 \pm 0.01	9.29 \pm 1.12	32.42 \pm 2.29	
G5	Apical	5 min	143.89 \pm 156.33	32.74 \pm 31.40	46.21 \pm 45.46	222.84 \pm 233.19
		30 min	95.28 \pm 22.69	38.05 \pm 8.69	39.96 \pm 6.70	173.29 \pm 38.07
		60 min	87.69 \pm 42.03	37.41 \pm 16.79	38.32 \pm 20.14	163.42 \pm 78.96
		Total concentration	326.86 \pm 221.05	108.20 \pm 56.87	124.49 \pm 72.31	559.55 \pm 350.23
	Basolateral	5 min	2.74 \pm 3.45	1.17 \pm 1.43	1.50 \pm 1.78	5.41 \pm 6.67
		30 min	4.62 \pm 2.19	2.94 \pm 1.68	2.61 \pm 0.81	10.17 \pm 4.67
		60 min	8.67 \pm 1.06	6.32 \pm 2.65	5.95 \pm 0.64	20.94 \pm 4.36
	Total concentration	16.03 \pm 6.70	10.43 \pm 5.76	10.06 \pm 3.23	36.53 \pm 15.69	

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859 **Table S9.** BCAAs (Leu, Ile and Val) concentration of the (80C:20W)2% sample in the apical and
 860 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 861 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(20C:80W)2%				
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)	
G1	Intestinal digestion time					
	Apical	5 min	87.16 \pm 1.41	17.49 \pm 2.15	22.61 \pm 0.41	127.25 \pm 3.96
		30 min	223.22 \pm 6.49	59.55 \pm 4.60	76.94 \pm 2.15	359.72 \pm 0.26
		60 min	268.57 \pm 23.96	97.39 \pm 2.99	111.88 \pm 3.84	477.84 \pm 23.11
		Total concentration	578.95 \pm 29.05	174.43 \pm 3.76	211.43 \pm 6.40	964.81 \pm 18.89
	Basolateral	5 min	0.34 \pm 0.14	0.16 \pm 0.02	0.22 \pm 0.02	0.73 \pm 0.18
		30 min	6.52 \pm 3.78	3.96 \pm 2.99	3.49 \pm 1.82	13.97 \pm 8.59
60 min		17.04 \pm 8.41	6.86 \pm 3.29	9.89 \pm 5.85	33.79 \pm 17.55	
	Total concentration	23.91 \pm 12.33	10.98 \pm 6.31	13.61 \pm 7.69	48.50 \pm 26.32	
G5	Apical	5 min	46.61 \pm 0.19	16.43 \pm 2.24	14.18 \pm 5.85	77.22 \pm 3.80
		30 min	64.01 \pm 15.13	27.10 \pm 4.27	28.40 \pm 4.02	119.51 \pm 23.41
		60 min	91.94 \pm 12.24	45.01 \pm 3.37	43.08 \pm 2.64	180.03 \pm 18.25
		Total concentration	202.55 \pm 27.56	88.54 \pm 5.40	85.66 \pm 12.50	376.75 \pm 45.45
	Basolateral	5 min	0.23 \pm 0.01	0.13 \pm 0.02	0.18 \pm 0.03	0.54 \pm 0.06
		30 min	4.01 \pm 1.00	1.64 \pm 0.04	2.29 \pm 0.04	7.94 \pm 1.08
		60 min	9.56 \pm 1.08	4.56 \pm 0.32	5.73 \pm 0.45	19.85 \pm 0.96
	Total concentration	13.80 \pm 2.09	6.32 \pm 0.38	8.20 \pm 0.37	28.32 \pm 2.10	

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Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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863 **Table S10.** BCAAs (Leu, Ile and Val) concentration of the (50C:50W)2% sample in the apical and
 864 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 865 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(50C:50W)2%				
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)	
G1	Apical	Intestinal digestion time				
		5 min	66.57 \pm 4.77	13.71 \pm 2.76	16.58 \pm 1.30	96.86 \pm 8.84
		30 min	189.64 \pm 7.59	53.96 \pm 1.51	68.90 \pm 1.15	312.51 \pm 10.25
		60 min	230.52 \pm 18.80	81.15 \pm 3.37	94.27 \pm 4.44	405.94 \pm 26.61
		Total concentration	486.73 \pm 21.62	148.82 \pm 2.11	179.75 \pm 4.29	815.31 \pm 28.02
	Basolateral	5 min	0.45 \pm 0.28	0.24 \pm 0.14	0.34 \pm 0.20	1.03 \pm 0.62
		30 min	5.82 \pm 2.23	2.68 \pm 1.05	3.84 \pm 1.64	12.34 \pm 4.91
		60 min	14.37 \pm 1.29	7.38 \pm 0.77	10.16 \pm 2.44	31.91 \pm 4.51
			Total concentration	20.64 \pm 3.80	10.29 \pm 1.96	14.34 \pm 4.29
	G5	Apical	5 min	83.24 \pm 24.10	17.06 \pm 4.89	20.92 \pm 8.48
30 min			173.16 \pm 13.56	47.77 \pm 4.53	58.89 \pm 4.80	279.82 \pm 22.89
60 min			207.23 \pm 20.68	73.93 \pm 6.36	84.93 \pm 8.92	366.08 \pm 35.96
			Total concentration	463.62 \pm 58.34	138.76 \pm 15.78	164.73 \pm 22.20
Basolateral		5 min	0.38 \pm 0.10	0.19 \pm 0.03	0.29 \pm 0.04	0.86 \pm 0.18
		30 min	4.41 \pm 1.78	2.18 \pm 0.72	2.83 \pm 0.76	9.43 \pm 3.26
		60 min	12.05 \pm 6.16	7.21 \pm 0.65	7.82 \pm 1.88	27.07 \pm 7.39
			Total concentration	16.84 \pm 8.05	9.58 \pm 0.10	10.94 \pm 2.68

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867 **Table S11.** BCAAs (Leu, Ile and Val) concentration of the (80C:20W)2% sample in the apical and
 868 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 869 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(80C:20W)2%				
		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)	
G1	Apical	Intestinal digestion time				
		5 min	51.96 \pm 6.02	15.56 \pm 0.24	16.68 \pm 2.74	84.20 \pm 9.00
		30 min	94.91 \pm 3.02	34.43 \pm 3.93	39.65 \pm 2.98	168.98 \pm 3.90
		60 min	122.21 \pm 11.29	48.75 \pm 6.58	59.22 \pm 8.47	230.17 \pm 26.33
		Total concentration	269.08 \pm 2.25	98.74 \pm 10.27	115.54 \pm 8.71	483.36 \pm 21.23
	Basolateral	5 min	0.17 \pm 0.03	0.10 \pm 0.01	0.16 \pm 0.01	0.43 \pm 0.05
		30 min	2.88 \pm 1.70	1.27 \pm 0.42	1.81 \pm 0.59	5.96 \pm 2.72
		60 min	7.59 \pm 3.33	3.50 \pm 1.05	5.22 \pm 1.30	16.30 \pm 5.69
			Total concentration	10.64 \pm 5.06	4.87 \pm 1.48	7.18 \pm 1.91
	G5	Apical	5 min	104.77 \pm 9.98	15.81 \pm 2.09	22.35 \pm 3.59
30 min			227.06 \pm 15.36	58.45 \pm 2.12	74.59 \pm 1.64	360.10 \pm 14.88
60 min			284.44 \pm 17.22	89.57 \pm 0.15	105.69 \pm 5.16	479.70 \pm 12.22
			Total concentration	616.26 \pm 42.56	163.82 \pm 4.05	202.63 \pm 0.08
Basolateral		5 min	0.45 \pm 0.28	0.18 \pm 0.07	0.25 \pm 0.07	0.87 \pm 0.43
		30 min	6.42 \pm 2.72	3.00 \pm 0.99	3.23 \pm 0.20	12.66 \pm 1.93
		60 min	15.14 \pm 5.43	8.58 \pm 2.48	8.65 \pm 0.00	32.38 \pm 2.95
			Total concentration	22.01 \pm 8.43	11.76 \pm 3.40	12.13 \pm 0.27

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Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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871 **Table S12.** BCAAs (Leu, Ile and Val) concentration of the (50C:50W)4% sample in the apical and
 872 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 873 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(50C:50W)4%			
G1		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	72.00 \pm 9.52	16.19 \pm 3.75	19.07 \pm 3.34	107.27 \pm 16.61
	30 min	164.15 \pm 3.81	46.98 \pm 8.67	62.00 \pm 6.21	273.14 \pm 18.69
	60 min	216.79 \pm 8.28	75.18 \pm 0.28	86.69 \pm 2.00	378.66 \pm 10.01
	Total concentration	452.95 \pm 5.04	138.36 \pm 12.69	167.76 \pm 7.55	759.07 \pm 25.29
Basolateral	5 min	0.37 \pm 0.12	0.18 \pm 0.06	0.24 \pm 0.03	0.79 \pm 0.22
	30 min	4.69 \pm 1.55	2.91 \pm 1.57	2.84 \pm 0.53	10.44 \pm 3.65
	60 min	12.06 \pm 3.39	9.87 \pm 0.80	7.99 \pm 0.69	29.92 \pm 4.88
	Total concentration	17.12 \pm 5.06	12.96 \pm 2.44	11.08 \pm 1.25	41.15 \pm 8.74
		G5			
Apical	5 min	81.54 \pm 4.76	15.33 \pm 3.68	18.16 \pm 3.65	115.03 \pm 12.08
	30 min	168.94 \pm 9.28	43.72 \pm 11.18	55.95 \pm 11.44	268.61 \pm 31.90
	60 min	196.43 \pm 17.75	60.76 \pm 3.37	70.20 \pm 1.92	327.38 \pm 23.04
	Total concentration	446.91 \pm 3.71	119.81 \pm 11.49	144.31 \pm 13.16	711.02 \pm 20.94
Basolateral	5 min	0.27 \pm 0.09	0.14 \pm 0.05	0.19 \pm 0.03	0.60 \pm 0.17
	30 min	4.45 \pm 1.18	1.95 \pm 0.23	2.74 \pm 0.39	9.14 \pm 1.80
	60 min	11.33 \pm 1.86	6.96 \pm 2.55	7.42 \pm 0.22	25.71 \pm 0.46
	Total concentration	16.05 \pm 3.13	9.05 \pm 2.27	10.35 \pm 0.64	35.45 \pm 1.51

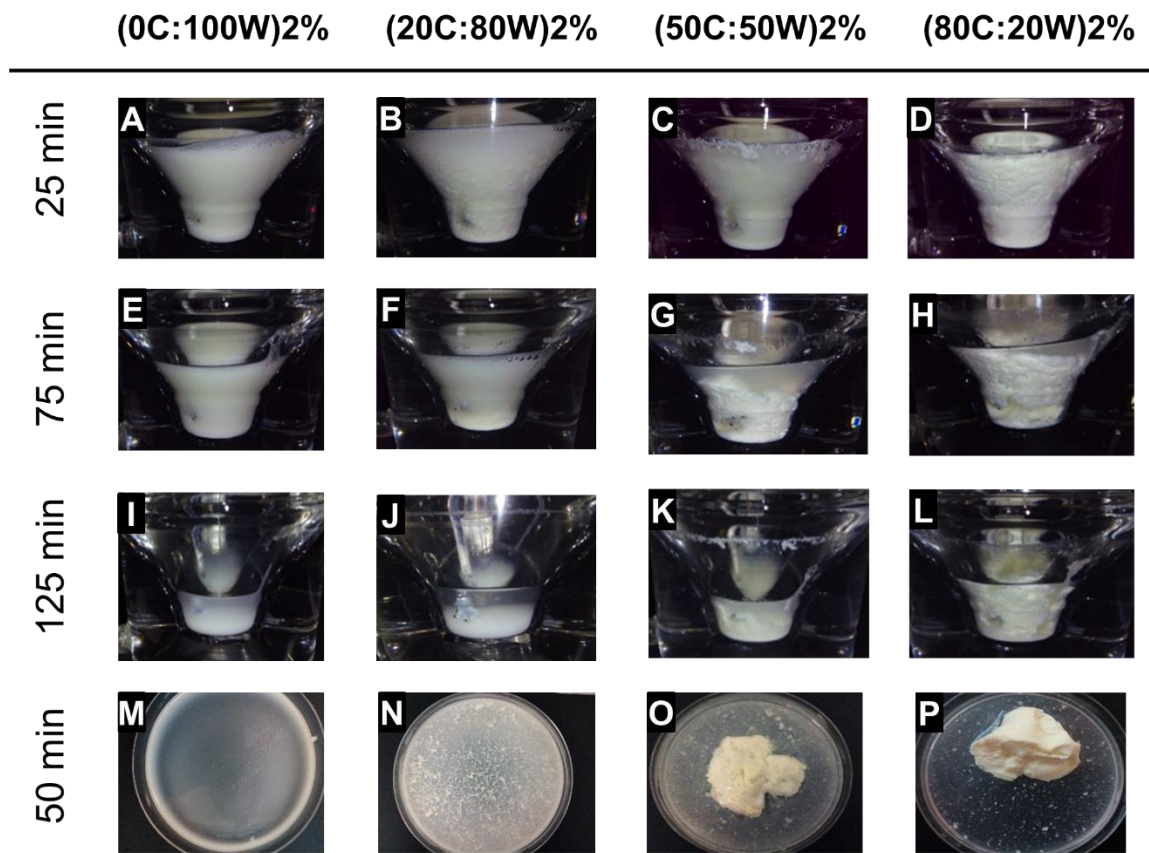
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875 **Table S13.** BCAAs (Leu, Ile and Val) concentration of the (50C:50W)8% sample in the apical and
 876 basolateral side of the Ussing chamber experiment during 5, 30 and 60 min of intestinal digestion in
 877 the G1 and G5 emptied aliquot. Values are presented as means \pm SD of two independent replicates.

		(50C:50W)8%			
G1		Leu ($\mu\text{g/mL}$)	Ile ($\mu\text{g/mL}$)	Val ($\mu\text{g/mL}$)	BCAAs ($\mu\text{g/mL}$)
		Intestinal digestion time			
Apical	5 min	72.37 \pm 7.46	15.07 \pm 5.92	18.00 \pm 3.02	105.43 \pm 16.40
	30 min	165.14 \pm 1.19	51.46 \pm 9.27	60.75 \pm 11.77	277.35 \pm 19.85
	60 min	217.82 \pm 11.68	80.73 \pm 19.51	86.42 \pm 15.85	384.98 \pm 47.04
	Total concentration	455.33 \pm 17.95	147.26 \pm 34.69	165.17 \pm 30.65	767.76 \pm 83.29
Basolateral	5 min	0.64 \pm 0.33	0.33 \pm 0.16	0.43 \pm 0.16	1.40 \pm 0.65
	30 min	5.39 \pm 2.09	2.54 \pm 0.81	3.31 \pm 0.76	11.25 \pm 3.66
	60 min	12.25 \pm 2.19	7.89 \pm 2.48	8.27 \pm 0.29	28.41 \pm 0.01
	Total concentration	18.28 \pm 4.62	10.76 \pm 1.52	12.01 \pm 1.21	41.05 \pm 4.31
		G5			
Apical	5 min	54.60 \pm 27.90	11.20 \pm 9.08	12.43 \pm 8.81	78.23 \pm 45.80
	30 min	126.34 \pm 15.82	38.29 \pm 0.97	46.00 \pm 0.14	210.63 \pm 14.99
	60 min	170.96 \pm 0.16	60.55 \pm 7.11	65.75 \pm 12.28	297.26 \pm 19.23
	Total concentration	351.90 \pm 11.92	110.04 \pm 17.16	124.18 \pm 20.95	586.12 \pm 50.03
Basolateral	5 min	0.42 \pm 0.03	0.21 \pm 0.01	0.26 \pm 0.01	0.89 \pm 0.05
	30 min	4.82 \pm 0.41	3.01 \pm 1.58	3.05 \pm 0.58	10.89 \pm 2.58
	60 min	11.12 \pm 1.31	5.08 \pm 0.83	7.58 \pm 1.82	23.78 \pm 3.96
	Total concentration	16.37 \pm 1.69	8.30 \pm 2.41	10.90 \pm 2.39	35.56 \pm 6.49

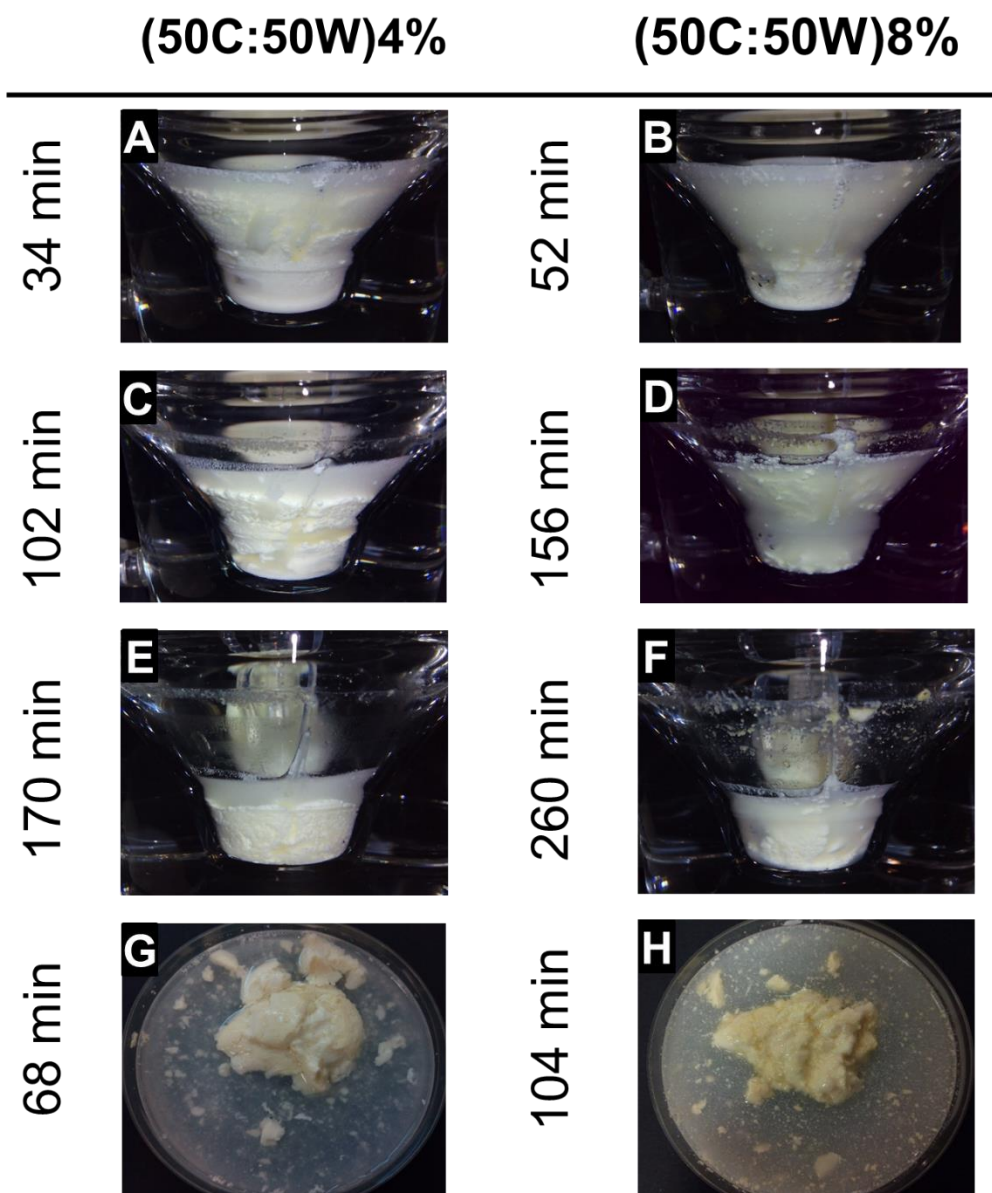
878

Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodtkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514



880

881 **Figure S1.** Gastric behaviour of the emulsion samples displayed in the vessel of the gastric
 882 model at 25, 75 and 125 min, corresponding to the GE1, GE3 and GE5 time points, respectively.
 883 Figures from M to P correspond to the gastric behaviour displayed in a petri dish at 50 min (GE2 time
 884 point). The images correspond to the behaviour immediately before emptying.

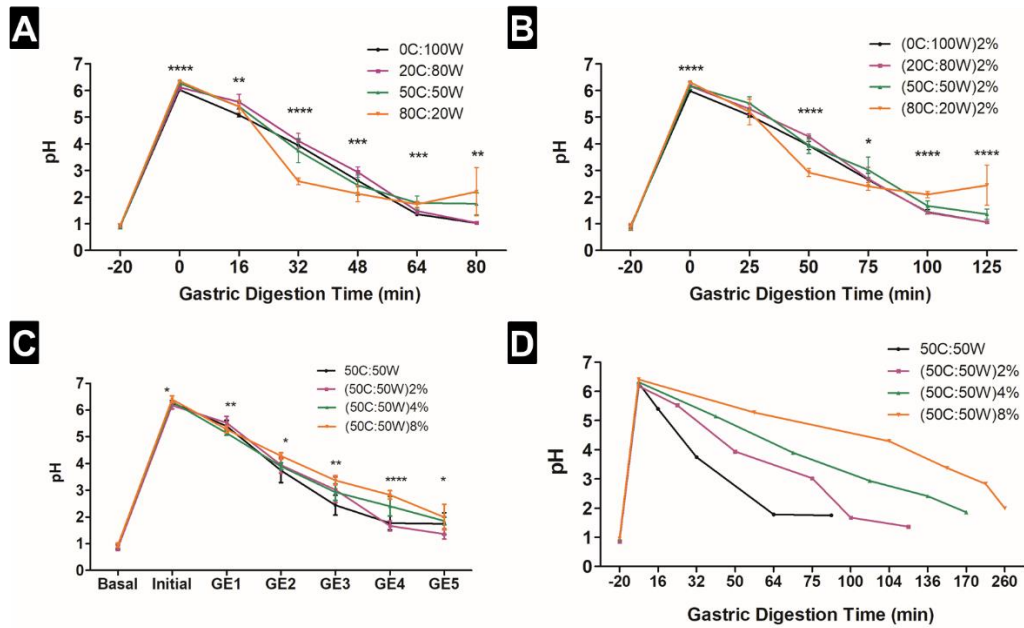


885

886 **Figure S2.** Gastric behaviour of emulsion samples with higher lipid content (4% and 8%)
 887 displayed in the vessel of the gastric model at GE1, GE3 and GE5 points. Figures G and H
 888 correspond to the gastric behaviour displayed in a petri dish at 68 and 104 min (GE2 time point). The
 889 images correspond to the behaviour immediately before emptying.

Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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890

891 **Figure S3.** Change in pH during gastric digestion using the semi-dynamic model, measured in the
 892 emptied aliquots, of (A) protein solution samples, (B) emulsion samples with 2% lipid, (C) samples
 893 with C:W ratio of 1 (i.e. formulation 50C:50W) containing 0%, 2%, 4% and 8% lipid in the emptied
 894 aliquots expressed as function of the GE points, and (D) same pH values than in graph (C) but
 895 expressed as function of the actual gastric digestion time. Gastric digestion time is as indicated in A,
 896 B and D graphs and the time before the start of the digestion (-20 min) corresponds to the basal
 897 stage. In C, the pH values are referred to the basal stage (before gastric digestion), initial (t=0, sample
 898 including oral phase and basal volumes) and the different GE samples (GE1-GE5) corresponding to
 899 each gastric emptying (GE) point. The time values are displayed in Table . Values are presented as
 900 means \pm SD (n=6). Significance difference in pH between samples in each GE point was determined
 901 by one-way ANOVA, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Mulet-Cabero, A.-I., Torcello-Gómez, A., Saha, S., Mackie, A. R., Wilde, P. J., & Brodkorb, A. (2020). Impact of caseins and whey proteins ratio and lipid content on in vitro digestion and ex vivo absorption. *Food Chemistry*, 319, 126514-126525. doi:10.1016/j.foodchem.2020.126514

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